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Application of environmental DNA to survey Bathurst Harbour (Tasmania) for the Endangered Maugean Skate (*Zearaja maugeana*)

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Cover images: Left: Image of Bathurst Harbour by David Moreno; Right: Image of Maugean skate (*Zearaja maugeana*) in Macquarie Harbour by Neville Barrett

This report is available on the NESP Marine and Coastal Hub website: www.nespmarinecoastal.edu.au

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Executive summary

The Maugean Skate *Zearaja maugeana* is a micro-endemic species known from only two isolated estuaries, Bathurst and Macquarie Harbours in southwestern/western Tasmania. This constitutes one of the most limited distributions of any known extant elasmobranch. As a result, the species is listed as 'Endangered' under the *Threatened Species Protection Act* (Tasmania) and the *Environmental Protection and Biodiversity Conservation Act* (Commonwealth).

Even though it was first discovered in Bathurst Harbour, most of what is known about the species comes from the Macquarie Harbour population. In fact, only four individuals have ever been reported in Bathurst Harbour, with the last known sighting occurring in 1992. Environmental conditions in Macquarie Harbour have changed markedly in recent times, due to the influence of various anthropogenic activities in and around the estuary (e.g., mining, hydro-electric generation and alteration of natural river flows, and marine fish farming). Recent research has shown clear signs of population stress and evidence of detrimental impacts of degraded environmental conditions on the Maugean skate in Macquarie Harbour.

Therefore, there is a critical need to elucidate the status of the Maugean skate in Bathurst Harbour to inform the development of updated and effective conservation plans and specific recovery actions for this unique micro-endemic skate.

This study aimed to use eDNA to determine the presence of the Maugean skate in Bathurst Harbour on the southwest coast of Tasmania.

Sampling in Bathurst Harbour occurred across two surveys in November 2021 and February 2022 and positive eDNA controls were collected in Macquarie Harbour in December 2021. Water within 1 m from the seafloor was collected at various sites in Bathurst Harbour and filtered using a self-preserving eDNA sampling system. Following each survey, DNA from the samples was extracted and analysed through qPCR amplification. Mitochondrial primer pairs from two gene regions were used to detect the presence of Maugean skate DNA in the samples. Where possible, positive detections were sequenced, and their identity verified.

Maugean skate DNA was successfully extracted and amplified. Detection from environmental samples in Macquarie Harbour confirms that eDNA assays and field sampling protocols as employed here can be used as a tool to monitor the presence of rare and cryptic elasmobranchs in remote or challenging environments.

Sufficient concentrations of Maugean skate DNA were not found anywhere in Bathurst Harbour to allow positive identification based on eDNA alone. There were multiple putative detections in the second survey, but only four of these yielded sufficient material to allow sequencing. Sequencing confirmed that these four samples were extremely low quantity Maugean skate DNA detections. The total number of DNA copies per qPCR reaction in the Bathurst Harbour samples (4-6) was two orders of magnitude lower than concentrations in environmental samples from Table Head in Macquarie Harbour (approximately 2000 copies of DNA per reaction).

There are various possible explanations for the extremely low traces of Maugean skate DNA seen in Bathurst Harbour: 1) Only very few individuals exist in Bathurst Harbour. 2) Latent DNA left over in the sediments from biological material, such as egg cases, was released due to the unusual environmental conditions in Bathurst Harbour during the summer of 2022.

3) Maugean skate are rare and transient within the system, although this scenario does not align with their known adaptability, resilience, and preference for estuarine environments.

Regardless of which of the scenarios presented above is correct, it is now clear that the vast majority, if not all, of the remaining Maugean skate live in Macquarie Harbour. Therefore, the findings of this study highlight the vulnerability of the species and the need for urgent conservation action and continued research focused on the Macquarie Harbour population to ensure the persistence of this unique species.

Keywords:

eDNA, Maugean skate, *Zearaja maugeana*, Bathurst Harbour, Macquarie Harbour, threatened species.

1. Introduction

Determining the presence of endangered marine species is important for the implementation of effective management strategies to minimise impacts on their populations and conserve the species. Confirming presence relies on locating the animals, which can prove challenging for species with low population numbers. A variety of methods have been used to determine the presence of rare marine species, including fishing and underwater visual surveys. Genetics has proven to be a viable alternate technique for detecting the presence of rare or cryptic species in the wild, by seeking DNA evidence in environmental samples of sediments, ice, or water (e.g., Jerde, 2021; Pederson et al., 2015; Sepulveda et al., 2019; Thomsen et al., 2012). Environmental DNA (eDNA) has been used for over a decade to investigate the presence of a variety of organisms, including microbes (e.g., Patil et al., 2005), plants and animals (e.g., Foote et al., 2012; Thomsen et al., 2012), delivering unique information on past and present biodiversity (Pederson et al., 2015). Vertebrate eDNA is DNA that is deposited in the environment through a variety of bodily processes, including the shedding of skin, hair, or feathers, or through defecation, urination, or excretion of saliva.

In the aquatic environment, the presence of a rare species can be assessed by taking a water sample and testing whether the DNA fingerprint of the target species is present. Using eDNA to determine presence of rare or cryptic species can be more efficient than detecting the animal itself, and eliminates the risks associated with capture techniques that may be harmful to the individuals. Additionally, developing a species-specific eDNA assay requires only a single DNA sample of the target species from which genetic primers (short nucleic acid sequences that provide a starting point for DNA synthesis) are designed. This species-specific approach uses real-time, or quantitative, polymerase chain reaction (PCR) tests (qPCR) to target individual eDNA sequences of the focal species and is confirmed through Sanger nucleotide (building blocks of DNA) sequencing (Patil et al., 2005). eDNA techniques have been applied in the marine environment to detect marine mammals, teleosts, and elasmobranchs in the wild (e.g., Foote et al., 2012; Thomsen et al., 2012; Simpfendorder et al., 2016; Sisgaard et al., 2015; Weltz et al., 2017).

This study used eDNA to determine the presence of the endangered Maugean skate *Zearaja maugeana* in Bathurst Harbour and Channel (hereinafter referred to as Bathurst Harbour) on the southwest coast of Tasmania. *Zearaja maugeana* has been classified as endangered under the *Australian Environment Protection and Biodiversity Conservation* (EPBC) *Act* (1999) and the *Tasmanian Threatened Species Protection Act* (1995) based on its small population size and restricted distribution, as it has only been reported from two remote estuarine systems in Tasmania, Bathurst and Macquarie Harbours on the southwest/west coast (Edgar et al., 2010). Initially discovered in Bathurst Harbour in 1988, *Z. maugeana* has not been recorded in that locality since 1992. Notably, only four individuals were ever sighted in Bathurst Harbour, despite extensive fishing and underwater visual surveys conducted over a number of years, with the most recent survey in 2016 (Last & Gledhill, 2007; Bell et al., 2016; Treolar et al., 2016). The lack of confirmed sightings has raised considerable uncertainty as to the status of the Bathurst Harbour population, implying either an exceptionally small population size or even localised extinction.

Bathurst Harbour has been a marine protected area since 2005 and, being in a wilderness area, is subject to minimal anthropogenic disturbance. Unlike Bathurst Harbour, Macquarie Harbour has a number of anthropogenic influences, including historic mining, salmon farming and river flow into the harbour being influenced by hydroelectric power generation. As a result, the environmental conditions have undergone significant changes in recent decades,

particularly in respect to dissolved oxygen levels, which have declined (Ross & MacLeod, 2016).

In contrast to Bathurst Harbour, *Z. maugeana* has traditionally been more readily encountered in Macquarie Harbour. However, it is not considered abundant in this location, with an estimated population of only 3000 individuals in 2016 (Bell et al., 2016). Additionally, recent research has highlighted the vulnerability of early life stages to the changing environmental conditions, long-term changes in the size structure of the population, and the mortality of individuals following significant environmental events (Moreno et al., 2020). Collectively these issues emphasise the vulnerability of the Maugean skate in Macquarie Harbour and the need to consider further conservation action to support the persistence of this unique micro-endemic skate in Tasmania. Given the vulnerability of *Z. maugeana* in Macquarie Harbour, it is important to determine if the species still exists in Bathurst Harbour, as this will inform the current conservation status and influence appropriate conservation actions for the species.

2. Methods

2.1 Sample collection

Sampling locations for eDNA in Bathurst Harbour were primarily selected based on the reported location of the four Maugean skate individuals captured, and other sites targeted in previous biodiversity surveys. Additional sample sites were selected based on the presence of 'suitable Maugean skate habitat' established from long-term observations of the species in Macquarie Harbour. Within Bathurst Harbour, there was a total of 20 sample sites in the Harbour, five sites in the Channel and one site in Melaleuca Lagoon (see Fig. 1 and Table 2). All sites were sampled in both November 2021 and February 2022, with each survey lasting approximately three days. Bathurst Harbour is a remote site that is inaccessible by land. Sampling trips required access by air to the nearby Melaleuca airstrip and basecamp, where a small outboard vessel was used for sampling and transport to the harbour through the Melaleuca Creek.

Both Macquarie and Bathurst Harbours are catchments for large rivers with a segregated water profile that limits mixing between layers. Given that the Maugean skate is a predominantly benthic organism, DNA traces are far likelier to be found near bottom waters. Water samples were collected from 1 m above the seafloor using a camera bottle. Each sample was thoroughly shaken to ensure homogeneous mixing of eDNA collected. Filtration occurred immediately after collection to ensure no degradation of eDNA. Self-preserving Smith & Root filters, which consist of a filter membrane, with either 0.45 or 5 µm pore sizes, encased in a plastic casing that preserves the samples through desiccation. After filtration, the filter requires no transfer, thus reducing handling (i.e., cross-contamination) and requires no cold or chemical storage, which can be logistically complex when working in remote areas. The filter cannisters were connected to a Smith & Root eDNA sampler, a negative pressure filtration system that measures filtration volume, flow rate and GPS location of sampling. Water was sampled at each site until a target volume was reached (20 L for 5 µm filters and 3 L for 0.45 µm) or the filter was clogged (< 0.2 L/min flow rate), using a maximum pressure of 10 PSI. If the filter was clogged, the filtered volume was recorded. Two replicate samples were collected at each site.

In the November 2021 survey, 5 µm filters were used across all 26 sites and 0.45 µm filters at a subset of five sites (see Table 2). To increase detection sensitivity (see below), only 0.45 µm filters were used for the second survey in February 2022 across all sites. Control samples were collected in Macquarie Harbour in December 2021. Two Maugean skate were captured and kept in a 750 L tank for approximately 10 minutes during a population survey (IMAS Sustainable Marine Research Collaboration Agreement Project: 115815). Water from this tank was filtered to function as a positive control. Environmental control samples were collected at two Macquarie Harbour sites replicating the methodology used in Bathurst Harbour (see Fig. 2). The first site, Table Head, represents an area of high occupancy for the species (Moreno et al. 2020), and the second site, Macquarie Heads, is an area not used by the species. To compare the sensitivity of filter size, both sites and the positive control were sampled using both 0.45 and 5 µm filters.

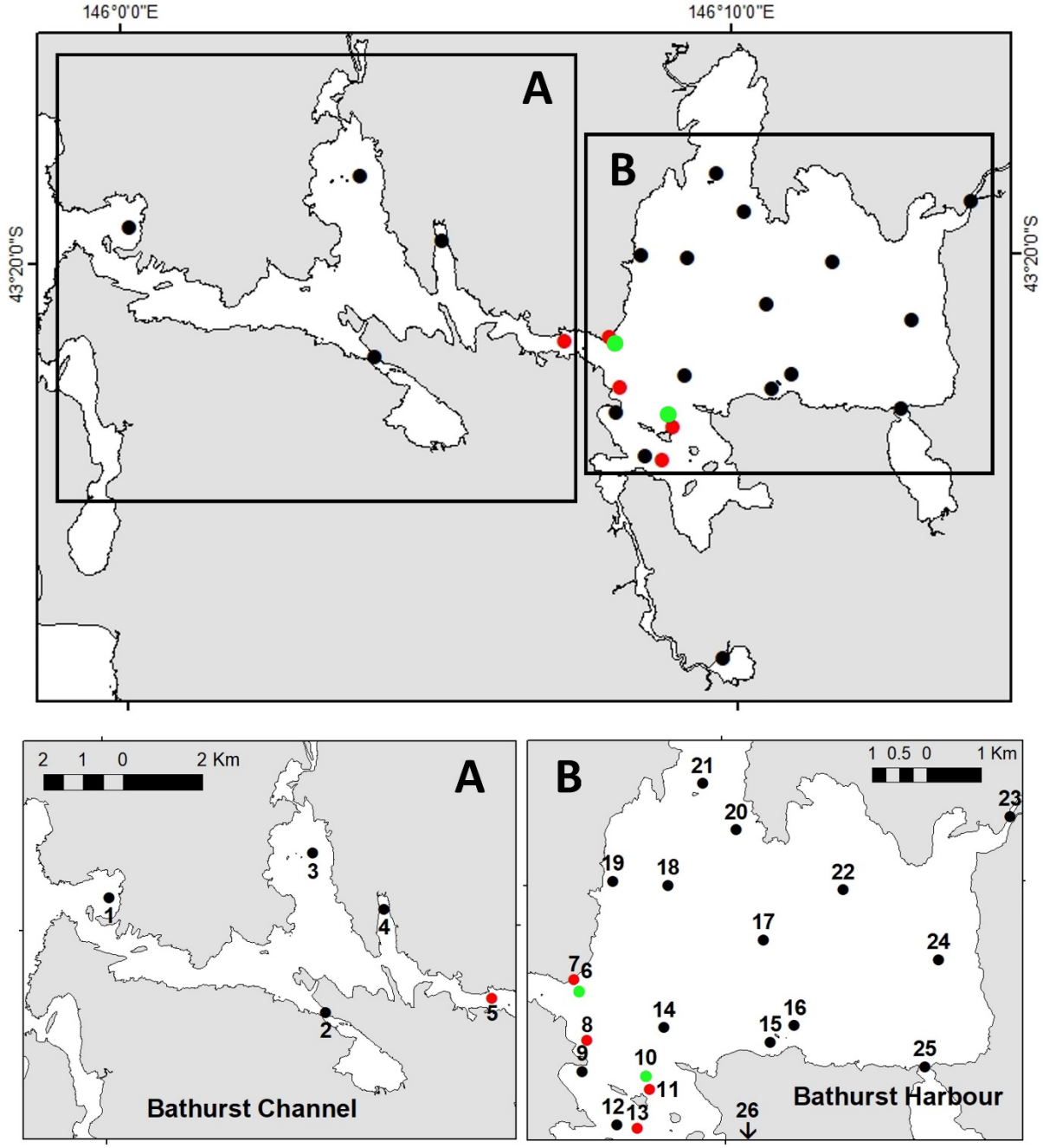

Figure 1. Bathurst Channel, Bathurst Harbour, and Melaleuca Lagoon Tasmania. Insets A and B in the top panel are expanded in the bottom left and right panels, respectively. Melaleuca Lagoon is the most southerly site in the top panel and is indicated by site 26 in the lower right panel, with an arrow indicating that the site is outside of the map boundary. Black circles denote water sampling sites, red circles denote both water and ROV sampling sites and green circles denote sites where Maugean skate were captured between 1989 and 1992, as described in Last & Gledhill, 2007. See Table 2 for DNA presence/absence details for each site.

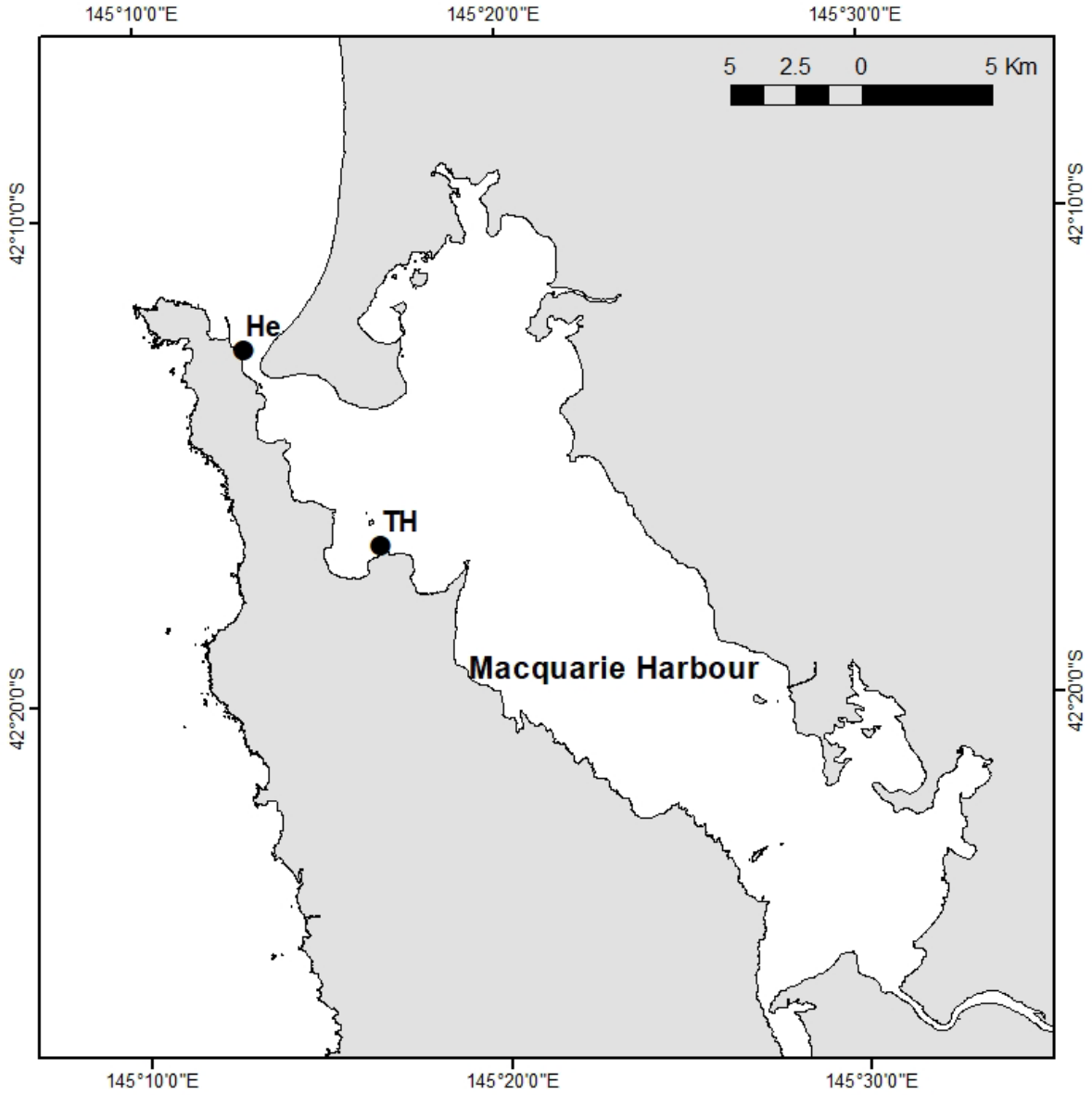

Figure 2. Macquarie Harbour, Tasmania. Black circles denote environmental control water sampling sites, Macquarie Heads (He) at the northern entrance to the harbour and Table head (TH). See Table 2 for DNA presence/absence details for each site.

2.2 Video surveys

A blue robotics remotely operated vehicle (ROV) was used to conduct a visual assessment of a subset of five sites during both Bathurst Harbour surveys to help independently verify eDNA results. The visual surveys mainly targeted the sites where Maugean skate were recorded in the past (see Fig. 1 and Table 2). While approximately the same five sites were investigated in both surveys, dive duration and dive profile where highly dependent on weather and current conditions at the time of the visual assessment. Videos were captured with the onboard navigation camera and an external GoPro Hero8 camera. Recordings from both surveys (approximately 380 minutes) were later analysed for potential sightings of Maugean skate, indirect signs of their presence (e.g., egg cases or feeding indentations) and known prey species (based on Macquarie Harbour dietary analysis, Weltz et al., 2019).

2.3 Environmental data

Like Macquarie Harbour, Bathurst Harbour has a unique environmental profile. Past work in Macquarie Harbour has shown a complex link between environmental conditions and the behaviour and habitat use of the Maugean skate (Moreno et al., 2020). Therefore, environmental data (salinity, temperature, dissolved oxygen, and depth) were collected at each water sampling and ROV site during the February 2022 survey.

2.4 eDNA analysis

Sample preparation, DNA extraction and qPCR amplification were conducted as per Weltz et al., (2017). Two mitochondrial primer pairs (herein referred to as probes) from the Maugean skate gene regions (loci) nicotine adenine dinucleotide dehydrogenase subunit 4 (NADH4; Weltz et al., 2017) and cytochrome oxidase subunit I (CO1; this study) were used in species-specific qPCR assays for detecting *Z. maugeana* DNA (see Table 1 for probe details). The detection and quantification limits for both probes were experimentally determined by establishing standard curves, both with and without background DNA, with 30 replicates per concentration.

Table 1. The two target mitochondrial loci and their primers used to detect and quantify Maugean skate DNA.

Loci	**Primer names and sequences**	**Reference**
NADH4	ZmForward (qF1): 5'-CTTCCTAATTCTAGCTATTTGAGGC-3' ZmReverse (qR1): 5'-AGGGGGCAAGGCGAGGTTAGCC-3'	Weltz et al., 2017
CO1	ZmCO1F1: 5'-CAATTATAATCGGCGGGTTTGAT-3' ZmCO1R1: 5'-GTGGAGAGAGAAAATTGTTAAGTCTATG-3'	This study

Three qPCR technical replicates were run for each sample. Each assay included positive (Maugean skate sample from Macquarie Harbour) and negative controls. Positive controls were included to avoid false negatives (i.e., Type II error) and were added last to the reactions to avoid false positives (i.e., Type I error). Negative controls included reagent controls and DNA samples from chondrichthyans (sharks, rays, skates, and chimaeras) common in Bathurst Harbour, elephantfish *Callorhinchus milii* and whitespotted dogfish *Squalus acanthias*, as well as Melbourne skate *Spiniraja whitleyi* and southern eagle ray *Myliobatis australis,* which occur in southwest Tasmania.

The number of copies of DNA per qPCR reaction averaged across the positive technical replicates was estimated for all samples from Macquarie and Bathurst Harbours with positive Maugean skate DNA detections. All positive reactions were also visualised on 1.2% agarose gel (gel electrophoresis) and the amplified products were purified using a commercially available gel purification kit (Qiagen). If the amplified product was visible in gel electrophoresis and a sufficient quantity (≥ 0.5 pg/uL) could be recovered, it was sent to an external facility for sanger sequencing. All sequences were aligned and compared with the Maugean skate target region to verify the detection.

3. Results

All positive qPCR reactions, including the positive controls, showed target amplification below 35 Ct (cycle threshold), and negative controls exhibited no amplification (Fig. 3). The efficiency of each qPCR assay was high (99– 102%), with R^2 values between 0.986 and 0.99.

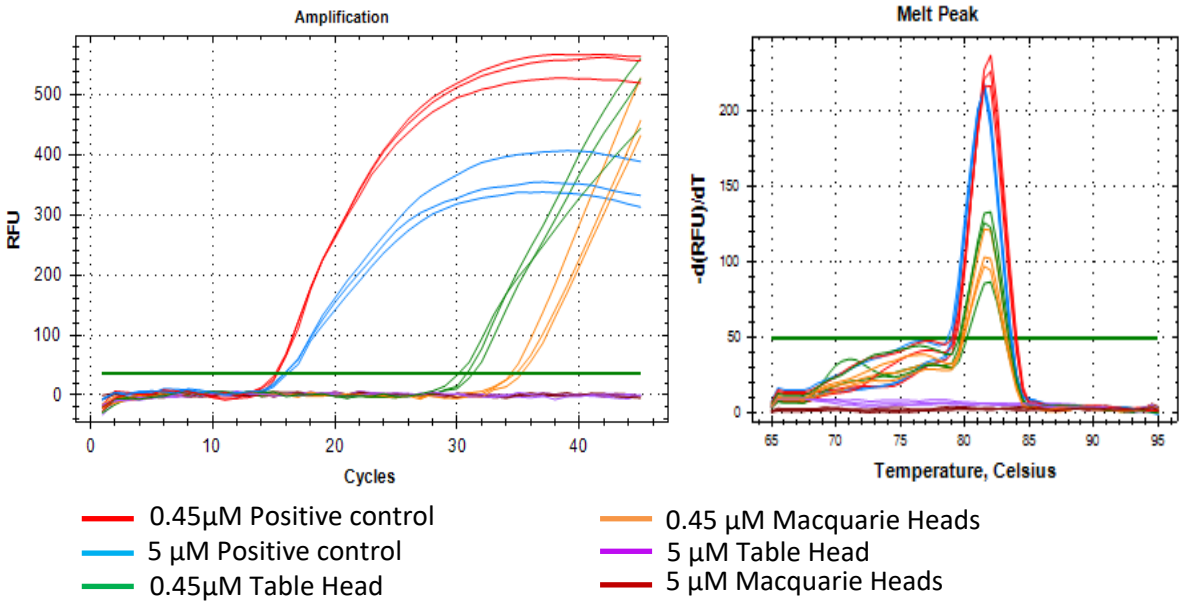

Figure 3. Real-Time qPCR amplification for Macquarie Harbour samples. The plot on the left shows the fluorescence versus qPCR cycle for the NADH4 probe. The plot on the right shows changes in fluorescence ratio at increasing melting temperatures (C°). The horizontal green line in the plots represents the minimum threshold. The coloured plot lines denote the positive and environmental control eDNA samples for the two filter sizes (see figure legend for details).

3.1 Controls

Negative controls using other chondrichthyans present in Bathurst Harbour or southwest Tasmania (see methods) showed no amplification for any of the species assayed (Fig. 3). The NADH4 and CO1 probes detected Maugean skate DNA in the positive control and the environmental controls (Table Head and Macquarie Heads, Macquarie Harbour) and the DNA could be sequenced (see Table 2).

Maugean skate DNA was detected from both 5 µm and 0.45 µm filters for the positive control, with mean yields of approximately 2,000,000 (NADH4 probe only) and 6,000,000 copies of DNA/reaction, respectively (see Table 2). Overall, despite the reduced filtered volume, 0.45 µm filters had higher target DNA yield and improved efficiency than the 5 µm filters for the positive control sample.

Positive detection occurred only in the 0.45 µm sample for the Table Head and Macquarie Heads environmental control sites (see Table 2). Maugean skate are known to be most abundant within Macquarie Harbour at Table Head, as such, although significantly lower than the positive control, mean yields from the positive detection for the NADH4 and CO1 probes were approximately 2,000 and 2,400 copies of DNA/reaction, respectively (see Table 2). Maugean skate have never been recorded at Macquarie Heads, however, it has a high current flow, so any DNA present is likely to be the result of flushing from within the Harbour,

as it was present only in relatively small amounts, based on the high Ct value and low DNA copy numbers (see Table 2). Despite the relatively low amount of DNA detected at Macquarie Heads, the DNA was successfully sequenced (see Table 2).

3.2 Bathurst Harbour eDNA

A total of approximately 500 L across all 26 sites were filtered for the November 2021 survey. There were no positive detections using the NADH4 probe, regardless of filter size (only five sites had 0.45 µm filters; see Table 2).

A total of approximately 75 L across all 26 sites were filtered for the February 2022 survey, with the use of 0.45 µm filters only explaining the reduced volume of water filtered compared to the first survey (see Section 2.1). Seven sites showed putative Maugean skate DNA detections using the NADH4 probe, however, these were all at extremely small concentrations (≤ 2 copies of DNA/reaction) and present in only one of three technical qPCR replicates (see Table 2). All samples fell well below the predefined cycle threshold for a positive detection (i.e., concentration where >95% runs give positive results; Ros-Garcia et al., 2012), which all Macquarie Harbour samples met. None of these seven samples had enough material for gel electrophoresis or sequencing (see Table 2), whereas again, all Macquarie Harbour samples did. Accordingly, all seven of these Bathurst Harbour samples were considered to be negative.

Given the status of the species, it was decided to further analyse these weak signals. A new CO1 probe was developed (see methods) to discount the possibility of contamination of qPCR products. To account for potential sources of contamination from lab equipment or during the extraction process, the second (still sealed) replicate 0.45 µm filter sample for each sampling site for both surveys was analysed in a different laboratory at a separate location.

As for the NADH4 probe, there were no positive detections from the analysis of the second filter from the first survey using the CO1 probe. For the second filter from the second survey, there were once again no sites that met the predefined detection threshold or showed positive detection across all three qPCR technical replicates. However, 11 sites showed weak putative detections in at least one technical replicate using the CO1 probe (Table 2). Interestingly, only three of the seven sites with putative detections using the NADH4 probe for the first filter sample were also part of the 11 sites found using the CO1 probe on the second filter sample (Table 2).

As for the NADH4 probe, all sites examined with the CO1 probe had extremely low concentrations of DNA (mean of 4-6 copies/reaction) and fell well below the predefined threshold for a positive detection, however, four samples had enough material for sequencing. For these samples, qPCR products of the assay were sequenced and aligned with the known target Maugean skate DNA sequence. There was a 100% match between the qPCR product and the reference Maugean skate target, i.e., Maugean skate DNA in extremely low levels was confirmed to be detected at these four sites (see Table 2).

Table 2. Putative Maugean skate DNA detections for both probes and each qPCR technical replicate across both sampling periods. Trep1-3 = technical qPCR replicates 1-3. * indicates sites that also had a 0.45um water sample collected during the first survey. ✓ and ✖ Indicate positive and negative DNA detections, respectively. ✓ and ✖ indicate positive detections that could or could not be sequence confirmed, respectively. – indicates a procedure that was not undertaken for a particular sample. ** indicates that sequencing was not undertaken.

Sample ID	ROV	Probe targeting the NADH4 Locus									Probe targeting the CO1 Locus							
		1st filter									2nd filter							
		1st sampling (5µM)				2nd sampling (0.45µM)					1st sampling (5µM)			2nd sampling (0.45µM)				
		Trep1	Trep2	Trep3	Copies/reaction	Trep1	Trep2	Trep3	Copies/reaction	Sequenced	Trep1	Trep2	Trep3	Trep1	Trep2	Trep3	Copies/reaction	Sequenced
Bathurst Harbour																		
Channel																		
1*	No	✖	✖	✖		✖	✖	✖			✖	✖	✖	✓	✖	✓	4	✖
2	No	✖	✖	✖		✖	✖	✖			✖	✖	✖	✖	✖	✖		
3	No	✖	✖	✖		✖	✖	✖			✖	✖	✖	✖	✖	✖		
4	No	✖	✖	✖		✖	✖	✖			✖	✖	✖	✖	✖	✖		
5*	Yes	✖	✖	✖		✖	✖	✖			✖	✖	✖	✖	✓	✓	6	✓
Harbour																		
6	No	✖	✖	✖		✖	✖	✖			✖	✖	✖	✓	✓	✖	5	✓
7	Yes	✖	✖	✖		✖	✖	✖			✖	✖	✖	✖	✖	✖		
8	Yes	✖	✖	✖		✖	✖	✖			✖	✖	✖	✓	✖	✓	6	✓
9	No	✖	✖	✖		✖	✓	✖	≤ 2	✖	✖	✖	✖	✖	✓	✓	5	✖
10	No	✖	✖	✖		✖	✖	✖			✖	✖	✖	✖	✖	✖		
11*	Yes	✖	✖	✖		✓	✖	✖	≤ 2	✖	✖	✖	✖	✖	✖	✖		
12	No	✖	✖	✖		✓	✖	✖	≤ 2	✖	✖	✖	✖	✖	✖	✖		
13	Yes	✖	✖	✖		✓	✖	✖	≤ 2	✖	✖	✖	✖	✖	✖	✖		
14	No	✖	✖	✖		✖	✖	✖			✖	✖	✖	✖	✖	✖		
15	No	✖	✖	✖		✖	✖	✖			✖	✖	✖	✖	✖	✖		
16	No	✖	✖	✖		✖	✖	✖			✖	✖	✖	✖	✖	✖		
17*	No	✖	✖	✖		✖	✖	✖			✖	✖	✖	✓	✓	✖	5	✖
18	No	✖	✖	✖		✖	✖	✖			✖	✖	✖	✓	✖	✓	6	✓
19	No	✖	✖	✖		✓	✖	✖	≤ 2	✖	✖	✖	✖	✖	✖	✖		
20	No	✖	✖	✖		✖	✖	✓	≤ 2	✖	✖	✖	✖	✖	✓	✓	4	✖
21	No	✖	✖	✖		✖	✖	✓	≤ 2	✖	✖	✖	✖	✖	✖	✖		
22	No	✖	✖	✖		✖	✖	✖			✖	✖	✖	✓	✖	✓	5	✖
23*	No	✖	✖	✖		✖	✖	✖			✖	✖	✖	✓	✖	✓	5	✖
24	No	✖	✖	✖		✖	✖	✖			✖	✖	✖	✖	✖	✖		
25	No	✖	✖	✖		✖	✖	✖			✖	✖	✖	✖	✓	✖	5	✖
Melaleuca Lagoon																		
26*	No	✖	✖	✖		✖	✖	✖			✖	✖	✖	✖	✖	✖		
Macquarie Harbour																		
Table Head	NA	✖	✖	✖		✓	✓	✓	~ 2000	✓	-	-	-	✓	✓	✓	~ 2400	-
Macquarie Heads	NA	✖	✖	✖		✓	✓	✓	~ 60	✓	-	-	-	✓	✓	✓	~ 60	-
Positive Control	NA	✓	✓	✓	~ 2000000**	✓	✓	✓	~ 6000000	✓	-	-	-	✓	✓	✓	~ 6000000	-

3.3 *Video surveys*

A total of 80 min of video across the five sites were recorded in the first survey and approximately 300 mins in the second survey. Dives ranged from 3 m to a maximum of 35 m in depth. Substrate type and biodiversity at the different sites were consistent with previous records (Barrett et al., 2010; Edgar, 1991). Bathurst Channel was in general deeper than Bathurst Harbour, and characterised by hard substrate, high water flow and high diversity of invertebrate fauna (e.g., see Fig. 4 top right panel). By contrast, sites inside Bathurst Harbour were relatively shallow (mean depth of 7 m) with a silty, soft bottom and evidence of some borrowing infauna (e.g., see Fig. 4 top left panel). No Maugean skate were recorded and there were no discernible indirect signs of their presence (e.g., egg cases, feeding indentations). A female thornback skate (*D. lemprieri*) was recorded at sample site 11 (see Fig. 1), at a depth of 5 m (see Fig. 4).

Figure 4. Image from ROV survey sites in Bathurst Channel and Bathurst Harbour, Tasmania. The top left and right images show the substrate types at sampling sites 7 (Bathurst Harbour; 10 m) and 5 (Bathurst Channel; 32 m), respectively. The bottom image shows a female thornback skate (*D. lemprieri*) at sampling site 11 (Bathurst Harbour; 5 m). See Figure 1 for site locations.

3.4 Environmental data

February 2022 was an extremely dry period, with limited freshwater input into the estuary, as such water conditions were largely marine throughout. Bathurst channel had marine salinities (mean 33.5 ‰) and high dissolved oxygen levels regardless of depth (90-100 %). Water inside Bathurst Harbour was also marine (mean 32.5 ‰). At shallow depths, dissolved oxygen levels inside Bathurst Harbour were generally like those of Bathurst Channel (90-100 %), however, they declined below the halocline (approximately 7 m) to approximately 80%. Melaleuca Lagoon was brackish at 27 ‰ salinity (marine salinity is ≥ 30 ‰). Temperature was on average 20 °C across the estuary.

4. Discussion

Maugean skate DNA in Bathurst Harbour was not present in concentrations above the threshold required for a positive detection based on eDNA analysis alone. However, enough

material was present in four samples to allow sequencing, which confirmed that these where extremely low-quantity Maugean skate DNA detections. The concentrations of target DNA in these samples (mean of 4-6 copies of DNA/reaction) are inconsistent with eDNA signatures detected at Table Head Macquarie Harbour (mean of approximately 2,000 copies of DNA/reaction), where a population of approximately 3000 Maugean skate individuals was estimated in 2016 (Bell et al., 2016). Firstly, this result demonstrates that at present there is not a population of Maugean skate in Bathurst Harbour that is equivalent to that of Macquarie Harbour. Instead, the evidence presented herein suggests that the species is either absent from Bathurst Harbour, or only an extremely small number of individuals or remnant DNA with no live animals remains.

In this study, Maugean skate DNA was successfully extracted and amplified using two probes each targeting two distinct loci. Detection from environmental samples in Macquarie Harbour confirms that eDNA assays and field sampling protocols as used here can be used as a tool to monitor the presence of rare and cryptic elasmobranchs in remote or challenging environments.

Based on long term catch and tracking data, Maugean skate in Macquarie Harbour are most common in Table Head. By contrast, skate do not go to Macquarie Heads, and no individuals have ever been recorded there (Bell et al., 2016). Therefore, any skate DNA present at Macquarie Heads is likely to have originated inside the harbour having become very diluted by the time it has made its way there. It is important to note, however, that despite its dilution, DNA concentrations at Macquarie Heads were 10-15 times higher than those of the four samples from Bathurst Channel and Harbour that could be sequenced (mean of approximately 60 Cf. 4-6 copies of DNA/reaction).

When comparing the performance of both filters for the positive environmental control samples in Macquarie Harbour, it appears that despite the reduced filtration volume, the 0.45 µm filters have a higher detection sensitivity, detecting Maugean skate at both Table Head and Macquarie Heads. Five µm filters were able to positively detect Maugean skate DNA in the positive control but failed to detect DNA at Table Head and Macquarie Heads. By exploiting the different sensitivity levels of different filter sizes, it may be possible to use eDNA to answer questions over different spatial scales. For example, the higher sensitivity of the smaller filters is ideal for studies like this one, where the target species are extremely rare, and it may be useful to document the occurrence of even minute traces of the target marker. Likewise, in some studies, the larger, less sensitive filters (5 µm) may be a useful monitoring tool, allowing researchers to infer occupancy and relative abundance across high abundance areas.

Environmental concentrations of DNA have been shown to correlate with density of the target species. Therefore, when using eDNA to detect rare or endangered species, it is reasonable to assume that target concentration of DNA will be exceptionally low. These conditions increase the chance of false negatives, i.e., instances where the species is present, but DNA traces are not detected in the sample. Recent studies have shown that changes to key aspects of the sampling protocol can strongly improve detection sensitivity in eDNA studies (Schultz & Lance, 2015; Zhiqiang et al., 2021). These recommendations were incorporated when formulating the experimental design in the present study to help maximise detection sensitivity and minimize the chance of false negatives (i.e., the inclusion of positive controls, high filtered water volume per site, multiple sample collection per site and multiple technical replicates during qPCR). Likewise, to ensure that sampling was spatially representative, the placement and density of sampling sites was informed by knowledge from the species in Macquarie Harbour. For example, within Bathurst Harbour all sites were closer than 2.5 km from the nearest site given that long term tracking of the skate in Macquarie Harbour shows

that the species have a high degree of site attachment, with individuals occupying persistent home ranges with an extent of approximately 5 km^2 on average (Bell et al., 2016; Moreno et al., 2020).

Analysis of eDNA can also be affected by a range of factors that could result in the detection of false positives. Given the low levels of detection, poor probe design can result in non-target sections, or the DNA of closely related species being amplified. To account for this, all assays in this study included appropriate negative (multispecies and reagent) controls that showed no amplification. Another potential source of error is contamination from qPCR products in the laboratory, although this can be discounted here, given that the second probe targeting a different region was developed and corroborated our findings in a separate laboratory. Additionally, positive controls were added last to the reactions to avoid false positives. Lastly, external contamination during sampling (gear), extraction and analysis (laboratory) could result in false positives.

To account for the possibility of laboratory or analysis contamination, analysis using the CO1 probe was conducted at a separate site in a laboratory where no samples from either Macquarie or Bathurst Harbours had been present. All field sampling equipment was thoroughly cleaned using a 2% Sodium hypochlorite solution before and after every sampling event. The eDNA filters are encased in a desiccant plastic casing that preserves the DNA sample and are shipped in a hermetically sealed bag. Bags were only opened immediately before use, the filters were handled using nitrile gloves, and the filters re-sealed in their shipping bag immediately after use, and not handled again until returned to the lab. As such, it is extremely unlikely that any of these potential issues could be the cause for the traces of DNA seen in the second Bathurst sample, suggesting that what was detected was a real extremely low quantity detection (mean of 4-6 copies of DNA/reaction) of Maugean skate DNA.

The Maugean skate was first discovered in Bathurst Harbour in 1988, and since its recognition as a new species and discovery in Macquarie Harbour, it has been widely assumed that two populations exist. However, despite consistent effort since its discovery, only four individuals have ever been seen in Bathurst Harbour, with the last of them captured in 1992 (Last & Gledhill, 2007). Furthermore, despite significant fisheries activity in Port Davey and the shelf off the southwestern/western Tasmanian coasts, no records of the species outside either Macquarie or Bathurst Harbour exist, noting that this is very unlikely regardless, given the species specialisations to live its entire life in estuarine conditions (Morash et al., 2020; Moreno et al., 2020). Given the findings in this study, it is unclear if the Maugean skate were ever abundant in Bathurst Harbour. Answering this question may be crucial for the conservation of the species, as it may provide insight into the causes for its possible disappearance from the area if it was abundant or help determine if Bathurst Harbour could represent a valuable refuge for the species in the future. It should be noted that dissolved oxygen levels in Bathurst Harbour at the depths at which Maugean skate occur in Macquarie Harbour are significantly higher than those of that Harbour (see section 3.4).

One of the primary limitations of eDNA to assess species occurrence is that a successful detection only indicates the presence of the target DNA in the area, not of the animal itself. The extremely low level Maugean skate DNA traces detected in Bathurst Harbour are likely to be the result of one of three possible scenarios:

1. The first would suggest that Maugean skates exists in Bathurst Harbour, but only in exceptionally small numbers. If this were the case, there may be seasonal movement

occurring around the Harbour. Seasonal movements would explain the lack of detections in the first sampling period in November 2021.

2. The second proposes that Maugean skate no longer exist in Bathurst Harbour, while their DNA is still present. This may occur where egg cases or other genetic material are found or released in the sediment of the seabed. There were clear seasonal differences in Bathurst Harbour between the sampling periods, where November 2021 was very wet, with 2021 having the wettest Tasmanian Spring since 2016 (Spring rainfall was 18% above the state average; http://www.bom.gov.au/). In comparison, February 2022 was incredibly dry, with summer 2021/22 the driest Tasmanian Summer since 1980-81 (Summer rainfall was 42 per cent below the state average; http://www.bom.gov.au/). Bathurst Harbour likely would have experienced increased seawater influx during summer 2021/22, as suggested by the marine salinity values across all sites in the Harbour and Channel in February 2022 (see Section 3.4), with Last and Gledhill (2007) stating that salinity in the Harbour is usually less than 10 ‰ (Cf. 32.5 ‰). This increased influx of seawater may have disturbed the sediment in the seabed and released genetic material to be detected.

3. Lastly, Maugean skate could be a transient resident in Bathurst Harbour. This would align with the small number of historical captures in Bathurst Harbour, although it does not align with the species' known adaptability, resilience, and preference to estuarine environments for all stages across its entire life cycle. This is clearly observed in the Macquarie Harbour population, which do not travel beyond the harbour. As such, this is the most unlikely of the scenarios.

Unlike the world heritage protected Bathurst Harbour, Macquarie Harbour has a long-documented history of anthropogenic impacts that have resulted in considerable degradation of the environment. Recent work on the Maugean skate in Macquarie Harbour has shown clear signs of population stress and detrimental impacts of the degraded environment (Moreno et al., 2020). Regardless of which of the scenarios presented above is correct, it is now clear that the vast majority, if not all, of the remaining Maugean skate live in Macquarie Harbour. Therefore, the findings of this study highlight the vulnerability of the species and the need for urgent conservation action and continued research focused on the Macquarie Harbour population to ensure the persistence of this unique species.

5. Recommendations

Despite its limited range, the current listing status of the species under the EPBC Act (1999) assumes that two distinct populations exist. However, Macquarie Harbour is the only site where the species remains in any substantial number. Therefore, a reassessment of the conservation status of the species should be considered.

It is vital that research, conservation, and management efforts are prioritised for the remaining population in Macquarie Harbour. It is essential that the population be monitored to improve our understanding of physiology, environmental ecology, and population dynamics and trends. Improved knowledge will confirm the level of vulnerability of the population and facilitate future conservation and remediation plans for *Z. maugeana* in Macquarie Harbour.

There is a need for immediate conservation action that is informed by science, particularly given the large uncertainties that still exist regarding the biology, ecology, and population dynamics of the species. Future solutions are likely to be driven by novel methods and

technologies. Given the eDNA of *Z. maugeana* was still present even in minute quantities in Bathurst Harbour, other retrospective research techniques, such as ancient DNA, could be applied to describe the genetic composition of prior residents. This could be useful to investigate more specific population genetic characteristics of historic (Bathurst Harbour) and remaining populations (Macquarie Harbour) and provide insight into the genetic relationship between such populations. It may also be useful for determining if Bathurst Harbour could be used as a refuge for Maugean skate.

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