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COTS Control Innovation Program | A research and development partnership to better predict, detect and respond to crown-of-thorns starfish outbreaks

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This report should be cited as

Uthicke S, Gomez Cabrera M, Patel F, Lawerence E, Tabor B, Foster S, Doyle J (2025) *Operationalising and implementing crown-of-thorns starfish (COTS) environmental (eDNA) monitoring on the Great Barrier Reef*. A report to the Australian Government by the COTS Control Innovation Program (97 pp).

Funding Acknowledgement

The COTS Control Innovation Program aims to accelerate the development of innovative surveillance and control methods to manage outbreaks of coral-eating starfish on the Great Barrier Reef. The Program is a collaboration between the Great Barrier Reef Foundation, Australian Institute of Marine Science, Commonwealth Scientific and Industrial Research Organisation, James Cook University and The University of Queensland. The Program is funded by the partnership between the Australian Government's Reef Trust and the Great Barrier Reef Foundation.

Traditional Owner Acknowledgement

The COTS Control Innovation Program extends its deepest respect and recognition to all Traditional Owners of the Great Barrier Reef and its Catchments, as First Nations Peoples holding the hopes, dreams, traditions and cultures of the Reef.

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Acronyms and Abbreviations

AIMS	Australian Institute of Marine Science
ATL	A Qiagen DNA extraction buffer, not an acronym
CCIP	Crown-of-thorns starfish Control Innovation Program
COTS	Crown-of-thorns starfish
CPUE	Catch per unit effort
ddPCR	Digital droplet PCR
DNA	Deoxyribonucleic acid
eDNA	Environmental DNA
FPC	False Positive Correction
GBR	Great Barrier Reef
GBRMPA	Great Barrier Reef Marine Park Authority
LTMP	AIMS Long-Term Monitoring Program
MCE	Mixed cellulose ester
NTC	No Template Control
PCB	Princess Charlotte Bay
PCR	Polymerase Chain Reaction
PMG	Pacific Marine Group
PP	Proportion positive
QC	Quality Control
QPWS	Queensland Parks and Wildlife Service
RJFMP	Reef Joint Field Management Program
SALAD	Scooter-Assisted Large Area Diver-based surveys
SAP	Sampling and Analysis Plan
SE	Standard Error
SOP	Standard Operating Procedure
SP	Self-Preserving
TES	A chemical buffer, rN-[Tris(hydroxymethyl)methyl]-2-aminoethanesulfonic acid
TSRA	Torres Strait Regional Authority
UV	Ultra Violet

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EXECUTIVE SUMMARY

Efficient crown-of-thorns starfish (COTS) control relies on early detection of outbreaks. Our previous studies demonstrated that COTS-specific environmental DNA (eDNA) analyses can detect the build-up of COTS populations prior to COTS being detected using standard in-water methods such as manta tows. The main objective of the COTS Control Innovation Program's (CCIP) project CCIP-D-03 was to finalise the development of COTS eDNA methods and provide recommendations to operationalise COTS eDNA monitoring. We aimed to: i) collect and analyse eDNA from up to 42 reefs per annum over a period of 3 years, ii) establish a sentinel station on Lizard Island and investigate small scale patterns, iii) provide recommendations on how eDNA methods can be used as a monitoring tool as part of a comprehensive COTS monitoring strategy (in collaboration with projects CCIP-D-01 and CCIP-D-02), and iv) provide a mapped out workflow and expected timeframes for sample collection, sample processing and subsequent delivery of data into the COTS Control Program decision support system (project CCIP-R-02).

We collected and analysed annual eDNA data from >20 reefs (up to 42 reefs) over 3 years, comprising over 3,500 eDNA samples (aim i). COTS eDNA increased in Cape Grenville, Princess Charlotte Bay and Lizard Island from 2021 to 2023. COTS eDNA in the currently culled area in the Townsville region remained high, with low levels in the Whitsundays. Reefs between Cairns and Lizard Island occasionally presented high eDNA signals between 2021 and 2023, making it unclear if this was caused by the 5th outbreak or a flare up of the 4th outbreak. However, fine scale data at the established sentinel station at Lizard Island (aim ii) from 2019–2023 unambiguously demonstrated that COTS eDNA has increased since 2019 and is highly correlated with observational data provided by Scooter Assisted Large Area Diver-based (SALAD) surveys. Hence, analyses of COTS eDNA have detected and documented increasing COTS populations, suggesting the beginning of the 5th outbreak wave.

The sampling strategy utilised for the three years consisted of 12 eDNA samples collected from each of three sites at each reef (total 36 samples per reef). Statistical modelling revealed that substantial costs can be saved, while maintaining or even improving error estimates, by increasing the number of sites and decreasing the number of samples taken per site. For example, a sampling strategy of 4 sites per reef and 6 samples per site (total 24 samples) has the same standard error as the current sampling strategy, while saving 30% of laboratory costs (aim iii). Further, comparisons between COTS estimates collected via eDNA monitoring and SALAD surveys were available from 42 reef/time combinations enabling a direct comparison of eDNA data with observational data. A regression of these parameters was highly significant, confirming that the amount of eDNA is positively related to observed in-water estimates of COTS densities, even at very low and ecologically relevant levels. Thus, eDNA provides an effective method with no inter-observer variation for early warning monitoring of increasing COTS populations.

Additional tests conducted during this project trialled different collection and preservation methods (aim iii) with the aim to improve on-water sampling and collection workflows, especially if non-research vessels were used for collection. A 'Standard method' was translated to several ships of opportunity, and staff on COTS control vessels, Reef Joint Field Management Program (RJFMP) vessels and a site-based tourist operation were trained to collect and preserve eDNA samples. Clean collections (zero contamination) were performed

on RJFMP vessels and RJFMP staff successfully collected eDNA samples from eight reefs in the Whitsunday region in 2023 and eDNA identified several reefs above a threshold of concern not identified by parallel manta tows. We also trialled eDNA collection from COTS control vessels and found cross contamination in some control samples. However, statistical analysis suggested issues arising from cross contamination may be overcome by additional training and increasing the number of negative controls. This needs to be tested in further research.

Overall, we achieved all objectives, provided a clear workflow (from collection to analysis of eDNA samples, aim iv) and demonstrated a translation of eDNA protocols to on-water operators (i.e. operationalised). For early outbreak detection and understanding causes and patterns of COTS outbreaks, we recommend regular backbone monitoring on fixed reefs with eDNA samples collected using research vessels, supplemented by samples on a larger number of reefs collected from ships of opportunity such as RJFMP cruises. **We recommend piloting the suggested improved sampling design (increasing sites to 4–6 and decreasing samples per site) in the first year of monitoring.** The monitoring should be complemented by ongoing annual monitoring on the sentinel sites studied at Lizard Island. We demonstrated the early detection of COTS through eDNA monitoring has scalable capacity. The ability to detect early population build up on a reef and regional scale is critical to early response and the suppression of future outbreaks.

Analysis presented here unambiguously demonstrated that eDNA is a suitable monitoring tool to detect and quantify very low densities of COTS. The eDNA techniques to detect COTS developed here are versatile and can be adapted to a variety of operational applications, ranging from **quantification** at pre outbreak densities (early detection, several sites and replicated samples needed) to **presence/absence** determination once outbreaks are established or post culling (few samples per reef/site needed).

1. INTRODUCTION

Acanthaster spp. are corallivorous seastar species distributed throughout the tropical Indo-Pacific (Uthicke et al. 2024a) . Several of these species exhibit population explosions leading to significant coral loss. For example, population outbreaks of the western Pacific crown-of-thorns starfish (COTS), *Acanthaster* cf. *solaris*, have occurred in French Polynesia (Kayal et al. 2012), Indonesia (Baird et al. 2013), Australia's Great Barrier Reef (GBR) (Moran 1986; Pratchett 2005), and Okinawa, Japan (Nakamura et al. 2014).

COTS can contribute up to 40% of coral loss (De'ath et al. 2012), although in recent years, losses through climate change induced bleaching are likely to assume a higher proportion (Bozec et al. 2022). The GBR is currently experiencing its 4th COTS outbreak wave since the 1960s and recent findings (partially funded through the COTS Control Innovation Program, CCIP) confirmed a 5th outbreak wave has now started in the northern GBR (Chandler et al. 2023; Uthicke et al. 2024b).

An accurate and large-scale surveillance and monitoring program for COTS is key to enabling a rapid identification of reefs under threat and subsequent response, but is difficult to achieve with conventional observational technologies (e.g. manta tow). During outbreaks, manta tow methods are suitable for large area surveys that identify locations under impact from increased COTS populations. Outside of outbreaks however, COTS occur in low densities and are difficult to detect. A monitoring program that has a wider selection of tools to detect COTS is needed to enable the detection of COTS at low density and early outbreak phases that cannot be detected with current manta tow observation methods (Babcock et al. 2020).

Since 2013, the Australian Institute of Marine Science (AIMS) has been developing innovative environmental DNA (eDNA) monitoring technology for COTS. These techniques can be used to detect and quantify COTS larvae (Uthicke et al. 2015; Doyle et al. 2017; Uthicke et al. 2019), newly settled juveniles (Doll et al. 2021) and post settlement COTS on the reef at extremely low densities (Uthicke et al. 2018; Doyle & Uthicke 2020; Kwong et al. 2021; Uthicke et al. 2022). Specifically for post settlement COTS eDNA, AIMS has developed eDNA methods to detect and quantify post settlement individuals in small (2 L) water samples filtered directly on individual reefs (Uthicke et al. 2018) and demonstrated that a clear relationship exists between COTS eDNA and actual COTS densities on the reef (Uthicke et al. 2024b). We also demonstrated that the sensitivity of the method is extremely high (Uthicke et al. 2022), with detection at the suggested 'Allee threshold' (Rogers et al. 2017) of three COTS ha^{-1} or below the 'outbreak levels' (0.11 COTS per manta tow) used for COTS management by the Great Barrier Reef Marine Park Authority (GBRMPA). Detecting COTS at these or lower levels will be essential to allow intervention at early stages of the outbreaks. In addition, detection of outbreaks at early stages will assist to identify locations and extent of the "initiation box", which will allow focusing culling efforts in that area to prevent future outbreaks and aid our understanding of the causes of (primary) outbreaks.

Our eDNA method shows promise as a scalable, rapid and accurate monitoring platform for COTS on the GBR. As a main outcome of this project, we propose a plan for testing and implementing a next-generation adult (post settlement) COTS monitoring program based on eDNA at scale. We predict that the application of this method will significantly increase the responsiveness of control programs and allow early intervention. This would be achieved by conducting broadscale eDNA surveys on many reefs, detection at low densities and

detection at densities difficult to detect through other methods. In addition, this method is a safe way to conduct surveys on reefs where in-water surveys are not feasible due, for example, to environmental risks, such as crocodile risk on Far Northern reefs.

One of the advantages of using eDNA for monitoring is the possibility to obtain samples through non-scientists ('citizen scientists') to save overall costs and expensive ship time. Many different options for this exist in the GBR context. These include tourist operators, and 'ships of opportunity' such as survey voyages of the Reef Joint Field Management Program (RJFMP) or the COTS control vessels. The challenges of samples collected by citizen scientists are that training in sample collection and preservation needs to be provided, and a clean working environment needs to be available on the vessels. In addition, our methods depend on detection of individual gene copies. Hence, care needs to be taken not to contaminate samples, which may specifically be a challenge when collecting from vessels also used for culling or handling COTS. Several options exist to minimise contamination risk and simplify the on-board workflow. For instance, self-preserving filters or passive sampling units reduce the need for handling of samples after collection. We tested the sensitivity of these methods and also tested how long samples can be stored with different preservation methods.

1.1 Project aims

eDNA monitoring to inform COTS management

The first two aims are to conduct eDNA monitoring at many reefs over the three years of the study as a baseline for potential later ongoing monitoring. This monitoring mainly focuses on the 'initiation box' (the area primary COTS outbreaks are currently presumed to start in) and is designed for early detection of an overall outbreak and to discern outbreaks on individual reefs.

- Aim (i) Collection and analysis of eDNA data from representative GBR reefs ('backbone reefs') over three years. These reefs are mainly located in the initiation zone, and many of these are in parallel to AIMS Long-Term Monitoring Program (LTMP) reefs or have a known COTS history.
- Aim (ii) Continue data collection at a sentinel station on Lizard Island and test small scale patterns.

1.2 Linkages to other projects in CCIP

This project is part of the Detection (D) subprogram of the COTS Control Innovation Program (CCIP) (**Figure 1**) and has strong linkages to all other projects in that subprogram. Project staff were involved in CCIP-D-01 (Lawrence et al. 2025a) and CCIP-D-02 (Lawrence et al. 2025b) and contributed to designing the development monitoring guidelines. In addition, we took part in a calibration field trip for CCIP-D-02 assisting with developing calibrations between different monitoring tools to estimate COTS densities (e.g. Scooter-Assisted Large Area Diver surveys (SALAD), manta tow, Control dives, eDNA). The project is also strongly linked to projects in other subprograms (**Figure 1**). In project CCIP-P-04 (Pre-outbreak monitoring, Pratchett et al. 2025) and the early investment project **"Delineation of Initiation box"**, we collected parallel samples with the Pratchett lab to compare eDNA results to

SALAD densities and found a good correlation between these, confirming that our method is quantitative even at low densities (see results). In project CCIP-P-05 (Benthic predation in rubble, Wolfe et al. 2025a) we developed an eDNA-based method to detect whether predators (crabs) have consumed COTS juveniles. In addition, we closely collaborated with the COTS Control Program decision support system (CCIP-R-02, Fletcher et al. 2025) and managers at GBRMPA, and annually delivered our data for integration in the COTS dashboard.

Via the Activities **"Assess Ships of Opportunity"** and **"Operationalise eDNA post settlement"** it has a clear impact pathway through **'Extended toolbox for COTS detection**" (Output), and **"Improved detection and monitoring (outcome) to the ultimate impact of Coral Protection in this project**" (**Figure 1**).

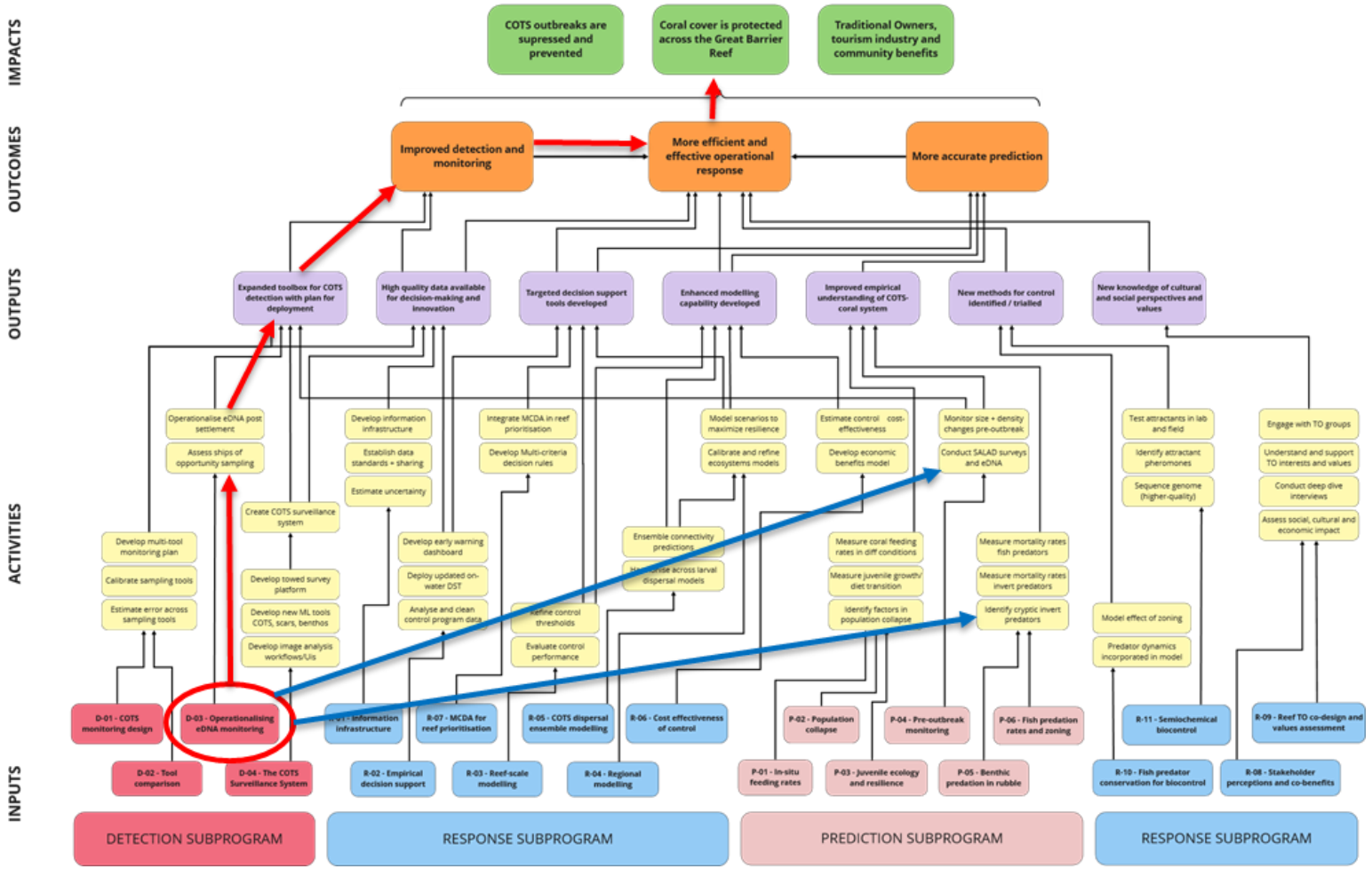

Figure 1. CCIP program logic, highlighting D-O3 (red circle) and the impact pathway (red arrows). Linkages and collaborations to other project/activities are indicated by blue arrows.

Improve workflow towards operationalising towards COTS eDNA, statistical modelling

The final aims of the project are to provide recommendations on how eDNA methods can be used as a monitoring tool as part of a comprehensive COTS monitoring strategy (aim iii), and to provide a mapped-out workflow and expected timeframes for sample collection (aim iv).

An important aspect of this project was to engage with operators to investigate the translation of eDNA procedures to operators. This would enable the use of existing logistics to collect eDNA samples and facilitate collection on a wide range of reefs. Given ship time is a major factor in resourcing marine monitoring and the collection side of eDNA lends itself to citizen science, we focus on testing the workflow for eDNA sampling to support operational uptake by non-scientists.

Specifically, we focus on:

- Testing different sample collection methods to find if methods other than filtration provide time and logistic efficiencies.
- Testing sample preservation methods that could reduce processing effort and contamination risks.
- Training non-scientists in sample collection.

To assess the efficacy of the eDNA sampling protocols and resulting data, we also used statistical tools to:

- Conduct a regression analysis for calibrating eDNA results with COTS densities derived from underwater scooter surveys.
- Estimate the number of sampling sites and samples per reef that is expected to provide an acceptable variance for the lowest cost.
- Assess the sampling contamination risk and investigate statistical means to manage false positives due to contamination.

Using the insights gained, we then provide recommendations on how this eDNA tool could be implemented as part of a structured COTS monitoring program (in collaboration with CCIP-D-01 Lawrence et al. 2025a, CCIP-D-02 Lawrence et al. 2025b). We also provide a mapped-out workflow and expected timeframes for sample collection, sample processing and subsequent delivery of eDNA monitoring data into the COTS Control Program decision support system (CCIP-R-02 Fletcher et al. 2025).

2. METHODS

2.1 eDNA monitoring to inform COTS management

2.1.1 Ongoing large-scale monitoring

We identified a collection of reefs to establish a ‘backbone’ monitoring. Reefs are located in several regions from Cooktown to Townsville (see **Appendix A** and **Figure 6**). In the first year of sampling (2021), we collected eDNA samples from 15 reefs between Lizard Island in the North and Bowden reef in the South. Reefs were chosen based on known COTS history, accessibility (e.g. from Lizard Island, Port Douglas or Cairns), and in part because they are AIMS Long-Term Monitoring Program (LTMP) reefs. Two reefs in Townsville area (Davies reef and Bowden reef) were included because we have obtained COTS eDNA data from previous years from those locations. In this and the following years, 7–8 reefs in Princess Charlotte Bay (PCB) and Cape Grenville area were collected under CCIP-P-04 (Delineation

of the Initiation Box, Pre-outbreak monitoring, Pratchett et al. 2025). We conducted additional eDNA sampling at outer Ribbon reefs as these do not usually experience COTS outbreaks, so served as 'field controls' for low COTS densities. Sample sites in the first year were chosen in the backreef habitat of most reefs. This was because at the time of collection (winter, outside spawning season) strong southeast trade winds can prevail, making other sites exposed. Some exceptions to this were in Cape Grenville and PCB where sites were matched to SALAD survey sites.

Presentation of the data from 'backbone' monitoring reefs were complemented by additional eDNA collections in Princess Charlotte Bay and Cape Grenville through CCIP-P-04 (Delineation of the Initiation Box), and additional eDNA collections in the Townsville and Whitsunday regions as part of CCIP-D-02 (Monitoring tool calibration, Lawrence et al. 2025b), and trial samples collected by RJFMP vessels (see **section 2.2.3**). Sampling locations from 2021 to 2023 can be found in **Appendix A**.

Sample collection consisted of three sites per reef, with sites within a reef separated by 0.5–1 nautical miles and typically located at the back reef and/or lagoon. At each site, 12 replicate eDNA samples were taken. Environmental DNA samples were collected by pumping 2.5 L water directly through an eDNA housing (Smith-Root, USA) containing a 47 mm, 1.2 μm mixed cellulose ester (MCE) membrane filter using an eDNA sampling device (Grover-Pro™, Grover Scientific, Townsville Australia). Membrane filters (kept cold in an esky on board the tender or fridge on the mothership) were removed from the housings within 2 hours of collection, folded carefully into eighths using bleach cleaned plastic forceps and placed in a 1.5 ml screw cap tube containing 540 µl of Qiagen buffer ATL as a preservative. The above description constitutes our 'Standard method' of eDNA sample collection and preservation for laboratory analysis.

2.1.2 Fine scale sampling at Lizard Island, a sentinel location for outbreak initiation

Environmental DNA sampling was undertaken at five sites around Lizard Island between August and September annually from 2019 until 2023, thus, the initial collections at these sites predate the CCIP (**Figure 2**). These sites were included in the results section to illustrate the overall temporal trend at this location, given the extended time series compared to the backbone monitoring established during this project. To obtain higher resolution and information on short term temporal variability, we collected eDNA samples twice per trip from each of the Lizard Island sites. A total of 30 replicate filters (15 per collection event) were collected at each site/year combination. Sample collection and fixation were conducted as described in section 2.1.1. Further details of Lizard Island eDNA experimental design and activities can be found in Uthicke et al. 2024b.

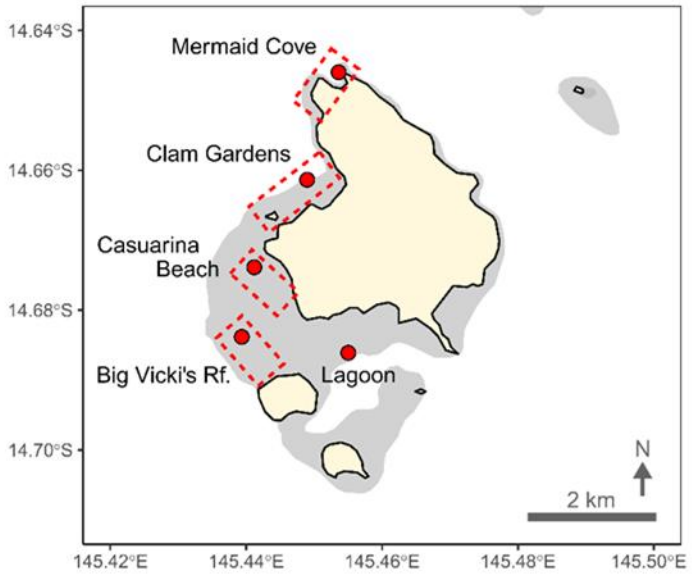

Figure 2. Location of eDNA sampling sites (red dots) and complementary SALAD survey areas (see section 2.3.1 red dashed boxes) at Lizard Island, northern Great Barrier Reef.

2.1.3 Laboratory methods

Laboratory methods for eDNA testing have been described previously (Uthicke et al. 2018; Uthicke et al. 2022). Briefly, eDNA extraction from filters was completed with a Qiagen Blood and Tissue DNeasy kit. The eDNA extracts were analysed using a COTS specific digital droplet PCR (ddPCR) assay developed at AIMS. Controls were conducted to monitor for any contamination introduced during the eDNA workflow. These included, 1) field controls – purified water filtered at regular intervals throughout a collection campaign, 2) extraction controls – blank filters extracted in the laboratory alongside eDNA samples and 3) ddPCR no template controls (NTC) – water added to ddPCR reactions instead of extracted DNA. Positive detection is defined as PCR amplification that is above controls. Extractions and ddPCR analysis were conducted at AIMS.

2.1.4 Permit

Environmental DNA sampling only requires non-destructive water samples; thus all sampling was conducted under the general AIMS permit (G 38062.1) with a Sampling and Analysis Plan (SAP) approved by GBRMPA. No ethical permit was required.

2.2 Improve workflow towards operationalising COTS eDNA monitoring

2.2.1 Laboratory eDNA sample preservation trials

Self-preserving filter units minimise handling time and reduce the risk of contamination. However, it is not known whether these units preserve samples for the same length of time as the method currently deployed as standard. Thus, a preservation trial was conducted over six months to ascertain the stability of eDNA samples collected and preserved via our 'Standard method' and samples collected using self-preserving filter housings. This standard method uses 47 mm, 1.2 µm mixed cellulose ester (MCE) membrane filter. We compared this method to self-preserving (SP) filter housings (Thomas et al. 2019) which contained the

same membrane filters. However, after collection these need no further processing until DNA extraction because the captured eDNA is preserved by drying the membrane filter with an in-built desiccant.

Experimental conditions

To obtain relatively stable COTS eDNA concentrations, we used a 10,000 L tank at the AIMS Sea Simulator with a seawater flow rate of approximately 14 L per min (two turnovers per day). Water temperatures ranged from 25°C to 27°C. After equilibration for two weeks, five COTS of approximately 25 cm diameter were introduced to the tank. The tank was allowed to further equilibrate for four days prior to eDNA collection.

As described above, two sample preservation methods were trialled.

1) 'Standard method'. Water is filtered through a nylon/silicon housing available from Smith-Root (USA) containing a 47 mm 1.2 µm MCE filter membrane. Once filtration is completed, the filter membrane is removed from the housing and placed in Qiagen ATL buffer to preserve captured eDNA.
2) Self-preserving method. Water is filtered through a housing similar to the standard method, except these biodegradable housings contain an in-built desiccant (available from Smith-Root, USA) that dries water from the filter membrane (Thomas et al. 2019).

For each method, 56 x 1 L water was filtered using an eDNA sampling device (Grover-Pro™, Grover Scientific, Townsville Australia). All samples were collected on the same day over a period of two hours. Samples were randomised, and four replicate samples for each preservation were extracted immediately as time zero (T_0). For the next 26 weeks replicate samples (n = 4) for both preservation methods were extracted approximately every two weeks. COTS specific eDNA was measured using digital droplet PCR (ddPCR) as previously described (Doyle et al. 2017; Uthicke et al. 2018).

Data was analysed with a generalised additive model (Hastie & Tibshirani 1986) using the `mgcv` package in R to determine significant differences between preservation methods.

2.2.2 Field comparison of four different collection and preservation methods

We conducted further preservation trials in the field together with testing different sampling methods, aiming to test methods to potentially improve sampling efficiency in the field and contamination risk. Time efficiency and reduced-contamination gains may be offset with higher costs (cost estimates at present day rates will be presented for comparison). Collection and preservation comparisons were conducted at a single site (DREDNA2) on Davies reef on the 22nd May 2022. Four collection and preservation methods were compared: three filtration methods and one passive collection method. For the filtration methods, 12 x 2.5 L eDNA samples were collected directly from the water through 47 mm, 1.2 µm MCE membrane filter using an eDNA sampling device (Grover-Pro™, Grover Scientific, Townsville Australia). The first two methods are the 'Standard Method' (A$6.70 per sample) and Self-preserving eDNA housings (approximately $46 per sample) as described in 2.2.1. The third filtration method uses standard filters, but instead of ATL collection, the entire filter and housing were placed inside an individual ziplock bag with four silica pouches (10 g, each pouch) to desiccate the filter (Kirtane et al. 2020; Allison et al. 2021; Cindy et al. 2021; Chen et al. 2022). This may provide a potential cost-efficient ($7 per sample) alternative to option 2.

The fourth method is a passive sampling approach, which is an alternative to filtration (Bessey et al. 2022). Passive sampling with Whirl-Pak® Speci-Sponges® is an effective method for collecting eDNA from marine waters (Jeunen et al. 2022) and we tested different soaking times of this sponge. Whirl-Pak® Speci-Sponges® are provided in a dried, sterile form in individual bags (Catalogue number B01245, 4.5 x 9 cm). Sponges were removed from their bags under clean conditions and fixed at 10 cm intervals to a 10 mm nylon braided rope using cable ties (**Figure 3**. A total of 12 Whirl-Pak® Speci-Sponges® per soaking time (5 minutes, 30 minutes, 3 hours and 24 hours) were fixed on individual ropes. At the location on Davies reef, a weight was fixed to one end of the rope and a small buoy to the other. Sampling units were deployed by carefully lowering the weighted end of the rope to the bottom (approximately 5–7 m depth). Upon retrieval at the respective soaking time, individual sponges were removed from the rope and placed into individual zip-lock bags, then kept cold on ice until returned to the main ship for preservation (< 2 hours). A 1 cm^2 section was cut from each sponge using clean scissors and placed into a 5 ml tube containing 900 µl Qiagen buffer ATL. DNA extraction utilised the same Qiagen Blood and Tissue extraction method as for filters but with proportional volume changes. Total cost for collection equipment was approximately $7 per sample.

The COTS eDNA capture efficiency was compared between the filtrations methods to determine if desiccation methods 2) and 3) above are comparable to the 'Standard method' (method 1). Passive sampling was compared to identify if an optimal soaking time is required to capture similar amounts of COTS eDNA to the 'Standard method'. Significance testing used Kruskal-Wallace non-parametric testing followed by Dunn's test for multiple comparisons.

2.2.3 Training non-scientists in sample collection

Throughout this project several reef operators and managers were trained, including research station staff, tourism operators, staff of COTS control vessels and QPWS staff. In this report, we focus on a training trial on Moore Reef pontoon, two COTS control vessel training sessions and training on a QPWS vessel. Controls consisting of clean, 1 L laboratory grade water (MilliQ) were routinely included in all eDNA collections in this project. The water from these controls was filtered through similar equipment used to collect samples and provides an indication of background contamination that may be inadvertently introduced to the sample.

Moore Reef 'Marine World', October 2022

We visited the Moore Reef 'Marine World' pontoon in October 2022 to train Marine World staff in eDNA collection methods (**Figure 3**). Subsequent to training, we collected parallel collections. A total of 12 samples and 4 controls were collected each by Marine World staff and by AIMS staff.

Figure 3. Environmental eDNA collections at Moore Reef, October 2022. Photo: M. Gomez Cabrera, AIMS.

Pacific Marine Group (PMG), Odyssey. May 2022

COTS control vessels are likely to have a high background level of COTS DNA due to the culling operations undertaken. As such, overlaying eDNA as a sensitive detection method requires understanding when/where inadvertent contamination may occur, in order to provide interpretable results. This activity employed control testing only throughout the vessel during a standard culling voyage in May 2022 (targeting Lynchs reef and Keeper reef). For this study on the Odyssey, a total of 23 control water samples were filtered at 12 locations around the main vessel (Outside: Aft deck [9 samples], Bow [2], Top Deck [2], Swim platform [1], Live Tank [1], Hatch [2]; Inside: Galley [1], Bathroom [1], Cabin [4]) and tenders (n = 13) to establish a 'background' reference of COTS eDNA during a routine COTS control vessel trip.

Pacific Marine Group, Odyssey. October 2022

After training of the PMG crew by AIMS staff (**Figure 4**), the crew independently collected eDNA and negative control samples on two reefs. COTS eDNA sampling was undertaken using the 'Standard method' at Hopkinson reef (n = 6) and Lynchs reef (n = 17) in October 2022 (**Table A 3**). These reefs were undergoing active culling by PMG during this time which enabled an opportunity to test eDNA procedures during operations involving culling at high COTS density reefs. A total of eight controls were also conducted throughout this trip.

Figure 4. Jason Doyle conducting eDNA training with crew from Pacific Marine Group on board the Odyssey. Photo: M. Gomez Cabrera, AIMS.

Reef Joint Field Management Program (RJFMP). November 2023

Reef Joint Field Management Program voyages were identified as another important potential sampling opportunity for COTS eDNA collection. A voyage on the Queensland Parks and Wildlife Service (QPWS) Patrol Vessel, Reef Resilience (voyage number FMPCRS2023-05), was identified to trial eDNA collections and coincided with routine COTS monitoring activities in reef areas adjacent to the Whitsunday region of the Tropical North Queensland Coast in November 2023 (**Figure 5**). During this voyage, eight reefs were visited for routine COTS monitoring activities (manta tow) and eDNA samples collected by the field team at each reef using the 'Standard method' described above. No COTS culling activities occurred during this trip. Three sites per reef were visited with 12 replicate eDNA samples collected at each site (See Appendix A, **Table A 3**). Prior to the voyage, a training session was conducted with the RJFMP field team and detailed protocols provided for ad-lib referencing whilst underway (Appendix B). For convenience, all eDNA collection equipment was bleach cleaned and pre-packaged in sets such that for any given site at a reef, a set of samples constituting 12 replicates was readily available. Field negative controls were conducted in duplicate for each reef giving a total of 16 field controls. Samples were analysed by ddPCR.

Figure 5. Training session with QPWS staff for eDNA collections in November 2023 as part of the Reef Joint Field Management Program (RJFMP). Photo: Sascha Taylor, QPWS.

2.3 Evaluating and enhancing sampling protocols and data analysis

2.3.1 COTS density and eDNA

Validating emerging eDNA monitoring methods against established visual survey techniques, like SALAD, is critical to ensure confidence in their reliability to estimate COTS densities. SALAD surveys employ sea scooters to enable divers to search larger reef areas than traditional methods. Working in pairs across different depths, divers record COTS and feeding scars within 5 m-wide belts along approximately 1 km transects, tracked by GPS. These visual surveys provide direct observational data of COTS presence and abundance, creating a benchmark against which to evaluate the accuracy of eDNA detections, particularly at low population densities where early intervention is most effective. We analysed the SALAD data aggregated to the reef level, alongside the eDNA data (also aggregated to the reef level) to determine whether eDNA measures are a useful indication of COTS density. We used data only at reefs where the SALAD and eDNA measures were collected within a similar timeframe (within 6 months). The sites sampled at the reef level

were not always the same but by aggregating the measures across the reef, they are taken to be a broad representation of what is happening at that reef at that time point.

We fitted a range of models to determine how well the eDNA can predict COTS density recorded on the SALAD surveys (**Appendix C**), which included both direct observations and feeding scars of COTS. Here, we present densities of actual COTS observations in the main text (but see **Appendix C**, and CCIP-D-02 (Lawrence et al. 2025b) and CCIP-P-04 (Pratchett et al. 2025) for analysis of inferred densities (COTS+ scars)). We also describe the model fitting for a model that can be used to relate the eDNA proportion positive (PP) metric to the SALAD density estimates. The response variable was the log transformation of the observed COTS density, and the predictor variable was the proportion of positive samples, where a sample is positive if either of two replicates analysed via ddPCR is deemed to be positive for COTS eDNA. There was a single case of no COTS (0 density) recorded on SALAD and we replaced that value with half the minimum observed non-zero density as an ad hoc means of including it in the analysis (i.e. to avoid taking logs of zero). This is standard practice and likely not to be misleading for our purposes here. A simple linear model was then fitted in R. The model predictions were back transformed to the natural scale (including a bias correction) and the predicted mean and associated confidence intervals plotted in R.

2.3.2 Sampling design analysis

To assess the efficiency and effectiveness of the current eDNA sampling strategy, we conducted a statistical modelling exercise (**Appendix D**). Data used for this analysis were all three sampling years from the 'backbone' reefs and three years of data from Lizard Island, giving 79 reef/trip combinations. Given the nested structure of our data (repeats within samples within sites within reefs) and the skewed distribution of positive counts (with approximately 60% of observations having zero positive droplets), a multilevel Poisson mixed-effects model was required to accurately capture the variance components at each sampling level. An offset of log(accepted) accounted for the variation in the number of accepted droplets in the digital PCR for each observation. Reef-Trip, with 79 levels, was the fixed effect variable of interest. The random effects were coded as repeat within sample within site within reef. The model was implemented using the glmmTMB package in R.

To evaluate optimal sampling strategies, we used the fitted model to simulate sampling under various protocols. The proportion positive (PP) metric refers to the fraction of samples that contain at least one COTS eDNA molecule (i.e. that yield at least one positive droplet in ddPCR). We focused on two target PP values with ecological significance: PP = 0.4, which corresponds to approximately 7.2 COTS eDNA copies x L^{-1} and is associated with COTS densities of around three per hectare (see correlation with SALAD presented in 3.3.1); and PP = 0.8, which corresponds to approximately 45.0 eDNA copies x L^{-1} and is associated with COTS densities of around 10 per hectare. These density thresholds correspond to important management thresholds in the COTS Control Program. We conducted 100,000 simulations at each combination of sites per reef (L), samples per site (S), and repeats per sample (R) at different model quantiles corresponding to these target PP values. All simulations assumed two repeats (technical duplicates) per sample (R = 2), reflecting the current assay design.

Standard error (SE) was used as our primary measure of precision for each sampling strategy. The simulations allowed us to explore the relationship between sampling effort allocation and statistical precision, with a focus on identifying strategies that could maintain current precision levels while potentially reducing the total number of samples required. We paid particular attention to the relative importance of increasing the number of sites versus increasing the number of samples per site.

Based on the experience of AIMS eDNA field collections and laboratory processing, time estimates for field activities pertaining to eDNA sample collection and processing was completed along with time and cost estimates for laboratory processing of eDNA samples. This information can be found in **Appendix H**. Cost estimates for field collection activities were not included as this is variable depending on vessel type and operating costs. Costs associated with labour were also not included in estimates however time provides a proxy to labour costs.

2.3.3 Testing if mean concentrations are significantly above control values

To determine whether COTS eDNA signals at each site were significantly above background contamination levels, we employed a bootstrap resampling approach (**Appendix E**). This method was selected due to the discrete nature of the data, the prevalence of zero counts in both control and low-concentration site samples, and the hierarchical structure of the sampling design, which made traditional parametric statistical methods inappropriate.

The statistic of interest was defined as the difference in means between control samples and field samples from each site. Our bootstrap procedure was executed as follows:

1. For each trip, control samples were paired with site samples collected during the same trip.
2. For each site comparison, we repeatedly drew samples with replacement:
    - Nc samples were drawn from the Nc controls
    - Ns samples were drawn from the Ns site samples
3. The difference between the means of these two bootstrap samples was calculated for each iteration.
4. This process was repeated 10,000 times to generate an empirical distribution of the difference in means between site samples and their matched controls.
5. From this empirical distribution, we determined whether the observed difference in means was statistically significant by calculating the proportion of bootstrap samples where the site mean was greater than the control mean.

A site was considered to have a COTS eDNA signal significantly above background contamination when the bootstrap analysis indicated that the site samples had significantly higher positive droplet counts than the control samples. Specifically, a significant signal was indicated when:

1. The lower bound of the 95% confidence interval for the difference in means was greater than zero, or
2. At least 95% of the bootstrap iterations showed a positive difference between site and control means.

This approach provides a robust statistical framework for detecting eDNA signals above background contamination levels without requiring arbitrary threshold corrections that could introduce bias.

3. RESULTS

3.1 eDNA monitoring to inform COTS management

3.1.1 Three years of COTS eDNA data in a backbone monitoring

The results of the three-year monitoring are summarised in **Figure 6**, with data presented as the proportion of positive samples on the reef level. In 2021, only one reef (McSweeney Reef) had a high (nearly 100%) proportion of positive samples in the Far North (Cape Grenville). All other reefs in the Cape Grenville region and Princess Charlotte Bay (PCB) region had relatively low values (~<0.4). Some reefs in the Lizard Island area and Batt Reef also had high values. Davies Reef and Bowden Reef, near the edge of the southern distribution of the 4th outbreak, consistently had high values until the end of the study in 2023.

In 2022, we added several more reefs to our eDNA program and surveyed 26 reefs. In that year, all reefs in Cape Grenville and PCB surveyed had high proportions of positive samples (PP > 0.9), suggesting a COTS outbreak was building up in those areas. No large changes occurred on reefs further south compared to the previous year (**Figure 6**).

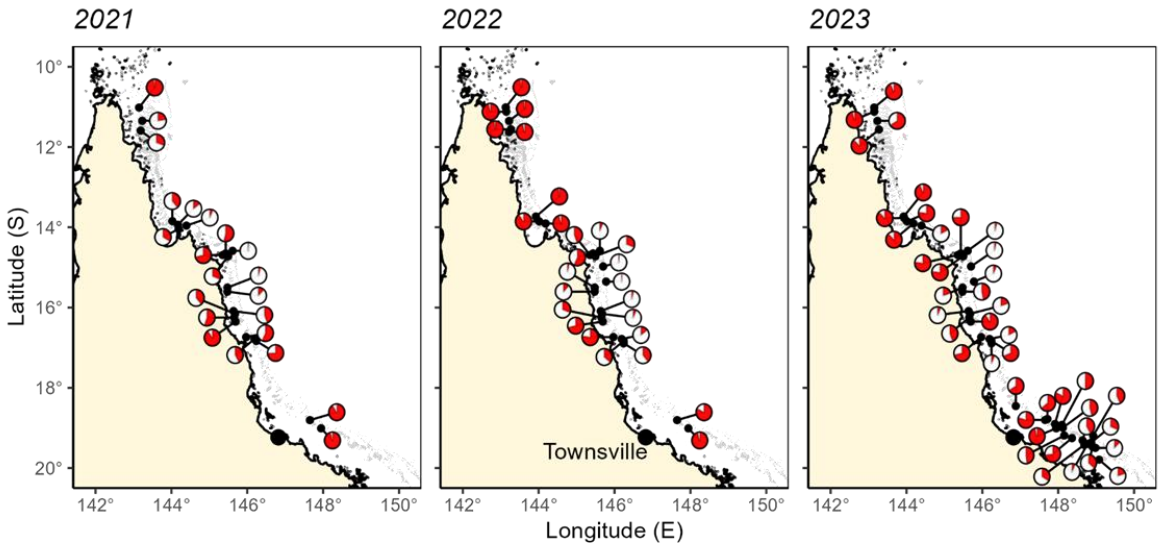

Figure 6. Pie charts indicating the proportion of samples positive (PP) for COTS eDNA (red portion of the pie charts) on reefs of the GBR targeted in the three years of the project.

In 2023, several additional reefs were sampled for a method comparison in CCIP-D-02 (Lawrence et al. 2025b), and reefs collected through QPWS/RJFMP (see below). The proportion of positive samples in far northern reefs (Cape Grenville and PCB) remained high, albeit slightly reduced compared with the previous year. Reefs in the Lizard Island area showed increasing values. In the Cairns area, numbers remained high on Batt Reef, and some other reefs were also showing a build-up. All outer Ribbon reefs (Davie Reef, Yonge Reef, Ribbon Reef No. 9, Ribbon Reef No 5) investigated showed very low proportions of positive samples. Being outer ribbon reefs, the expectation was that the COTS eDNA in these locations would represent a low density, non-outbreaking reef eDNA signal. The proportion of positive samples remained high on most reefs in the Townsville region, whereas reefs to the south of Bowden Reef (i.e. mainly the reefs collected by QPWS, see below) exhibited low proportions of positive samples (**Figure 6**).

3.1.2 Fine scale sampling at Lizard Island, a sentinel location for outbreak initiation

We started eDNA monitoring at Lizard Island in 2019, thus, including samples collected prior to CCIP, we now have five years of data from five locations at Lizard Island with matching SALAD survey data (Uthicke et al. 2024b). We observed an increase in the proportion of positive samples from 2019 to 2023 across Lizard Island (**Figure 7A**). Similar to larger scale patterns observed in Cape Grenville and PCB, the proportion of positive samples averaged for all stations sharply increased in 2023 (**Figure 7A**). This increase was also detectable at each individual station (**Figure 7B**) although samples at the lagoon generally had low values.

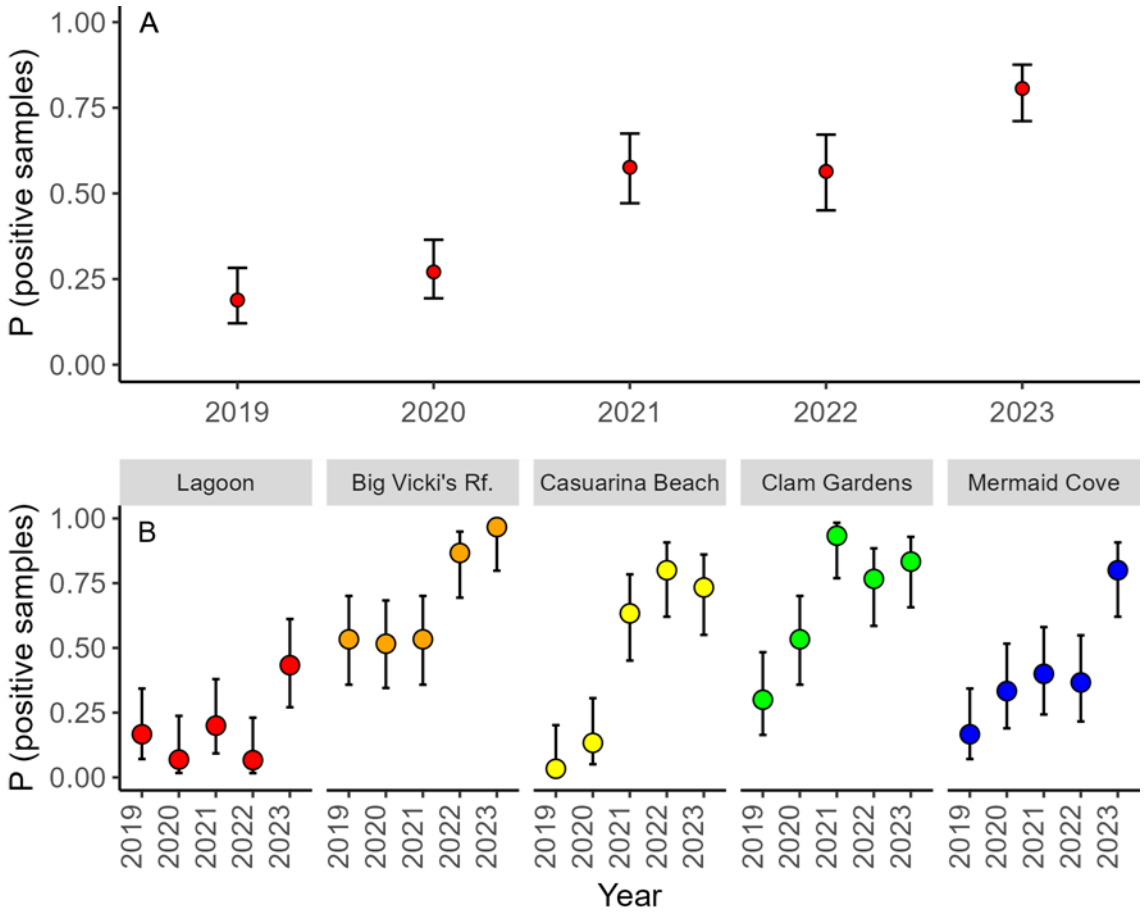

Figure 7. Proportion of positive samples for *Acanthaster* cf. *solaris* eDNA on Lizard Island, **(A)** averaged for five years and **(B)** separated by sampling site for each year. Coloured dots represent model fit and vertical bars represent the upper and lower bounds of the 95% confidence interval.

SALAD data on Lizard Island confirmed the sensitivity of the eDNA method, as COTS densities in 2019 and 2020 were low (< 3 COTS ha^{-1}). We found that the COTS density determined via SALAD and the proportion of positive eDNA samples were correlated, providing an important connection between eDNA and observed COTS densities (Uthicke et al. 2024b). The correlation of eDNA with SALAD surveys are discussed in more detail in subsequent sections, and the CCIP-D-02 (Lawrence et al. 2025b) and CCIP-P-04 (Pratchett et al. 2025) final reports.

3.2 Improve workflow towards operationalising COTS eDNA monitoring

3.2.1 eDNA sample preservation trials

We tested two preservation methods ('Standard method' and self-preserving filters) for eDNA over a six-month period. In general, both methods preserved most of the eDNA over this time period. A Generalised Additive Model (**Figure 8**) illustrates that the standard method is more effective than the self-preservation method; there was significantly (p = 0.0013) slower DNA degradation when using the standard method. While for the first 80 days of the study the 95% confidence intervals of the DNA overlaps, the difference increases towards the end of the trial. By day 180 the self-preservation method shows a decline in DNA concentration 4 times larger than the one for the standard method (32% to 8% respectively).

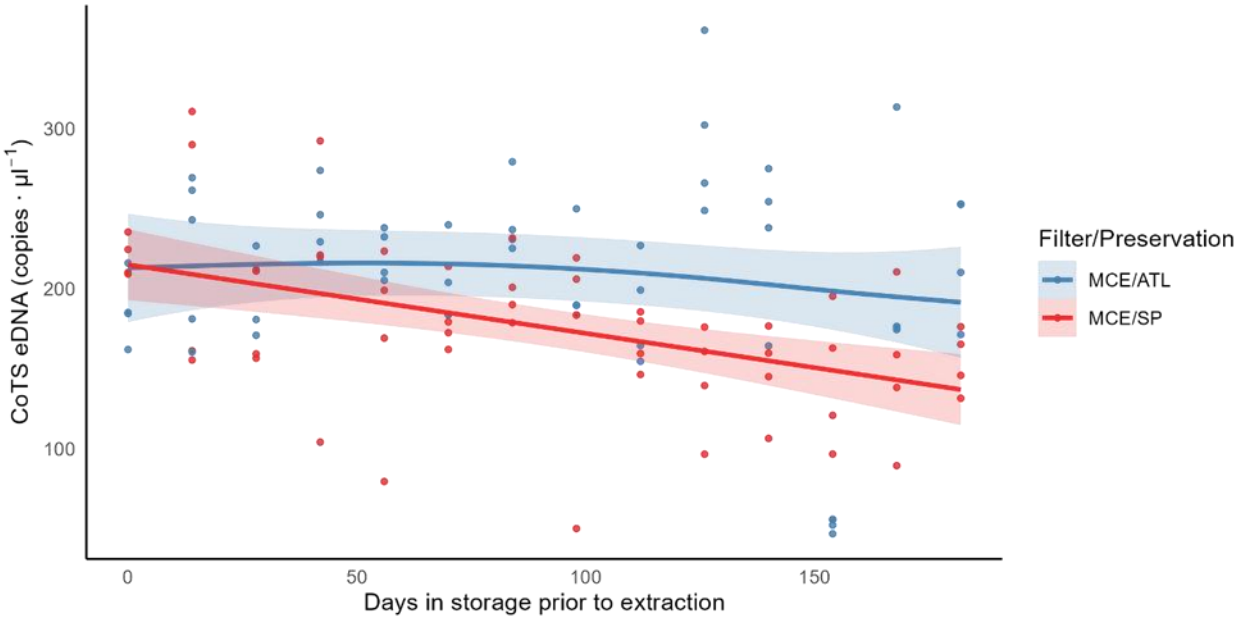

Figure 8. eDNA preservation efficacy of the two methods over time. Lines represent the GAM predicted concentration of COTS eDNA over time. Ribbons show 95% confidence interval. Concentration from individual filter samples shown as dots. Blue indicates samples stored in ATL for the duration of the experiment, red indicate samples stored in self-preserving housings.

3.2.2 Field comparison of four different collection/preservation methods

In addition to laboratory tests, we trialled three different preservation methods for filtration based eDNA collections and a passive sampling method in the field.

Amongst the filtration methods, we detected a statistical difference across the three different filtration eDNA capture methods tested (χ^2 = 18.59, p <0.0001), which was due to the 'Standard method' capturing and preserving more COTS eDNA on average compared to either of the desiccation methods (p < 0.05, **Figure 9, left panel**). The mean ± SE COTS eDNA captured by the 'Standard method' (113.2 ± 15.1 copies L^{-1}) was approximately double that captured by commercially available self-preserving filters (61.1 ± 20.6 copies L^{-1}), and four times that captured by standard filter housing with desiccant pouches (26.1 ± 6.5 copies L^{-1}) (**Figure 9**). Except for one of the replicates from the standard filter housing with desiccant pouches, all replicates from the filtration methods contained detectable COTS eDNA. Thus, the proportion of positive samples (PP) was close to 1 for all filter samples.

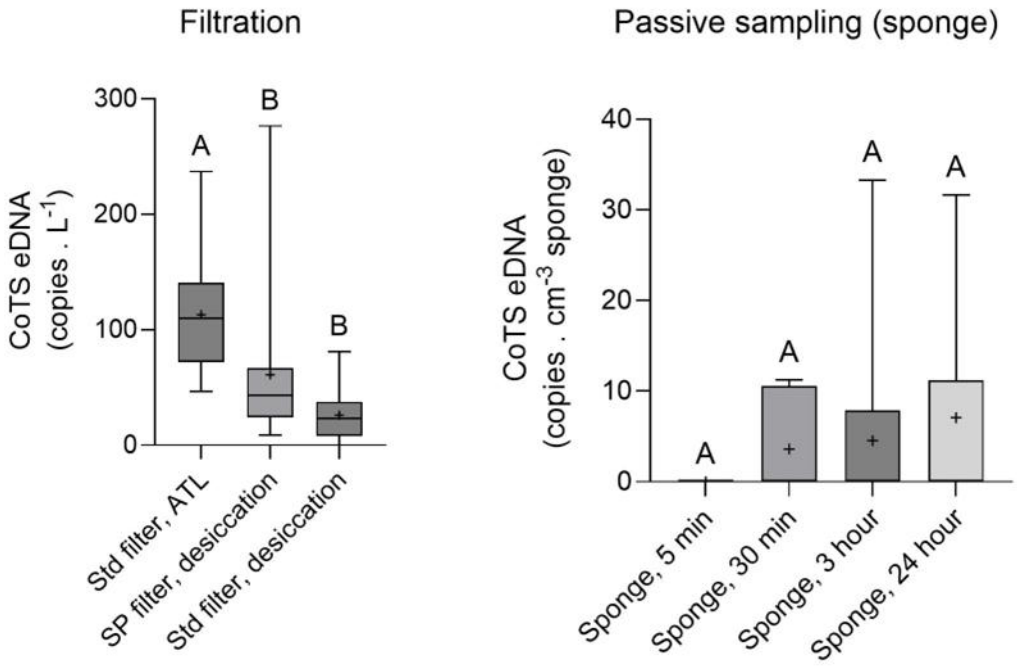

Figure 9. Total COTS eDNA copies per capture method in three filtration-based collection methods (left panel) and passive collection using Whirl-Pak® Speci-Sponges® (right panel). The limits of the boxes are the interquartile range (25% and 75% interquartile), minimum and maximum are represented by the whiskers, the median and mean is the horizontal line and '+' within the box respectively.

Passive sampling was trialled using artificial sponges. Sponges soaked for 5 minutes did not capture any detectable COTS eDNA (**Figure 9**, **right panel**). Sponges soaked for 30 minutes, 3 hours and 24 hours however captured COTS eDNA with mean ± SE of 3.6 ± 1.5, 4.5 ± 2.9 and 7.1 ± 3.5 copies per cm^{-3} of sponge respectively. However, likely due to the high variance, there was no significant difference in eDNA concentration with different soak times (χ^2 = 4.94, p = 0.176) (**Figure 9, right panel**)**.** Due to the different sampling units, it is difficult to compare filtration methods and sponge results when eDNA concentration is considered, but concentrations were higher and less variable in the filtered samples. Four of the replicate sponges (n = 12 for each soaking time) soaked for 30 minutes, three of the sponges soaked for 3 hours and four of the sponges soaked for 24 hours were positive. Thus, overall, the proportion of positive samples for sponges soaked longer than 30 min was about 0.3.

3.2.3 Training non-scientists in sample collection on 'ships of opportunity'

Moore Reef 'Marine World', October 2022

Training was successfully provided to pontoon staff. The trials at the Moore Reef 'Marine World' pontoon revealed all controls (n = 4) conducted by AIMS staff were negative whereas three of the four controls conducted by Marine World staff were positive for COTS eDNA, albeit at a low level (a single positive ddPCR droplet in each of the 3 negative controls). Three of the 12 samples processed by AIMS staff were identified as positive for COTS eDNA whereas five of the 12 samples processed by Marine World staff were positive. Although at the low end of detection, the concentration of COTS eDNA was three times more in the Marine World collected samples compared to the AIMS collected samples (**Table 1**). It is uncertain how the controls returned a positive result for COTS eDNA. All

positive detections across samples and controls were at the low end of detectability, therefore it is possible that even the slightest deviation from clean operating procedures influenced the outcome. This exercise demonstrated the significance of appropriate training and clean area preparation.

Table 1. Summary of non-scientist eDNA collection activities conducted in 2022 with Marine World staff at Moore reef tourist pontoon and Pacific Marine Group staff from the COTS control vessels Odyssey.

eDNA Collection trial	Date	Location	Sample/ Control	No. collected	No. positive	COTS eDNA copies x L^{-1} (mean ± SE)
Moore Reef 'Marine World', October 2022	24-Oct-22	Moore reef	S[a]	12	5	5.9 ± 3.3
			C[a]	4	3	14.9 ± 5.0
			S[b]	12	3	2.0 ± 1.0
			C[b]	4	0	0
Pacific Marine Group, Odyssey. May 2022[c]	3 to 09 May-22	Main ship	C	23	1	0.9 ± 0.9
	3 to 09 May-22	Tender	C	13	4	22.0 ± 11.1
Pacific Marine Group, Odyssey. October 2022[d]	7-Oct-22	Lynchs reef	S	5	5	1299.8 ± 655.6
	22-Oct-22	Hopkinson reef	S	6	6	691.2 ± 603.2
	26-Oct-22	Lynchs reef	S	12	12	350.0 ± 67.9
	07, 22 & 26/Oct/2022	Hopkinson & Lynchs reef	C	8	5	81.9 ± 31.4

[a] Collected by Marine World staff; [b] Collected by AIMS staff; [c] Testing controls only while culling at Lynchs & Keeper reef, [d] High COTS density reefs

Pacific Marine Group, Odyssey. May 2022

A total of 36 negative controls were collected by Pacific Marine Group staff with 23 controls collected at various locations around the main ship and 13 controls processed on the tender during culling operations. Only one control conducted on the aft deck out of the 23 processed around the main ship returned positive, whereas 4 out of the 13 controls processed on the tender returned positive for COTS eDNA. The concentration of COTS eDNA was approximately 20 times higher in controls obtained from tenders compared to those conducted around the main ship (**Table 1**). These results indicate that tenders are a potential source of inadvertent contamination of COTS eDNA.

Pacific Marine Group, Odyssey. October 2022

Odyssey staff collected field eDNA samples and controls in October 2022. Of the eight controls collected during this trip, five contained measurable amounts of COTS eDNA with a concentration of 81.9 ± 31.4 (mean ± SE) COTS eDNA copies L^{-1}. All field samples collected from Hopkinson reef and Lynchs reef contained high amounts of COTS eDNA relative to the controls, with concentrations ranging from 350.0 ± 67.9 at Lynchs reef on the 26th Oct 2022 to 1,299.8 ± 655.6 at Lynchs reef on the 7th Oct 2022.

Reef Joint Field management Program (RJFMP). November 2023

Communications with QPWS and GBRMPA identified that collecting eDNA as part of the RJFMP is one of the most promising options to increase the number of reefs that can be monitored for early detection. Discussions with RJFMP staff suggested the collection of

eDNA samples fitted well with the routine operations of the field team, and required approximately two hours per reef. This timing compares to the timing on AIMS research trips. The proportion of positive samples (PP) at each reef ranged from 0.06 at Gould reef No. 1 to 0.43 at Jacqueline reef (data incorporated in three-year results, section 3.1) indicating general low densities of COTS. Regression analysis of eDNA data with SALAD data (see section 3.3.1) demonstrates that a proportion of positive eDNA samples of 0.4 corresponds to an observed COTS density near the Allee threshold of three COTS ha^{-1}. It is possible therefore that some of the reefs with the highest numbers detected on this trip may have the beginnings of COTS population build ups. All reefs on this voyage except for U/N Rf 19-065 were surveyed using manta tow. No COTS were observed during manta tows at any of the reefs surveyed. Feeding scars were identified during two manta tows at Bait reef, one manta tow at Gould reef No. 1 and during three manta tows at Jacqueline reef. All field controls (n = 16) conducted for this trip were found negative for COTS eDNA, highlighting the eDNA training and collection workflow was successful. These results demonstrate the successful translation of eDNA sampling within the operational environment of the RJFMP for monitoring of low COTS density reefs.

3.3 Evaluating and enhancing sampling protocols and data analysis

3.3.1 COTS density and eDNA

The regression for observed COTS densities from in-water SALAD surveys against the eDNA proportion of positive samples was significant ($p < 0.001$) with 53% of the variance being explained (**Figure 10**). The confidence intervals (CI) represent the uncertainty around the mean prediction. (We note that if we were to plot the prediction interval, where we would expect approximately 95% of future observations to fall, it would be wider than the CI).

The model assists to translate eDNA monitoring data into approximate COTS SALAD density equivalents to identify management relevant metrics. For example, for observed densities at proportion of positive samples of 0.4 the mean SALAD density prediction is approximately 3.5 (lower CI = 2.7, upper CI = 4.5) COTS ha^{-1} (Allee threshold, Rogers et al. 2017). At a proportion of positive samples of 0.8 the mean SALAD density prediction is approximately 8.9 (lower CI = 7.0, upper CI = 11.3) COTS ha^{-1}, approximately the lower limit of current outbreak description threshold (Keesing & Lucas 1992).

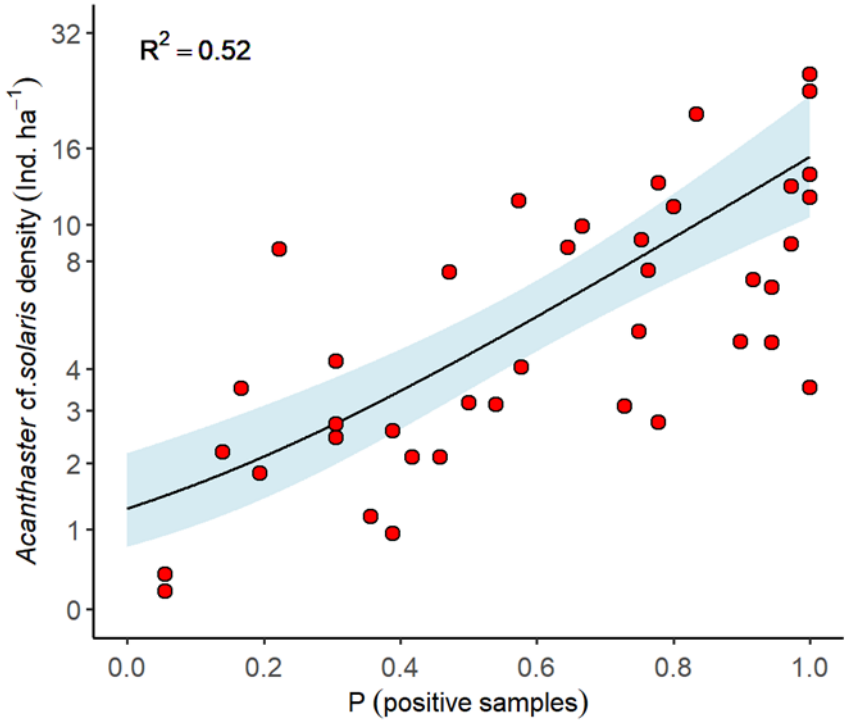

Figure 10. Linear model of proportion of positives from eDNA data (predictor) and COTS densities observed using SALAD surveys (response variable). Red points are the raw data and estimated predicted mean from the linear model (solid line) with associated confidence intervals (blue area).

3.3.2 Sampling design analysis

Based on the estimated relationship between observed COTS density via SALAD surveys and eDNA (section 3.3.1) we simulated standard errors (SE) for two proportion positive levels: PP = 0.4 and PP = 0.8, corresponding to observed densities of ~3 COTS x ha^{-1} and ~10 COTS. ha^{-1} respectively for different numbers of sites per reef and samples per site (**Appendix D**, **Figure 11** and **Figure 12**). Regardless of the PP value, there is a sharp peak in SE at one site per reef and one sample per site (L = 1, S = 1) from which errors initially decline rapidly for additional sites and/or samples. The standard error declines more rapidly with the addition of sites, compared to samples. This indicates there is more value in increasing sites per reef than increasing samples per site.

Based on a cost benefit analysis presented in **Table 2**, several options can be considered for an optimised sampling design when considering error. For example, sites per reef (L) and samples per site (S) may be traded off to achieve the same error which is graphically represented by the "iso-error" line in **Figure 11** and **Figure 12**. These simulations suggest that the current sampling strategy (36 samples, L = 3, S = 12), while effective for the desired outcome, could be improved in terms of the total number of samples required to achieve the same precision. For example, other combinations that achieve the same error as the current sampling strategy require only 24 (L = 4, S = 6), 20 (L = 5, S = 4), or 18 (L = 6, S = 3) samples (**Table 2, Appendix H**). The biggest gain in sampling efficiency (i.e. reduction in total samples) was achieved by adding one site per reef and halving the number of samples per site (L = 4, S = 6). The sampling option with the smallest error presented in **Table 2 (Appendix H)** was 12 sites and 3 samples per site, however there is a large increase in field time required to collect from 12 sites therefore this scenario is likely to remain theoretical.

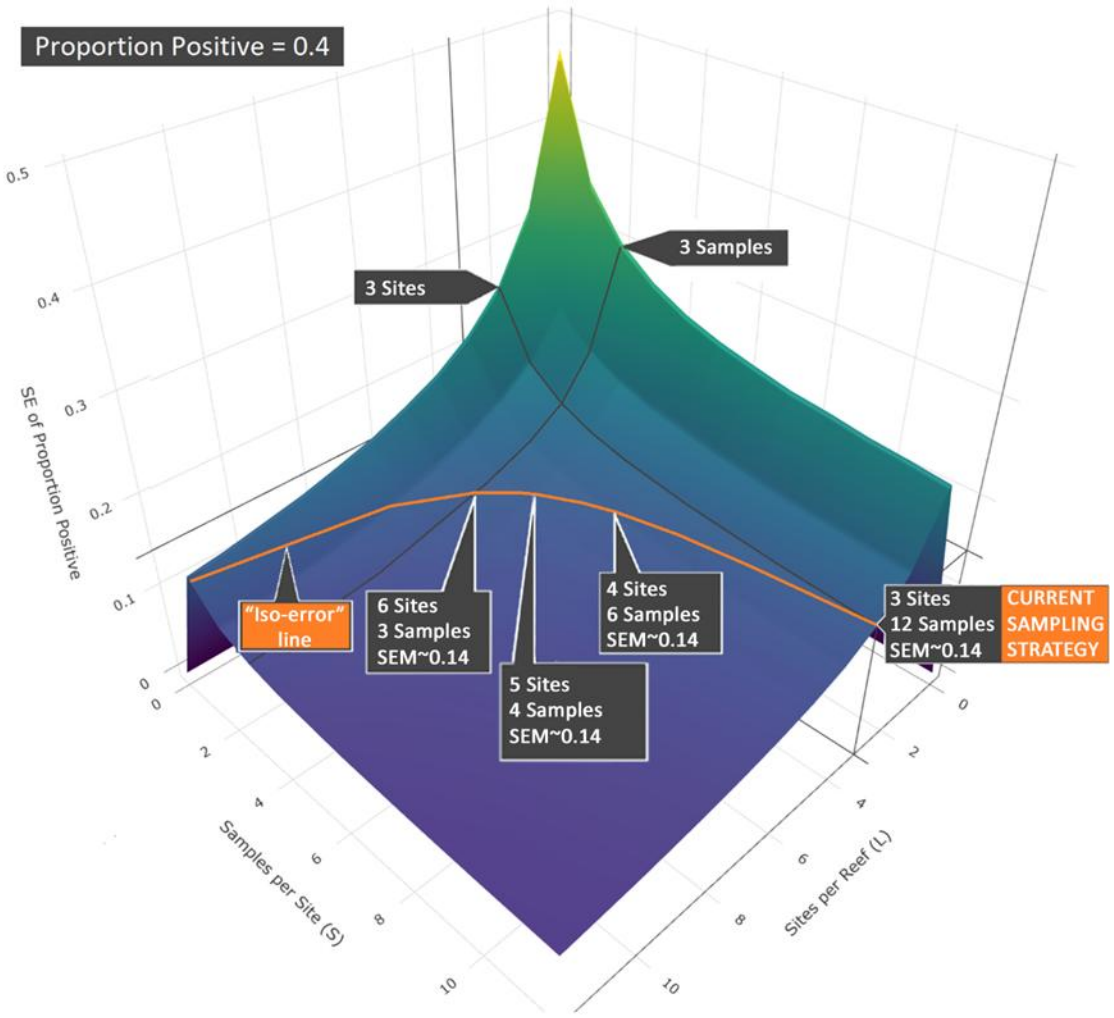

Figure 11. Three-dimensional surface plot showing the relationship between sampling effort (sites per reef (L) and samples per site (S)) and standard error of proportion positive (0.4). The orange "iso-error" line connects sampling design combinations that yield equivalent precision (SEM ~0.14). Various sampling strategies are highlighted, including the current approach (3 sites, 12 samples per site) and alternative configurations requiring fewer total samples while maintaining statistical power. The gradient coloration represents the magnitude of standard error, with cooler colours (blue/purple) indicating lower standard error values.

Alternatively, a lower error can be achieved by maintaining the total number of samples collected under the current sampling strategy (36 samples, L = 3, S = 12). For example, L = 4 / S = 9, L = 6 / S = 6 and L = 12/ S = 3 provide a 10%, 20% and 30% improvement in error respectively with no change in total samples required (**Table 2, Appendix H**).

When we consider reducing both laboratory costs and field time in addition to reducing error, several options also present. For example, collecting eDNA samples from 5 sites per reef and 6 samples per site results in a 12% improvement in error, a 17% reduction in laboratory time and cost, and a 3% reduction in field time required to collect samples. Alternatively, a sampling strategy that has 4 sites per reef and 8 samples per site results in a 7% improvement in error, an 11% reduction in laboratory time and cost, and a 5% reduction in field time required to collect samples (**Table 2, Appendix H)**.

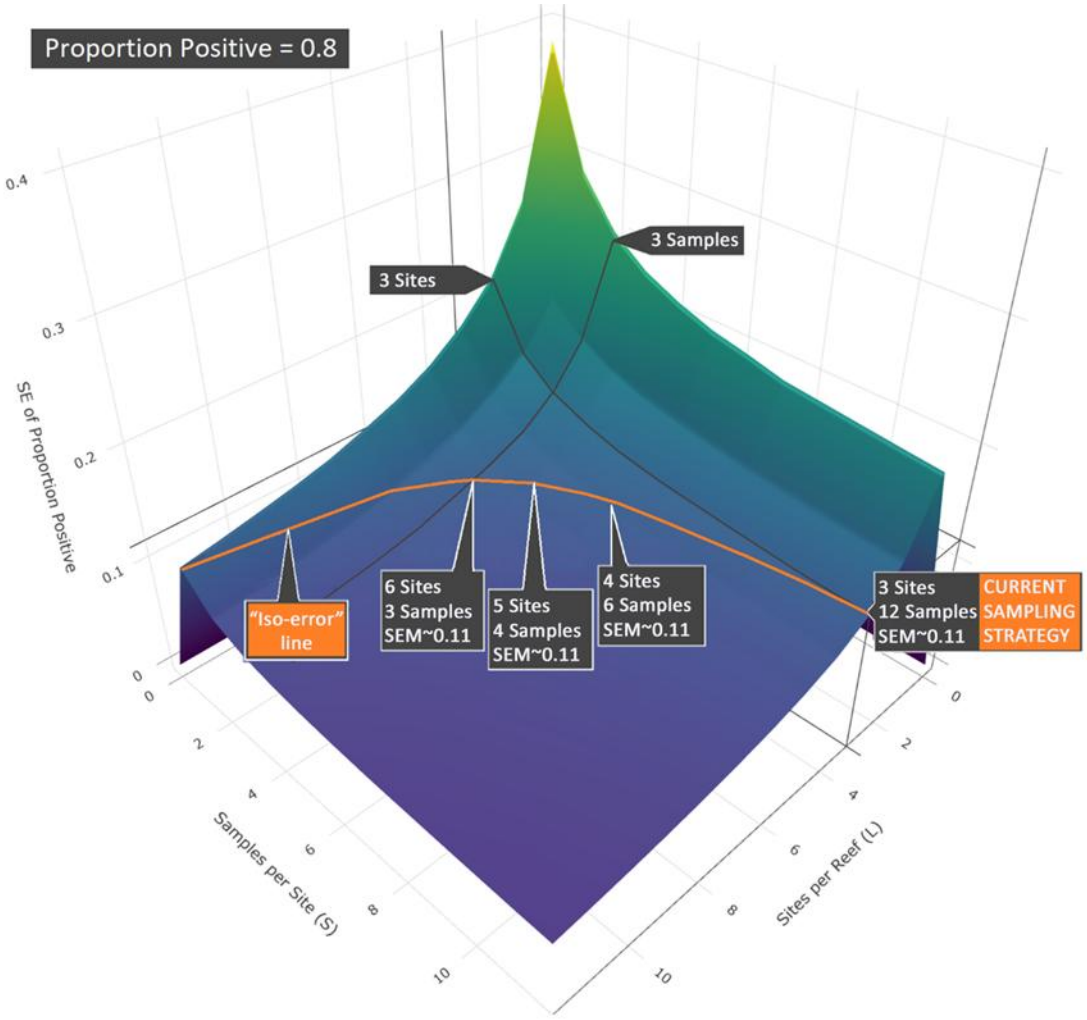

Figure 12. Three-dimensional surface plot showing the relationship between sampling effort (sites per reef (L) and samples per site (S)) and standard error of proportion positive (0.8). The orange "iso-error" line connects sampling design combinations that yield equivalent precision (SEM ~0.14). Various sampling strategies are highlighted, including the current approach (3 sites, 12 samples per site) and alternative configurations requiring fewer total samples while maintaining statistical power. The gradient coloration represents the magnitude of standard error, with cooler colours (blue/purple) indicating lower standard error values.

This analysis is focused on the reef level, but selection of a sampling strategy that increases the number of sites per reef may provide information that also increases the resolution of eDNA detection to the site scale. However, a different sampling regime would need to be implemented if site specific information was required.

Table 2. Estimated standard errors, times and costs for various combinations of sites per reef (L) and samples per site (S) at the modelled proportion positive of 0.4 (~3 COTS ha^{-1}) and 0.8 (~10 COTS ha^{-1}). The yellow highlighted row is the current sampling strategy used for this project, from which data was modelled. Rows highlighted in green are sampling combinations on the "iso-error" line depicted in **Figure 11** and **Figure 12** that have similar errors to the current sampling strategy. Rows highlighted in blue are sampling combinations that maintain the total sample number as for the current sampling strategy. Rows that are indicated with an asterisk (*) indicate sampling strategies with overall improvement (lower error, lab cost and field time) compared to the current sampling strategy

Sites/ Reef	Samples/ Site	Total Samples	Field Time (est. min)	Lab Time (est. min)	Analysis consumable cost (approx. $AU/ Reef)	Standard Error (PP = 0.4)	Standard Error (PP = 0.8)
3	12	36	138	468	1,476	0.138	0.115
*4	*6	24	112	312	984	0.136	0.112
4	9	36	148	468	1,476	0.125	0.104
*5	*4	20	110	260	820	0.134	0.111
*6	*3	18	114	234	738	0.134	0.110
6	6	36	168	468	1,476	0.111	0.092
12	3	36	198	468	1,476	0.095	0.078

The current sampling strategy of 3 sites per reef and 12 samples per site on average enabled statistical separation of PP values of 0.4 (~low COTS density) and 0.8 (~intermediate density). By targeting a similar or smaller error as achieved in the current sampling design for PP 0.4 (0.138) and considering cost and effort, the following sampling strategies for detection of COTS at reef level for low COTS density and intermediate COTS density are recommended:

a) low level, ~3 COTS ha^{-1} (PP = 0.4)

- 4 sites, 6 samples per site, total 24 samples (SE = 0.136)
- 5 sites, 4 samples per site, total 20 samples (SE = 0.134)
- 6 sites, 3 samples per site, total 18 samples (SE = 0.134)

b) intermediate level, ~10 COTS ha^{-1} (PP = 0.8)

- 3 sites, 5 samples per site, total 15 samples (SE = 0.135)
- 4 sites, 3 samples per site, total 12 samples (SE = 0.134)
- 5 sites, 3 samples per site, total 15 samples (SE = 0.121)

However, it should be noted that reducing samples at the site level, further reduces the inference which can be drawn on that level. If reducing laboratory costs is of lesser concern, the following scenarios for low levels are attractive because they keep the current sampling regime (option a) or decrease error further, thus making the assay more sensitive (b, c, d) while keeping a high level of site replication.

a) 3 sites, 12 samples per site, total 36 samples (SE = 0.138) (main advantage: continuation of current design).
b) 4 sites, 9 samples, total of 36 samples (SE = 0.125)
c) 5 sites, 8 samples, total of 40 samples (SE = 0.115)
d) 6 sites, 6 samples, total of 36 samples (SE = 0.111)

Given reefs surveyed vary in size, a sampling strategy with flexible site numbers based on reef size could also be considered. Further consideration should be given after piloting suggested improved monitoring design.

The sensitivity of eDNA requires a high quality of sampling effort, and care needs to be taken not to contaminate samples. Hence, we consider collections from research vessels and QPWS vessels as most suitable. However, training for other ships of opportunity and citizen scientists can easily be achieved. For COTS control vessels or other operators involved in handling or culling COTS, we recommend the collection of a larger amount of negative control samples to analyse for possible contamination.

3.3.3 Testing if mean concentrations are significantly above control values

Analysis of eDNA samples across the majority of reef sites demonstrated that the current False Positive Correction (FPC) method produced consistent and reliable results for management decision-making. As shown in **Table A 10** and **Table A 11** (**Appendix E**), most reef-trip combinations maintained their status relative to decision thresholds after correction. Sites with substantial eDNA signals, including McSweeney Reef (PP = 1.00), Corbett Reef (PP = 0.97), and multiple sites from the JCU trip, showed minimal change in their proportion positive values after the correction process was applied. This consistency across most sites suggests that at current contamination levels, the specific correction approach has limited practical impact on management decisions for reefs with clear signals.

The bootstrap approach presented in **Appendix E** (**Table A 12**) provides a statistical method for determining COTS presence that accounts for background contamination in a systematic way. For the vast majority of sites (>90%), the approach of not correcting samples for controls and bootstrap testing if measured values are above the control yielded the same outcomes as the current approach, with clear statistical significance levels allowing for confident management decisions. However, the current FPC approach significantly reduces the PP in some instances, as seen at North Direction Island Reef where one site (NDEDNA2) dropped from 0.42 to 0.08 after correction (**Table A 12**), pushing the entire reef below the PP = 0.4 threshold associated with 3 COTS ha^{-1}. The bootstrap analysis (p = 0.124) confirms this signal is not significantly above background noise, suggesting both approaches would lead to the same management decision if the controls accurately represent contamination. This highlights the tension between statistical rigour and risk management in COTS monitoring.

4. DISCUSSION

The overarching aims of this project (CCIP-D-03) were to conduct eDNA monitoring to inform COTS management and to improve the workflow towards operationalising COTS eDNA monitoring, using field trials, training and statistical modelling.

The specific aims of the project were:

- Aim (i) Collection and analysis of eDNA data from representative GBR reefs ('backbone reefs') over three years. These reefs were mainly located in the initiation zone, and many of these were in parallel to AIMS Long-Term Monitoring Program Reefs or have a known COTS history.
- Aim (ii) Continue data collection at a sentinel station on Lizard Island and test small scale patterns.
- Aim (iii) to provide recommendations on how eDNA methods can be used as a monitoring tool as part of a comprehensive COTS monitoring strategy
- Aim (iv) provide a mapped-out workflow and expected timeframes for sample collection

4.1 eDNA monitoring to inform COTS management

Long term sampling of COTS eDNA over three years and eDNA analysis on up to 42 reefs per year was achieved in this project. This included the training and successful collection of eDNA samples by the QPWS/RJFMP in November 2023, and numerous staff from multiple organisations involved in the March 2023 CCIP-D-02 (Lawrence et al. 2025b) calibration voyage. Data were reported to GBRMPA for incorporation into the GBRMPA dashboard, assisting with reef prioritisation for culling. Analysis presented here unambiguously demonstrated that eDNA is a suitable monitoring tool to detect and quantify very low densities of COTS.

Monitoring of COTS densities using eDNA was demonstrated to be a quantitative, non-visual method that provides high sensitivity, reef level detection of low-density COTS populations that has a good correlation with SALAD surveys (see also: Pratchett et al. 2022; Lawrence et al. 2025b). Thus, the aim to develop an early detection method based on eDNA was achieved.

By combining previous data from 2019 and 2020 with data from the backbone reefs and others analysed in the current CCIP project (2021–2023), we were able to identify an increase in the COTS eDNA at Lizard Island, congruent with observed increases in COTS densities via SALAD surveys over the same time (**Figure 10** and Pratchett et al. 2025). This also extended to small scale patterns around Lizard Island with most locations experiencing the same increasing trend in measured COTS eDNA from 2019–2023 (Chandler et al. 2023). The continued monitoring of Lizard Island as a sentinel site will be critical going forward to ensure early warning tools such as eDNA provide timely information for on-water decisions.

The detection of an early outbreak in areas of Lizard Island and other reefs to the north provided strong support for the maturity of the method and sampling regime, and provide important data to inform COTS outbreak response. Both eDNA and SALAD clearly suggest that a new outbreak wave seems to have initiated in 2021/22 in the entire area from Cape Grenville to Lizard Island (Pratchett et al. 2022; Chandler et al. 2023; Uthicke et al. 2024b). However, size measurements at Lizard Island suggested that multiple recruitment pulses

contributed to the new outbreak (Chandler et al. 2023). It should also be noted that COTS outbreaks have been reported in the Torres Strait (AIMS 2022).Given the closeness of the Torres Strait to Cape Grenville reefs, it cannot be excluded that those outbreaks are connected.

4.2 Improve workflow towards operationalising COTS eDNA monitoring

Determining the relationship between COTS densities (as determined by SALAD) and eDNA results was critical to this project. We found that eDNA as a proportion of positive samples was an effective predictor of COTS densities at levels well below those measurable by standard manta tow monitoring. The combination of SALAD and eDNA now provides a solid foundation as a monitoring tool for early warning of COTS outbreaks. Subsequently, we modelled scenarios to determine optimal eDNA sampling regimes that had improvement in detection error, time and analysis cost.

We developed an optimal workflow for collecting eDNA samples (details in Section 7, **Appendix B**), which allowed samples to be delivered directly into an efficient laboratory workflow (**Appendix F, Appendix G**) to analyse each for COTS eDNA.

Initial trials at the Moore Reef 'Marine World' pontoon showed that deviations from clean operating procedures may cause contamination in negative controls. This demonstrated the significance of appropriate training and clean area preparation and prompted a revision of workflow requirements to undertake eDNA sampling.

Initial discussion with QPWS and GBRMPA identified that collecting eDNA as part of the RJFMP is one of the most promising options to largely increase the number of reefs which can be monitored for early detection. Subsequently, the standard eDNA collection workflow was successfully achieved on board two different QPWS vessels, the *Reef Ranger* and *Reef Resilience*. Results demonstrated that we were able to identify low density COTS populations in eDNA collections from these trips. Indeed, some reefs in the Whitsunday region were already reaching COTS densities (based on SALAD – eDNA correlations) that may indicate a build-up of COTS. These reefs were also surveyed using manta tow and returned zero sightings, highlighting the utility of eDNA as an early warning monitoring method in this context.

We also worked with Pacific Marine Group (a Townsville based COTS control contractor) to trial eDNA collections during culling voyages from their vessel, *Odyssey*. Although we detected some carryover of COTS eDNA into controls (and hence actual samples) mainly in controls collected on the tender, we were able to successfully implement the workflow on board. These results demonstrate that the main ship provides a relatively clean environment to process eDNA samples, whereas the tenders have higher chance of introducing background contamination to test samples. Because samples must be collected from the tender, further research is required to ensure operationalisation of monitoring from COTS control vessels. In addition to these results, operational information was discussed as part of a debrief, particularly:

- The timing of conducting eDNA sampling from a COTS control tender would be critical as COTS cull divers returning in dripping wetsuits pose a higher chance of contaminating eDNA samples, and,
- Live boating is more common than anchoring a tender. During live boating, the driver of the tender is unable to divert attention away from diver monitoring to conduct eDNA sampling, thus for eDNA sampling activities to occur, additional resource

management from the team is required (e.g. extra non-diving person on board tender, delay diving to conduct eDNA sampling, etc.).

These results demonstrate that it is possible for COTS control vessels to collect eDNA samples, however, there is an increased risk of contamination of samples leading to false positive determinations. Consideration of the use of eDNA and respective controls from COTS control vessels is required to ensure results can be correctly interpreted. The modelling results suggest that minor contamination in eDNA samples collected on COTS vessels do not hinder correctly determining eDNA concentration if sufficient control samples are collected (see section 3.2.3, also see **Appendix D**).

The development and testing of alternative eDNA collection and preservation methods was integral to this project and linked closely with the transfer of the method to on-water Operators. We confirmed that collection of eDNA samples via commercially available self-preserving filters is possible. The use of self-preserving filter housing also eliminates workflow steps (e.g. removal of the filter on the main vessel and transfer to preservation buffer) that may introduce cross contamination compared to our 'Standard method'. However, self-preserving housings are single use items and have an on-going cost of approximately $46 per sample collection. This is nearly seven times the cost per sample than eDNA samples collected and preserved via our 'Standard method'. Using the latter method, used filter housings can be bleach cleaned and re-used multiple times, resulting in an approximate cost of $6.70 per sample. The self-preserving filter housing is produced from a biodegradable plastic, and we found in conditions where eDNA sampling resulted in the filter housing being regularly submerged (waves, swell, etc.), the self-preserving filter housing tended to begin the degradation process and clog the filter membranes. Both methods allowed for the laboratory processing of samples at later date. Samples stored in self-preserving filter housing enable stable preservation of captured eDNA for up to 84 days (approximately three months), while samples preserved using our 'Standard method' remained stable for longer periods (up to six months). Degradation trials were not conducted with the third filter method tested (standard filters and desiccation). Although we detected less COTS eDNA on those filters than with the standard method, results showed some promise and further testing may be warranted.

We also trialled a passive eDNA sampling method that utilises artificial sponges (Jeunen et al. 2022) and found that even though our 'Standard method' is the optimal method for COTS eDNA capture, soaking these sponges in the water for a minimum of 30 minutes also captured COTS eDNA. However, variation between replicates was high and a lower proportion of samples was positive with the method trialled. However, upon further developments this passive sampling method could still be useful providing a simple way to obtain eDNA samples whilst other operational activities are underway.

4.3 Evaluating and enhancing sampling protocols and data analysis

Data collected for the present project and previous projects were used in statistical modelling to i) provide a calibration between eDNA and SALAD derived COTS densities, ii) improve sampling design by testing if increased site numbers or replicates within sites are beneficial, and iii) propose a test for the presence of COTS at a site in case of positive droplets in control samples.

We demonstrated that > 50% of the variance in eDNA (using the proportion positive metric) was explained by COTS densities. The function and proportion of variance explained is

similar to previously published data based on lower sample numbers (Pratchett et al. 2022; Uthicke et al. 2024b). Thus, eDNA can be approximated to COTS densities to improve the utility for management decisions, based on thresholds in a similar way to the current use of manta tow. In general, lower densities (~< 10 ha^{-1}) are best estimated using the proportion positive (PP) metric. At higher densities, this metric saturates (i.e. most values are 1 because all filters have detectable COTS eDNA), and the average eDNA copy number becomes a better estimator for density (see **Appendix C**). Thus, combined interpretation allows estimation if densities are above or below pre-determined threshold values.

Modelling data under two different scenarios (proportion of positive equals 0.4 or 0.8, i.e. values corresponding to important reproductive and ecological threshold values for COTS densities), provided valuable insights, and a cost-benefit analysis allowed proposing an optimised sample design where reduced field and laboratory costs yielded improved efficiency. In general, increasing the number of sites is more beneficial that increasing the number of replicates per site. Our initial sample size of 3 sites x 12 samples can be regarded as very high, and alternative strategies (e.g. 4 sites x 6 samples) yielded the same standard error with 33% less samples to be analysed.

The current low levels of contamination have little impact on inference about reef COTS densities based on eDNA proportion of positives. It is not clear how to adjust for this contamination without introducing bias and statistical issues, nor is it clear how this contamination may be attributed to procedures in the workflow. Until further development, it is recommended to continue using the current method of false positive correction. While the correction appears statistically justified, if management are risk-averse they still may want to investigate such sites where potentially meaningful signals are being reduced. These decisions ultimately depend on the relative costs of false positives versus missed early detections in the COTS Control Program. We recommend that control samples are routinely collected. eDNA activities occurring on COTS control vessels or other platforms with a high risk of COTS DNA contamination, should increase the number of controls allowing for more elaborate correction procedures. This approach should be tested in future projects.

5. CONCLUSIONS

The eDNA methods developed and operationalised here are important, complementary, new tools enabling early detection of COTS population increases, thereby allowing for early and effective intervention through targeted population control. On Lizard Island and in other samples, we demonstrated that eDNA detects COTS outbreaks prior to manta tows. Thus, using eDNA as an early detection method ‘buys time’ for interventions like culling. During the timeframe of this project, we have highlighted results from a ‘backbone’ sampling of reefs from Lizard Island to the Townsville region. We were able to extend this monitoring to both Princess Charlotte Bay and Cape Grenville through collaboration with CCIP-P-04 (Pratchett et al. 2025), resulting in novel insight about the initiation of new outbreaks. Early detection of population outbreaks is crucial to improve understanding of the factor(s) that contribute to the initiation of recurrent outbreaks on the GBR. For instance, to evaluate whether recruitment enhancement through increased runoff (Birkeland 1982; Brodie et al. 2005) or oceanographic events play a significant role in the initial population build-up (Wooldridge & Brodie 2015), it is important to pinpoint the start of these outbreaks in time and space. Similarly, to understand the role of predation on juveniles (Balu et al. 2021; Desbiens et al. 2023; Wolfe et al. 2025b) and adult COTS (Cowan et al. 2020; Caballes et al. 2022) it is necessary to quantify predator densities and understand predation pressure on reefs where COTS populations begin and continue to build, as opposed to reefs with well-established

populations of adults. Overall, the analysis presented here unambiguously demonstrates that eDNA is a suitable monitoring tool for early detection of COTS outbreaks.

6. RECOMMENDATIONS FOR IMPLEMENTING EDNA MONITORING

Monitoring COTS using eDNA surveys is a method that has been developed primarily for integration within an early warning system. However, this method is versatile and can be adapted to other operational applications (see **Table 3**). These include presence and absence detection only, or quantification at higher levels. We demonstrated that with appropriate preparation and training, eDNA collection can be successfully undertaken within an operational setting. We recommend incorporation of eDNA surveys as part of a broadened COTS monitoring toolbox to contribute to proactive planning for reef prioritisation.

With regards to early detection monitoring, we recommend the following actions:

- Continuation of eDNA monitoring at backbone reefs identified within this report, with a particular emphasis on the continued monitoring of reefs in the far northern sector. Consultation with the monitoring design sub-project CCIP-D-01 (Lawrence et al. 2025a) will be critical to provide the most effective application of COTS eDNA monitoring. Practically, this can be achieved through a combined effort involving, for example, research agencies such as AIMS, COTS control vessels, the Reef Joint Field Management Program, collection based on Research Stations, and by Tourism Operators.
- Implement sampling strategies suggested to reduce cost/effort or to further improve precision, specifically options for reducing sampling effort by adding more sites per reef but a lower number of replicate samples.
- Continuation of detailed site monitoring at Lizard Island as a sentinel site to assist early detection and location of outbreaks in this important area. Reduce sample size on that island to 2 x 10 samples per site.
- eDNA monitoring is a suitable method for remote settings. Given the findings of increasing COTS populations in higher latitudes and preliminary evidence of high COTS population within some areas of the Torres Strait, we suggest engagement with the Torres Strait Regional Authority (TSRA) to discuss COTS monitoring via eDNA in the Torres Strait.
- Use eDNA monitoring as a first instance to survey many reefs and conduct focused SALAD surveys where eDNA results suggest a population build up.
- Continue to deliver workflow improvements both on- and off-water to deliver eDNA monitoring data in a timely manner to GBRMPA dashboard for decision support.
- Given the wide access that COTS control vessels have to reefs, further research should test options to avoid contamination from those vessels. These may include changes in sampling time (e.g. collect eDNA prior to any culling efforts on each day) or collecting a larger number of negative control samples to allow statistical correction for false positives.

Table 3. Indicative COTS eDNA collection scenarios for different management options.

	Purpose / Operational Application	Sample size recommended (sites X samples)	On-water collection time estimate	Laboratory costs (per reef or site)	Collection by	Comment
Reef level	Quantify densities at low levels (< 3 COTS ha^{-1})	(3 x 12 = 36) If maintaining consistency was priority) 4 x 6 = 24 5 x 4 = 20 6 x 3 = 18	~2 h	$2-4k	Research Vessel, QPWS/RJFMP, (Citizen science)	Reduced sampling options from our current sampling design with similar error. Recommended for the backbone monitoring of early detection.
	Detect presence at low (< 3 COTS ha^{-1}) levels	~ 10 samples per reef*	30 min	$1k	(Research Vessel), QPWS/RJFMP, Citizen science, COTS control vessels	Might be possible on control vessels, e.g. by supplying more negative controls.
	Quantify densities at intermediate levels (>10 COTS ha^{-1})	3 x 5 = 15 4 x 3 = 12 5 x 3= 15	1.5 h	$1.5-2k	(Research Vessel), QPWS/RJFMP, Citizen science, COTS control vessels	Might be possible on control vessels, e.g. by analysing more negative controls (water samples supplied which have no COTS eDNA).
	Detect presence at intermediate levels	~5 samples per reef*	20–30 min	$0.5k	(Research Vessel), QPWS/RJFMP, Citizen science. Possibly COTS control vessels	Might be possible on control vessels, e.g. by supplying more negative controls.
Site level	Detect re-occurrence after culling	6–12 per time, need baseline before last culling	15 min (per point in time)	$1k Depends on time and frequency	COTS control vessels (trial needed)	Need discussion if this is needed on site or reef level. It should be possible to run from control vessels.
	Quantify low levels (~3 COTS ha^{-1})	2 x time points x 10 samples = 20	2 h	$2k	Research stations, Research Vessel, QPWS/RJFMP	More development required. Was not a priority, only explored at Lizard Island sentinel site.
	Fast on-board detection for day-to-day decision on culling	4–5 per site	15 min	Developmental costs, but low ongoing	COTS control vessels	Dipstick is available, but further development needed for fast analysis on board the control vessels.

* sample size for presence absence analyses at low and intermediate levels inferred from occupancy analysis given in Uthicke et al. (2022).

7. OPTIMISED WORKFLOW AND TIMELINES FOR FIELD COLLECTION, LABORATORY PROCESSING AND DATA DELIVERY

We developed a standard operating procedure (SOP) for eDNA collection that was successfully implemented on 1) Pacific Marine Group, October 2022 *Odyssey*, 2) CCIP-D-02 (Lawrence et al. 2025b) calibration trip, March 2023, *Reef Ranger*, 3) QPWS/RJFMP, trip November 2023, *Reef Resilience*. This SOP can be found in **Appendix B**. We have developed standard laboratory processing procedures that enable the seamless processing of eDNA samples to a result for interpretation. Based on current activities, we provide an estimated workflow and expected timeframes to complete different steps within the COTS eDNA workflow (**Table 4**, **Figure 13**). We also present scenarios for decision support (**Table 3**) that is discussed in more detail in the project reports from CCIP-D-02 (Lawrence et al. 2025b) and CCIP-R-02 (Fletcher et al. 2025). We have opted to include several site/samples scenarios as it would be prudent to include more site options for larger reefs and smaller site options for smaller reefs. Although the current monitoring design was focused on the reef level, we provide several likely site level applications and their constraints in **Table 4**. We believe a continuous re-monitoring of culled sites is an important potential application and could be relatively easily achieved as time-series data would be available for each individual site. Lastly, fast on-board detection was a developmental activity not prioritised in this project, however methods for this approach can be refined and further developed if needed.

A project co-ordination role should be established to co-ordinate and facilitate activities surrounding eDNA monitoring and work closely with GBRMPA in the reef prioritisation planning to optimise activities. Equipment and eDNA collection gear preparation would be completed pre-trip and refresher training (if required) conducted accordingly with the collection provider. Once on water, we anticipate up to two hours of time required to conduct eDNA collection and sample processing for each reef. Once a collection provider has returned all samples and equipment post voyage, we anticipate a laboratory processing time of approximately 5 days (per 100–200 samples). This includes data analysis and interpretation, with final data uploads available soon after.

By way of example, a 10-day voyage should be able to collect eDNA samples from up to 20 reefs, assuming favourable weather and proximity, providing approximately 600–800 samples (including controls) under a low-level quantification sampling strategy (See **Table 3**). Assuming *ad-lib* access to laboratory processing, it would be feasible to complete the processing of this volume of samples within four weeks and have data uploaded to the early warning dashboard within that time frame.

Table 4. Workflow and expected timeframes for sample collection, sample processing and subsequent delivery of data into COTS Control Program decision support system (CCIP-R-02 Fletcher et al. 2025). #On-water collection time is dependent on the specific application as detailed in **Table 2**.

Step	Step in workflow	Who	Timeframe
1	Preparation of eDNA collection equipment, training (or refresher training), SOP's, permit, risk assessment, etc.	Project Co-ordinator, Collection provider (e.g. QPWS)	Approximately 1 week prior to trip
2	Field collection and sample processing	Collection provider	Up to 2 hours per reef[#]
3	Post-trip collection of eDNA gear and samples, debrief and clean-up of eDNA collection equipment	Project Co-ordinator, Collection provider (e.g. QPWS)	3 days
4	Laboratory analysis (DNA extraction, ddPCR, Quality Checks (QC), data analysis and interpretation)	Laboratory technician	Approximately 4 days turnaround time per 100 samples (=approximately 3 reefs worth of samples), increasing efficiencies with batch processing
5	Delivery of data to COTS dashboard by GBRMPA.	Project Co-ordinator / GBRMPA	1 day

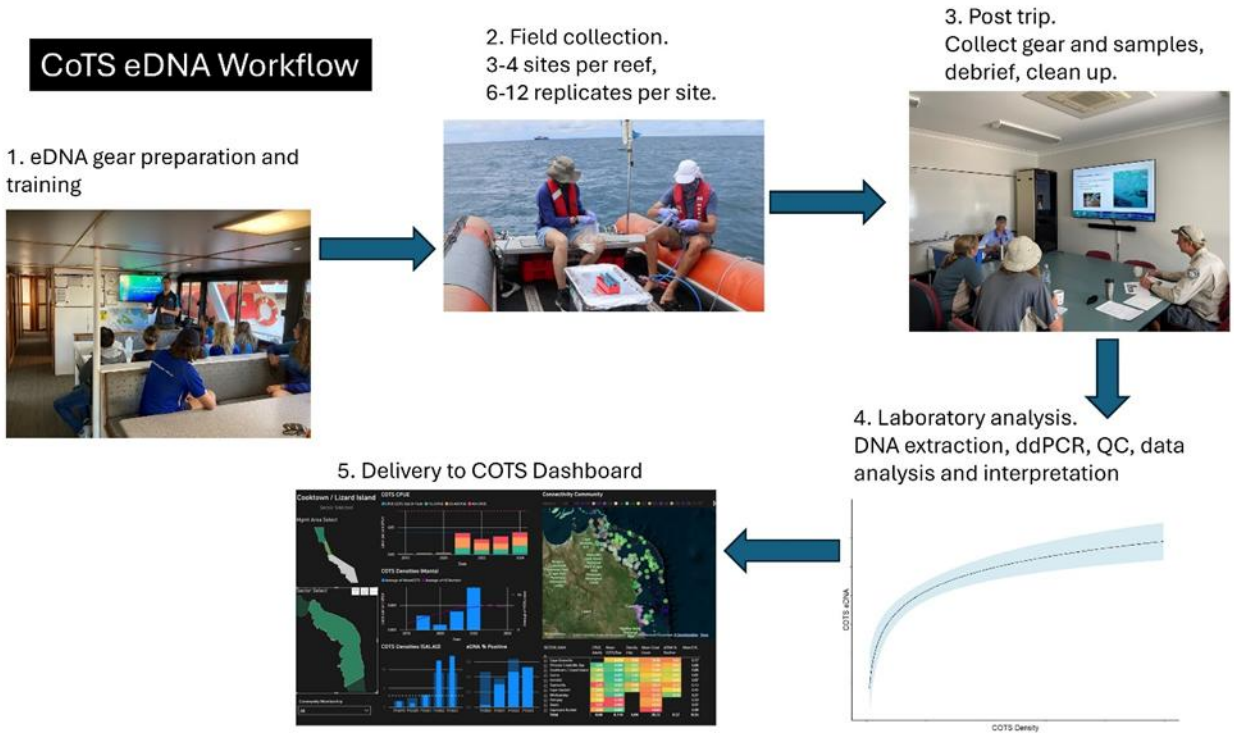

Figure 13. Graphic illustration of the workflow to collect and analyse COTS eDNA presented in **Table 4**.

8. OUTPUTS

This project has delivered the following outputs:

- Recommendations on eDNA sampling design and cost to achieve various objectives and provided clear recommendations for sampling design.
- Refinement of sampling methodology and statistical analyses for eDNA monitoring and provided details of the analysis, descriptions of workflow and SOPs.
- Exploring alternative methods for sample collection (e.g. passive sampling sponges) by testing several methods and summarising outcomes in this report.
- Training of non-scientists in sample collection including, tourism and COTS Control operators and QPWS staff. Successful trial collection implemented and integrated into results.
- Annual monitoring data (2021–2023) collected using eDNA method from 12 reefs, three years of monitoring data submitted to GBRMPA and taken up into COTS dashboard. **Figure 14** is an example of dashboard output.

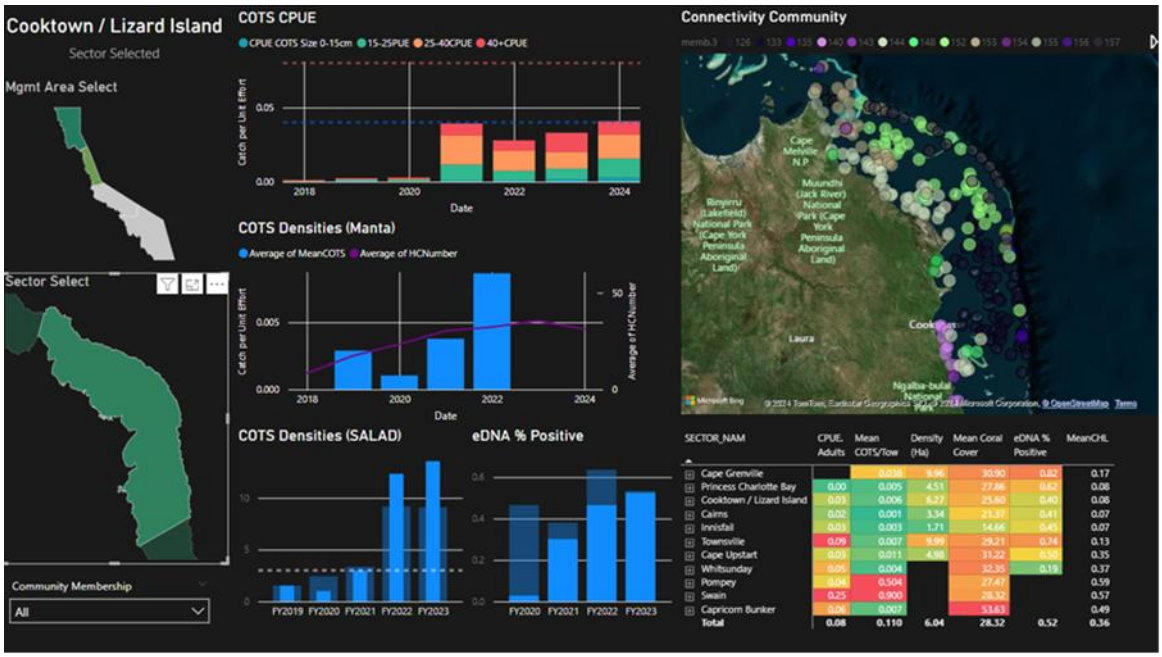

Figure 14. Example of dashboard output from project CCIP-R-02 (Fletcher et al. 2025, image provided by Sam Matthews, GBRMPA).

9. RESEARCH SYNERGIES AND NEXT STEPS

Research synergies revolved around the central Sub-program theme of Detection, with an emphasis on early warning detection. As such, we developed significant synergies with the complimentary early warning monitoring method of SALAD surveys detailed within CCIP-P-04 (Pratchett et al. 2025) and the early investment project, “Delineation of the outbreak area”. This allowed coverage of up to 42 reefs annually, including 7 reefs in the Princess Charlotte Bay and Cape Grenville area.

Collaborative research on using eDNA to investigate invertebrate predation has been continued through CCIP-P-03 (Byrne et al. 2025), and to an add on project to CCIP-P-03. Project staff also collaborated with CCIP-D-01 (Lawrence et al. 2025a) and CCIP-D-02 (Lawrence et al. 2025b) to conduct cross method calibration and contribute to monitoring design.

We continue to collaborate with the Queensland Parks and Wildlife Service through the Reef Joint Field Management Program and all COTS Control Vessels.

Next steps for the implementation of the suggested monitoring design are presented in previous sections. We recommend field validation of statistically modelled sampling strategies with reduced samples. If management objectives other than early detection are to be achieved, further R&D is required, e.g. developing new tools for fast detection on board, identifying sources of contamination on COTS control vessels or developing an appropriate sampling design and statistical analysis tools for monitoring changes in populations on reefs after culling.

Future research priority areas:

- Field validation of statistically modelled sampling strategies with similar errors to the current sampling strategy, but with fewer overall samples required.
- Translation of operational development to on-water monitoring activities, including priority reef planning.
- Develop fast methods allowing immediate detection to use eDNA results in tactical decision making (e.g. Biosensors).
- Develop and test alternative sampling tools for faster on water collection.
- Extend dynamic range of eDNA surveys (e.g. intermediate to high COTS density) by incorporating a concentration metric. This may be important where workplace health and safety issues prevent diving or manta tows (e.g. crocodiles).

10. MANAGEMENT IMPLICATIONS AND IMPACT

The data from COTS eDNA surveys are currently being integrated at the GBRMPA as part of a prototype Early Warning System for COTS outbreaks at regional scale (**Figure 14**). This leverages the ability of the eDNA survey method to observe COTS at low densities and to collect large volumes of data from across the GBR Marine Park. The data are also incorporated as part of the yearly reef prioritisation process to help identify and predict reefs where outbreaks may be occurring.

This research contributes to achieving the overarching outcomes and impacts identified in CCIP's Research Impact Plan:

- Outcomes – Improved detection and monitoring; more efficient and effective operational response; more accurate prediction. Early detection of COTS is an imperative to early intervention to prevent the downstream impact of COTS outbreaks.
- Impacts – COTS outbreaks are suppressed and prevented; Coral cover is protected across the GBR; Traditional Owners, tourism industry and community benefit. We suggest that an eDNA monitoring activity is a critical additional monitoring tool to collect information about the status of COTS densities on the GBR.

11. ACKNOWLEDGEMENTS

We recognise the contributions of staff and crew of the COTS control vessels, particularly Pacific Marine Group who assisted with practical evaluations of the methods. We are grateful for the willingness and assistance provided by the Queensland Parks and Wildlife Service as part of the Reef Joint Field Monitoring Program to undertake eDNA collection, in particular Sascha Taylor for managing the interactions and providing boundless enthusiasm to trial eDNA collections in regular operational activities. We are also grateful to the Moore Reef 'Marine World' pontoon for their assistance trialling some of the methods. AIMS and JCU provided in-kind contributions towards this work. We are indebted to the crew of the AIMS research vessels and appreciate the support from the Lizard Island Research Station Directors, Drs. Anne Hoggett and Lyle Vail.

12. DATA ACCESSIBILITY

Data is stored within data repositories at the Australian Institute of Marine Science. Data is available upon request and relevant data supplied to the Great Barrier Reef Marine Park Authority. Project data are available from: https://doi.org/10.25845/2XC1-XJ49.

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APPENDIX A: LIST OF REEFS WHERE ENVIRONMENTAL DNA COLLECTIONS OCCURRED FROM 2021–2023. SPECIFIC CCIP PROJECTS HAVE BEEN IDENTIFIED AND TOTAL SAMPLE NUMBERS FROM EACH REEF GIVEN.

Table A 1. COTS eDNA sampling locations and sample numbers from 2021. Data from CCIP-EIP-02 are published in Pratchett et al. (2022).

2021	CCIP-EIP-02	CCIP-D-03	Total
11-049	36		36
11-162	36		36
13-124	36		36
Batt Reef		36	36
Bowden Reef		36	36
Cairns Reef		36	36
Clack Reef	36		36
Corbett Reef	36		36
Davie Reef	36		36
Davies Reef		36	36
Eyrie Reef		36	36
Green Island		35	35
Lizard Island		150	150
McSweeney Reef	36		36
Moore Reef		36	36
North Direction Island		36	36
Osterlund Reef		36	36
Rudder Reef		36	36
Thetford Reef		36	36
Tongue Reef		36	36
Undine Reef		36	36
Yonge Reef		36	36
Grand Total	252	653	905

Table A 2. COTS eDNA sampling locations and sample numbers from 2022.

2022	CCIP-EIP-02	CCIP-D-03	Total
11-049	36		36
11-160	24		24
11-164	12		12
13-093a	36		36
13-124	36		36
Batt Reef		36	36
Bowden Reef		36	36
Cairns Reef		36	36
Corbett Reef	36		36
Davies Reef		36	36
Elford Reef		36	36

2022	CCIP-EIP-02	CCIP-D-03	Total
Eyrie Reef		36	36
Green Island		36	36
Lizard Island		150	150
McSweeney Reef	36		36
Monsoon Reef	36		36
Moore Reef		36	36
North Direction Island		36	36
Osterlund Reef		36	36
Ribbon Reef 5		36	36
Ribbon Reef 9		36	36
Rudder Reef		36	36
Thetford Reef		36	36
Tongue Reef		36	36
Undine Reef		36	36
Yonge Reef		36	36
Grand Total	252	762	1014

Table A 3. COTS eDNA sampling locations and sample numbers from 2023.

2023	CCIP-EIP-02	CCIP-D-02	CCIP-D-03	Total
11-049	36			36
11-160	36			36
13-093a			36	36
13-124			36	36
19-065[#]			36	36
Bait Reef[#]			36	36
Banfield Reef		24		24
Batt Reef			36	36
Bowden Reef			36	36
Cairns Reef			36	36
Cobham (North) Reef[#]			36	36
Corbett Reef			36	36
Darley Reef		48		48
Davie Reef			36	36
Davies Reef		24	36	60
Elford Reef			36	36
Eyrie Reef			36	36
Faith Reef		12		12
Gould No.1 Reef[#]			36	36
Green Island			36	36
Hedge Reef			36	36
Jacqueline Reef[#]			35	35
Lizard Island			150	150
Lynchs Reef		48		48
McSweeney Reef	36			36
Monsoon Reef	36			36
Moore Reef			36	36
North Direction Island			36	36
Osterlund Reef			36	36
Prawn Reef		24		24
Rib Reef			36	36
Ribbon Reef 5			36	36
Ribbon Reef 9			36	36
Rudder Reef			36	36
Seagull Reef[#]			36	36
Showers Reef[#]			36	36
Shrimp Reef		12		12
Thetford Reef			36	36
Tongue Reef			36	36
Undine Reef			36	36
Wallaby Reef[#]			36	36
Yonge Reef			36	36
Grand Total	144	192	1265	1601

[#]eDNA collections conducted by the Reef Joint Field Management Team as part of translating eDNA collection methods to on-water Operators (see Methods Section 2.2.3).

APPENDIX B: STANDARD OPERATING PROCEDURE FOR COTS EDNA COLLECTIONS: A WORKFLOW FOR COLLECTION BY NON SCIENTISTS AND OPERATORS

Materials

- Pump
- Charging cable
- Rigid blue inlet tubing (plus a spare)
- Flexible outlet tubing with small blue connector (plus a spare)
- Filter housings – pre-assembled, packs of 12
- Large inlet tubing
- Forceps
- Filter housing for controls (pre-prepared sets for 2 controls)
- Control water bottles
- 1.5 ml pre-label sample tubes
- Tube rack
- Gloves
- Centrifuge
- Esky with ice/ice pack for keeping samples cool while on site

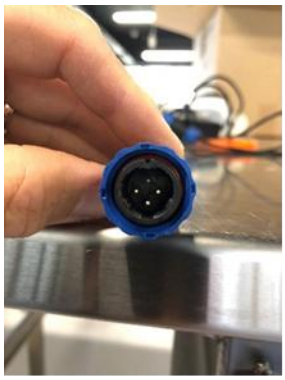
Site selection

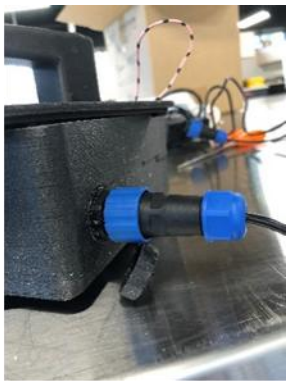
- Select 3 sites per reef, arbitrarily assign these sites 1, 2 and 3.
- Not all reefs are the same and it can be challenging to select site for large and ill defined reefs, however, sites should be back reef and ideally separated by 0.5–1 nm, e.g.:

- eDNA sample collection requires 12 x water samples per site (36 samples total per reef).

Pump setup

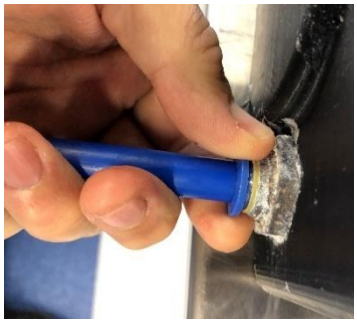
- Ensure pump is fully charged overnight before the day of use. Use the pin location on the plug connections to connect the charger to 240 volts.





- Connect rigid blue tube to the inlet side of the pump (follow arrows on the pump for water flow).
- Connect flexible tubing with small blue connector to the outlet side (follow the arrows on the pump for water flow).

- Please be sure to seat the tubing into the pump completely then pull on the blue collar to secure the tubing in place. To release the tubing, push on the blue collar then gently pull the tubing free of the pump.



Attaching filter cartridge

- Filter housings and large tubing come clean and in packs of 12. Currently, we are aiming for 12 samples per site.
- Once on site, join all the filter housings with the large inlet tubes. They look like a very bad bouquet of flowers!

- Attach the barbed end of the black filter housing to the rigid blue tube. There will be a small piece of flexible tubing on the end of the rigid blue tube to push on to the barbed end.

- The completed setup should look like this:

Collecting a sample

- Place the large tube attached to the filter cartridge into the water. Please try not to immerse the filter cartridge in the water. This is difficult if there are rough conditions so do the best you can.
- Ensure the outlet tubing is going into an outflow bottle – this is used to obtain a consistent volume of sample.
- Press the on-button on the pump to start pumping. Ensure the flow rate knob is turned all the way to the right to maximise flow rate.
- Continue pumping water until the water level in the outflow bottle is in line with the tape. The outflow collection bottles are 2.5 lts at the tape (plus/minus a small amount) which is a standard volume that we filter across the eDNA program.
- At this point, lift the filter cartridge out of the water and invert it in the air to let all the water run through the filter. Allow the pump to continue pumping air for a few seconds after all the water has flowed through.

- With the pump still going, disconnect the vacuum inlet tubing from the barbed end of the filter housing. Removing the filter housing with the pump still going ensures as much residual seawater is removed as possible.
- Remove the large tubing from the soft silicon side.
- Place the filter housing back in the bag it came from and continue the process with the remaining filter housing for each site. All 12 filters housing can go back into the same bag for each site.
- Please keep all samples cool in an esky with ice/ice block while in the tender. They can be put in the fridge once back on board the main ship.
- Record sample number, date, time and location details (reef, site lat and long):

Sample number	Date	Time (24 hours)	Reef/location	Latitude	Longitude

Preserving DNA (back on the main ship)

- The kit contains pre-filled, pre-labelled 1.5 ml sample tubes containing a preservation solution.
- Set up a clean space to complete this operation.
- Gloves are supplied as well as bleach tablets to prepare bleach solutions for area cleaning and DNA Erase for general DNA cleaning of gloved hands.
- Put a set of gloves on for the next steps.
- Preparation of bleach solution: 1 tablet per spray bottle filled with tap water and allow to dissolve.
- Spray work area with bleach and wipe with paper towel.
- Spray gloved hands with DNA erase, use clean paper towel to absorb excess if needed.
- Centrifuge the required number of 1.5 ml sample tubes for 1 minute at 10,000 x g to ensure all liquid is at the bottom of the sample tube.
- Open filter housing to expose the gridded membrane filter.
- Using a new set of forceps, carefully fold the membrane filter in half three times.
- Ensure you do not take the backing nylon support mesh. Please retain as this can be reused.
- Once folded into a neat cone shape, transfer the membrane filter to the 1.5 ml samples tube.
- Use the forceps to gently push the filter into the 1.5 ml sample tube.
- Place the cap of the sample tube back on tightly.
- Shake the 1.5 ml sample tube for 5 seconds, then centrifuge for 1 minute.
- Complete data table ensuring sample numbers are assigned.
- All components of the filter housings are reusable. Please rinse with fresh water and allow them to dry before returning to the box.

Controls

- Controls enable us to identify any cross contamination of samples from the general workflow. To do this, there are ‘Control kits’ prepared for filtering purified water through equipment that was cleaned at the same time as the standard sampling gear. For this trip, please conduct duplicate control sampling once per day. This will require 2 x clean water control bottles and 1 x set of control sampling equipment.
- Controls can be conducted on the main ship at any time of the day.
- Clean water controls are 1 lt bottles that have been bleach cleaned and filled with purified water.
- Control sampling equipment bags containing all the equipment for collection of control samples are also provided. These bags contain 2 x filter housings, 2 x large inlet tubes and 2 x forceps.
- Assemble filter housing and large inlet tube as for a sample including connection to the pump.
- Open clean water control bottle and insert the large tube. Begin filtration of the clean water by turning the pump on.
- As for a sample, once all the water from the bottle has been filtered, raise the inlet tubing in the air and allow residual water to run through the filter housing.
- Return the filter housing to the bag it came from and continue with the preservation of the DNA as described above for samples.

At the end of the day

- All sampling gear can be re-used. Please fresh water rinse the filter housing, large tube and nylon support mesh in fresh water. If possible spread out (e.g. in the sun on top deck) to dry on drying trays provided, using a tea towel to absorb excess fresh water on the bottom of the tray. Once dry, sampling gear can be packed into a spare nally bin in any way it fits. Sampling gear will be bleached cleaned and re-loaded once back at AIMS.
- Please run fresh water through the inlet tubing, pump and outlet tubing (no filter cartridge) to rinse the salt water from the pump internals.
- Disconnect the inlet tubing and outlet tubing and run the pump once more to remove as much water as possible from the internal plumbing of the pump.
- Connect pump to charge overnight.

Packing it all back up

- All sampling gear can be re-used. Please fresh water rinse the filter housing, large tube and nylon support mesh in fresh water. If possible spread out (e.g. in the sun on top deck) to dry on drying trays provided, using a tea towel to absorb excess fresh water on the bottom of the tray. Once dry, sampling gear can be packed into a spare nally bin in any way it fits. Sampling gear will be bleached cleaned and re-loaded once back at AIMS.
- Please run fresh water through the inlet tubing, pump and outlet tubing (no filter cartridge) to rinse the salt water from everything.
- Disconnect the inlet tubing and outlet tubing and run the pump once more to remove as much water as possible from the internal plumbing of the pump.
- Put the pump back in the pelican case
- Send me (j.doyle@aims.gov.au) a copy of the datasheet and/or leave a hard copy with the samples.

Sample number (or number range)	Date	Time (24 hours)	Sample / Control	Reef/location	Site No.	Latitude	Longitude	Volume water filtered (L)	Comment

APPENDIX C: REEF LEVEL MODELLING OF SALAD AND EDNA DATA (CSIRO)

Models at the Reef Summary Scale

CSIRO Data61

2024-06-19

Contents

1 Introduction

In this analysis we look at the SALAD data aggregated to the reef level, alongside the eDNA data (also aggregated to the reef level) to determine whether eDNA measures are a useful indication of how many COTS or COTS + scars are present at a reef. We have used data only at reefs where the SALAD and eDNA measures were collected within a similar timeframe (often within days). The sites sampled at the reef are not always the same but by aggregating the measures across the reef they are taken to be a broad representation of what is happening at that reef. In trying to "predict" the COTS density on SALAD using the eDNA measures, we acknowledge that our response variable (COTS density) is also imperfect given that COTS are patchy across whole reefs so we do not expect a precise prediction.

2 Modelling strategy

It is convenient to begin with some definitions and concepts.

2.1 Proportions and odds

If P is a *proportion*, i.e. on the $0 \le P \le 1$ range then the corresponding *odds*, O, is the ratio

$$O = \frac{P}{1-P} \qquad \text{and hence} \qquad \log O = \log P - \log(1-P)$$

Assuming $P \neq 1$ note that $0 < O < \infty$ and if also $P > 0$ then $-\infty < \log O < \infty$.

In the models that follow `raw_prop`, the proportion of positive samples, will be considered as a predictor of CoTS density. One difficulty with this is that, since P is confined to a finite range, the predictions, or estimates, to come from the model can also only be on a limited range, as we shall see. For this and other reasons, precictors on an unbounded range, like $\log O$, are often more appropriate, and more convenient to use as precictors in linear, or linear-like models.

2.2 Response variables

The response variable in our models will be either the *observed CoTS density*, i.e. a density based on the actual number of animals seen during SALAD tows, or the *inferred CoTS density*, the corresponding measure including both scars and animals.

We will consider only linear models with a transformed response and possibly transformed predictors, as detained progressively below.

2.3 Zero responses: an *ad hoc* protocol

Since the response variable will require log transformation, and as the data contains at least one 0 density, *zero densities will be promoted to half the minimum observed non-zero density* as an ad hoc means of including them in the analysis, i.e. to avoid taking logs of zero. This is standard and likely not to be misleading for our purposes here.

2.4 Back transforming from a log-transformed response variable

If a model is fitted with a log-transformed response, but estimates are later required in the natural scale, back transforming from the log scale is often not enough. Simple back transforming will provide an estimate of the *median* on the natural scale. If an estimate of the *mean* on the natural scale is needed then a *bias correction* is required, as the median is less than the mean, and often considerably less.

In the case of CoTS densiies the mean on the natural scale *is* required. This is because to estimate the total number of CoTS in a defined region, the *mean* density, not the *median* density, has to be multiplied by the area of the region.

There is a large literature on how to estimate the mean in this situation, but in what follows we will only consider a simple bias correction that should suffice for now.

3 A simple model

3.1 Observed CoTS density

A promising model previously considered by the AIMS group takes the following form, using *R* code to define it with obvious notation.

```
AIMS <- lm(log(obs_den) ~ raw_prop, data = reef_summary)
summary(AIMS)
```

```
Call:
lm(formula = log(obs_den) ~ raw_prop, data = reef_summary)

Residuals:
    Min      1Q  Median      3Q     Max
-1.6656 -0.4009  0.0641  0.5893  1.6202

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept) -0.02309    0.25612  -0.090    0.929
raw_prop     2.49803    0.37163   6.722 5.86e-08 ***
---
```

```
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.6976 on 38 degrees of freedom
Multiple R-squared:  0.5432,    Adjusted R-squared:  0.5312
F-statistic: 45.18 on 1 and 38 DF,  p-value: 5.864e-08
```

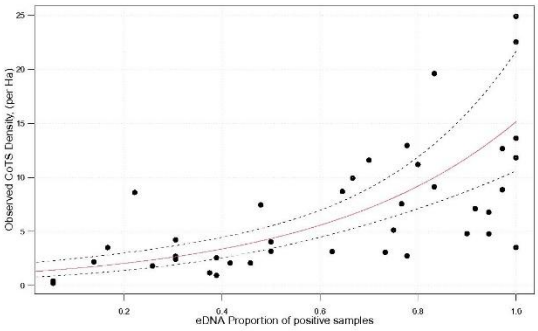


Figure 1: Data and predictions in the natural scale: Observed CoTS density

The predictors are both significant and the adjusted $R_a^2 = 53.1\%$ looks promising. The Figure shows the data with the predicted *mean* together with 95% confidence limits, for values of `raw_prop` spanning its entire achievable range. The estimates include the simple bias correction mentioned above; the details are simple but tedious and hence the code is not shown.

A limitation of the modelling strategy is immediately clear. Since the proportion, plotted along the $x-$axis, is strictly limited to the range $0 \leq P \leq 1$, and since the curve is strictly increasing over this range, the estimates of CoTS density cannot rise above its maximum of $\sim 14.3\,\text{Ha}^{-1}$ at $P = 1$, nor fall below its minimum of $\sim 1.5\,\text{Ha}^{-1}$ at $P = 0$. If an estimate of the number of CoTS in a region one Ha is required, this is restricted to lie between these limits.

The Figure below shows the same data but with the $y-$axis on a log scale.

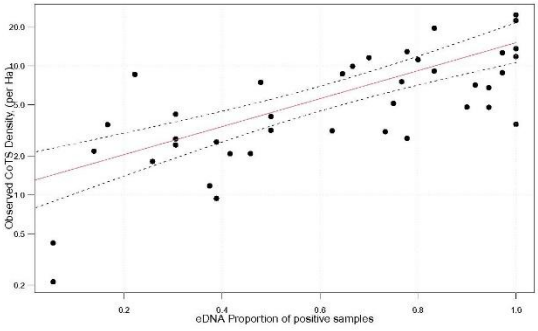


Figure 2: Data and predictions with a log y scale

3.1.1 Model disgnostics

We now consider some standard model diagnostics, graphically. The diagram on the left shows the (standardised) residuals plotted against the fitted values as a visual both for possible outliers and for variance heterogeneity (on

the log scale).

The diagram on the right shows a normal scores plot, as a visual check for meaningful departures from the underlying Gaussian assumption (on the log scale).

Neither indicate much cause for concern. The extreme points are flagged by their SALAD identifiers.

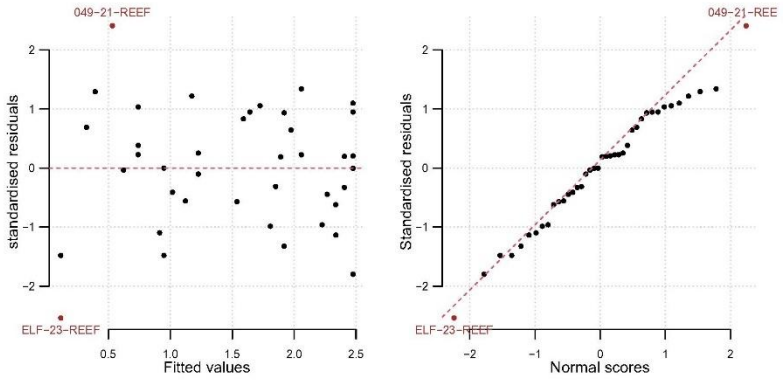


Figure 3: Two diagnostic plots

3.2 Inferred CoTS density

This response adds the number of scars, as a proxy for occluded animals, to the observed number of CoTS in each SALAD tow. The analysis proceeds on parallel lines to the previous case.

```
AIMS2 <- lm(log(inf_den) ~ raw_prop, data = reef_summary)
summary(AIMS2)
```

```
Call:
lm(formula = log(inf_den) ~ raw_prop, data = reef_summary)

Residuals:
     Min       1Q   Median       3Q      Max
-1.26228 -0.35218 -0.09815  0.51897  1.31394

Coefficients:
            Estimate Std. Error t value Pr(>|t|)
(Intercept)   1.2271     0.2310   5.313 4.98e-06 ***
raw_prop      1.8524     0.3351   5.527 2.53e-06 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.6291 on 38 degrees of freedom
Multiple R-squared:  0.4457,    Adjusted R-squared:  0.4311
F-statistic: 30.55 on 1 and 38 DF,  p-value: 2.534e-06
```

The adjusted $R_a^2 = 43.1\%$ is down on the previous model, but still fairly high. The Figure shows the (bias corrected) estimates of the mean density at all levels of the `raw_prop` predictor, with 95% confidence limits. In this case the adherence to the model is much less tight than in the observed density case, as hinted it would be by the reduced R_a^2.

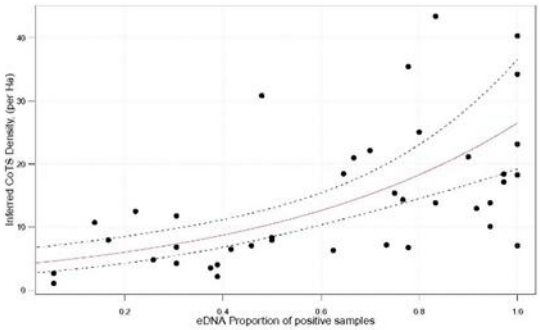


Figure 4: Data and predictions in the natural scale: Inferred CoTS density

The Figure below shows the same data but with the $y-$axis on a log scale.

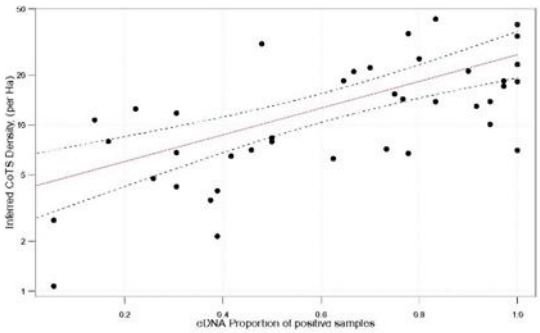


Figure 5: Data and predictions with a log y scale: Inferred CoTS density

3.2.1 Model disgnostics

We now consider some standard model diagnostics, graphically, using the same protocol as used in the observed density analysis given above.

Neither indicate much cause for concern. The extreme points are flagged by their SALAD identifiers.

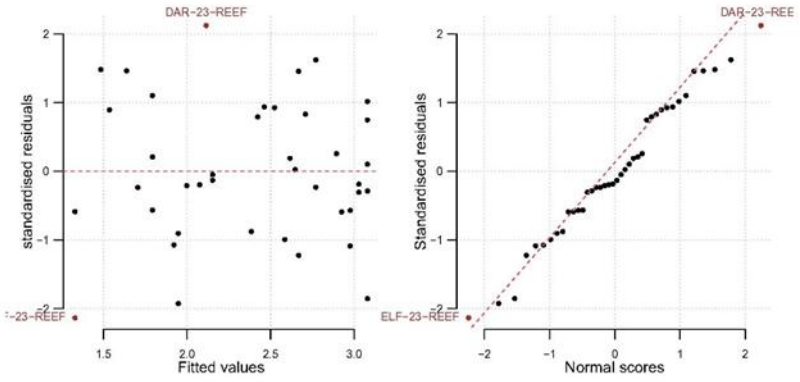


Figure 6: Two diagnostic plots

4 Alternative models explored by CSIRO

We now consider a small variant on the modelling strategy detailed above where we consider using `eDNS_concentration` as an alternative predictor, as well as using `raw_prop` on the *odds* scale to obviate the prediction limitation noted above when it is confined to the $(0, 1)$-scale. Both predictors will in fact be log-transformed for incorporation in the model, commensurate with the log-transformed response.

Initially both predictors will be considered, but ultimately only the most effective of the two will be used in the final model to be recommended.

4.1 Initial data exploration

We begin by looking at a plot of the log-transformed observed density against each of the log-transformed candidate predictors separately.

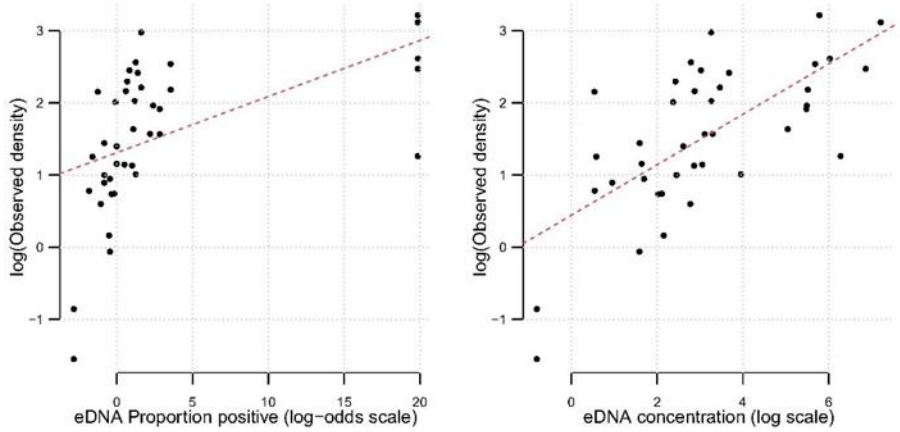


Figure 7: Exploratory plots of log-density vs each candidate predictor

Opening up the proportion positive to the unbounded log-odds scale, in the hope of finding a predictor that covers a wider range of values, immediately uncovers an apparent anomaly in the data. A group of 5 points have a positive proportion so close to unity that they remain within a finite range only by chance. They form a group of very high-leverage points for the regression and slightly out of kilter with a dependency pattern suggested by the remaining points. This feature is covered up when the predictor is not expanded in this way.

The log-concentration predictor, on the other hand, appears to have a strong and consistent relationship with the log-transformed response.

4.2 Initial models

An exploratory fit of a linear model using both candidate predictors accentuates the effect of the high-leverage points. The results for both response variables are shown below

```
CSIRO_0o <- lm(log(obs_den) ~ eDNA_pp_log_odds + eDNA_log_conc, reef_summary)
CSIRO_0i <- lm(log(inf_den) ~ eDNA_pp_log_odds + eDNA_log_conc, reef_summary)
summary(CSIRO_0o)
```

```
Call:
lm(formula = log(obs_den) ~ eDNA_pp_log_odds + eDNA_log_conc,
    data = reef_summary)

Residuals:
     Min       1Q   Median       3Q      Max
-1.67327 -0.44765  0.03191  0.56193  1.54352

Coefficients:
                 Estimate Std. Error t value Pr(>|t|)
(Intercept)       0.398691   0.274920   1.450 0.155425
eDNA_pp_log_odds -0.008465   0.029304  -0.289 0.774296
eDNA_log_conc     0.371690   0.098521   3.773 0.000566 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.7644 on 37 degrees of freedom
Multiple R-squared:  0.466, Adjusted R-squared:  0.4371
F-statistic: 16.14 on 2 and 37 DF,  p-value: 9.125e-06
```

```
summary(CSIRO_0i)
```

```
Call:
lm(formula = log(inf_den) ~ eDNA_pp_log_odds + eDNA_log_conc,
    data = reef_summary)

Residuals:
    Min      1Q  Median      3Q     Max
-1.3433 -0.4291 -0.1065  0.6189  1.3470

Coefficients:
                 Estimate Std. Error t value Pr(>|t|)
(Intercept)       1.600035   0.252400   6.339 2.18e-07 ***
eDNA_pp_log_odds -0.006073   0.026904  -0.226  0.82264
eDNA_log_conc     0.256088   0.090451   2.831  0.00746 **
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1
```

```
Residual standard error: 0.7017 on 37 degrees of freedom
Multiple R-squared:  0.3284,    Adjusted R-squared:  0.2921
F-statistic: 9.045 on 2 and 37 DF,  p-value: 0.0006338
```

In both cases the log-concentration predictor stands out as the most effective, though the adjusted R_a^2 values are lower. The coefficients for the log-odds proportion positive precictor are, in both cases, convincingly non-significant and the values are even slightly negative.

4.3 Final models

An obvious model refinement is, as anticipated, to remove the log-odds predictor and focus on how well the log-concentration does by itself in the models.

```
CSIRO_o <- update(CSIRO_0o, . ~ . - eDNA_pp_log_odds)
CSIRO_i <- update(CSIRO_0i, . ~ . - eDNA_pp_log_odds)
summary(CSIRO_o)
```

```
Call:
lm(formula = log(obs_den) ~ eDNA_log_conc, data = reef_summary)

Residuals:
     Min       1Q   Median       3Q      Max
-1.71219 -0.42186  0.03845  0.57103  1.52103

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)    0.44370    0.22375   1.983   0.0546 .
eDNA_log_conc  0.34948    0.06084   5.744 1.28e-06 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.7551 on 38 degrees of freedom
Multiple R-squared:  0.4647,    Adjusted R-squared:  0.4507
F-statistic: 32.99 on 1 and 38 DF,  p-value: 1.278e-06
```

```
summary(CSIRO_i)
```

```
Call:
lm(formula = log(inf_den) ~ eDNA_log_conc, data = reef_summary)

Residuals:
     Min       1Q   Median       3Q      Max
-1.37121 -0.40558 -0.08913  0.60669  1.35683

Coefficients:
             Estimate Std. Error t value Pr(>|t|)
(Intercept)    1.63233    0.20533   7.950 1.33e-09 ***
eDNA_log_conc  0.24015    0.05583   4.301 0.000114 ***
---
Signif. codes:  0 '***' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1

Residual standard error: 0.6929 on 38 degrees of freedom
Multiple R-squared:  0.3274,    Adjusted R-squared:  0.3097
F-statistic:  18.5 on 1 and 38 DF,  p-value: 0.0001144
```

The adjusted R^2 statistics are now $R_a^2 = 45.1\%$ (observed density) and $R_a^2 = 31\%$ (inferred density) are small, but

the benefit is that these models are capable of estimating higher CoTS densities than the models using the limited-range positive proportion predictor, and hence the job they are doing is more difficult.

The mean CoTS density estimation curves, with pointwise confidence limits are shown in the following Figure.

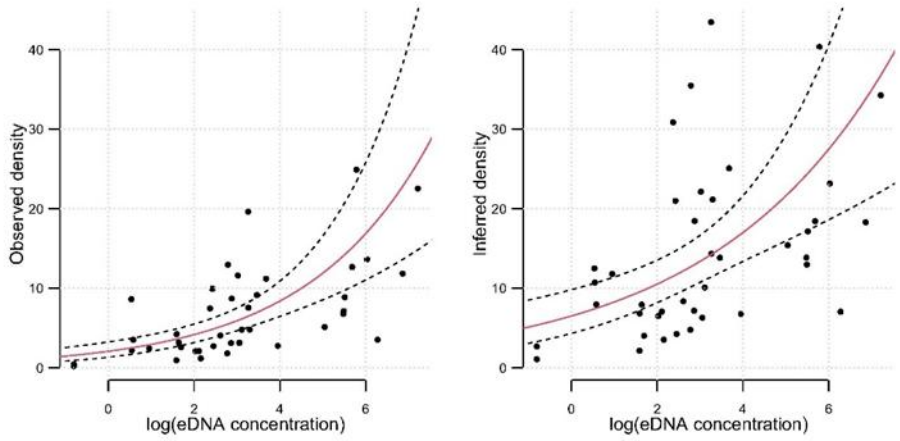


Figure 8: Density and estimated mean density plots: Observed left, Inferred right

The same data with logarithmic y-axes are shown in the Figure below.

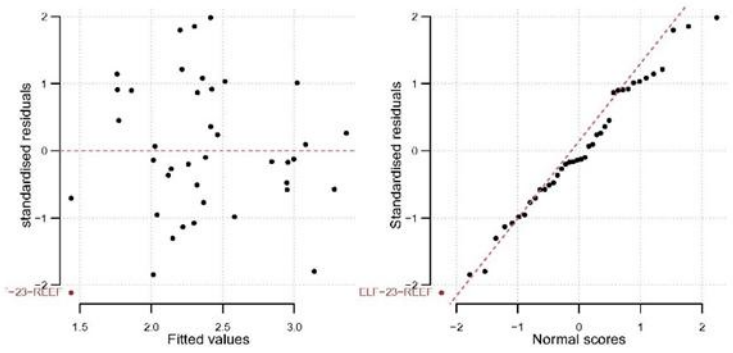


Figure 11: Two diagnostic plots, Inferred density model

5 Discussion

We have explored a range of modeling strategies using both the proportion of positive samples (and transformations) and mean eDNA concentrations (and transformations) from the eDNA data as predictors and the observed and inferred COTS densities from the SALAD data as the response variables. Comparing the modeling strategies highlights the trade-offs between simplicity and flexibility in statistical modeling. While the initial approach of modeling proportion of positive samples vs observed COTS densities offers straightforward interpretation and decent predictive performance within a limited range, the final approach allows for a broader range of predictor values, albeit with increased complexity. Ultimately, the choice between these strategies depends on the specific research objectives and the trade-offs between model simplicity and predictive accuracy.

The choice of model should ultimately depend on the purpose of the eDNA data collection. For low to medium densities of CoTs, the simple model with proportion of positive samples as the predictor, shows a promising relationship with the observed CoTS densities on SALAD surveys (less so the inferred densities). However, as noted this model is not suitable for predicting densities below approximately 1.5 COTS per hectare and above 14 COTS per hectare. This is not a huge limitation as accurately detecting CoTS densities below 1.5 COTS per hectare is not currently a primary objective of the CoTS monitoring program and other methods are better suited for monitoring CoTs at the higher density range.

The models may be used to help translate eDNA monitoring data into approximate CoTS SALAD density equivalents eg. at proportion of positive samples of 0.4 the mean SALAD density prediction is approximately 3.5 COTS per Ha. (Lower CI = 2.7, Upper CI = 4.5). At a proportion of positive samples of 0.8 the mean SALAD density predition is approximately 8.9 COTS per Ha. (Lower CI = 7.0, Upper CI = 11.3). It should be noted though, that the inferred COTS densities are almost double the observed COTS densities at the equivalent proportion of positive samples thresholds. The SALAD estimates are an average of the obsevered (and inferred) densities along three 1km tracks around the perimeter of a reef. Depending on the size of the reef, this may only represent a small fraction of the reef and so care must be taken in not considering the SALAD densities as absolute COTS densities but the best estimate of in-water COTS densites that we have to compare the eDNA measurements to. Collecting data in the future to help improve the translation of COTS measurements into absolute densities would be beneficial. Such data may include collecting Reefscan around the entire perimeter of a reef where eDNA measurements are taken or culling entire reefs (where practical) following eDNA sampling.

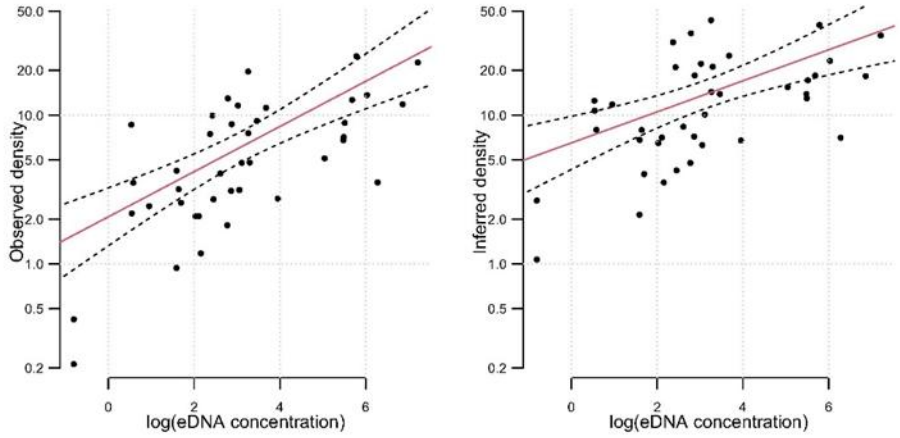


Figure 9: Density and estimated mean density plots: Observed left, Inferred right, (log y-scale)

4.3.1 Diagnostic plots

For completeness we provide the same diagnostic plots here as were given above for the AIMS models.

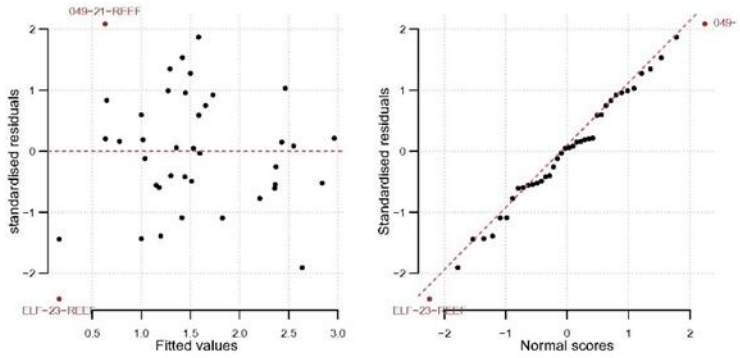


Figure 10: Two diagnostic plots, Observed density model

APPENDIX D: STATISTICAL MODELLING FOR EVALUATING EDNA SAMPLING STRATEGY (CSIRO)

Introduction

This appendix summarises a study to evaluate the sampling strategy for reefs on the Great Barrier Reef (GBR). On a given trip each reef is sampled at several sites (sites per reef, L), with several samples per site (S), each of which is subdivided into several repeats (R).

The current sampling strategy is L = 3, S = 12 and R = 2.

Given finite resources, is this an optimal sampling strategy or can it be improved, viz.

1. Can similar precision, or sampling error, be achieved with fewer total samples or repeats?
2. What is the best strategy for further reducing sampling error?
3. How much scope is there for further reduction in sampling error?

The concentration of eDNA in control samples was very low (see Appendix G), hence controls can be ignored without loss of generality.

Data Structure

The data have a hierarchical structure.

A typical trip of approximately a week may involve visits to several reefs. At each reef several sites (usually 3) were chosen for sampling. Typically, 12 – but sometimes more – 2.5 litre sea water samples were taken at each site. The samples were filtered (concentrated) and processed, before being split into two repeats for eDNA digital droplet PCR analysis. Hence repeats are the lowest observational level for data analysis.

For each observation in droplet-digital PCR (ddPCR) a sample volume is fractionated into many (~20,000) uniformly sized droplets, each of 0.85 nanolitres. After PCR and fluorescent tagging, these “accepted” droplets are read to determine whether they contain COTS DNA (“positive” droplets) or don’t (“negative” droplets).

For each observation, the total number of accepted droplets was near uniform, with a mean of close to 22,000 (median ~ 22,500): 1% had fewer than 14,500 and 1% had more than 24,100. In contrast, about 60% of observations had zero positive droplets; 5% had 15 or more and 1% had 58 or more positive droplets. Repeats within samples were similar, but otherwise the positive counts were highly skewed (**Table A 4**). The highest positive count was 11,921 in a repeat with 19,591 accepted droplets. It was paired with a repeat with 11,921 positives in 19,591 accepted.

A total of 37 reefs were studied over 9 trips. There were 79 reef-trip combinations, with 13 reefs visited only once, 7 visited twice, 16 visited thrice, and one visited four times. On most reefs three sites were sampled: usually the same three sites on each visit. Five sites were sampled at Lizard Island. Except for Lizard Island, twelve samples were taken at each site. Fifteen samples were taken at each of the five Lizard Island sites.

Table A 4. Number of observations (repeats) by range of positive counts for each trip.

	Positive Counts																
Trip	0	1	2	3-4	5-7	8-11	12-16	17-24	25-34	35-49	50-69	70-99	100-169	170-249	250-499	500-999	>1000
7594	777	144	68	57	31	23	19	17	14	7	3	0	2	0	2	0	0
7708	363	103	45	24	11	4	2	0	0	0	0	0	0	0	0	0	0
7816	875	117	47	52	23	12	10	9	1	0	0	0	0	0	0	0	0
7913	309	89	28	27	13	2	1	1	0	0	0	0	0	0	0	0	0
8036	281	94	37	29	9	2	2	0	0	0	0	1	1	0	0	0	0
8081	1042	221	108	117	85	37	21	6	3	0	0	0	2	0	0	0	2
8198	225	125	53	49	18	12	3	1	0	0	0	0	0	0	0	0	0
Init	141	22	5	0	1	5	3	11	14	8	8	6	13	3	0	0	0
JCU	55	30	18	51	83	62	69	47	49	38	22	4	8	0	3	1	2

Modelling

The data were modelled with a multilevel Poisson mixed model. An offset of log(accepted) accounted for the variation number in accepted droplets for each observation. Reef-Trip, with 79 levels, was the (fixed effect) variable of interest. The random effects were coded as repeat within sample within site within reef.

As R-code:

```
fit <- glmmTMB(Positive ~ 0 + offset(log(Accepted)) + ReefTrip +
                    (1|(ReefTrip:Site_No)/Site_sample/Repeat),
                family = poisson, data = eDNA)
```

The variance between repeats (i.e. observations) within samples was small. The variance between samples within sites was similar to the variance between sites within reefs (**Table A 5**).

Table A 5. Hierarchical model (fit) variance components.

```
Conditional model:
 Groups                              Name             Std.Dev.
 Repeat:Site_sample:Site_No:ReefTrip (Intercept)           0.063293
 Site_sample:Site_No:ReefTrip        (Intercept)           0.882360
 Site_No:ReefTrip                    (Intercept)           0.915753
```

The 79 Reef-Trip levels may be ordered by their estimated coefficient and assigned a percentile. A predicted sample proportion positive (PP) and seawater eDNA concentration (in molecules/L) may be estimated using the default L = 3, S = 12, R = 2 sampling protocol (**Table A 6**).

Table A 6. Reef-Trip effects ordered by magnitude with the percentile used for modelling sampling errors. The corresponding predicted sample proportion positive and eDNA concentration (molecules/L sea water) are also given. The orange lines indicate PP = 0.4 and PP = 0.8, which are of interest as cutoffs for COTS densities of 3 ha^{-1} and 10 ha^{-1}.

Reef-Trip	Coefficient	Percentile	Proportion	eDNA
Yonge_7708	-30.3	1.3	0.00	0.0
Ribbon_9_7816	-30.3	2.5	0.00	0.0
Ribbon_5_7816	-30.3	3.8	0.02	0.1
Ribbon_9_8081	-15.0	5.1	0.03	0.3
Osterlund_7816	-15.0	6.3	0.03	0.3
Yonge_8081	-15.0	7.6	0.03	0.3

Reef-Trip	Coefficient	Percentile	Proportion	eDNA
Osterlund_7594	-14.9	8.9	0.04	0.3
Undine_7816	-14.6	10.1	0.05	0.5
Ribbon_5_8081	-14.3	11.4	0.05	0.5
Elford_8081	-14.3	12.7	0.06	0.5
Yonge_7816	-14.3	13.9	0.06	0.5
Undine_8081	-14.2	15.2	0.06	0.6
Rudder_7816	-14.2	16.5	0.06	0.6
Davie_7594	-14.2	17.7	0.11	1.1
Cairns_7816	-13.3	19.0	0.14	1.5
Cairns_7594	-13.2	20.3	0.15	1.6
Thetford_8081	-13.1	21.5	0.16	1.8
Davie_8081	-13.0	22.8	0.16	1.8
Thetford_7816	-13.0	24.1	0.22	2.7
NorthDirl_7913	-12.5	25.3	0.24	3.2
Osterlund_8081	-12.4	26.6	0.26	3.6
11_049_Init	-12.3	27.8	0.29	4.2
Corbett_7594	-12.1	29.1	0.31	4.6
Elford_7816	-12.0	30.4	0.34	5.3
Tongue_7816	-11.9	31.6	0.36	5.9
13_124_7594	-11.8	32.9	0.37	6.1
Rudder_7594	-11.8	34.2	0.37	6.2
Moore_7816	-11.8	35.4	0.38	6.5
11_162_Init	-11.7	36.7	0.38	6.7
Clack_7594	-11.7	38.0	0.40	7.1
Rudder_8081	-11.6	39.2	0.41	7.3
Cairns_8081	-11.6	40.5	0.41	7.5
Undine_7594	-11.6	41.8	0.42	7.9
Shrimp_8036	-11.5	43.0	0.43	8.1
Tongue_8081	-11.5	44.3	0.43	8.2
NorthDirl_7708	-11.5	45.6	0.44	8.7
Eyrie_7913	-11.4	46.8	0.45	9.1
Prawn_8036	-11.4	48.1	0.46	9.3
Thetford_7594	-11.4	49.4	0.46	9.5
Davies_8036	-11.3	50.6	0.48	10.5
Darley_8036	-11.2	51.9	0.52	12.0
Tongue_7594	-11.0	53.2	0.54	13.1
Green_7594	-11.0	54.4	0.55	13.9
Lizard_7913	-10.9	55.7	0.58	15.7
Lizard_7708	-10.8	57.0	0.59	16.9
Green_8081	-10.8	58.2	0.61	17.8
Eyrie_7708	-10.6	59.5	0.62	19.3
Lynchs_8036	-10.6	60.8	0.64	20.6
NorthDirl_8198	-10.5	62.0	0.66	22.4
Eyrie_8198	-10.5	63.3	0.68	24.2
Batt_7816	-10.3	64.6	0.71	28.1
Green_7816	-10.2	65.8	0.72	29.8

Reef-Trip	Coefficient	Percentile	Proportion	eDNA
Banfield_8036	-10.2	67.1	0.73	31.8
Lizard_8198	-10.1	68.4	0.75	34.1
13_124_8081	-10.0	69.6	0.76	36.1
Moore_8081	-10.0	70.9	0.76	36.8
13_093a_8081	-10.0	72.2	0.76	37.3
Rib_Reef_8081	-10.0	73.4	0.79	42.3
Davies_7816	-9.5	74.7	0.84	58.3
Faith_8036	-9.5	75.9	0.84	59.3
Batt_8081	-9.5	77.2	0.85	62.1
Corbett_8081	-9.4	78.5	0.85	64.4
Batt_7594	-9.4	79.7	0.86	67.0
HedgeReef_8081	-9.3	81.0	0.87	72.4
Bowden_8081	-9.3	82.3	0.88	78.0
Moore_7594	-9.1	83.5	0.90	92.6
Davies_8081	-9.0	84.8	0.90	93.8
Davies_7594	-9.0	86.1	0.91	97.9
Bowden_7816	-9.0	87.3	0.92	108.1
Bowden_7594	-8.3	88.6	0.96	190.4
Corbett_JCU	-8.3	89.9	0.96	196.4
13_124_JCU	-8.1	91.1	0.97	244.1
McSweeney_JCU	-7.7	92.4	0.98	356.1
11_160_JCU	-7.7	93.7	0.99	372.7
Monsoon_JCU	-7.6	94.9	0.99	418.3
11_049_JCU	-7.2	96.2	0.99	568.8
13_093a_JCU	-7.2	97.5	0.99	576.5
11_164_JCU	-6.8	98.7	1.00	916.6
McSweeney_Init	-6.4	100.0	1.00	1363.5

In the dataset (as opposed to model predictions), the first three reef-trips had zero positive counts. The following five had only one positive count each.

Ecological Significance of Target PP Values

The selection of specific proportion positive (PP) values for our sampling strategy evaluation was based on their established relationship with COTS population densities and their importance in management decision-making. Two key threshold values were identified from previous ecological studies: PP = 0.4 (corresponding to model quantile 0.382) represents a critical ecological threshold where COTS populations reach approximately 3 individuals per hectare. At this concentration (mean of 7.2 molecules/L or median of 6.0 molecules/L of eDNA in seawater), COTS populations are beginning to approach levels that may indicate early outbreak conditions. This serves as an important early warning threshold for the COTS Control Program. PP = 0.8 (corresponding to model quantile 0.736) indicates COTS densities of approximately 10 individuals per hectare. At this higher concentration (mean of 45.0 molecules/L or median of 36.8 molecules/L of eDNA), COTS populations have reached active outbreak status, typically triggering direct intervention measures. This represents a critical management action threshold in the COTS Control Program.

These density thresholds were established using a separate analysis correlating eDNA measurements with visual COTS surveys. The relationship between eDNA concentration and PP is non-linear across the full range (**Figure A 2**), but is approximately linear in the PP range of 0.2 to 0.8, making PP an effective metric for quantifying COTS abundance within this ecologically relevant range. Beyond PP = 0.8, the relationship becomes increasingly non-linear as PP approaches its upper asymptote of 1.0, making high PP values less useful for discriminating between very high COTS densities. The simulations described below focus specifically on these two ecologically significant thresholds (PP = 0.4 and PP = 0.8) to determine optimal sampling strategies for accurately assessing whether reefs are above or below these important management decision points.

Standard Error Simulation

The point estimates (or corresponding percentiles, **Table A 6**) and variance structure (**Table A 5**) allow multiple draws of samples from with a defined sampling protocol to be simulated.

We illustrate, three examples of positive counts with the default L = 3, S = 12, R = 2 protocol, assuming accepted 22,000 droplet. The target quantile is 73.6% which corresponds to a mean sample proportion positive (PP) of 0.8 in a large sample (**Table A 7**).

Standard error simulations involve many simulations. In the results 100,000 simulations at each L, S, R and quantile level were used to get smoothed three-dimensional graphs. This is an example with only three simulations ("draws").

Table A 7. Positive counts after three simulated draws of 22,000 accepted droplets from the model at the 73.6% quantile. L = site, R = repeat and S = sample.

Draw 1, calculated concentration = 25.6 molecules/L and PP = 0.833

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
L1 R1	4	2	1	0	0	2	1	2	6	3	0	1
L1 R2	1	1	3	0	0	0	0	1	6	0	0	1
L2 R1	2	0	3	0	0	2	1	3	1	1	2	1
L2 R2	2	1	1	0	1	0	0	0	0	1	5	3
L3 R1	4	1	7	4	2	0	0	0	3	1	5	0
L3 R2	1	1	0	16	0	0	0	0	1	1	3	0

Draw 2, calculated concentration = 20.1 molecules/L and PP = 0. 556

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
L1 R1	1	0	0	2	1	0	0	4	0	1	2	0
L1 R2	1	0	0	2	1	0	0	8	0	2	0	1
L2 R1	0	0	0	1	2	0	0	6	0	0	2	7
L2 R2	0	0	0	1	3	0	0	11	0	3	0	8
L3 R1	1	0	1	2	1	1	1	0	2	1	0	1
L3 R2	1	0	3	1	0	2	0	1	0	1	0	0

Draw 3, calculated concentration = 34.5 molecules/L and PP = 0. 778

	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12
L1 R1	4	0	0	0	4	4	9	2	2	0	1	2
L1 R2	3	1	0	0	3	2	6	0	1	0	1	4
L2 R1	1	0	1	1	5	5	9	5	0	0	1	0
L2 R2	0	0	4	0	4	5	9	2	0	0	0	0
L3 R1	0	5	3	0	1	6	0	4	0	4	2	1
L3 R2	0	2	4	0	1	11	0	7	0	0	2	1

The three draws have concentrations of 25.6, 20.1 and 34.5 DNA molecules/L respectively. For this small sample, the mean concentration is 26.7 molecules/L, with a standard error of 7.3.

Concentration measurements, which are a multiple of positive counts, are highly right skewed. Hence, for a sample with 100,000 draws the mean concentration was 45.1 molecules/L, with a standard error of 32.3.

Expressed as sample proportion positive, the three draws have PPs of 0.833, 0.556 and 0.778. The mean is 0.722 with a standard error of 0.147. For a sample with 100,000 draws the mean PP was 0.7978 ~ 0.80, with a standard error of 0.115.

The model can readily be used to simulate other sampling protocols (**Table A 8** and **Table A 9**) by varying the L, S, R and quantile parameters.

Table A 8. A single draw with an L = 4, S = 6, R = 2 protocol at the 38.2% quantile, corresponding to a PP = 0.4 in large samples.

	S1	S2	S3	S4	S5	S6
L1 R1	1	1	1	0	0	2
L1 R2	0	2	0	0	0	0
L2 R1	0	0	2	0	0	0
L2 R2	0	0	1	0	0	0
L3 R1	1	0	0	0	0	0
L3 R2	0	0	0	0	0	0
L4 R1	0	1	0	0	1	0
L4 R2	0	0	0	0	1	0

Table A 9. A single draw with an L = 5, S = 8, R = 1 protocol at the 90.0% quantile.

	S1	S2	S3	S4	S5	S6	S7	S8
L1 R1	44	4	4	8	1	11	11	14
L2 R1	3	5	12	3	7	4	16	60
L3 R1	5	10	0	25	2	29	10	8
L4 R1	6	1	17	22	3	18	24	2
L5 R1	2	2	0	5	3	69	18	58
L1 R1	44	4	4	8	1	11	11	14

A large number of draws (100,000) were simulated at the sampling protocols and quantiles of interest. From these, a mean and standard error were calculated. While the calculated standard errors are representative of the precision of each sampling strategy, the underlying distributions are skewed (**Figure A 1**). This is evidenced by the difference between two measures of precisions: the standard error, and the boundaries of the lower 15% and upper 85% of the draws from the simulation. (One standard deviation from the mean of a Normal Distribution corresponds to the 15.9% and 84.1% quantiles.).

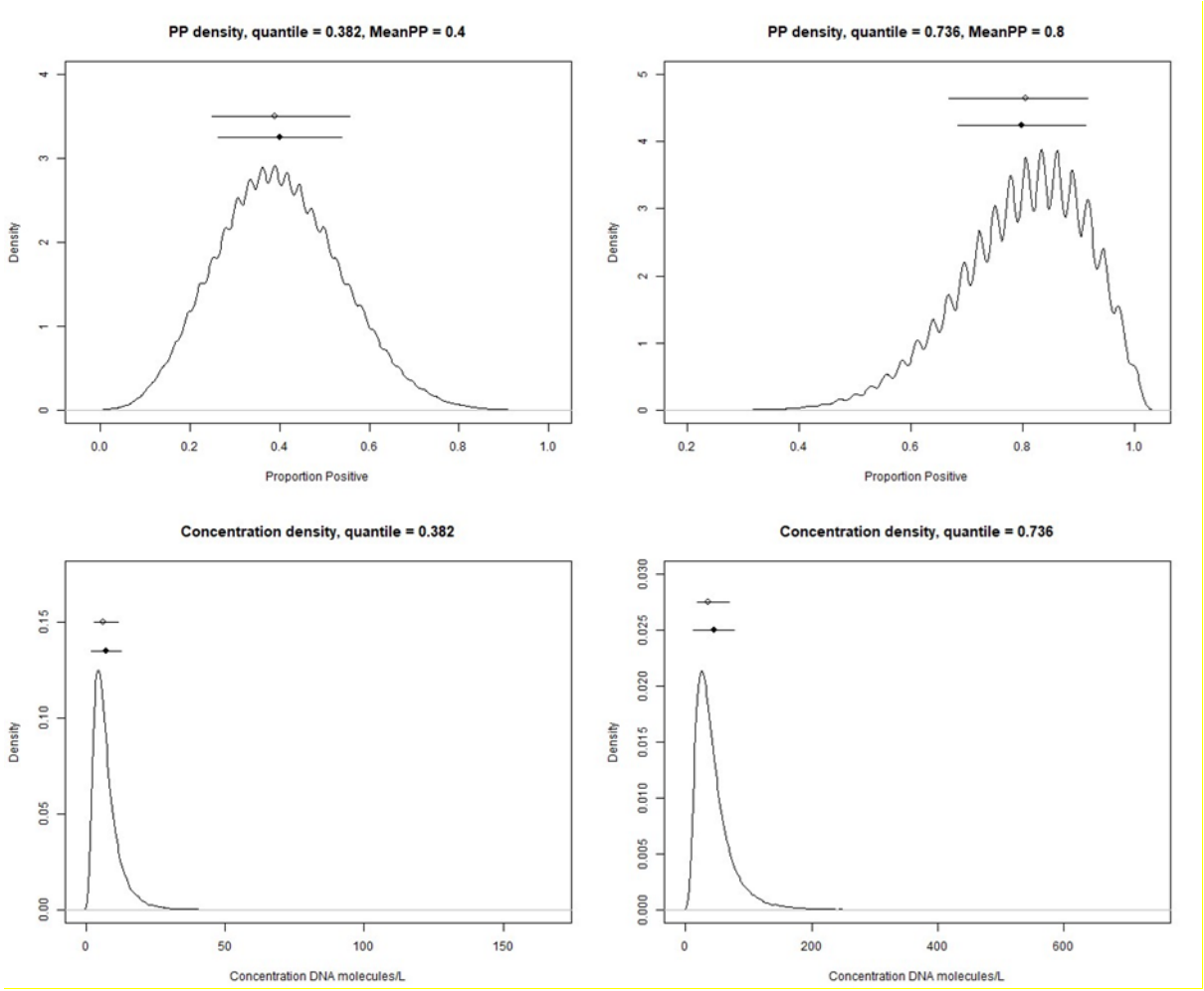


Figure A 1. Simulation of 100,000 draws (each equivalent to a reef) at two model quantiles: 0.382 (left) and 0.736 (right), which correspond to two proportion of positive samples (PP) of interest, viz PP = 0.4 and PP = 0.8. The hollow circle is the median with bars covering ±35% of samples (15% to 85%). The filled circle is the mean with bars covering ±1 standard error (mean ± SE). This simulation is for the 3 sites per reef, 12 samples per site and 2 repeats per sample design (L = 3, S = 12, R = 2). The "bumps" in the PP (top) are because this measure is discrete the 36 samples per reef result in 37 discrete values. The graphing software (the density() function in R) has smoothed these values. The concentration density (bottom) is highly skew, resulting in both the mean and standard error being greater than the corresponding median and 15% to 85% range. The concentration (x) axes on the concentration density plots cover the full range of draws from 100,000 simulations. Small numbers of larger values distort the mean and SE.

The simulated concentrations and sample proportion positives (PP) have a non-linear relationship (**Figure A 2**). The relationship is approximately linear over the PP = 0.2 to PP = 0.8 range. Beyond this, and particularly at high concentrations, the relationship is non-linear, reaching asymptotes at PP = 0 and PP = 1. At high concentrations PP is not useful as a measure of the amount of COTS DNA in the water samples.

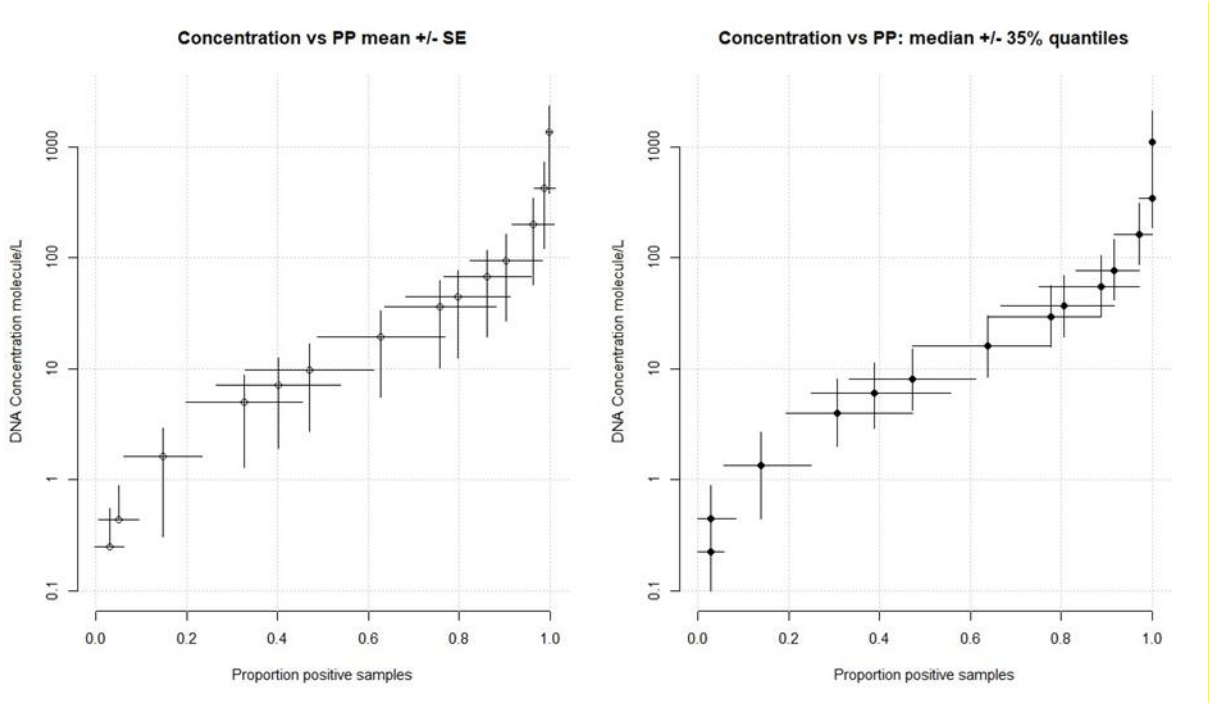


Figure A 2. Simulated concentrations vs proportion positives (PP) based on mean ± SE (left) and median ± 35% quantiles (right). Again, the sampling design is 3 sites per reef, 12 samples per site and 2 repeats per sample design (L = 3, S = 12, R = 2) and varying quantile between 0% and 100% (0.0 to 1.0).

Optimal Sampling Strategies

Simulations were performed over a large range of sampling strategies. Those presented in the body of the report (**Figure 11** and **Figure 12**) are at COTS eDNA reef concentrations and mean sample proportions positives of interest to AIMS, viz PP = 0.4 and PP = 0.8 (equivalent to DNA mean reef concentrations of 7.2 and 45.0 molecules/L, and median reef concentrations of 6.0 and 36.8 molecules/L). The simulations were also restricted to two repeats per sample, i.e. they assume the current laboratory practice of duplicating the sample analysis by preparing two plate wells per sample.

The model quantiles of 0.382 and 0.736 corresponded to mean sample PPs of 0.40 and 0.80, and median sample PPs of 0.39 and 0.81.

The standard error (SE) was used as a single measure of precision for each of the sampling strategies (**Figure 11** and **Figure 12** in the main report). Unlike quantile measurements (like 15% to 85%), it is symmetrical. It is also closely related to other measures of spread of the data.

To estimate the relationship between SE and reef sampling strategies at each target PP, we simulated standard errors across 1 to 12 sites per reef (L = 1 to 12) at each of 1 to 12 samples per site (S = 1 to 12). All simulations assumed two repeats (duplicates) per sample (R = 1).

For each of the 144 combinations of L and R, 100,000 simulations were done. From the distribution of these, a mean and standard error were calculated (e.g. see **Figure A 1** above for an example at quantiles 0.382 and 0.736, corresponding to PP = 0.4 and PP = 0.8, and see **Table A 7** and the accompanying text for an illustration of the calculation with 3 simulations/draws).

The findings follow a consistent pattern across many combinations of L, S, R and quantile investigated (e.g. see **Figure 12** in the main report for the typical pattern).

Unsurprisingly the greatest error (least precision) was found when sampling one site per reef and one sample per site (i.e. one sample per reef). The SE initially dropped rapidly as the number of sites and/or samples increased (**Figure 11** and **Figure 12**). The fall was faster with increases in sites per reef (L) than with increases in samples per site (S). Although the fall continued, eventually the addition of more total samples (L x S) lead to diminishing returns. Compared to the current sampling strategy (L = 3, S = 12, R = 2; 36 samples), twelve sites per reef and twelve samples per site (L = 12, S = 12; 144 samples) were required to approximately halve the sampling error in both PP and concentration at PP = 0.8. At PP = 0.4, the L = 12, S = 12 (144 sample) strategy produced only about a 30% reduction in sampling error compared to L = 3, S = 12 (36 samples).

An important finding was that the simulated error (i.e. precision) under the current sampling strategy (L = 3, S = 12) could be maintained with fewer total samples (**Figure 11** and **Figure 12** in the main text). Currently 3 x 12 = 36 total samples are taken at each reef. Sampling protocols of L = 4 and S = 6; L = 5 and S = 4; and L = 6 and S = 3 – requiring 24, 20 and 18 samples respectively – had approximately the same SE as the current L = 3, S = 12 protocol. The biggest gain in sampling efficiency (i.e. reduction in total samples) was achieved by adding one site per reef and halving the number of samples per site.

APPENDIX E: CONSIDERATIONS FOR SAMPLES IN THE PRESENCE OF POSITIVE DROPLETS IN NEGATIVE CONTROLS

Executive Summary

Adjusting for background contamination and testing whether the COTS eDNA are above a certain threshold are related. Contamination is measured using control samples of two types: two-thirds were filtered from distilled water in the field, while the remainder were “extraction controls” prepared in the lab. These corrections and calls are only important i.e. may affect management decisions, at the lowest eDNA concentrations in field samples.

The current method of false-positive correction (FPC) subtracts positive counts from field samples in proportion to the number found in control samples, with the assignment of samples to be “corrected” made at random. By chance, more correction is applied to some sites and/or reefs, introducing bias. If such sites have low positive counts they may be moved below a threshold that would normally trigger further investigation or reef culling, which happened with North Direction Island Reef in this study.

Rather than applying a FPC we used statistical bootstrapping on the raw data to infer (“call”) the presence or absence of eDNA in site samples. This is a robust way to declare the presence of eDNA above a certain threshold whilst also taking into account any contamination.

Determining the best way to account for contamination when using the data for other purposes e.g. trend analysis is more difficult. At low concentrations, both control and reef samples consisted entirely or almost entirely of zeroes for this CCIP study. The current low levels of contamination have little impact on inference about reef COTS densities. It is not clear how to adjust for this contamination without introducing bias and statistical issues. Nor is it clear how this contamination may be attributed to procedures in the workflow, an important consideration when correcting for background contamination. We recommend using the raw reef sample measurements without correction for the low concentrations found in the control samples.

It is possible that “operationalising” reef eDNA measurements on cull vessels may lead to higher levels of contamination. If this is the case, one approach may be to take controls between each reef on a trip. This would enable determination of the point at which contamination intruded on the sampling. If contamination intrudes part-way during a sampling trip, statistical methods can be used to adjust the limit of detection. It is possible contamination occurs primarily in the lab, as there was no significant difference between controls prepared entirely in the lab (extraction controls) and field controls.

In the absence of “real world” data from the control vessels it is difficult to determine appropriate analytical techniques. We suggest revisiting this issue if it becomes a concern.

Introduction

The Australian Institute of Marine Science (AIMS) is seeking a method of false positives correction (FPC) and method for testing whether COTS eDNA in reef water samples is above potential background contamination. This report presents a proposed bootstrap method for declaring a statistically significant COTS eDNA signal and a discussion around issues of

correcting for false positives. It also highlights the effects and potential downsides of the existing FPC framework.

Background

From a statistical perspective, analysis of low levels of eDNA is difficult because of its discrete nature and small counts in each sample. Control samples may be entirely zero or have only a few positives from thousands of counted droplets. Some site samples are similar to the controls, while others have counts spanning an order of magnitude or more. Sites with low eDNA concentrations consist almost entirely of zero counts, as do control samples. Such sparse data are difficult to compare using traditional parametric statistical methods.

Approach

To test whether COTS eDNA is above any background contamination at a site we consider a bootstrap approach. The statistic of interest is the difference in the means between control samples and field samples. Bootstrapping involves repeatedly drawing samples with replacement. The number of draws from each sample set – control or field – is the same as the number of samples in the respective set. In a single draw, N_c samples are drawn from the N_c controls and N_s samples from the N_s site samples. In each draw the same sample may be drawn multiple times or not at all. The difference between the means of these two bootstrap samples is then calculated. This is repeated many times (10,000 total here) which results in an empirical distribution of the statistic of interest, in this case the difference in means between site samples and matched controls.

Considerations

In practice, controls were taken on each *trip* on which many *reefs* and *sites* per *reef* were sampled. Controls from a given *trip* are paired with each set of *site* samples (from the same trip) for analysis.

About two-thirds of controls have been sampled in the field – to represent real-world sampling – with one-third prepared in the lab – as a test of process contamination.

Whilst all field samples were filtered from 2.5 litres of sea water, field controls were filtered from either 2.5 litres (for the first three trips) or 1.0 litres of distilled water (subsequently). Laboratory “extraction” controls did not involve volumetric filtration.

For consistency, it would be preferable to compare positive droplet concentrations per litre of water, whether sea water or distilled water used for controls. However, this would involve excluding one-third of controls (the extraction controls). Furthermore, contamination may occur on either a per-unit-volume or a per-sample basis. Any contamination is almost certainly due to COTS DNA in solution or on surfaces as eDNA analysis is highly specific. Volumetric carry-over between samples may cross-contaminate COTS DNA, as might equipment repeatedly used to process samples. It was the view of the experimental scientists that this second form of contamination was most likely, meaning that treating the contamination on a per sample basis is justified.

This treatment of controls assumes that sample handling is the primary cause of contamination. This is borne out by the presence of contamination in both extraction and field controls (**Table A 10**). Whilst there is no statistical difference in contamination between extraction and field controls ($p = 0.92$), the amount of contamination was too low to draw a statistical conclusion. It is possible contamination occurs primarily in the laboratory.

Table A 10. Positive droplets per observation (repeat) by control type

	Control Type	
Positive drops	Extraction	Field
0	152	363
1	2	3
2	0	2

Data Structure

The data have a hierarchical structure and are correlated within each level. Bootstrap analysis is difficult for hierarchical data.

A typical *trip* of approximately a week may involve visits to several *reefs*. At each *reef,* several *sites* (usually 3) are chosen for sampling. Typically, 12 – but sometimes more – water *samples* are taken at each site. In essence, each sample is split into two *repeats* for eDNA digital drop analysis. Hence *repeats* are the *lowest observational level* for data analysis.

For each observation in droplet-digital PCR (ddPCR) a sample volume is partitioned into many uniformly sized droplets (each 0.85 nanolitres). These "accepted" droplets are divided into those which contain COTS DNA ("positive") and those that don't ("negative"). At low DNA concentrations almost all droplets are negative.

For each observation, the total number of *accepted* droplets was near uniform, with a mean of close to 22,000 (median ~ 22,500), 1% had fewer than 14,500 and 1% had more than 24,100. In contrast, about 60% of observations had zero positive droplets; 5% have 15 or more and 1% had 58 or more positive droplets.

On a given trip, many sites had few observations with non-zero positive counts.

Here we consider testing the significance of the eDNA signal on a *site* basis. Is there statistical evidence that site eDNA measurements exceed the contamination levels evidenced in the control samples?

Site data have two levels: *samples* and *repeats*. *Repeats* are correlated, especially when counts are high. Analysis at the *repeat* level treats repeats as independent and is *more likely* to detect differences when none exist if correlation is considered. Analysis at the *sample* level (by pooling *repeats*) is more conservative and is *less likely* to detect differences when they exist. Given many comparisons are made, a conservative approach is appealing, but both *sample*-based and *repeat*-based will be presented.

The Existing False Positive Correction (FPC)

Of the nine *trips* considered here (7594, 7708, 7816, 7913, 8036, 8081, 8198, Init and JCU), only three (7708, Init and JCU) had any detection of contaminants (**Table A 11**).

Table A 11. Positive droplets per observation (repeat) for trip controls.

	Trip								
Positive drops	7594	7708	7816	7913	8036	8081	8198	Init	JCU
0	86	34	66	26	72	132	42	21	36
1	0	1	0	0	0	0	0	3	1
2	0	1	0	0	0	0	0	0	1

For example, in Trip 7708, 3 positive counts occurred across 36 control *repeats* (18 *samples*), two in one repeat and another in a second repeat (proportion of 1 in 12). There were 516 field *repeats* (258 *samples*) on this *trip*, of which 187 had positive (> 0) counts. One-twelfth of 516 is 43, hence in proportional terms 3 contaminating drops in 36 repeats is proportionally 43 contaminating drops in 516 repeats. The correction consists of subtracting 43 positives from 516 *repeats*. However, only 187 of these *repeats* are positive. Repeats with zero counts are clearly not contaminated. Hence 43 of the 187 positive *repeats* are randomly chosen and one positive drop is subtracted from each.

This random allocation tends to distribute FPC unevenly across *sites*. The most extreme example is Site NDEDNA2 at North Direction Island. Initially a total of 6 positive drops were measured from 24 *repeats* (12 *samples*). Five positives were subtracted from these as a result of the random assignment process, leaving only a single positive drop in the final data for NDEDNA2 (**Table A 12**). When site results are expressed as proportion positive, 2 sites (11-049EDNA2, and NDEDNA2) drop below the PP = 0.4. and PP = 0.8 cutoffs. Again, NDEDNA2 is the most notable, having fallen from PP = 0.42 to PP = 0.08.

After summing all three sites, North Direction Island Reef falls from a raw PP = 0.44 to a corrected PP = 0.31, below the PP = 0.4 threshold associated with 3 COTS ha^{-1}, again mostly due to the NDEDNA2 site (**Table A 13**).

The pre-correction and post-correction positive counts and proportion positive by *site* are summarised in **Table A 12** and by Reef in **Table A 13**.

Table A 12. Before and after False Positive Correction by Site-Trip combinations for sites with non-zero controls. The table compares raw positive counts and proportion positive (PP) values with their corresponding corrected values after applying the current false positive correction (FPC) method. Data shown only for trips where controls exhibited some level of contamination (7708, Init, and JCU).

Reef	Site	Trip	Raw Positive Counts	Corrected Positive Counts	Raw Proportion Positive	Corrected Proportion Positive
11-049	NW point	JCU	827	825	1.00	1.00
11-049	SW Corner	JCU	392	390	1.00	1.00
11-049	Western edge	JCU	269	268	1.00	1.00
11-049	11-049EDNA1	Init	3	2	0.25	0.17
11-049	11-049EDNA2	Init	5	4	0.42	0.33
11-049	11-049EDNA3	Init	4	2	0.25	0.17
11-160	NW point	JCU	1416	1414	1.00	1.00
11-160	Western point	JCU	257	254	1.00	1.00
11-162	11-162EDNA1	Init	10	10	0.50	0.50
11-162	11-162EDNA2	Init	43	43	0.42	0.42
11-162	11-162EDNA3	Init	0	0	0.00	0.00
11-164	Western point	JCU	842	840	1.00	1.00
13-093a	Bommie	JCU	381	380	1.00	1.00
13-093a	Channel entrance	JCU	1321	1318	1.00	1.00
13-093a	North Point	JCU	220	219	1.00	1.00
13-124	NW point	JCU	840	838	1.00	1.00
13-124	Southernmost point	JCU	82	81	0.75	0.75
13-124	Western bay	JCU	187	185	1.00	1.00
Corbett	Lighthouse outside	JCU	235	232	1.00	1.00
Corbett	SE corner	JCU	270	269	0.92	0.92
Corbett	SE edge	JCU	119	115	1.00	1.00
Eyrie	EREDNA1	7708	10	9	0.75	0.67
Eyrie	EREDNA2	7708	12	9	0.67	0.58
Eyrie	EREDNA3	7708	44	39	0.92	0.92
Lizard Island	Big Vickis Reef	7708	56	54	0.53	0.53
Lizard Island	Casuarina Beach	7708	48	44	0.73	0.63
Lizard Island	Clam Gardens	7708	170	159	0.97	0.93
Lizard Island	Lagoon	7708	8	6	0.27	0.20
Lizard Island	Mermaid Cove	7708	25	19	0.50	0.40
McSweeney	McREDNA1	Init	3057	3054	1.00	1.00
McSweeney	McREDNA2	Init	941	934	1.00	1.00
McSweeney	McREDNA3	Init	409	406	1.00	1.00
McSweeney	Northern edge	JCU	201	199	1.00	1.00
McSweeney	SW corner	JCU	276	272	1.00	1.00
McSweeney	Western shoal	JCU	5551	5551	1.00	1.00
Monsoon	NW point	JCU	351	349	1.00	1.00
Monsoon	Southern edge	JCU	401	400	0.92	0.92
Monsoon	SW Bay (near Cay)	JCU	599	598	1.00	1.00
North Direction Island	NDEDNA1	7708	22	19	0.75	0.67

North Direction Island	NDEDNA2	7708	6	1	0.42	0.08
North Direction Island	NDEDNA3	7708	4	3	0.17	0.17
Yonge	YREDNA1	7708	0	0	0.00	0.00
Yonge	YREDNA2	7708	0	0	0.00	0.00
Yonge	YREDNA3	7708	0	0	0.00	0.00

Table A 13. Proportion Positive Before and after False Positive Correction by Reef-Trip. The table compares raw proportion positive (PP) values with their corresponding corrected values after applying the current false positive correction (FPC) method.

Reef	Trip	Raw Proportion Positive	Corrected Proportion Positive
11-049	JCU	1.00	1.00
11-049	Init	0.31	0.22
11-160	JCU	1.00	1.00
11-162	Init	0.31	0.31
11-164	JCU	1.00	1.00
13-093a	JCU	1.00	1.00
13-124	JCU	0.92	0.92
Corbett	JCU	0.97	0.97
Eyrie	7708	0.78	0.72
Lizard Island	7708	0.60	0.54
McSweeney	Init	1.00	1.00
McSweeney	JCU	1.00	1.00
Monsoon	JCU	0.97	0.97
North Direction Island	7708	0.44	0.31
Yonge	7708	0.00	0.00

Bootstrap Procedure and Results

The bootstrap procedure for FPC is illustrated for the site NDEDNA2 on North Direction Island Reef during Trip 7708.

Each observation was expressed as a proportion of droplets which are positive (positive/accepted). These proportions were normalised by multiplying by the average number of accepted droplets per observation (22,000). When repeats were combined at the sample level, a simple mean was used.

The 12 NDEDNA2 field sample proportions, normalised to 22,000 accepted droplets, were:

0.00, 0.00, 0.47, 0.00, 0.48, 0.00, 0.46, 0.00, 0.97, 0.47, 0.00, 0.00

Four of these samples had approximately 0.5 positive droplets when normalised to 22,000 accepted droplets, which corresponded to one of the two repeats in the sample having a single positive droplet.

The 18 field control sample normalised proportions for *trip* 7708 were,

0.48, 0.00, 0.00, 0.00, 0.00, 0.00, 0.00, 0.00, 0.00, 0.00, 0.00, 0.00 1.41, 0.00, 0.00, 0.00, 0.00, 0.00

The raw means for the sample and control are 0.238 and 0.104 positive droplets per 22,000 respectively, with a difference of 0.133.

By inspection, for the sample mean to exceed the control mean on bootstrap, any random selection that includes the control value of 1.41 at least once must include at least two non-zero values from the field sample set to have a higher mean.

Choosing 12 observations with replacement gives an example of one bootstrap field sample:

0.00, 0.46, 0.47, 0.00, 0.00, 0.00, 0.47, 0.47, 0.00, 0.00, 0.00, 0.00

An example bootstrap sample of the control values is:

0.00, 0.00, 0.00, 0.48 1.41, 0.00, 0.00, 0.00, 0.00, 0.00, 0.00 1.41, 0.00, 0.00, 0.00, 0.00, 0.00, 0.00

The means of these samples are 0.156 and 0.183 respectively and their difference is -0.0275, an example where the bootstrap control mean exceeds the bootstrap sample mean.

Repeating this process many times (10,000 here) gives a distribution of the difference between the two means (**Figure A 3**). Although the mode, median and mean of this distribution are greater than zero, 12% of results are less than zero (p = 0.12).

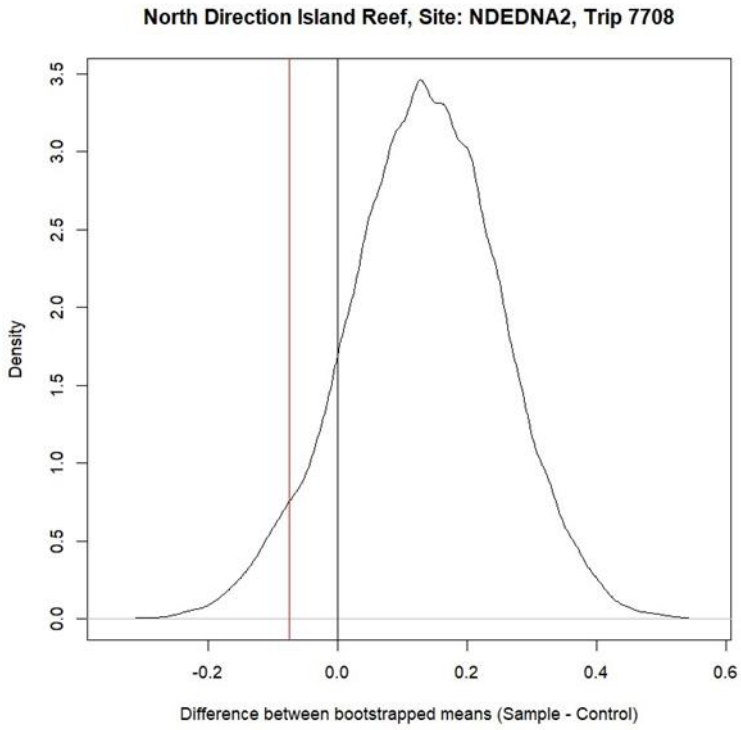


Figure A 3. 5% of samples are less than the red line.

Based on this test we would not be confident we have detected eDNA above the background contamination at this site.

An eDNA concentration adjusted for false positives would be their difference of 1.94 copies/L, although this is not statistically significant.

This false positive corrected concentration and bootstrap test is designed for inference on a site basis. It is preferable to use the uncorrected field samples for such purposes as: mapping, detecting changes with time, or detecting differences between sites. Though individual site-based tests may reject evidence of COTS above background contamination, that does not mean that COTS is not present. And since contamination controls are not specific to each site, the best measurement of COTS concentration at a site is the mean concentration.

If the previous procedure is repeated for the NDEDNA2 site (Trip 7708), the result is almost identical (p = 0.12).

When applied across all *Site-Trips* conclusions about presence/absence are very similar when the analysis is at the repeat or sample level (**Table A 14**

Proposed Approaches to Background Contamination or False Positive Correction (FPC)

With current levels of contamination, the eDNA concentrations of interest are somewhat above noise levels.[1] Intuitively we would simply subtract control from field sample eDNA measurements. However, this is complicated by several issues.

1. Only 1 L of water is used to prepare field controls, compared to 2.5 L for reef samples. The natural quantitative approach would be to subtract control from sample values in the final units of measurement, i.e. concentrations. But it seems more likely contamination occurs on a per sample basis.
2. Laboratory extraction controls have no volume associated with them, hence correcting on a concentration basis is not possible. Excluding them removes one-third of the controls.
3. Randomly subtracting positive counts (as per the current technique) can potentially eliminate reefs from consideration for further investigation, especially when "proportion positive" (PP) is used to guide culling decisions.
4. Subtracting an average of (control) positive counts neglects the denominator; the small number of positive droplets are among many accepted (mostly negative) droplets, averaging 22,000, but ranging over a factor of two.
5. Subtracting an average contamination is not compatible with the sample proportion positive metric.
6. Many reef sites have zero detected eDNA. Uniformly subtracting a control will result in negative positive counts or concentrations. This causes statistical problems as well as being physically impossible.

Another set of issues arises from attribution of contamination. Where does the contamination occur?

1. Both laboratory and field controls show low levels of contamination. There is no significant difference between the two in this large study. Potentially most contamination occurs in the laboratory.
2. Several sites and 3 reef-trips (of 79) had zero measured eDNA, demonstrating that field eDNA concentrations can be below the limit of detection, including any background contamination from the sampling process.
3. The controls are more akin to quality controls rather than experimental controls. For example, experimental controls would normally be prepared from seawater sampled during the trip, rather than distilled water taken aboard. Seawater controls could be

[1] For context, a COTS density of 3 individuals ha^{-1} – the lower threshold of interest – corresponds to approximately a proportion positive (PP) of 0.4; an average of 4.8 positive samples per site. Using simulations explained elsewhere in this report, that corresponds to an average of about 10.8 positive droplets per site. At the low levels of contamination recorded in this study, only 2 or sometimes 3 positive drops per site are sufficient to be confident of a detection above the noise level. With current levels of contamination, the eDNA concentrations of interest are well above noise levels.

away from the reef if eDNA is localised. Experimental controls are usually paired with test samples, which is not practical.

4. Contamination is likely from specific events during sample handling, either in the field or lab. It seems more likely to occur after (either immediately or delayed) handling samples (in the field or lab) with high eDNA concentration. It is not clear how these events could be detected or corrected with the current procedures.

The current low levels of contamination have little impact on inference about reef COTS densities. It is not clear how to adjust for this contamination without introducing bias and statistical issues, nor is it clear how this contamination may be attributed to procedures in the workflow.

We recommend using the raw reef measurements, with the following caveat.

It is possible that “operationalising” reef eDNA measurements on cull vessels may lead to higher levels of contamination.

One approach may be to take controls between reefs. This would enable determination of the point at which contamination intruded on the sampling. Statistical time-series analysis could then be used to adjust for contamination. If contamination intrudes part-way during a sampling trip, the limit of detection would change from this time onwards.

We suggest revisiting this issue if it becomes a concern.

Summary of Bootstrap Results for Presence/Absence

Table A 14. Statistical comparison of eDNA signals between reef sites and control samples. This table presents results from bootstrap analyses comparing COTS eDNA levels at different sampling locations with their corresponding control samples. For each reef-trip-site combination, the table shows: sample mean (normalised positive droplets per 22,000 accepted droplets), control mean, mean difference, and p-values indicating statistical significance at both sample and repeat levels. Significance is indicated as: NS (not significant), * (p<0.05), ** (p<0.01), or *** (p<0.001). Results with p<0.05 represent sites where COTS eDNA signal is statistically distinguishable from background contamination.

REEF	TRIP	SITE	Sample mean	Control mean	Difference	p-value samples		p-value repeats	
11-049	JCU	NW point	33.859	0.079	33.78	0	***	0	***
11-049	JCU	SW Corner	15.951	0.079	15.872	0	***	0	***
11-049	JCU	Western edge	11.069	0.079	10.99	0	***	0	***
11-049	Init	11-049EDNA1	0.122	0.12	0.002	0.433	NS	0.43	NS
11-049	Init	11-049EDNA2	0.203	0.12	0.083	0.175	NS	0.195	NS
11-049	Init	11-049EDNA3	0.159	0.12	0.039	0.368	NS	0.384	NS
11-160	JCU	NW point	55.671	0.079	55.592	0	***	0	***
11-160	JCU	Western point	10.342	0.079	10.263	0	***	0	***
11-162	Init	11-162EDNA1	0.413	0.12	0.293	0.017	*	0.023	*
11-162	Init	11-162EDNA2	1.756	0.12	1.636	0.035	*	0.006	**
11-162	Init	11-162EDNA3	0	0.12	-0.12	1	NS	1	NS
11-164	JCU	Western point	34.091	0.079	34.012	0	***	0	***
13-093a	8081	13-093aEDNA 1	0.906	0	0.906	0	***	0	***
13-093a	8081	13-093aEDNA 2	2.192	0	2.192	0	***	0	***
13-093a	8081	13-093aEDNA 3	1.096	0	1.096	0	***	0	***
13-093a	JCU	Bommie	15.283	0.079	15.204	0	***	0	***
13-093a	JCU	Channel entrance	53.652	0.079	53.573	0	***	0	***
13-093a	JCU	North Point	8.721	0.079	8.642	0	***	0	***
13-124	8081	13-124EDNA MP1	0.742	0	0.742	0	***	0	***
13-124	8081	13-124EDNA MP2	8.702	0	8.702	0	***	0	***
13-124	8081	13-124EDNA MP3	0.289	0	0.289	0.001	***	0	***
13-124	JCU	NW point	34.391	0.079	34.312	0	***	0	***
13-124	JCU	Southernmost	3.3	0.079	3.221	0	***	0	***
13-124	JCU	Western bay	7.685	0.079	7.605	0	***	0	***
13-124	7594	13-124EDNA1	0.043	0	0.043	0.342	NS	0.362	NS
13-124	7594	13-124EDNA2	0.747	0	0.747	0	***	0	***
13-124	7594	13-124EDNA3	0.228	0	0.228	0.031	*	0.015	*
Banfield	8036	BAN_CCIPD02_1	2.693	0	2.693	0	***	0	***
Banfield	8036	BAN_CCIPD02_2	0.543	0	0.543	0	***	0	***
Batt	7594	BAEDNA1	1.553	0	1.553	0	***	0	***
Batt	7594	BAEDNA2	1.064	0	1.064	0	***	0	***
Batt	7594	BAEDNA3	7.242	0	7.242	0	***	0	***
Batt	7816	BAEDNA1	0.742	0	0.742	0	***	0	***
Batt	7816	BAEDNA2	0.524	0	0.524	0	***	0	***
Batt	7816	BAEDNA3	2.59	0	2.59	0	***	0	***
Batt	8081	BAEDNA1B	2.739	0	2.739	0	***	0	***
Batt	8081	BAEDNA2B	6.605	0	6.605	0	***	0	***
Batt	8081	BAEDNA3	0.922	0	0.922	0	***	0	***
Bowden	7594	BREDNA1	13.253	0	13.253	0	***	0	***
Bowden	7594	BREDNA2	1.443	0	1.443	0	***	0	***
Bowden	7594	BREDNA3	12.15	0	12.15	0	***	0	***
Bowden	7816	BREDNA1	1.686	0	1.686	0	***	0	***
Bowden	7816	BREDNA2	14.07	0	14.07	0	***	0	***
Bowden	7816	BREDNA3	1.508	0	1.508	0	***	0	***
Bowden	8081	BREDNA1	6.352	0	6.352	0	***	0	***
Bowden	8081	BREDNA2	2.267	0	2.267	0	***	0	***
Bowden	8081	BREDNA3	1.292	0	1.292	0	***	0	***
Cairns	7594	CREDNA1	0.086	0	0.086	0.352	NS	0.363	NS
Cairns	7594	CREDNA2	0.045	0	0.045	0.341	NS	0.36	NS
Cairns	7594	CREDNA3	0.081	0	0.081	0.117	NS	0.121	NS
Cairns	7816	CREDNA1	0.199	0	0.199	0.028	*	0.013	*
Cairns	7816	CREDNA2	0.04	0	0.04	0.351	NS	0.355	NS

Cairns	7816	CREDNA3	0	0	0	1	NS	1	NS
Cairns	8081	CREDNA1	0.166	0	0.166	0.007	**	0.013	*
Cairns	8081	CREDNA2	0.325	0	0.325	0.002	**	0.001	**
Cairns	8081	CREDNA3	0.451	0	0.451	0	***	0	***
Clack	7594	CLREDNA1	0.215	0	0.215	0.029	*	0.013	*
Clack	7594	CLREDNA2	0.212	0	0.212	0.008	**	0.003	**
Clack	7594	CLREDNA3	0.467	0	0.467	0.001	***	0.001	***

REEF	TRIP	SITE	Sample mean	Control mean	Difference	p-value samples		p-value repeats	
Corbett	7594	COREDNA1	0.217	0	0.217	0.11	NS	0.125	NS
Corbett	7594	COREDNA2	0.045	0	0.045	0.355	NS	0.351	NS
Corbett	7594	COREDNA3	1.654	0	1.654	0.11	NS	0.041	*
Corbett	8081	CorReDNA MP1	4.167	0	4.167	0	***	0	***
Corbett	8081	CorReDNA MP2	2.884	0	2.884	0	***	0	***
Corbett	8081	CorReDNA MP3	1.247	0	1.247	0	***	0	***
Corbett	JCU	Lighthouse	9.573	0.079	9.494	0	***	0	***
Corbett	JCU	SE corner	11.124	0.079	11.045	0	***	0	***
Corbett	JCU	SE edge	4.783	0.079	4.704	0	***	0	***
Darley	8036	DAR_CCIPD02_1	1.204	0	1.204	0	***	0	***
Darley	8036	DAR_CCIPD02_2	0.493	0	0.493	0.002	**	0	***
Darley	8036	DAR_CCIPD02_3	0.971	0	0.971	0	***	0	***
Darley	8036	DAR_CCIPD02_4	0	0	0	1	NS	1	NS
Davie	7594	DAVEDNA1	0.043	0	0.043	0.354	NS	0.362	NS
Davie	7594	DAVEDNA2	0	0	0	1	NS	1	NS
Davie	7594	DAVEDNA3	0.04	0	0.04	0.362	NS	0.371	NS
Davie	8081	DAVEDNA1	0.04	0	0.04	0.341	NS	0.361	NS
Davie	8081	DAVEDNA2	0	0	0	1	NS	1	NS
Davie	8081	DAVEDNA3	0.338	0	0.338	0.002	**	0.001	***
Davies	7594	DREDNA1	8.437	0	8.437	0	***	0	***
Davies	7594	DREDNA2	8.934	0	8.934	0	***	0	***
Davies	7594	DREDNA3	0.794	0	0.794	0	***	0	***
Davies	7816	DREDNA1	1.464	0	1.464	0	***	0	***
Davies	7816	DREDNA2	7.055	0	7.055	0	***	0	***
Davies	7816	DREDNA3	0.951	0	0.951	0	***	0	***
Davies	8036	DR_CCIPD02_1	1.453	0	1.453	0	***	0	***
Davies	8036	DR_CCIPD02_2	0.041	0	0.041	0.352	NS	0.355	NS
Davies	8081	DREDNA1	4.134	0	4.134	0	***	0	***
Davies	8081	DREDNA2	5.433	0	5.433	0	***	0	***
Davies	8081	DREDNA3	1.241	0	1.241	0	***	0	***
Elford	7816	ErEDNA1	0.755	0	0.755	0	***	0	***
Elford	7816	ErEDNA2	0.12	0	0.12	0.032	*	0.037	*
Elford	7816	ErEDNA3	0.039	0	0.039	0.357	NS	0.367	NS
Elford	8081	ErEDNA1B	0.084	0	0.084	0.111	NS	0.123	NS
Elford	8081	ErEDNA2	0	0	0	1	NS	1	NS
Elford	8081	ErEDNA3	0	0	0	1	NS	1	NS
Eyrie	7708	EREDNA1	0.396	0.105	0.291	0.008	**	0.019	*
Eyrie	7708	EREDNA2	0.475	0.105	0.37	0.006	**	0.01	**
Eyrie	7708	EREDNA3	1.732	0.105	1.627	0	***	0	***
Eyrie	7913	EREDNA1	1.09	0	1.09	0	***	0	***
Eyrie	7913	EREDNA2	0.2	0	0.2	0.007	**	0.012	*
Eyrie	7913	EREDNA3	0.242	0	0.242	0.002	**	0.004	**
Eyrie	8198	EREDNA1	2.332	0	2.332	0	***	0	***
Eyrie	8198	EREDNA2	0.367	0	0.367	0	***	0	***
Eyrie	8198	EREDNA3	0.71	0	0.71	0	***	0	***
Faith	8036	FAI_CCIPD02_1	9.62	0	9.62	0	***	0	***
Green Island	7594	GIEDNA1	0.131	0	0.131	0.032	*	0.047	*
Green Island	7594	GIEDNA2	0.682	0	0.682	0	***	0	***
Green Island	7594	GIEDNA3	1.176	0	1.176	0	***	0	***
Green Island	7816	GIEDNA1	0.201	0	0.201	0.008	**	0.014	*
Green Island	7816	GIEDNA2	2.663	0	2.663	0	***	0	***
Green Island	7816	GIEDNA3	1.774	0	1.774	0	***	0	***
Green Island	8081	GIEDNA1	0.695	0	0.695	0	***	0	***
Green Island	8081	GIEDNA2	0.994	0	0.994	0	***	0	***
Green Island	8081	GIEDNA3	0.359	0	0.359	0	***	0	***
Hedge	8081	HedEDNA 1	3.017	0	3.017	0	***	0	***
Hedge	8081	HedEDNA 2	1.687	0	1.687	0	***	0	***
Hedge	8081	HedEDNA 3	3.53	0	3.53	0	***	0	***

REEF	TRIP	SITE	Sample mean	Control mean	Difference	p-value samples		p-value repeats	
Lizard Island	7708	Big Vickis Reef	0.942	0.105	0.837	0	***	0	***
Lizard Island	7708	Casuarina Beach	0.791	0.105	0.686	0	***	0	***
Lizard Island	7708	Clam Gardens	3.532	0.105	3.428	0	***	0	***
Lizard Island	7708	Lagoon	0.129	0.105	0.024	0.356	NS	0.351	NS
Lizard Island	7708	Mermaid Cove	0.433	0.105	0.328	0.007	**	0.007	**
Lizard Island	7913	Big Vickis Reef	2.058	0	2.058	0	***	0	***
Lizard Island	7913	Casuarina Beach	1.016	0	1.016	0	***	0	***
Lizard Island	7913	Clam Gardens	1.732	0	1.732	0	***	0	***
Lizard Island	7913	Lagoon	0.049	0	0.049	0.132	NS	0.132	NS
Lizard Island	7913	Mermaid Cove	0.297	0	0.297	0	***	0	***
Lizard Island	8198	Big Vickis Reef	3.438	0	3.438	0	***	0	***
Lizard Island	8198	Casuarina Beach	1.95	0	1.95	0	***	0	***
Lizard Island	8198	Clam Gardens	1.365	0	1.365	0	***	0	***
Lizard Island	8198	Lagoon	0.437	0	0.437	0	***	0	***
Lizard Island	8198	Mermaid Cove	1.211	0	1.211	0	***	0	***
Lynchs	8036	LYN_CCIPD02_1	3.035	0	3.035	0	***	0	***
Lynchs	8036	LYN_CCIPD02_2	0.617	0	0.617	0	***	0	***
Lynchs	8036	LYN_CCIPD02_3	0.404	0	0.404	0.006	**	0.001	**
Lynchs	8036	LYN_CCIPD02_4	0.362	0	0.362	0	***	0.001	***
McSweeney	Init	McREDNA1	123.716	0.12	123.596	0	***	0	***
McSweeney	Init	McREDNA2	38.277	0.12	38.157	0	***	0	***
McSweeney	Init	McREDNA3	16.66	0.12	16.54	0	***	0	***
McSweeney	JCU	Northern edge	8.468	0.079	8.388	0	***	0	***
McSweeney	JCU	SW corner	11.251	0.079	11.172	0	***	0	***
McSweeney	JCU	Western shoal	224.414	0.079	224.335	0	***	0	***
Monsoon	JCU	NW point	14.328	0.079	14.249	0	***	0	***
Monsoon	JCU	Southern edge	16.257	0.079	16.178	0	***	0	***
Monsoon	JCU	SW Bay (near Cay)	24.441	0.079	24.362	0	***	0	***
Moore	7594	MOEDNA1	0.265	0	0.265	0.008	**	0.004	**
Moore	7594	MOEDNA2	1.816	0	1.816	0	***	0	***
Moore	7594	MOEDNA3	70.812	0	70.812	0	***	0	***
Moore	7816	MOEDNA1	0.04	0	0.04	0.355	NS	0.355	NS
Moore	7816	MOEDNA2	0.161	0	0.161	0.03	*	0.013	*
Moore	7816	MOEDNA3	1.489	0	1.489	0	***	0	***
Moore	8081	MOEDNA1	0.54	0	0.54	0	***	0	***
Moore	8081	MOEDNA2	6.364	0	6.364	0	***	0	***
Moore	8081	MOEDNA3	0.658	0	0.658	0	***	0	***
North Direction Island	7708	NDEDNA1	0.892	0.105	0.787	0	***	0.001	***
North Direction Island	7708	NDEDNA2	0.238	0.105	0.133	0.124	NS	0.123	NS
North Direction Island	7708	NDEDNA3	0.156	0.105	0.052	0.361	NS	0.372	NS
North Direction Island	7913	NDEDNA1	0.279	0	0.279	0	***	0	***
North Direction Island	7913	NDEDNA2	0	0	0	1	NS	1	NS
North Direction Island	7913	NDEDNA3	0.206	0	0.206	0.007	**	0.003	**
North Direction Island	8198	NDEDNA1	2.01	0	2.01	0	***	0	***
North Direction Island	8198	NDEDNA2	0.535	0	0.535	0	***	0	***
North Direction Island	8198	NDEDNA3	0.493	0	0.493	0	***	0	***
Osterlund	7594	OREDNA1	0.045	0	0.045	0.357	NS	0.363	NS
Osterlund	7594	OREDNA2	0	0	0	1	NS	1	NS
Osterlund	7594	OREDNA3	0	0	0	1	NS	1	NS
Osterlund	7816	OREDNA1	0	0	0	1	NS	1	NS
Osterlund	7816	OREDNA2	0.043	0	0.043	0.355	NS	0.359	NS
Osterlund	7816	OREDNA3	0	0	0	1	NS	1	NS
Osterlund	8081	OREDNA1	0.297	0	0.297	0.031	*	0.041	*
Osterlund	8081	OREDNA2	0.165	0	0.165	0.032	*	0.037	*
Osterlund	8081	OREDNA3	0.041	0	0.041	0.352	NS	0.355	NS
Prawn	8036	PRA_CCIPD02_1	0.123	0	0.123	0.032	*	0.039	*
Prawn	8036	PRA_CCIPD02_2	0.905	0	0.905	0	***	0	***
Rib	8081	RibEDNA1	0	0	0	1	NS	1	NS
Rib	8081	RibEDNA2	2.216	0	2.216	0	***	0	***
Rib	8081	RibEDNA3	9.276	0	9.276	0	***	0	***

REEF	TRIP	SITE	Sample mean	Control mean	Difference	p-value samples		p-value repeats	
Ribbon 5	7816	RR5EDNA1	0	0	0	1	NS	1	NS
Ribbon 5	7816	RR5EDNA2	0	0	0	1	NS	1	NS
Ribbon 5	7816	RR5EDNA3	0	0	0	1	NS	1	NS
Ribbon 5	8081	RR5EDNA1	0.081	0	0.081	0.112	NS	0.122	NS
Ribbon 5	8081	RR5EDNA2	0	0	0	1	NS	1	NS
Ribbon 5	8081	RR5EDNA3	0	0	0	1	NS	1	NS
Ribbon 9	7816	RR9EDNA1	0	0	0	1	NS	1	NS
Ribbon 9	7816	RR9EDNA2	0	0	0	1	NS	1	NS
Ribbon 9	7816	RR9EDNA3	0	0	0	1	NS	1	NS
Ribbon 9	8081	RR9EDNA1	0.039	0	0.039	0.359	NS	0.362	NS
Ribbon 9	8081	RR9EDNA2	0	0	0	1	NS	1	NS
Ribbon 9	8081	RR9EDNA3	0	0	0	1	NS	1	NS
Rudder	7594	RREDNA1	0.481	0	0.481	0.001	***	0	***
Rudder	7594	RREDNA2	0.173	0	0.173	0.007	**	0.011	*
Rudder	7594	RREDNA3	0.191	0	0.191	0.007	**	0.013	*
Rudder	7816	RREDNA1	0.042	0	0.042	0.354	NS	0.366	NS
Rudder	7816	RREDNA2	0.04	0	0.04	0.356	NS	0.357	NS
Rudder	7816	RREDNA3	0	0	0	1	NS	1	NS
Rudder	8081	RREDNA1	1001.136	0	1001.136	0	***	0	***
Rudder	8081	RREDNA2	0	0	0	1	NS	1	NS
Rudder	8081	RREDNA3	0.04	0	0.04	0.349	NS	0.364	NS
Shrimp	8036	SHR_CCIPD02_1	0.321	0	0.321	0	***	0.001	***
Thetford	7594	THEDNA1	0.9	0	0.9	0	***	0	***
Thetford	7594	THEDNA2	0.18	0	0.18	0.034	*	0.041	*
Thetford	7594	THEDNA3	0.34	0	0.34	0.007	**	0.003	**
Thetford	7816	THEDNA1	0.243	0	0.243	0.007	**	0.013	*
Thetford	7816	THEDNA2	0.079	0	0.079	0.362	NS	0.357	NS
Thetford	7816	THEDNA3	0	0	0	1	NS	1	NS
Thetford	8081	THEDNA1	0	0	0	1	NS	1	NS
Thetford	8081	THEDNA2	0.294	0	0.294	0.002	**	0.001	**
Thetford	8081	THEDNA3	0.041	0	0.041	0.355	NS	0.357	NS
Tongue	7594	TonEDNA1	2.202	0	2.202	0	***	0	***
Tongue	7594	TonEDNA2	0	0	0	1	NS	1	NS
Tongue	7594	TonEDNA3	0.66	0	0.66	0	***	0	***
Tongue	7816	TonEDNA1	0.401	0	0.401	0.001	**	0	***
Tongue	7816	TonEDNA2	0.4	0	0.4	0.008	**	0.005	**
Tongue	7816	TonEDNA3	0.082	0	0.082	0.114	NS	0.129	NS
Tongue	8081	TonEDNA1	1.96	0	1.96	0	***	0	***
Tongue	8081	TonEDNA2	0	0	0	1	NS	1	NS
Tongue	8081	TonEDNA3	0.282	0	0.282	0	***	0	***
Undine	7594	UREDNA1	0.568	0	0.568	0	***	0	***
Undine	7594	UREDNA2	0.088	0	0.088	0.113	NS	0.124	NS
Undine	7594	UREDNA3	0.436	0	0.436	0.002	**	0	***
Undine	7816	UREDNA1	0.042	0	0.042	0.355	NS	0.359	NS
Undine	7816	UREDNA2	0	0	0	1	NS	1	NS
Undine	7816	UREDNA3	0	0	0	1	NS	1	NS
Undine	8081	UREDNA1	0.038	0	0.038	0.352	NS	0.36	NS
Undine	8081	UREDNA2	0.041	0	0.041	0.355	NS	0.367	NS
Undine	8081	UREDNA3	0	0	0	1	NS	1	NS
Yonge	7708	YREDNA1	0	0.105	-0.105	1	NS	1	NS
Yonge	7708	YREDNA2	0	0.105	-0.105	1	NS	1	NS
Yonge	7708	YREDNA3	0	0.105	-0.105	1	NS	1	NS
Yonge	7816	YREDNA1	0.04	0	0.04	0.346	NS	0.355	NS
Yonge	7816	YREDNA2	0	0	0	1	NS	1	NS
Yonge	7816	YREDNA3	0.04	0	0.04	0.358	NS	0.356	NS
Yonge	8081	YREDNA1	0	0	0	1	NS	1	NS
Yonge	8081	YREDNA2	0.039	0	0.039	0.347	NS	0.358	NS
Yonge	8081	YREDNA3	0	0	0	1	NS	1	NS

APPENDIX F: QIACUBE PROTOCOL WORKFLOW FOR EXTRACTION OF EDNA FROM 47 MM FILTERS FOR EDNA FILTERS STORED IN QIAGEN ATL BUFFER

Filter preservation

Filters are folded carefully into eighths and then placed into a 1.5 ml screw cap tube. It is particularly important that you do not 'scrunch' the filter into the tube. Rather, using forceps, carefully place the filter into the tube, point end first and gently twist so that the filter slightly curls around the inside of the tube. The filter can then be gently tapped to ensure the 'point' of the folded filter reaches the bottom of the tube.

Add 540 µl Qiagen buffer ATL.

Vortex for 5 seconds and

Centrifuge at 10,000 x G for 1 minute

NOTE: Please aliquot roughly the amount of Qiagen buffer ATL you need into a separate 50 ml falcon tube.

NOTE: Our preservation trials have indicated DNA captured on filters is stable in Qiagen buffer ATL for up to 6 months at room temperature.

NOTE: This extraction method uses the same "200 µl samples" method on the Qiacube. The exception is that only a single 50 µl elution is done. It is important to check the number of elution steps in the Qiacube protocol. See Jason for a demonstration on how to do this.

Part A – Sample Lysis (Day 1)

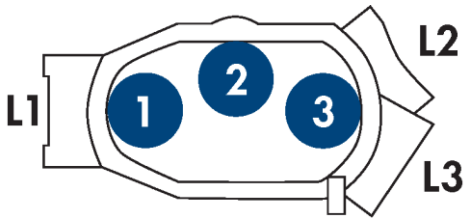
1. Filters are stored in the field in 1.5 ml screw cap tubes containing 540 µl Qiagen buffer ATL.
2. Remove required number of tubes from storage.
3. If required, prepare Proteinase K (10 mg/ml) by mixing pre-weighed Prot K (-20 freezer) and Molecular grade water (e.g. 110 g of Prot K + 11 ml of Nuclease Free Water), vortex until dissolved and store in -20 freezer in ~1 ml aliquots until use.
    a. NOTE: Freeze thawing of Proteinase K is only recommended up to two times.
    b. Always keep proteinase K on ice when in use.
4. Centrifuge each screw cap tube at 10,000xg for 1 minute to ensure all liquid is away from the top of the tube.
5. Add 60 µl Proteinase K (10 mg/ml) to each tube using a new tip each time.
6. Cap tightly and vortex for five seconds.
7. Incubate at 56ºC overnight with rotation (30 rpm) in the hybridisation oven.

Part B – Loading Qiacube (Day 2)

1. The order of preparing the Qiacube deck is important so as not to cross contaminate. Always start with new gloves.
2. ALWAYS WORK FROM CLEAN TO 'DIRTY', THAT IS, PREPARE ALL THE CLEAN PLASTIC WARE AND REAGENTS BEFORE HANDLING THE SAMPLES.
3. Centrifuge each screw cap tube at 10,000xg for 1 minute to ensure all liquid is away from the top of the tube.

4. Label sample tubes (2 ml Biosphere tubes) and elution tubes (1.5 ml tubes) that come with the rotor adaptors).
5. Prepare Qiacube Rotor Adaptors using the 12 position rotor adaptor guide. Careful attention must be paid to the correct seating of the tubes and lids in the rotor adaptor. Always double check correct seating of tubes and lids before loading the prepared rotor adaptor into Qiacube.

    - Position 1/L1 – Spin column
    - Position 2 – EMPTY
    - Position 3/L3 – Elution tube (1.5 ml tube)



6. Prepare Qiacube reagent bottles; AL, 100% ethanol, AW1, AW2, $TE_{0.1}$. Ensure Qiacube reagent bottles are topped up to the fill line. NOTE: 3 complete runs using the 200 µl protocol can be done with the reagent bottles filled to the fill line.
7. Fill the tip racks with new tips. Note, 1 x tip rack is sufficient for a 12 sample run.
8. Carefully place the reagent holder with reagents into the reagent position on the Qiacube deck.
9. Centrifuge samples that were incubated overnight at 10,000xg for 1 minute.
10. Using a 1 ml pipette and **reverse pipetting**, aspirate 0.2 ml of lysed sample (ATL/proteinase K digest) and dispense to a 2 ml sample tube.
11. Carefully place the sample tubes in their respective positions in the samples rack. Be careful to match the numbering of the rotor adaptors to the sample tubes, **i.e. Sample tube position 1 goes with rotor adaptor position 1.**
12. Press the power button on the Qiacube to start the instrument.
13. Press “DNA”.
14. Press “DNeasy Blood and Tissue”.
15. Press “Tissues and Rodent Tails”.
16. Press “200 µl samples”
    - NOTE: THE FOLOWING IS REQUIRED IF ELUTION VOLUME OF LESS THAN 150 ml IS NEEDED.
    - Press “Edit”
    - Scroll to 2. ElutionVolume, Select “-“ (= 0 ul), Select Save
    - Scroll to Inc.2nd AE, Select “-“ (= 0 sec), Select Save
    - Scroll to Centr.2nd AE, Select “-“ (= 0 sec), Select Save
    - Scroll to 3. ElutionVolume, Select “-“ (= 0 ul), Select Save
    - Scroll to Inc.3rd AE, Select “-“ (= 0 sec), Select Save
    - Scroll to Centr.3rd AE, Select “-“ (= 0 sec), Select Save
17. Press Start.
18. Press Next (4 times) – these are prompts to check the loading you have already done.
19. Press “Start”.

20. Protocol will take approximately 75 minutes to complete.

NOTE: Once the protocol has been modified, the same modified protocol can be run again by pressing the Quickstart button on the home screen. You will see that the modified protocol Quickstart button has red highlights indicating a modification has been made. I would recommend checking the elution steps describe above on every run, just to be sure.

Part C – Finalising DNA Extracts (Day 2)

21. Carefully remove the completed DNA extracts from the Qiacube. The Rotor adaptors will now have the elution tube with the spin column inserted. There will be the filtrate from all of the various washes in the bottom of the rotor adaptor.
22. Remove the spin column and discard into waste (waste category: Guanidine salts, no free liquid).
23. Remove elution tube (1.5 ml tube) containing purified DNA extract. Cap and place in a rack.
24. Pour off the liquid from the rotor adaptor into a waste collection bottle (waste category: Guanidine salts, free liquid).
25. Discard the rotor adaptor to waste (waste category: Guanidine salts, no free liquid).
26. Vortex the elution tubes and quick spin to ensure contents are at the bottom of the tube.
27. Transfer entire 50 µL of purified DNA from elution tube into pre-UV'd (15 min) labelled PCR tubes and place in labelled PCR tray in Jason's Nally bin in the glass door walk in fridge.

APPENDIX G: BIO-RAD DIGITAL DROPLET PCR (DDPCR) WORKFLOW FOR MEASUREMENT OF COTS EDNA

Prepare samples and ddPCR mastermix (in main lab area)

1. UV treat 2 x Biorad 96-well assay plates. Two plates are needed per assay. The first is used to set up the assay containing a total volume of 25 µl. The second plate is used to transfer generated droplets into.
2. Use the excel worksheet entitled "ddPCR Assay Template" to design your experiment.
3. Remove your DNA extracts and controls (positive and NTC, all of which should already be stored in PCR strip tubes) from storage. Ensure you vortex and briefly centrifuge everything to make sure its mixed and that any liquid within the tubes is located at the bottom of the tube, away from the lid.
4. For a full plate assay, arrange your samples/control tubes in a PCR plate holder such that your skip every second row. The reason being that samples/controls are analysed in duplicate.
5. Take out a pre-prepared ddPCR mastermix (***JD – need to add bulk master mix preparation protocol***) tube from the -20°C freezer and thaw. Mix by vortexing and centrifuge to remove bubbles.
6. Carefully pipette out the ddPCR master mix into a reagent reservoir. If you are using more than one tube of ddPCR master mix, gently mix the contents of each tube in the reagent reservoir by tipping the reservoir side to side a few times.
7. Prepare a 25 µl mastermix reaction for each sample. This can be done using the 12-channel 125 µl electronic pipette (Integra).
8. Use the repeat dispense function (*see note below*) of the 12-channel 125 µl electronic pipette to dispense 4 x 20 µl of ddPCR mastermix into rows A-D of an assay plate. Repeat this for rows E-H. Re-freeze any remaining ddPCR mastermix and mark the tube with a red line so that we know it has been thawed out.
    - NOTE: Repeat dispense aspirates a total volume of 84 µl. An initial dispense 2 µl goes back into the reagent reservoir, then it dispenses the 4 x 20 µl. A final volume of 2 µl remains which can be dispensed back into the reagent reservoir.
9. Use the pipette and mix function of the 12-channel 125 µl electronic pipette to aspirate 5 µl of your DNA and dispense it into each row of your assay plate. This function also mixes your reaction. **Note, each DNA sample is analysed in duplicate. For example, samples tube in Row A will end up in assay plate rows A and B.**
10. Centrifuge the completed assay plate in the Beckman Allegra Centrifuge (which requires a separate induction, see the PC2 lab manager) at 2,000 x g for 2 minutes. This is to remove any bubbles that may have formed in the assay plate wells from the mixing step prior.

Automatic Droplet Generation (AutoDG)

1. This part of the process occurs in the ddPCR lab.
2. Prepare the automatic droplet generator (AutoDG) for the process as follows:
    a. Touch the screen of the AutoDG to bring the instrument out of the idle mode.
    b. Open the door of the instrument by lifting up the handle at the front.

c. On the screen at the front of the AutoDG there is an “Oil Type” icon (see **Figure A 4**). Check that this matches the chemistry you need. If so, proceed with step ‘c’; if not follow steps below (i to vi) to change the bottle.

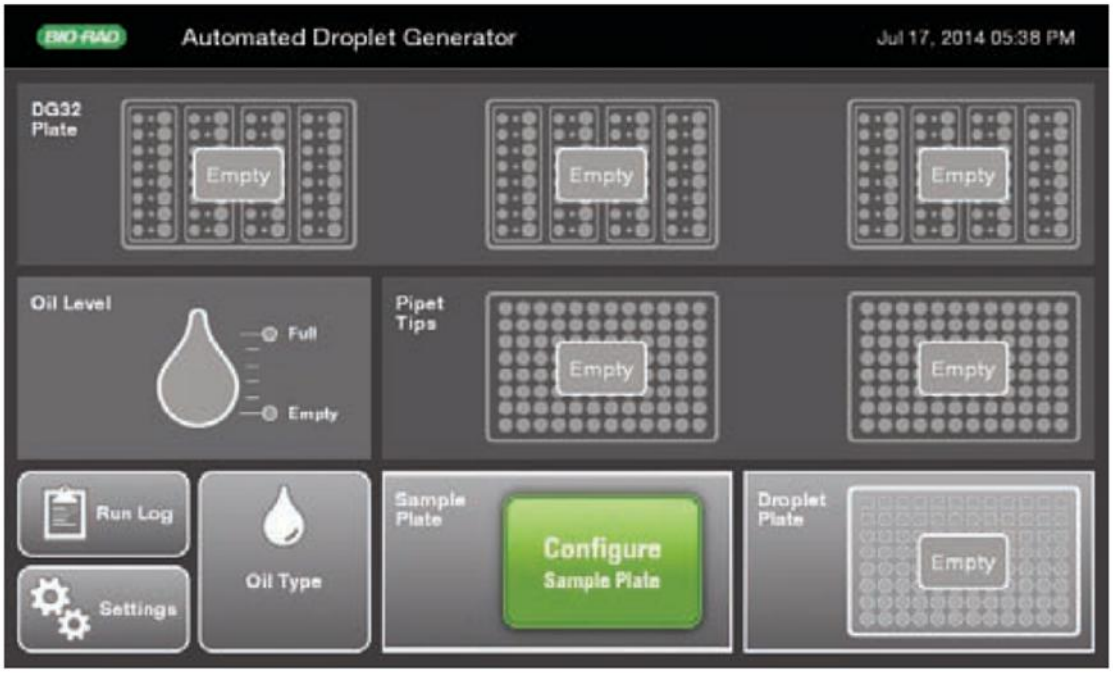


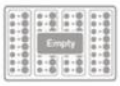
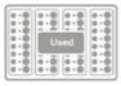
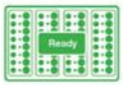
Figure A 4. Home screen, no consumables loaded.

i. Select the type of automated droplet generation oil (**Probes** or **EvaGreen**) by touching the “oil type” icon (**Figure A 4**). The droplet you select will turn blue. Touch OK to set the oil type.
ii. Once you have selected the type of oil, you will be prompted to remove the bottle of oil in the instrument and replace it with the new bottle of oil.
iii. Then you can remove the oil bottle from the delivery system at the front left corner of the instrument.
iv. Put away the bottle you just removed (there should be a box on the shelf with the cap for the bottle), cap the bottle and place inside the box.
v. Remove the cap from the bottle of Automated Droplet Generation Oil you want to use. Fasten the bottle into the delivery system (where you removed the previous bottle) by turning the bottle until it stops moving, **the label on the bottle should face outwards**. Touch “ok” to indicate you finish the exchange. The equipment will flush the lines with the new oil. This process may take a few minutes.
vi. Store the cap of the bottle in the box and put away until needed.
vii. You will receive a message saying “oil change successful” and the system will display the oil type at the bottom left of the screen.

Although the equipment has a display showing the level of oil, this won’t be correct anymore. Check the actual level of oil visually on the bottle. Exchange the bottle if it has less than 10 ml.

d. If the instrument deck is empty, the indicator lights on the deck of the AutoDG should be off, the corresponding areas of the touch screen will be grey (see **Table A 15**).

Table A 15. AutoDG Instrument status as indicated by deck lighting and touch screen.

Deck Lighting Status	Touch Screen Icon Status		Indication
Off	Gray, Empty		Ready to configure a new run
Off	Gray, Used		Ready to configure a new run; instrument will prompt for consumable replacement in used positions when the next run is configured
Green	Green, Ready		Ready to configure a new run; consumables in the green positions are ready to be used
Yellow	Yellow, Load		Run configured, load consumables as prompted (this status occurs only during run setup)
Blue	Blue, Complete		Run complete and droplets ready; occurs only at droplet plate position
Red	Red, ?		Consumable status unknown after power loss, please confirm manually

e. To configure sample plate, touch the “Configure Sample Plate” button (**Figure A 4**).
f. Touch or swipe across the screen to select columns in which your samples are located. Touching a selected column will deselect it. Any combination of columns can be selected. **Rows cannot be selected**. You don’t need to label the plate but this can be done if desired, along with adding notes. Touch OK when done.
g. The consumable icons will blink yellow (see **Table A 15**) to indicate where new consumables need to be loaded into the instrument. If the icon remains grey, that consumable is not needed for the run.
    i. **There is no icon to indicate that the pipette tip bin is in place**, nor whether it is full or empty. Please check that the bin is in place and empty before starting each run. The bin should be located to the left, in the middle row of the equipment deck, just behind the Droplet Generation Oil bottle.
h. Load consumables from the back to the front to avoid contamination.
i. Remove the plastic wrapping off the DG32 AutoDG cartridges and load them along the back row of the instrument. Orient the cartridges with the green gaskets to the right. If the cartridges are loaded correctly a green light will replace the yellow light. If this is not the case reposition the cartridge until the green light is lit.
j. Remove the plastic wrapping and the lid of the tip boxes and place in the middle row of the instrument. There is no front or back. If the boxes are

loaded correctly a green light will replace the yellow light. If this is not the case reposition the box until the green light is lit.

k. Place the assay plate you just centrifuged in the front left plate holder of the instrument, labelled in the screen as “Sample Plate”. The holder is designed to hold the plate in the right orientation (first row “A” to the back of the instrument and last row “H” to the front). If the plate is loaded correctly a green light will replace the yellow light. If this is not the case reposition the plate until the green light is lit.
l. To load the droplet plate assembly, remove the cooling block from the freezer underneath the AutoDG. Place it into the front right plate holder, labelled on the screen as “Droplet Plate”. If the cooling block is loaded correctly a green light will replace the yellow light. If this is not the case reposition the cooling block until the green light is lit.
m. Place a clean ddPCR 96-well plate for droplet collection into the cooling block. The cooling block should provide the right orientation for the plate. **There is no light to indicate that the plate has been loaded into the instrument, so be mindful to do this and check that the plate is sitting firmly on the cooling block.**
n. Once all the rows of the instruments are loaded with the necessary consumables, the icon of the “droplet plate” will turn blue and display a “START Droplet Generation” message. Touch the icon. You’ll be prompted to confirm the run. At this point, please check that the oil you need is the one specified and if you named the run and added notes, these are correct. Touch “confirm”.
o. The lid of the instrument will close automatically, and the run will start. After a few moments a message will be displayed with the length of time remaining for completion of the run.
p. Once completed, take the “droplet plate” and continue to the next step ‘Sealing the plate’.
q. After the run is finished make sure that all the used consumables are removed from the equipment and disposed of.
    i. Remove the cartridges and the assay plate and place in bin.
    ii. Remove the tips bin, empty in the bin, wipe with DNA erase and place back in the equipment. After two or three uses, this should be replaced by a new bin.
    iii. Remove the tip boxes and take them to the recycling plastic bin in the autoclave room.
    iv. Remove the cooling block and place **upside down** in the freezer under the bench.
    v. The equipment doesn’t require a clean up unless a spill is noticed. If this is the case, contact Jason Doyle in the first instance, if he is not available, contact K-le Gomez.
    vi. Close the lid of the AutoDG, it will return to idle mode on its own.

Sealing the plate

1. Pre-heat plate sealer to 180ºC (this is a preset temperature so turning the plate sealer on is all that is required).

2. Place the heating block inside the plate sealer.
3. Place the completed assay plate containing your generated droplets on the heating block.
4. Place foil seal on the PCR plate. Ensure the red line faces upwards. Foil seals sometimes stick together so ensure you only have a single foil seal.
5. Press green “seal” button.
6. Plate will be taken into the sealer and heat sealed. This takes about five seconds.
7. Remove plate and continue to PCR.

PCR

Standard ddPCR cycling protocol for COTS mtCOI is:

Cycling Step	Temperature (ºC)	Time	Ramp rate	Cycles
Enzyme activation	95	10 min	2ºC/sec	1
Denaturation	94	30 sec		40
Annealing/extension	60	1 min		
Enzyme deactivation	98	10 min		1
Hold (optional)	10	infinite		1

Thermocycler - BioRad C1000 Thermocycler

1. Place plate inside thermocycler.
2. Close lid and screw tightening knob until it just becomes tight, then turn another half turn. You may hear a rachet click on the half turn, this is normal.
3. Start run by:
    a. Saved files
    b. CTO26903
    c. Jason Doyle
    d. ddPCR PROBE
    e. press run
4. Ensure that the volume is set to 40 µl and 105°C temperature.
5. Press OK to start cycling.

Reading droplets on Droplet Reader

1. Open droplet reading software (Quantisoft).
2. Select new template.
3. Double click the first two cells in column 1.
    a. Enter sample name
    b. Check experiment – ABS
    c. Check supermix – Supermix for probes no dUTP
    d. Check target 1 – name COTS mtCOI
    e. Click type – unknown (for samples, positive for + control and NTC for – control)
4. Click ‘Apply’ after each step above - step 3b to 3e will be carried over to the next samples so only sample name needs to be changed.
5. Click ‘OK’.
6. Click ‘Save As’ to save the template.
7. Click ‘Run’.

   a. **NOTE: THE DROPLET READER MUST BE PRIMED IF NOT USED FOR 3 OR MORE DAYS- see Jason for further detail on priming if required.**
8. Run in columns and set dye set to FAM/VIC.
9. A full plate takes approximately 2.5 hours to read.

APPENDIX H: ESTIMATED STANDARD ERRORS, TIMES AND COSTS FOR VARIOUS COMBINATIONS OF SITES PER REEF (L) AND SAMPLES PER SITE (S) AT THE MODELLED PROPORTION POSITIVE OF 0.4 (~3 COTS PER HA) AND 0.8 (~10 COTS PER HA).

Example of error, time and cost estimates

Sites/ Reef	Samples/ Site	Total Samples	Field Time (est. min)	Lab Time (est. min)	Analysis consumable cost (approx. $AU/ Reef)	Standard Error (PP = 0.4)	Standard Error (PP = 0.8)
3	4	12	66	156	492	0.173	0.143
3	5	15	75	195	615	0.164	0.135
3	6	18	84	234	738	0.157	0.129
3	8	24	102	312	984	0.148	0.123
3	10	30	120	390	1,230	0.142	0.117
3	12	36	138	468	1,476	0.138	0.115
4	2	8	64	104	328	0.188	0.155
4	3	12	76	156	492	0.163	0.134
4	4	16	88	208	656	0.150	0.124
*4	*6	24	112	312	984	0.136	0.112
*4	*8	32	136	416	1,312	0.128	0.106
4	9	36	148	468	1,476	0.125	0.104
4	10	40	160	520	1,640	0.122	0.102
4	12	48	184	624	1,968	0.119	0.099
5	2	10	80	130	410	0.168	0.137
5	3	15	95	195	615	0.146	0.121
*5	*4	20	110	260	820	0.134	0.111
*5	*6	30	140	390	1,230	0.121	0.100
5	8	40	170	520	1,640	0.115	0.095
5	10	50	200	650	2,050	0.110	0.091
5	12	60	230	780	2,460	0.107	0.089
*6	*3	18	114	234	738	0.134	0.110
6	4	24	132	312	984	0.122	0.102
6	6	36	168	468	1,476	0.111	0.092
6	8	48	204	624	1,968	0.105	0.087
6	10	60	240	780	2,460	0.101	0.083
6	12	72	276	936	2,952	0.098	0.081
12	3	36	198	468	1,476	0.095	0.078

Time and cost estimates.

[#] cost estimate based on 2024 pricing.

Activity/Step in process	**Estimate**
Field time to travel to one site	10 min
Field time to collect and process one sample	3 min
Lab time to process one sample	13 min
Analysis cost for one sample[#]	$41

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COTS Control Innovation Program | A research and development partnership to better predict, detect and respond to crown-of-thorns starfish outbreaks