Author/s:
Coutts, A;O'Brien, A;Weeks, AR;Swearer, SE;van Rooyen, A;Branigan, S;Morris, RL

Title:
An environmental DNA approach to informing restoration of the functionally extinct oyster, Ostrea angasi

Date:
2022-11-01

Citation:
Coutts, A., O'Brien, A., Weeks, A. R., Swearer, S. E., van Rooyen, A., Branigan, S. & Morris, R. L. (2022). An environmental DNA approach to informing restoration of the functionally extinct oyster, Ostrea angasi. Aquatic Conservation Marine and Freshwater Ecosystems, 32 (11), pp.1732-1744. https://doi.org/10.1002/aqc.3886.

Persistent Link:
https://hdl.handle.net/11343/318315

Received: 20 March 2022 | Revised: 23 August 2022 | Accepted: 24 August 2022

DOI: 10.1002/aqc.3886

RESEARCH ARTICLE

WILEY

An environmental DNA approach to informing restoration of the functionally extinct oyster, *Ostrea angasi*

Alexander Coutts[1] | Allyson O'Brien[2] | Andrew R. Weeks[2,3] | Stephen E. Swearer[1] | Anthony van Rooyen[3] | Simon Branigan[4] | Rebecca L. Morris[1]

[1]National Centre for Coasts and Climate, Building 147, School of Biosciences, University of Melbourne, Parkville, Victoria, Australia

[2]School of BioSciences, Building 147, University of Melbourne, Parkville, Victoria, Australia

[3]CESAR, 293 Royal Parade, Parkville, Victoria, Australia

[4]The Nature Conservancy Australia, Suite 2.01, 60 Leicester Street, Carlton, Victoria, Australia

Correspondence
Alexander Coutts, National Centre for Coasts and Climate, Building 147, School of Biosciences, University of Melbourne, Parkville, Victoria, 3010, Australia.
Email: alexander.coutts@utas.edu.au

Funding information
This work was funded through the Port Phillip Bay Fund (awarded to The Nature Conservancy) by the Victorian Government's Department of Environment, Land, Water and Planning. The Nature Conservancy contributed to selection of field sites based on the results of the OysterWatch Project.

Abstract

1. The success of oyster reef restoration can be enhanced by data on the distribution of remnant populations to inform the selection of suitable restoration locations. A quantitative polymerase chain reaction-based environmental DNA (eDNA) assay was designed to provide distribution data for the oyster, *Ostrea angasi*, whose reefs are functionally extinct in Port Phillip Bay, Australia.
2. *Ostrea angasi* eDNA accumulation and decay was measured in aquaria containing oysters in low and high densities, prior to testing the efficacy of the eDNA approach for detection of oysters at 15 field sites. *Ostrea angasi* eDNA accumulated significantly faster in aquaria where more individuals were present, while eDNA became undetectable 2–6 days after oysters were removed in low-density treatments.
3. The eDNA samples were successful at detecting *O. angasi* in the field when taken in close proximity of an oyster population. Increasing the sample number and volume could maximize oyster detection, demonstrating the potential of eDNA to identify suitable sites for the restoration of functionally extinct marine ecosystems.

KEYWORDS
CO1, detectability, ecosystem engineer, environmental DNA, native species, oyster reef restoration

1 | INTRODUCTION

Oysters, and the reefs that they form, were once widespread and conspicuous features of the world's estuaries. Along with almost all marine ecosystems, oyster reefs have been altered or damaged by anthropogenic activities (Halpern et al., 2008). This has had a drastic effect on the global distribution of oyster reefs, with an estimated 85% loss, primarily from the effects of over-fishing and coastal development (Beck et al., 2011). The loss of oyster reefs represents the loss of a range of vital ecosystem services and socio-economic benefits, which include supporting biodiversity (Hadley et al., 2010) and fisheries (Peterson, Grabowski & Powers, 2003), coastal protection (Chowdhury et al., 2019) and water filtration (Ermgassen et al., 2013). Restoration ecology has become an important tool in the

effort to return the goods and services provided by oyster reef ecosystems.

Oyster reef restoration usually needs to overcome problems of substrate or recruitment limitation, or both. The deployment of new substrate for oysters to colonize is commonly the first step in an oyster reef restoration project. Recycled oyster shell is often used as the substrate, but projects have also used a combination of alternative materials including other bivalve shells, concrete, limestone, and granite (Hernandez et al., 2018). Natural colonization of this reef substratum may occur in areas where there are nearby remnant oysters and/or oyster farms to provide an adequate larval supply to support reef development. Where there is an inadequate larval supply, oysters can be seeded onto the reef as juveniles or adults, often from a costly hatchery-rearing process. Careful selection of restoration sites to maximize natural recruitment is therefore more cost-effective than seeding reef substratum where there is an adequate larval supply (Geraldi et al., 2013). Oyster reef restoration was pioneered on the US Pacific and Gulf of Mexico coasts (Hernandez et al., 2018), projects building on these have now been established in Europe (Pogoda et al., 2019), Asia (Bangladesh – Chowdhury et al., 2019; Hong Kong – The Nature Conservancy, 2022) and Australia (Gillies, Crawford & Hancock, 2017; McAfee et al., 2022).

In Port Phillip Bay, Australia, shellfish reef ecosystems formed by the native flat oyster *Ostrea angasi* and blue mussels *Mytlius edulis* were once extensive (Ford & Hamer, 2016). The existence of *O. angasi* in shell middens suggests they were an important part of the diet of Indigenous people for at least 10,000 years prior to European settlement (Godfrey, 1989). With European settlement, oysters were intensively fished for food and lime in the 1840s (Ford & Hamer, 2016). The fishery decimated oyster populations in all of Victoria's major estuaries and removed the reef substrate for oyster larvae to settle on and grow. Port Phillip Bay had one of the last remaining viable commercial shellfish fisheries in Victoria. While this fishery targeted scallops (*Pecten fumatus*) and mussels, it also affected *O. angasi* through bycatch and widespread removal of suitable *O. angasi* habitat by dredge fishing techniques. This fishery diminished in the 1990s, due to destructive dredge fishing and overexploitation, marking the end of substantial commercial shellfish harvesting in Port Phillip Bay (Ford & Hamer, 2016). Today, 90% of shellfish reefs in Port Phillip Bay have been lost and *O. angasi* is considered functionally extinct (Ford & Hamer, 2016). The population is probably too small to recover in the short-term, even in the absence of harvesting because the system is now both recruitment limited (insufficient brood stock remains) and substrate limited (insufficient suitable hard substrate remains) (Ford & Hamer, 2016; Gillies et al., 2018). Hence, shellfish reefs in their pre-colonial state, have been largely erased from the memory of much of the public and current coastal managers (Alleway & Connell, 2015).

Active shellfish reef restoration efforts are currently being undertaken in Port Phillip Bay (McAfee et al., 2022). A 12-ha limestone reef has been constructed to restore shellfish reef habitats and was seeded with oyster spat from the Victorian Shellfish Hatchery (The Nature Conservancy, 2018; Brannigan, Fitzsimons & Gillies, 2020). Widespread restoration of functional shellfish reef ecosystems in Port Phillip Bay will require expansion and replication of these newly constructed reefs. If shellfish reefs can be placed nearby to other suitable habitat and existing natural *O. angasi* brood stock, survival and expansion for both remnant and restored populations could be maximized through a reef network (Schulte, Burke & Lipcius, 2009; Gillies, Crawford & Hancock, 2017).

Accurate and widespread *O. angasi* distribution data, and knowledge of the species' habitat preferences are some of the key parameters needed for identifying optimal locations for *O. angasi* restoration in Port Phillip Bay. These parameters, along with spatial environmental data and other relevant ecological parameters such as food availability and predator intensity, can allow for more accurate habitat suitability modelling to determine the most appropriate locations for shellfish restoration (Theuerkauf & Lipcius, 2016). The ecology and distribution of natural *O. angasi* reefs has been understudied and thus knowledge of the reference ecosystem that should be aimed for is limited (except see, GIllies, Crawford & Hancock, 2017). While historical records (Ford & Hamer, 2016; Gillies et al., 2018), mapping (Crawford, 2015) and local fisher's knowledge (Hamer, Pearce & Winstanley, 2013) have been used to reconstruct former oyster reef distributions, few studies have investigated the contemporary distribution of *O. angasi*. Cohen, Currie & McArthur (2000) used dredge sampling to investigate the status of *O. angasi* at sites where they were historically abundant, and The Nature Conservancy conducted diver and camera surveys at a number of specific sites in Port Phillip Bay, but widespread surveys to map the extent of the remnant *O. angasi* reefs in Port Phillip Bay are still lacking. This knowledge gap presents a challenge as the collection of distribution data by diver surveys is expensive and time consuming. However, molecular survey techniques such as environmental DNA (eDNA) can provide an accurate and cost-effective alternative to traditional techniques (Huver et al., 2015; Smart et al., 2016).

Environmental DNA is genetic material extracted from an organism's environment, whether that be from a true environmental sample (e.g. soil, water, sediment), from trace material left behind by organisms (e.g. faeces, saliva) or from a host organism acting as the 'environment' for a parasite, pathogen, epiphyte or microbiome (Bass et al., 2015). Environmental DNA's application to informing conservation and wildlife management began when it was first applied in the detection of a single frog species in freshwater streams (Ficetola et al., 2008). Since then, eDNA has become a well-established tool for biodiversity monitoring and is widely used for single species detection and in combination with high throughput DNA sequencing technologies for multi-species biodiversity assessments (eDNA metabarcoding) (Thomsen & Willerslev, 2015). A major strength of eDNA applications over visual and trapping techniques is its ability to detect rare and cryptic species. Environmental DNA has repeatedly been proven to be more effective at detecting rare and cryptic species than other methods such as animal trapping, visual surveys and host necroscopy for parasites (Hunter et al., 2015; Huver et al., 2015; Smart et al., 2015; Lugg

et al., 2018). In freshwater systems large scale monitoring programmes of invasive (Biggs et al., 2015; Tingley et al., 2019) and endangered species (Xu et al., 2018) using eDNA from water samples have been successful. In the marine environment surveys of rare and cryptic species have been limited to smaller spatial and temporal scales (Miralles et al., 2016; Richardson et al., 2016; Gargan et al., 2017; Minamoto et al., 2017), but there have been recent attempts to upscale the use of eDNA tools across broader marine regions (West et al., 2021).

Using eDNA approaches to survey rare species in the marine environment is more challenging than in fresh water. Dispersion, stratification and dilution of eDNA by water movement in the marine environment occurs on larger spatial scales and is dictated by a unique and complex set of interacting forces including tides and variable currents. However, recent studies have shown promising results for detection of rare marine animals, including elasmobranchs (Gargan et al., 2017; Weltz et al., 2017), bivalves (Ardura et al., 2015) and cephalopods (Mauvisseau et al., 2017). Furthermore, the recent adoption of quantitative polymerase chain reaction (qPCR) as commonplace in eDNA workflows has increased the sensitivity of single and multi-species assays, allowing lower detection limits and more reliable occupancy data (Wilcox et al., 2013). With the continued advancement of molecular technologies and sampling methodologies, eDNA monitoring is becoming an increasingly powerful tool for detection of marine animals for use by scientists and managers alike.

The efficacy of an environmental DNA approach for informing oyster reef restoration is investigated with the aim to demonstrate that eDNA can be an effective tool in informing marine restoration projects where gaining distribution data can augment success. A species-specific qPCR assay for detection and quantification of the *O. angasi* CO1 mitochondrial gene in eDNA extracted from both laboratory and field derived water samples was developed. A survey of 15 sites was undertaken to determine the efficacy of the assay to provide accurate distribution data for *O. angasi* in Port Phillip Bay. To guide further development of a robust *O. angasi* eDNA sampling regime in the marine environment, the accumulation and decay rates of *O. angasi* eDNA were explored as well as the effects of oyster density and sampling direction and distance from a restored reef on eDNA concentrations.

2 | METHODS

2.1 | Probe development

2.1.1 | qPCR assay development

A species-specific qPCR assay was developed to target a 141-base pair region of the *O. angasi* CO1 mitochondrial gene. The nucleotide sequence of this region was obtained from the NCBI GenBank database in accession numbers AF540598 (Morton, Lam & Slack-Smith, 2003), AF112287 (Foighil et al., 1999) and DQ078638-DQ078668 (Hurwood, Heasman & Mather, 2005).

The assay consisted of a pair of primers; forward primer: TAGCGCTGTTCCCTTGATCT, reverse primer: GATCCCCACCAC CTACAGG, and a fluorescent probe: ACACTTCCAGTTCTGGCTGG. These were obtained as a custom PrimeTime assay from Integrated DNA Technologies.

2.1.2 | Limits of detection and quantification

To calculate the limit of detection (LOD) and limit of quantification (LOQ) of the *O. angasi* assay, qPCR reactions containing 10, 1, 0.1, 0.01, 0.001, 0.0005, 0.00025 and 0.000125 ng/µl *O. angasi* genomic DNA were performed. These concentrations were selected based on results from known DNA standards and samples taken in aquaria containing *O. angasi*. They were designed to span the entire range of concentrations we thought we could encounter in the field and lab experiments. LOD is defined as the lowest *O. angasi* DNA concentration at which qPCR can detect *O. angasi* 95% of the time, while LOQ is the lowest concentration at which the coefficient of variation (CV) of eDNA concentration between replicates is less than 0.35. There were 10 replicates of each concentration. The LOD and LOQ were calculated in R version 3.6.1 using a publicly available R code (Merkes et al., 2019). The script modelled frequency of detection and CV for concentration using the best fitting model parameter as per Klymus et al. (2020).

2.1.3 | Specificity tests

The specificity of the assay was verified by testing for amplification of off-target DNA extracted from the tissue of three closely related species: Pacific oyster (*Crassostrea gigas*), blue mussel (*Mytilus galloprovincialis*) and flat oyster (*Ostrea stentina*). The assay was further verified by testing that it can amplify DNA extracted from wild *O. angasi* individuals collected from two wild populations: 9 ft Bank (−38.1001, 144.4482); and Bird Rock (−38.09657, 144.43892; Figure 1). Two baseline comparison samples, which also acted as positive controls were included in these specificity tests. These baseline samples consisted of *O. angasi* genomic DNA extracted from individuals collected from a wild population in Blairgowrie (−38.357218, 144.773376) and a restored oyster reef near Geelong (−38.11885, 144.506717), which was seeded with oysters from an aquaculture population. All DNA samples used in these tests were normalized to 5 ng/L.

2.2 | Laboratory experiment

A laboratory experiment was conducted in a recirculating seawater system at the University of Melbourne in April 2019. The water in the system was collected directly from Port Phillip Bay. Seawater for the experiment was separated from the system 1 week prior to the start of the experiment, which allowed for any *O. angasi* DNA potentially

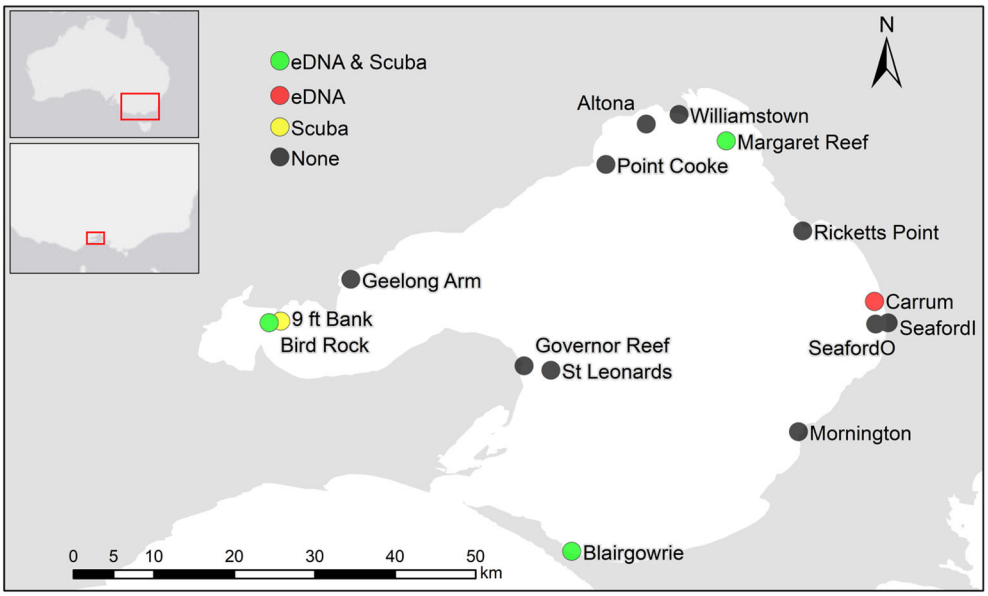

FIGURE 1 Map showing the results of the *Ostrea angasi* environmental DNA (eDNA) survey. Black corresponds to sites with no detection, green to sites with both eDNA and scuba survey detection, yellow to sites with scuba survey detection only and red to sites with eDNA detection only. Note the site names correspond to those listed in Table S1.

present in the system to degrade. The water was stored in nine 52.9-L experimental tanks during this time. To account for changes in volume and salinity caused by evaporation during this period, any evaporated water was replaced with reverse osmosis (RO) water before the experiment started.

Fifty-one oysters from Margaret Reef, a restored oyster reef in Port Phillip Bay, Victoria were used in this experiment. The nine experimental tanks were randomly assigned to one of three treatments: (1) control (no oysters); (2) low density (one oyster); and (3) high density (16 oysters). There were three replicates of each treatment. Oysters were assigned to tanks based on size class using shell length; each oyster's length was measured to the nearest 0.5 cm and grouped into four size classes (Spat <1 cm, Small 1–4 cm, Medium 4.5–5 cm, Large >5 cm). The low-density treatment tanks contained one large oyster, while the high-density treatment tanks contained two small oysters, eight medium oysters and six large oysters. The shell-free wet weight of each oyster was measured after the experiment. The mean ± *SE* total oyster wet weight was greater in the high oyster treatment (33.71 ± 3.91 g) than in the low oyster treatment (4.40 ± 1.53 g).

An eDNA sample was taken from each tank before the oysters were added, to test that any *O. angasi* DNA potentially present in the system had degraded. Further eDNA samples were taken immediately after oysters were added to the tanks and at 8, 24, 48 and 144 hours (one sample at each time point). Oysters were removed from all tanks immediately after the 24-hour sample was taken.

All eDNA samples were taken using sterile, 60-ml Luer lock syringes (Shandong Hapool Medical Technology, China) in combination with Sterivex 0.22-μm Luer lock filters (Merck, Germany). The syringes were used to push 100 ml of water through a separate filter for each sample. The samples were stored at −80°C until processing. Filtered water was returned directly to the tank that it came from to keep the volume of water in the tank constant throughout the experiment.

2.3 | Field survey

Fifteen sites in Port Phillip Bay were sampled between June and October 2019 to test the efficacy of the *O. angasi* eDNA assay in the field and to determine *O. angasi* occupancy at a range of sites within the bay (Table S1). Sites were chosen to maximize coverage of geographical locations, tidal regimes, habitat types and depths (Table S1). During site selection, an emphasis was placed on sites known to support historical oyster populations (Cohen, Currie & McArthur, 2000; Ford & Hamer, 2016) and sites identified as possible restoration targets by The Nature Conservancy through explorative surveys and settlement plate monitoring (The Nature Conservancy, 2018; Victorian National Parks Association, 2020). Some positive control sites where *O. angasi* is known to be present were also selected; these were at 9 ft Bank, Bird Rock (The Nature Conservancy, 2018) and Blairgowrie pier (B. Cleveland, personal communication).

At each site, four eDNA samples were taken, two from the water surface and two from the bottom immediately above the sea bed. Three surface samples and three bottom samples were taken at the positive control sites (Bird Rock, Blairgowrie and 9 ft Bank). At the positive control sites, all water samples were collected within five metres of live oysters. For all sites, if the water depth was less than two metres, the bottom samples were omitted. Each sample consisted of 10 150-ml aliquots which were all collected directly into one 2-L sample bottle, haphazardly within the site. All samples were taken within 20 m of a central point. This central point was chosen either by coordinates provided by The Nature Conservancy where applicable, or by haphazardly choosing a location at the site. The sampling bottle was cleaned before sampling at each site by washing with 70% ethanol, followed by RO (reverse osmosis) water and sea water from the site. From each 1.5-L sample, 1 L was filtered using 60-ml Luer lock syringes and Sterivex filters (0.22 μm). After filtration, the filters were stored on ice during transportation

to the University of Melbourne, where they were then stored at −80°C until processing.

At each site, the presence and abundance of oysters was established through diver surveys. The number of clumps or individual oysters was counted in 10 replicate quadrats (1 m^2). If no oysters were detected using this method, then a 5-minute, non-random timed search was performed to confirm the presence or absence of oysters. Depth was measured *in situ* using a dive computer.

One surface and one bottom sample from each site was tested for the presence of *O. angasi* DNA, while the other two samples were kept as a back-up. All samples were processed for 9 ft Bank and Bird Rock because the first two samples were negative, and *O. angasi* is known to occur at these sites. Furthermore, all samples at 9 ft Bank were negative, so these samples were retested (a second set of qPCR reactions was run). Only results from the second set of qPCR reactions are presented. Oysters were deemed present at a site if at least one eDNA sample was positive for *O. angasi* DNA. For the scuba survey, oysters were deemed present if any live oysters were found in a quadrat or during the 5-minute search.

2.4 | eDNA dispersion experiment

An existing *O. angasi* restoration site at Margaret's Reef was used to determine the spatial resolution of the *O. angasi* assay by measuring the distance from an oyster reef at which *O. angasi* eDNA becomes undetectable. The Margaret's Reef restoration site consisted of two restored reefs, the centres of the reefs were 77 metres apart. The centre of the site, around which the sampling was based, was defined as halfway between the centre point of the two reefs. Environmental DNA samples were taken at three distances (0, 200 and 500 m) along four transects radiating from the centre of the site in a cross formation (Figure 2). Two of the transects ran parallel to the shoreline, while the other two ran perpendicular to the shore. The bearings of the transects from the centre of the site were 350° (NNW), 80° (ENE), 170° (SSE) and 260° (WSW), Figure 2.

There was one sampling point at 0 m to represent the maximum *O. angasi* DNA concentration at the site. There were four sampling points located 500 m from the centre, one on each transect. Due to weather conditions during the sampling day there were only two sampling points located 200 m from the centre. These were on the SSE and ENE transects. At each sampling point two eDNA samples were taken, one from the surface and one from the bottom, 50 cm above the substrate. Surface samples were taken by collecting sea water directly from the source in a sterile 60-ml Luer lock syringe or in sterile WhirlPack sample bags before filtration. The water was passed directly through a 0.22-μm Sterivex filter. A total of 1 L of water was filtered for each sample, 60 ml at a time. Bottom water samples were taken using a Niskin bottle and transferred to WhirlPack bags before filtration.

The syringe, filter, WhirlPack bag and gloves were used only once. Before sampling at each site, the Niskin bottle was rinsed with a 10% bleach solution, followed by RO water and sea water from the site. Procedural control samples for the WhirlPacks and Niskin bottles were taken by repeating the eDNA sampling and filtering procedure using sterile RO water instead of sea water. All eDNA samples were stored on ice. Upon return to the University they were stored at −80°C.

2.5 | DNA extraction

DNA was extracted from the Sterivex filters using the Qiagen DNeasy Blood & Tissue Kit. The standard spin-column protocol was modified to suit extraction from the Sterivex filters (Tingley et al., 2019). Forty μl of proteinase K and 120 μl of ATL lysis buffer were added to the filter casing and sealed using a Bunsen burner and tweezers. The

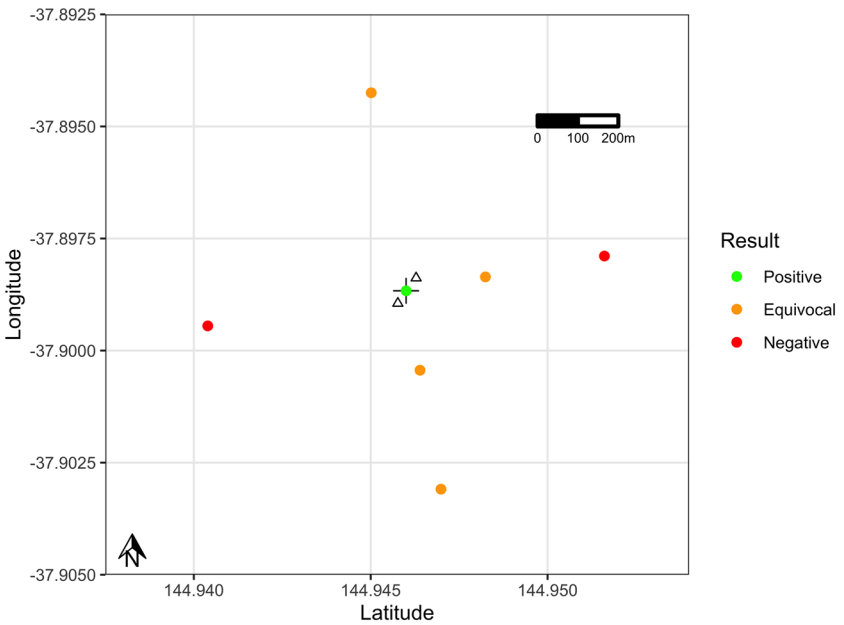

FIGURE 2 Map showing the results of the environmental DNA dispersion experiment. Four 1-L water samples were taken at each sampling point represented by coloured dots (two at the substratum and two at the water surface). Black triangles represent two artificial oyster reefs and the black cross represents the halfway point between the reefs. Sampling points were considered positive (green) if at least one sample was positive, equivocal (orange) if at least one sample was equivocal (one out of three quantitative polymerase chain reaction replicates exhibited amplification) and negative (red) if all samples were negative.

sealed filters were incubated at 55°C for 2 hours, while constantly rotating. The solution was removed from the filter casings using a Luer lock syringe. For the remainder of the extraction, the standard Qiagen DNeasy protocol for tissue samples was followed, with the exception of the volumes of AL buffer (500 μl), ethanol (500 μl) and AE buffer (100 μl) used. The extracted DNA was stored at −20°C. Negative controls were included in each extraction batch by following the same extraction protocol using an empty 2-ml tube rather than a Sterivex filter. Tissue DNA extractions were used for primer development and primer specificity testing. DNA extractions from tissue samples used the standard Qiagen DNeasy Blood & Tissue Kit spin column protocol.

2.6 | qPCR analysis

2.6.1 | qPCR protocol

The same qPCR protocol was used for amplification of DNA from all eDNA samples and tissue samples. All samples were run in triplicate on a Roche 480 light cycler unit, in a 384 well plate format. Each reaction had a volume of 10 μl and contained 0.5 μl 20X custom PrimeTime assay, 5 μl KAPA probe force master mix, 2.5 μl ddH_2O and 2 μl of DNA. Every 384-well plate included DNA standard reactions containing 5, 0.5, 0.05, 0.005 and 0.0005 ng/L of *O. angasi* DNA. Each plate also included negative controls with no DNA template, and internal PCR controls (IPCs) to test for the presence of PCR inhibitors in seawater samples. To ensure that sample DNA concentrations stayed within the range of the standard curve, all DNA from tissue samples was diluted to 5 ng/L before processing. The reaction conditions were 3 minutes at 98°C, 50 cycles of 10 seconds at 90°C, 20 seconds at 60°C and 1 minute at 37°C. A sample was considered positive for *O. angasi* DNA if at least two out of three technical replicates exhibited amplification. If a sample exhibited amplification in just one qPCR replicate, the result was considered equivocal.

2.6.2 | DNA quantification calculations

DNA concentration from each sample was obtained by comparing sample crossing point values to those of the DNA standards, using the second derivative max absolute quantification function of the Roche Light Cycler software; qPCR efficiency values were calculated from the DNA standards.

The Roche Light Cycler software calculated eDNA amount (ng) present in the 2-μl qPCR sample. For analysis this was converted to ng/L *O. angasi* eDNA concentration in the original seawater sample using the following equation:

$$\frac{eDNA\ amount\ (ng) \times 50}{Water\ sample\ volume\ (L)} = eDNA\ concentration\ (ng/L\ seawater).$$

Due to contamination detected in one sample from a control tank in the laboratory experiment, a contamination threshold (CoT) was calculated (0.22 ng/L of sea water). This CoT represents the lowest concentration at which we can be confident the detected eDNA was present due to the oyster treatments and not contamination. The CoT was calculated by taking the upper limit of the 95% confidence interval for the *O. angasi* DNA concentration detected in the contaminated sample.

2.7 | Data analysis

A linear mixed effects model tested the effects of time and oyster density on eDNA concentration using the R package lme4 (Bates et al., 2015). The control treatment was excluded from the analysis as time was irrelevant to this treatment. Time and oyster density were treated as fixed factors while tank was a random factor with repeated samples taken over time. Environmental DNA concentration was square-root transformed prior to analysis to achieve a normally distributed response variable. Post-hoc pairwise comparisons of group means were conducted for significant fixed effects (time and treatment) using the emmeans package (Lenth, 2020). To test for a relationship between oyster weight and eDNA concentration in experimental tanks a linear regression was fitted using the maximum eDNA concentration (24-hour sample) as the response variable and oyster weight as the explanatory variable. Each variable had one observation per tank. To test whether a positive eDNA result was more likely at sites where oyster presence was confirmed by scuba diver surveys, Fisher's exact test was used to compare the count data for presence and absence of oysters using both methods. Due to the lack of positive samples in the dispersion experiment, these data were not statistically analysed. All statistical analyses were conducted in R version 3.6.1.

3 | RESULTS

3.1 | Limits of detection and quantification

The LOD was calculated as 0.000887 ng/μl, the equivalent of 0.887 ng/L sea water in a 100-ml water sample (lab experiment) and 0.0887 ng/L in a 1-L water sample (field experiment). The LOQ was calculated as 0.0028 ng/μl or 2.8 ng/L (lab experiment) and 0.28 ng/L (field experiment).

3.2 | Specificity and comprehensiveness tests

Quantitative PCR reactions containing DNA from the baseline *O. angasi* samples reached their fluorescence threshold after an average of 20.34 cycles (n = 6, SD = 0.29). By comparison, reactions containing *C. gigas* and *M. galloprovincialis* DNA reached their fluorescence thresholds after an average of 36.13 (n = 3, SD = 0.93) and 36.56 (n = 3, SD = 1.17) cycles, respectively. The qPCR assay exhibited no amplification of *O. stentina* DNA. While there was amplification of off-target DNA, the qPCR assay underestimated *C. gigas* and *M. galloprovincialis* DNA concentrations by a magnitude

of approximately 10^5. Therefore, it is unlikely that these off-target sequences would be amplified at the concentrations at which eDNA is found in sea water. *Ostrea angasi* tissue DNA samples from 9 ft Bank and Bird Rock exhibited amplification after an average of 20.72 (n = 9, SD = 0.83) and 20.70 (n = 9, SD = 0.24) qPCR cycles, respectively.

3.3 | Laboratory experiment

Ostrea angasi eDNA was detected in all low and high oyster treatment tanks (Figure 3). In high oyster density treatment tanks, eDNA was detectable immediately after oysters were added to the tanks. In low oyster density treatment tanks, eDNA was detectable after 8 hours. All seawater system negative control samples were negative and all samples from control treatment tanks were negative, except for the 48-hour sample in the second control tank (mean concentration = 0.12 ng/L, SD = 0.10). *Ostrea angasi* eDNA in the low oyster density treatment tanks decayed to undetectable concentrations after 50–144 hours across the three replicate tanks. *Ostrea angasi* eDNA in the high oyster density treatment tanks decayed over time but remained at a detectable concentration for the duration of the experiment in all replicates.

There was a significant interaction between time and treatment ($F_{4,\ 16} = 5.5965$, $P < 0.05$; Table S2). In the high oyster treatment, there was a significant increase in eDNA concentration from hour 0 to 8 ($t_{16} = -4.869$, $P < 0.05$) and a significant decrease from hour 24 to 48 ($t_{16} = 4.745$, $P < 0.05$). However, there were no significant differences in eDNA concentrations between any time points in the low oyster treatment. Therefore, the interaction was due to a much larger magnitude of increase and decrease in eDNA concentration in the high oyster treatment compared to the low oyster treatment. Environmental DNA concentrations were significantly higher in the high oyster treatment than the low oyster treatment at hours 8 ($t_8 = 4.516$, $P < 0.005$) and 24 ($t_8 = 3.969$, $P < 0.005$). Maximum eDNA concentrations (concentration at 24 hours) were positively correlated with total oyster weights in tanks ($F_{1,\ 7} = 16.06$, $P < 0.05$, $R^2 = 0.6531$, n = 9; Figure 4).

3.4 | Field survey

Oysters were detected at Blairgowrie, Bird Rock and Margaret's Reef by the dive survey and eDNA assay, 9 ft Bank by the dive survey only, and Carrum by the eDNA assay only (Table 1; Figure 1). All other sites were negative for dive surveys and the eDNA assay (Figure 1). A positive eDNA result at any site was significantly more likely if the scuba survey site was also positive (odds ratio = 20.00, $P = 0.033$). Quantitative PCR efficiency did not fall below 90%, no PCR inhibition was detected, and all qPCR and DNA extraction negative controls were negative. Average eDNA concentrations per 1-L water sample ranged from 0 to 0.145 μg/L. Positive eDNA samples at Blairgowrie, Carrum and Bird Rock had eDNA concentrations below the LOD. Therefore, other samples at these sites could have contained similar eDNA concentrations that were not detected by the assay.

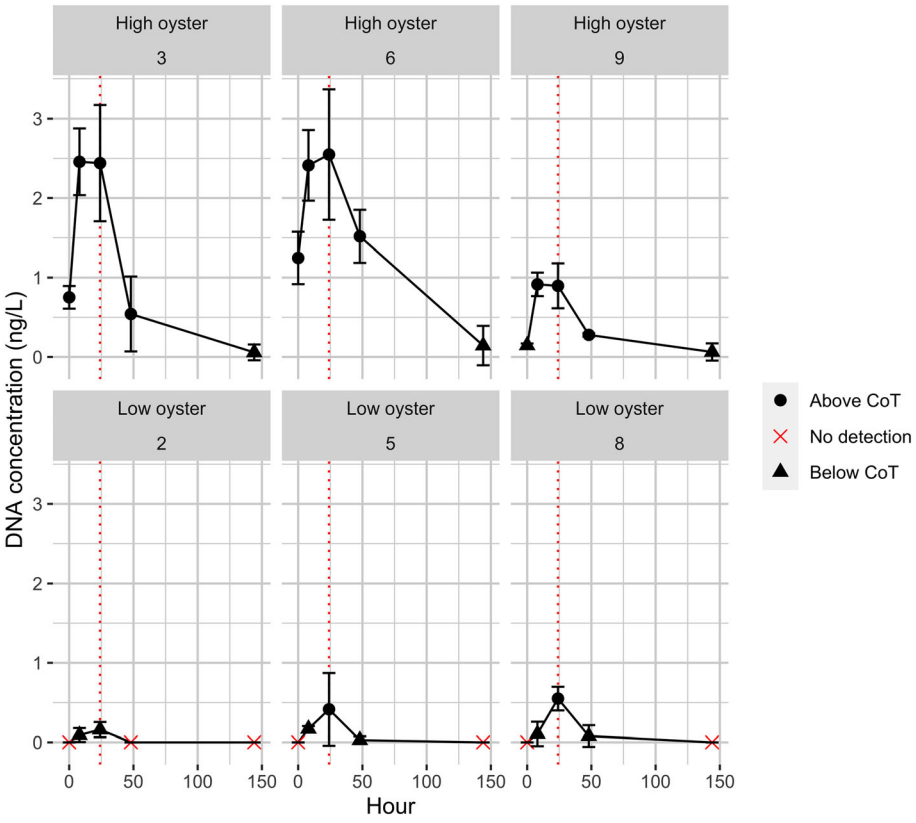

FIGURE 3 Average concentration of *Ostrea angasi* DNA in water samples of tanks with high (16 oysters) and low (one oyster) densities. Oysters were added to the tanks at 0 hours and removed from the tanks after 24 hours. The time of oyster removal is shown by a red dotted line. Each graph panel shows results from one experimental tank. Error bars shown are one standard deviation. DNA concentration is measured in nanograms of *O. angasi* DNA per 1-L water sample. Averages and SD are calculated from three quantitative polymerase chain reaction replicates for each water sample. CoT refers to contamination threshold.

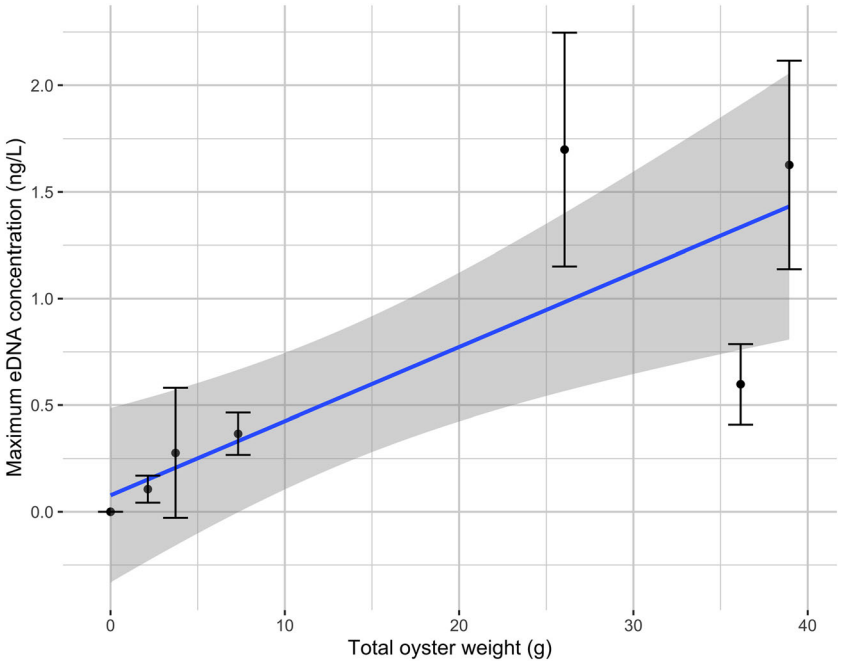

FIGURE 4 Relationship between the maximum environmental DNA (eDNA) concentration (concentration at 24 hours) and total oyster weight for each of nine laboratory experimental tanks. Blue line shows line of best fit. Grey shading shows 95% confidence interval of the fitted values of the linear regression. Error bars show SD for eDNA concentration from 3 quantitative polymerase chain reaction replicates.

TABLE 1 Quantitative polymerase chain reaction results for individual samples from survey sites where environmental DNA (eDNA) analysis returned positive or equivocal results for the presence of *Ostrea angasi*. Mean *O. angasi* eDNA concentration for each sample considered positive (i.e. two or three triplicates) is shown in brackets. Dashes indicate that no sample was taken, or the sample was not processed. Results from the eDNA dispersion experiment at Margaret Reef are not presented in this table

Site	Top sample 1	Top sample 2	Top sample 3	Bottom sample 1	Bottom sample 2	Bottom sample 3
Blairgowrie	1/3	-	-	3/3 (0.04041). ng/L)	-	-
Carrum	2/3 (0.0127 ng/L)	-	-	0/3	-	-
Margaret Reef	0/3	-	-	3/3 (0.145 ng/L)	-	-
Bird Rock	0/3	0/3	3/3 (0.0417 ng/L)	-	-	-
9 ft Bank	1/3	0/3	0/3	0/3	0/3	0/3

3.5 | Environmental DNA dispersion experiment

Of the 14 water samples taken, one was positive for *O. angasi* eDNA. This was one of the two bottom samples at the central point. Six samples exhibited equivocal results as only one out of three qPCR replicates exhibited amplification. These were: two top samples taken 500 m from the centre of the site on the NNW and SSE transects, one bottom sample taken 500 m from the centre of the site on the SSE transect, two bottom samples taken 200 m from the centre of the site on the SSE and ENE transects and one top sample from the centre of the site (Figure 2).

4 | DISCUSSION

Environmental DNA tools are a potentially more efficient solution (Huver et al., 2015; Smart et al., 2016) to collecting distribution data on rare species that would inform restoration efforts. For *O. angasi*, the eDNA assay was able to accurately determine eDNA concentration in seawater samples and quantify accumulation and decay of eDNA in the laboratory. The assay was also successful at detecting *O. angasi* eDNA in seawater samples from the laboratory and the field. The assay detected *O. angasi* eDNA at Carrum, Blairgowrie, Bird Rock and Margaret's Reef in Port Phillip Bay, these were all sites where *O. angasi* has been previously observed at or nearby to the sampling location. *Ostrea angasi* presence was confirmed at Blairgowrie, 9 ft Bank and Bird Rock by the scuba surveys but not at Carrum. The eDNA survey result was equivocal at 9 ft Bank where scuba divers found the highest oyster density. Evidence from the eDNA dispersal experiment suggest that the assay is only able to detect *O. angasi* eDNA close to its source, however, the false negative survey at 9 ft bank was an anomaly that indicates there is more research needed prior to industry adoption. An enhanced sampling methodology, for example through exploring the effect of the number and volume of replicates (Schabacker et al., 2020) or substrate selection (Koziol et al., 2019) and further knowledge of the dynamics of marine aqueous eDNA is needed to improve the reliability of the assay at oyster detection.

The no oyster treatment from the laboratory experiment acted as a negative control in two out of three replicates. However, positive results in one sample from tank four suggest that some contamination occurred during the experiment, potentially through accidental

transfer of water between experimental tanks during sampling as negative controls for qPCR were all negative. Many similar mesocosm experiments measuring the accumulation and decay of eDNA have not included empty mesocosm controls due to the low risk of contamination in a controlled laboratory environment (Sassoubre et al., 2016; Mauvisseau et al., 2017; Sansom & Sassoubre, 2017; Jo et al., 2019). The results here suggest that negative controls for the presence of the target species could help to assess the effect of contamination between mesocosms on the experimental results in future studies. Although the influence of contamination should be considered when interpreting the results of this experiment, we believe the ability to make inferences based on the results has not been wholly compromised as the low and high oyster treatments show a clear difference in pattern and eDNA concentration compared to the control.

The high oyster treatment tanks accumulated significantly more eDNA than the low oyster treatment tanks, over the same time period. Subsequently, significantly more eDNA decayed in the high oyster treatment tanks than in the low oyster treatment tanks. The high oyster treatment exhibited a pattern where eDNA accumulation and decay were not consistent across time steps. The lack of eDNA accumulation between 8 and 24 hours is probably due to eDNA concentration reaching an equilibrium. Similar experiments in other studies have shown that while the rate of eDNA shed by organisms is constant, the rate of decay is dependent on the amount of eDNA present in the system (Barnes et al., 2014; Sassoubre et al., 2016). Thus, the decay rate increases as more eDNA is shed, until the decay and shedding rates are equal, and equilibrium is reached. The amount of eDNA decay will be highest immediately after animals are removed from the system as eDNA concentration is at its maximum and eDNA shedding rate is effectively zero, this explains why, in the laboratory experiment, the majority of eDNA decay occurred between 24 and 48 hours in all tanks.

The maximum eDNA concentrations found in field samples were more comparable to those found in low oyster treatment tanks (all samples less than 0.5 ng/L), than in high oyster treatment tanks. The pattern of eDNA decay in the experimental tanks suggests that, once shed, *O. angasi* eDNA is only detectable for a matter of days, thus making it likely that any field detection of the oyster eDNA reflects their current local distribution. The results of the dispersion experiment are consistent with this conclusion – *O. angasi* eDNA was only detectable in one sample taken directly above the restored oyster reef. The observed small area of eDNA detectability is consistent with research showing that marine fish eDNA was only detectable within 30 m of the target organism (Murakami et al., 2019). Although these findings give us confidence in the spatial resolution of the eDNA assay in the field, they also suggest that water samples need to be taken in close proximity to an *O. angasi* population for positive detection.

Ostrea angasi eDNA detectability reduces quickly as distance from the source population increases. The occurrence of a false negative survey at 9 ft bank also suggests that, in some situations, detectability of *O. angasi* eDNA using our sampling design can be low even in close proximity to oyster populations. Three out of four positive eDNA samples from the field survey returned *O. angasi* DNA concentrations below the assay's LOD, suggesting that false negatives could be caused by eDNA concentrations being too low at sites where oysters are present. A possible cause for this is the effect that water movement can have on eDNA spatial and temporal distributions in marine systems. The distribution of eDNA in aquatic environments is the product of its persistence (determined by shedding and decay rates) and movement. For example, in a river system, the distance and direction eDNA moves downstream from its source is dependent on the speed and direction of flow in the river and the amount of time the eDNA takes to decay (Jane et al., 2015; Shogren et al., 2019). In marine systems, the direction of water flow is often variable, and there is no physical dispersion barrier like a river bank. This unpredictability and potentially greater advection and diffusion, and thus dilution, of eDNA reduces its detectability and spatial and temporal accuracy. The limited previous attempts to date at eDNA detections of rare or cryptic marine species have shown similar detectability issues to this study. For example, Gargan et al. (2017) detected devil rays at two out of three sites where they observed them in visual surveys and harbour porpoise eDNA was detected at one out of three sites where echolocation clicks were recorded (Foote et al., 2012). Both studies suggest that water movement, dilution and degradation are likely causes for the low detectability. These processes generate heterogeneity in the distribution of target eDNA throughout the water column.

Our study attempted to account for this heterogeneity by pooling 10 subsamples and taking samples at the top and bottom of the water column. However, each subsample only had a volume of 150 ml and these samples were pooled so that ultimately only two samples were processed and analysed per site. Studies on freshwater fish have shown that taking larger sample volumes of up to 6 L can reduce the effect of spatial heterogeneity in eDNA distributions and greatly increase the probability of detecting the target species compared to a 1-L sample volume (Schultz & Lance, 2015; Wilcox et al., 2016). Environmental DNA sample volume can be limited by filter clogging when using Sterivex filters (Tingley et al., 2019), however, we experienced little difficulty in filtering 1-L seawater samples by hand, so increasing water volume is a viable option for future *O. angasi* eDNA surveys. Schabacker et al. (2020) used a novel tow-net method to filter 3,000 L of water and improve detection sensitivity. Furthermore, Lugg et al. (2018) showed that increasing the number of separate samples taken can also increase detection probabilities of platypus. Increased sample volume and number taken at each site should increase the chances of the *O. angasi* eDNA assay detecting *O. angasi* when it is present. However, the effects of increased sample volume and sample number on eDNA detection probabilities are likely species and habitat specific, so further research into how eDNA sampling designs can be optimized for *O. angasi* specifically is recommended. Vertical heterogeneity in eDNA concentrations was also addressed by taking samples at the surface and the bottom of the water column. We hypothesised that *O. angasi* eDNA concentrations may be higher in bottom water samples because it is a benthic

species. A study on jellyfish found higher concentrations of eDNA close to the sea floor, potentially due to a high abundance of jellyfish in the bottom half of the water column (Minamoto et al., 2017). However, the current results suggest that whether a sample was taken at the surface or the bottom had no detectable influence on detectability. This was probably because the sites were mostly in shallow water where little vertical stratification occurs.

False negatives and thus apparent low detectability could be caused by the presence of PCR inhibitors in samples. DNA from environmental samples can be especially prone to PCR inhibition due to the higher chance of impurities being present and this dictates the need for IPCs (McKee, Spear & Pierson, 2015). In this study, PCR inhibition is an unlikely explanation for false negative results as all IPCs returned results within the normal range. Another explanation for false negatives could be variation in the *O. angasi* CO1 sequence between populations in Port Phillip Bay, leading to a mismatch between primer sequences and target eDNA sequences (Wilcox et al., 2015). The Corio Bay area of Port Phillip Bay, where the 9 ft Bank and Bird Rock sites were located, is hydrodynamically isolated from the rest of Port Phillip Bay and very little larval transport occurs in and out of this region (Jenkins et al., 1997). A haplotype of the CO1 sequence could potentially occur in this area. However, there is very little variation in the *O. angasi* CO1 sequence throughout southern Australia (Hurwood, Heasman & Mather, 2005). Furthermore, the results of the specificity tests show that amplification of *O. angasi* DNA taken from tissue samples is equally efficient whether oysters were collected from the Corio Bay region or other regions of Port Phillip Bay, suggesting that there are no differences between the CO1 sequences from each area.

Carrum was the only site where *O. angasi* eDNA was detected but no oysters were found during the scuba survey. No extant *O. angasi* were detected at this site in towed video surveys completed by The Nature Conservancy (2018). However, *O. angasi* has been observed on artificial reef structures nearby to the Carrum eDNA sampling site in recent years (B. Cleveland, personal communication, 2019). The detection of eDNA at Carrum strongly suggests that a nearby population of *O. angasi* exists, especially considering the high spatial and temporal accuracy of the assay seen in the laboratory and dispersion experiment, and the proximity of known nearby substrate suitable for *O. angasi*, including bivalve shells, artificial reef structures and natural rocky reefs (Mills, Hamer & Quinn, 2017). The detected eDNA could be from the small number of oysters previously observed on nearby artificial reef structures or another nearby unknown population. However, the chance of false positive results in eDNA studies has been well documented, thus another explanation could be that the detection at Carrum was caused by contamination. Environmental DNA samples were taken from Seaford on the same day that the Carrum samples were taken, while on the previous sampling day, 15 days earlier, samples were taken at Mornington. All samples from both days were negative, suggesting that there was little opportunity for contamination to cause a false positive at Carrum. While the assay did exhibit amplification of DNA from off-target species, this amplification was far less efficient than that of *O. angasi* DNA. The concentration of the positive DNA sample at Carrum was 0.01 ng/L, this result would require a *C. gigas* or *M. galloprovincialis* DNA concentration of 1,270 ng/L sea water (based on the calculated magnitude of underestimation for *C. gigas* and *M. galloprovincialis* DNA of $\sim 10^5$). Based on the presented evidence, it is unlikely that a false positive result has occurred at Carrum.

The eDNA samples analysed by the *O. angasi* assay probably represent a small spatial (less than 200 m radius) and temporal scale ($\sim$2–6 days). Due to the small spatial scale of oyster detection, without development the assay would not be efficient for surveying large seascapes. In freshwater eDNA studies, the habitat of interest within a landscape is often broken up into many small, discreet ponds or streams which can be sampled individually, so that eDNA surveys can cover the whole landscape and reliably detect the target species if it is present within sampling sites, with relatively few samples (Biggs et al., 2015; Secondi et al., 2016; Tingley et al., 2019). For the *O. angasi* eDNA assay in Port Phillip Bay a spatially comprehensive survey is not feasible as the area is large and cannot be broken up into small discrete patches. For example, historical mussel and oyster reefs are estimated to have covered up to 50% of Port Phillip Bay's sea floor (Crawford, 2015; Ford & Hamer, 2016), this represents a 965-km^2 area of potential remnant *O. angasi* habitat. Of this potential remnant habitat, 148 km^2 has been identified as suitable for shellfish restoration through restoration suitability modelling (The Nature Conservancy, 2018).

We provide early evidence for an *O. angasi* eDNA tool that has the potential to be used for efficient seascape-scale sampling to detect undocumented remnant shellfish reefs to inform restoration, when the accuracy is more reliable at distance. Without development, large-scale sampling using the assay is logistically and financially infeasible due to oyster detection only occurring in close proximity to the water sample so a high sampling resolution (e.g. a top and bottom water sample every 200 m) would be required to cover an area, and there were inconsistencies observed (e.g. 9 ft Bank – where the highest oyster densities were recorded). Thus, more research is needed before immediate industry adoption. The accuracy of the *O. angasi* eDNA tool at distance could probably be increased by quantifying the effect of water flow (e.g. through measurements using an Acoustic Doppler Current Profiler, or novel unmanned aerial vehicle techniques; Koutalakis, Tzoraki & Zaimes, 2019) relative to existing reefs and where the sample is taken, and by testing the effect of increasing the number and volume of replicate samples. The benefit to restoration of understanding the current distribution of target species is that sites can be chosen based on proximity to a larval source, and thus increase the likelihood of natural recruitment at the restoration site. An assay that could detect oysters in a larger sample area, relative to the dispersal distance of a species (Rodriguez-Perez, James & Sanderson, 2021), would therefore be more useful for sampling seascapes to aid restoration site selection. The precision of the assay, however, is useful for pinpointing the exact location of existing populations, as a positive result means that oysters are within a small distance of the sample location. This could be useful for oyster restoration if broodstock are required for culturing oysters for seeding

reefs, or if looking for remnant reefs as reference sites for monitoring. This would require much smaller areas of sampling interest, however. Nevertheless, this study has demonstrated the ability of eDNA tools to detect rare and cryptic marine species, that with continued development could be used to provide valuable distribution data to aid future restoration efforts.

ACKNOWLEDGEMENTS

We thank L. Adams, T. Graham, R. Sharma, D. Chamberlain, S. Suebsanguan, B. Cleveland, L. Barrett, S. Elliot-Kerr, E. Barbier and R. Zhu for their help with field work. We also thank S. Song, S. Licul and A. Ghodke for their help in the laboratory and Wayne O'Connor for providing the *O. stentina* sample used in probe validation. We additionally thank Kade Mills, Nicole Mertens and the Victorian National Parks Association's Reef Watch Programme who provided advice on site sampling through the OysterWatch Programme. This work was funded through the Port Phillip Bay Fund (awarded to The Nature Conservancy for the OysterWatch Project, sub-contracted to R.L.M. and S.E.S.) by the Victorian Government's Department of Environment, Land, Water and Planning. The National Centre for Coasts and Climate is funded through the Earth Systems and Climate Change Hub by the Australian Government's National Environmental Science Program. Open access publishing facilitated by The University of Melbourne, as part of the Wiley - The University of Melbourne agreement via the Council of Australian University Librarians. Open access publishing facilitated by The University of Melbourne, as part of the Wiley - The University of Melbourne agreement via the Council of Australian University Librarians.

CONFLICT OF INTEREST

The authors have no competing interests to declare.

AUTHOR CONTRIBUTIONS

Experimental design was conducted by A.C., A.O., R.L.M., A.W., S.B. and S.E.S. Laboratory sampling was performed by A.C. Probe development was performed by A.V., A.C., A.W. and R.L.M. Field sampling was performed by A.C., A.O. and R.L.M. Molecular analysis was performed by A.C. and A.V. Data analysis was performed by A.C., A.O. and R.L.M. The manuscript was written and prepared by all authors.

DATA AVAILABILITY STATEMENT

Data available on request from the authors.

ORCID

Alexander Coutts https://orcid.org/0000-0001-6011-2352
Rebecca L. Morris https://orcid.org/0000-0003-0455-0811

REFERENCES

Alleway, H.K. & Connell, S.D. (2015). Loss of an ecological baseline through the eradication of oyster reefs from coastal ecosystems and human memory. *Conservation Biology*, 29(3), 795–804. https://doi.org/10.1111/cobi.12452

Ardura, A., Zaiko, A., Martinez, J.L., Samulioviene, A., Semenova, A. & Garcia-Vazquez, E. (2015). eDNA and specific primers for early detection of invasive species - a case study on the bivalve *Rangia cuneata*, currently spreading in Europe. *Marine Environmental Research*, 112(Part B), 48–55. https://doi.org/10.1016/j.marenvres.2015.09.013

Barnes, M.A., Turner, C.R., Jerde, C.L., Renshaw, M.A., Chadderton, W.L. & Lodge, D.M. (2014). Environmental conditions influence eDNA persistence in aquatic systems. *Environmental Science & Technology*, 48(3), 1819–1827. https://doi.org/10.1021/es404734p

Bass, D., Stentiford, G.D., Littlewood, D.T.J. & Hartikainen, H. (2015). Diverse applications of environmental DNA methods in parasitology. *Trends in Parasitology*, 31(10), 499–513. https://doi.org/10.1016/j.pt.2015.06.013

Bates, D., Mächler, M., Bolker, B. & Walker, S. (2015). Fitting linear mixed-effects models using lme4. *Journal of Statistical Software*, 67(1), 1–48. https://doi.org/10.18637/jss.v067.i01

Beck, M.W., Brumbaugh, R.D., Airoldi, L., Carranza, A., Coen, L.D., Crawford, C. et al. (2011). Oyster reefs at risk and recommendations for conservation, restoration, and management. *Bioscience*, 61(2), 107–116. https://doi.org/10.1525/bio.2011.61.2.5

Biggs, J., Ewald, N., Valentini, A., Gaboriaud, C., Dejean, T., Griffiths, R.A. et al. (2015). Using eDNA to develop a national citizen science-based monitoring programme for the great crested newt (*Triturus cristatus*). *Biological Conservation*, 183, 19–28. https://doi.org/10.1016/j.biocon.2014.11.029

Brannigan, S., Fitzsimons, J.A. & Gillies, C.L. (2020). Modern middens: shell recycling for restoring an endangered marine ecosystem in Victoria, Australia. *Ecological Restoration and Mangement*, 21(3), 198–204. https://doi.org/10.1111/emr.12425

Chowdhury, M.S.N., Walles, B., Sharifuzzaman, S.M., Hossain, M.S., Ysebaert, T. & Smaal, A.C. (2019). Oyster breakwater reefs promote adjacent mudflat stability and salt marsh growth in a monsoon dominated subtropical coast. *Scientific Reports*, 9(1), 8549. https://doi.org/10.1038/s41598-019-44925-6

Cohen, B.F., Currie, D.R. & McArthur, M.A. (2000). Epibenthic community structure in port Phillip Bay, Victoria, Australia. *Marine and Freshwater Research*, 51(7), 689–702. https://doi.org/10.1071/MF00027

Crawford, M. (2015). Using participatory GIS to map historic oyster reefs in port Phillip Bay. Unpublished Masters Project Report. School of Environment, Science and Engineering, Southern Cross University, Lismore.

Ermgassen, P.S.E.Z., Spalding, M.D., Grizzle, R.E. & Brumbaugh, R.D. (2013). Quantifying the loss of a marine ecosystem service: filtration by the eastern oyster in US estuaries. *Estuaries and Coasts*, 36(1), 36–43. https://doi.org/10.1007/s12237-012-9559-y

Ficetola, G.F., Miaud, C., Pompanon, F. & Taberlet, P. (2008). Species detection using environmental DNA from water samples. *Biology Letters*, 4(4), 423–425. https://doi.org/10.1098/rsbl.2008.0118

Foighil, D.O., Marshall, B.A., Hilbish, T.J. & Pino, M.A. (1999). Trans-Pacific range extension by rafting is inferred for the flat oyster *Ostrea chilensis*. *Biological Bulletin*, 196(2), 122–126. https://doi.org/10.2307/1542557

Foote, A.D., Thomsen, P.F., Sveegaard, S., Wahlberg, M., Kielgast, J., Kyhn, L.A. et al. (2012). Investigating the potential use of environmental DNA (eDNA) for genetic monitoring of marine mammals. *PLoS ONE*, 7(8), e41781. https://doi.org/10.1371/journal.pone.0041781

Ford, J.R. & Hamer, P. (2016). The forgotten shellfish reefs of coastal Victoria: documenting the loss of a marine ecosystem over 200 years since European settlement. *Proceedings of the Royal Society of Victoria*, 128(1), 87. https://doi.org/10.1071/RS16008

Gargan, L.M., Morato, T., Pham, C.K., Finarelli, J.A., Carlsson, J.E.L. & Carlsson, J. (2017). Development of a sensitive detection method to survey pelagic biodiversity using eDNA and quantitative PCR: a case

study of devil ray at seamounts. *Marine Biology*, 164(5), 112. https://doi.org/10.1007/s00227-017-3141-x

Geraldi, N.R., Simpson, M., Fegley, S.R., Holmlund, P. & Peterson, C.H. (2013). Addition of juvenile oysters to enhance oyster reef development in Pamlico Sound. *Marine Ecology Progress Series*, 480, 119–129. https://doi.org/10.3354/meps10188

Gillies, C.L., Crawford, C. & Hancock, B. (2017). Restoring Angasi oyster reefs: what is the endpoint ecosystem we are aiming for and how do we get there? *Ecological Management & Restoration*, 18(3), 214–222. https://doi.org/10.1111/emr.12278

Gillies, C.L., McLeod, I.M., Alleway, H.K., Cook, P., Crawford, C., Creighton, C. et al. (2018). Australian shellfish ecosystems: past distribution, current status and future direction. *PLoS ONE*, 13(2), e0190914. https://doi.org/10.1371/journal.pone.0190914

Godfrey, M.C.S. (1989). Shell midden chronology in southwestern Victoria: reflections of change in prehistoric population and subsistence? *Archaeology in Oceania*, 24(2), 65–69. https://doi.org/10.1002/j.1834-4453.1989.tb00213.x

Hadley, N.H., Hodges, M., Wilber, D.H. & Coen, L.D. (2010). Evaluating intertidal oyster reef development in South Carolina using associated faunal indicators. *Restoration Ecology*, 18(5), 691–701. https://doi.org/10.1111/j.1526-100X.2008.00502.x

Halpern, B.S., Walbridge, S., Selkoe, K.A., Kappel, C.V., Micheli, F., D'Agrosa, C. et al. (2008). A global map of human impact on marine ecosystems. *Science*, 319(5865), 948–952. https://doi.org/10.1126/science.1149345

Hamer, P., Pearce, B. & Winstanley, R. (2013). *Towards reconstruction of the lost shellfish reefs of Port Phillip Bay. Recreational Fishing Grants Program Research Report*. Report number: Project SG/117.

Hernandez, A.B., Brumbaugh, R.D., Frederick, P., Grizzle, R., Luckenbach, M.W., Peterson, C.H. et al. (2018). Restoring the eastern oyster: how much progress has been made in 53 years? *Frontiers in Ecology and the Environment*, 16(8), 463–470. https://doi.org/10.1002/fee.1935

Hunter, M.E., Oyler-McCance, S.J., Dorazio, R.M., Fike, J.A., Smith, B.J., Hunter, C.T. et al. (2015). Environmental DNA (eDNA) sampling improves occurrence and detection estimates of invasive Burmese pythons. *PLoS ONE*, 10(4), e0121655. https://doi.org/10.1371/journal.pone.0121655

Hurwood, D.A., Heasman, M.P. & Mather, P.B. (2005). Gene flow, colonisation and demographic history of the flat oyster *Ostrea angasi*. *Marine and Freshwater Research*, 56(8), 1099–1106. https://doi.org/10.1071/MF04261

Huver, J.R., Koprivnikar, J., Johnson, P.T.J. & Whyard, S. (2015). Development and application of an eDNA method to detect and quantify a pathogenic parasite in aquatic ecosystems. *Ecological Applications*, 25(4), 991–1002. https://doi.org/10.1890/14-1530.1

Jane, S.F., Wilcox, T.M., McKelvey, K.S., Young, M.K., Schwartz, M.K., Lowe, W.H. et al. (2015). Distance, flow and PCR inhibition: eDNA dynamics in two headwater streams. *Molecular Ecology Resources*, 15(1), 216–227. https://doi.org/10.1111/1755-0998.12285

Jenkins, G.P., Black, K.P., Wheatley, M.J. & Hatton, D.N. (1997). Temporal and spatial variability in recruitment of a temperate, seagrass-associated fish is largely determined by physical processes in the pre- and post-settlement phases. *Marine Ecology Progress Series*, 148(1/3), 23–35. https://www.jstor.org/stable/24857469

Jo, T., Murakami, H., Yamamoto, S., Masuda, R. & Minamoto, T. (2019). Effect of water temperature and fish biomass on environmental DNA shedding, degradation, and size distribution. *Ecology and Evolution*, 9(3), 1135–1146. https://doi.org/10.1002/ece3.4802

Klymus, K.E., Merkes, C.M., Allison, M.J., Goldberg, C.S., Helbing, C.C., Hunter, M.E. et al. (2020). Reporting the limits of detection and quantification for environmental DNA assays. *Environmental DNA*, 2(3), 271–282. https://doi.org/10.1002/edn3.29

Koutalakis, P., Tzoraki, O. & Zaimes, G. (2019). UAVs for hydrologic scopes: application of a low-cost UAV to estimate surface water velocity by using three different image-based methods. *Drones*, 3(1), 14. https://doi.org/10.3390/drones3010014

Koziol, A., Stat, M., Simpson, T., Jarman, S., DiBattista, J.D., Harvey, E.S. et al. (2019). Environmental DNA metabarcoding studies are critically affected by substrate selection. *Molecular Ecology Resources*, 19(2), 366–376. https://doi.org/10.1111/1755-0998.12971

Lenth, R. (2020). emmeans: Estimated Marginal Means, aka Least-Squares Means.

Lugg, W.H., Griffiths, J., van Rooyen, A.R., Weeks, A.R. & Tingley, R. (2018). Optimal survey designs for environmental DNA sampling. *Methods in Ecology and Evolution*, 9(4), 1049–1059. https://doi.org/10.1111/2041-210X.12951

Mauvisseau, Q., Parrondo, M., Fernandez, M.P., Garcia, L., Martinez, J.L., Garcia-Vazquez, E. et al. (2017). On the way for detecting and quantifying elusive species in the sea: the *Octopus vulgaris* case study. *Fisheries Research*, 191, 41–48. https://doi.org/10.1016/j.fishres.2017.02.023

McAfee, D., McLeod, I.M., Alleway, H.K., Bishop, M.J., Branigan, S., Connell, S.D. et al. (2022). Turning a lost reef ecosystem into a national restoration program. *Conservation Biology*, 27, e13958. https://doi.org/10.1111/cobi.13958

McKee, A.M., Spear, S.F. & Pierson, T.W. (2015). The effect of dilution and the use of a post-extraction nucleic acid purification column on the accuracy, precision, and inhibition of environmental DNA samples. *Biological Conservation*, 183, 70–76. https://doi.org/10.1016/j.biocon.2014.11.031

Merkes, C.M., Klymus, K.E., Allison, M.J., Goldberg, C., Helbing, C.C., Hunter, M.E. et al. (2019). Generic qPCR limit of detection (LOD) /limit of quantification (LOQ) calculator, R script, U.S. Geological Survey code release.

Mills, K., Hamer, P. & Quinn, G. (2017). Artificial reefs create distinct fish assemblages. *Marine Ecology Progress Series*, 585, 155–173. https://doi.org/10.3354/meps12390

Minamoto, T., Fukuda, M., Katsuhara, K.R., Fujiwara, A., Hidaka, S., Yamamoto, S. et al. (2017). Environmental DNA reflects spatial and temporal jellyfish distribution. *PLoS ONE*, 12(2), e0173073. https://doi.org/10.1371/journal.pone.0173073

Miralles, L., Dopico, E., Devlo-Delva, F. & Garcia-Vazquez, E. (2016). Controlling populations of invasive pygmy mussel (*Xenostrobus securis*) through citizen science and environmental DNA. *Marine Pollution Bulletin*, 110(1), 127–132. https://doi.org/10.1016/j.marpolbul.2016.06.072

Morton, B., Lam, K. & Slack-Smith, S. (2003). First report of the European flat oyster *Ostrea edulis*, identified genetically, from oyster harbour, Albany, South-Western Western Australia. *Molluscan Research*, 23(3), 199–208. https://doi.org/10.1071/mr03005

Murakami, H., Yoon, S., Kasai, A., Minamoto, T., Yamamoto, S., Sakata, M. K. et al. (2019). Dispersion and degradation of environmental DNA from caged fish in a marine environment. *Fisheries Science*, 85(2), 327–337. https://doi.org/10.1007/s12562-018-1282-6

Peterson, C.H., Grabowski, J.H. & Powers, S.P. (2003). Estimated enhancement of fish production resulting from restoring oyster reef habitat: quantitative valuation. *Marine Ecology Progress Series*, 264, 249–264. https://doi.org/10.3354/meps264249

Pogoda, B., Brown, J., Hancock, B., Preston, J., Pouvreau, S., Kamermans, P. et al. (2019). The native oyster restoration Alliance (NORA) and the Berlin oyster recommendation: bringing back a key ecosystem engineer by developing and supporting best practice in Europe. *Aquatic Living Resources*, 32, 13. https://doi.org/10.1051/alr/2019012

Richardson, M.F., Sherman, C.D.H., Lee, R.S., Bott, N.J. & Hirst, A.J. (2016). Multiple dispersal vectors drive range expansion in an invasive marine

species. *Molecular Ecology*, 25(20), 5001–5014. https://doi.org/10.1111/mec.13817

Rodriguez-Perez, A., James, M.A. & Sanderson, W.G. (2021). A small step or a giant leap: accounting for settlement delay and dispersal in restoration planning. *PLoS ONE*, 16(8), e0256369. https://doi.org/10.1371/journal.pone.0256369

Sansom, B.J. & Sassoubre, L.M. (2017). Environmental DNA (eDNA) shedding and decay rates to model freshwater mussel eDNA transport in a river. *Environmental Science & Technology*, 51(24), 14244–14253. https://doi.org/10.1021/acs.est.7b05199

Sassoubre, L.M., Yamahara, K.M., Gardner, L.D., Block, B.A. & Boehm, A.B. (2016). Quantification of environmental DNA (eDNA) shedding and decay rates for three marine fish. *Environmental Science & Technology*, 50(19), 10456–10464. https://doi.org/10.1021/acs.est.6b03114

Schabacker, J.C., Amish, S.J., Ellis, B.K., Gardner, B., Miller, D.L., Rutledge, E.A. et al. (2020). Increased eDNA detection sensitivity using a novel high-volume water sampling method. *Environmental DNA*, 2(2), 244–251. https://doi.org/10.1002/edn3.63

Schulte, D.M., Burke, R.P. & Lipcius, R.N. (2009). Unprecedented restoration of a native oyster Metapopulation. *Science*, 325(5944), 1124–1128. https://doi.org/10.1126/science.1176516

Schultz, M.T. & Lance, R.F. (2015). Modeling the sensitivity of field surveys for detection of environmental DNA (eDNA). *PLoS ONE*, 10(10), e0141503. https://doi.org/10.1371/journal.pone.0141503

Secondi, J., Dejean, T., Valentini, A., Audebaud, B. & Miaud, C. (2016). Detection of a global aquatic invasive amphibian, *Xenopus laevis*, using environmental DNA. *Amphibia-Reptilia*, 37(1), 131–136. https://doi.org/10.1163/15685381-00003036

Shogren, A.J., Tank, J.L., Egan, S.P., Bolster, D. & Riis, T. (2019). Riverine distribution of mussel environmental DNA reflects a balance among density, transport, and removal processes. *Freshwater Biology*, 64(8), 1467–1479. https://doi.org/10.1111/fwb.13319

Smart, A.S., Tingley, R., Weeks, A.R., van Rooyen, A.R. & McCarthy, M.A. (2015). Environmental DNA sampling is more sensitive than a traditional survey technique for detecting an aquatic invader. *Ecological Applications*, 25(7), 1944–1952. https://doi.org/10.1890/14-1751.1

Smart, A.S., Weeks, A.R., van Rooyen, A.R., Moore, A., McCarthy, M.A. & Tingley, R. (2016). Assessing the cost-efficiency of environmental DNA sampling. *Methods in Ecology and Evolution*, 7(11), 1291–1298. https://doi.org/10.1111/2041-210X.12598

The Nature Conservancy. (2018). *Restoring the lost shellfish reefs of port Phillip Bay, final evaluation Report, stage 2 extension November 2017 to December 2018. Prepared for the Department of Environment, Land, Water and Planning*.

The Nature Conservancy. (2022). Hong Kong oyster restoration. Online at: https://www.tnc.org.hk/en-hk/what-we-do/hong-kong-projects/oyster-restoration/ [Accessed 28th January 2022]

Theuerkauf, S.J. & Lipcius, R.N. (2016). Quantitative validation of a habitat suitability index for oyster restoration. *Frontiers in Marine Science*, 3, 64. https://doi.org/10.3389/fmars.2016.00064

Thomsen, P.F. & Willerslev, E. (2015). Environmental DNA - an emerging tool in conservation for monitoring past and present biodiversity. *Biological Conservation*, 183, 4–18. https://doi.org/10.1016/j.biocon.2014.11.019

Tingley, R., Greenlees, M., Oertel, S., van Rooyen, A.R. & Weeks, A.R. (2019). Environmental DNA sampling as a surveillance tool for cane toad *Rhinella marina* introductions on offshore islands. *Biological Invasions*, 21(1), 1–6. https://doi.org/10.1007/s10530-018-1810-4

Victorian National Parks Association. (2020). *Final report: oyster watch 2018–2020*. Victorian National Parks Association.

Weltz, K., Lyle, J.M., Ovenden, J., Morgan, J.A.T., Moreno, D.A. & Semmens, J.M. (2017). Application of environmental DNA to detect an endangered marine skate species in the wild. *PLoS ONE*, 12(6), e0178124. https://doi.org/10.1371/journal.pone.0178124

West, K., Travers, M.J., Stat, M., Harvey, E.S., Richards, Z.T., DiBattista, J.D. et al. (2021). Large-scale eDNA metabarcoding survey reveals marine biogeographic break and transitions over tropical North-Western Australia. *Diversity and Distributions*, 27(10), 1942–1957. https://doi.org/10.1111/ddi.13228

Wilcox, T.M., Carim, K.J., McKelvey, K.S., Young, M.K. & Schwartz, M.K. (2015). The dual challenges of generality and specificity when developing environmental DNA markers for species and subspecies of *Oncorhynchus*. *PLoS ONE*, 10(11), e0142008. https://doi.org/10.1371/journal.pone.0142008

Wilcox, T.M., McKelvey, K.S., Young, M.K., Jane, S.F., Lowe, W.H., Whiteley, A.R. et al. (2013). Robust detection of rare species using environmental DNA: the importance of primer specificity. *PLoS ONE*, 8(3), e0059520. https://doi.org/10.1371/journal.pone.0059520

Wilcox, T.M., McKelvey, K.S., Young, M.K., Sepulveda, A.J., Shepard, B.B., Jane, S.F. et al. (2016). Understanding environmental DNA detection probabilities: a case study using a stream-dwelling char *Salvelinus fontinalis*. *Biological Conservation*, 194, 209–216. https://doi.org/10.1016/j.biocon.2015.12.023

Xu, N., Zhu, B., Shi, F., Shao, K., Que, Y., Li, W. et al. (2018). Monitoring seasonal distribution of an endangered anadromous sturgeon in a large river using environmental DNA. *The Science of Nature*, 105(11), 62. https://doi.org/10.1007/s00114-018-1587-4

SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

How to cite this article: Coutts, A., O'Brien, A., Weeks, A.R., Swearer, S.E., van Rooyen, A., Branigan, S. et al. (2022). An environmental DNA approach to informing restoration of the functionally extinct oyster, *Ostrea angasi*. *Aquatic Conservation: Marine and Freshwater Ecosystems*, 1–13. https://doi.org/10.1002/aqc.3886