Coral Reefs
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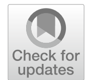

REPORT

eDNA monitoring detects new outbreak wave of corallivorous seastar (*Acanthaster* cf. *solaris*) at Lizard Island, Great Barrier Reef

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Abstract Crown-of-thorns seastar (CoTS, *Acanthaster* cf. *solaris*) outbreaks remain a significant cause of coral loss on the Great Barrier Reef (GBR) and across the West-Pacific Ocean. Previous outbreaks on the GBR have only been discovered once fully established, which constrains opportunities for effective control. Early detection of outbreaks would provide an important opportunity for early intervention and increase understanding of outbreak cause(s). Here, we assess the utility of environmental DNA (eDNA) monitoring to detect the initiation of a population outbreak at Lizard Island over five years (2019–2023), compared with density estimates obtained using Scooter-Assisted Large Area Diver-based (SALAD) surveys. At each of the five eDNA sampling sites, 30 replicate samples were collected annually and analysed with CoTS-specific primer sets and digital droplet PCR. Both methods detected distinct increases in CoTS densities from 2020/21 onwards, indicating the start of a new population outbreak. A large part of the observed variation in eDNA (expressed as the percentage of positive samples) was explained by changes in recorded CoTS density, confirming that eDNA data provide a quantitative estimate for adult CoTS abundance. SALAD surveys and eDNA are new and complementary monitoring methods that facilitate early detection of CoTS outbreaks, which will enable more effective management intervention.

Keywords *Acanthaster* · Coral reef crisis · Nuisance species · eDNA

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Introduction

Acanthaster spp. are corallivorous seastar species distributed throughout the tropical Indo-Pacific (Uthicke et al. 2024). Several of these species exhibit episodic population explosions ('outbreaks', 'irruptions') leading to significant coral loss. Population outbreaks of the western Pacific CoTS, *Acanthaster* cf. *solaris*, have occurred in French Polynesia (Kayal et al. 2012), Indonesia (Baird et al. 2013), Australia's Great Barrier Reef (Moran 1986; Pratchett 2005), and Okinawa, Japan (Nakamura et al. 2014). Similarly, outbreaks of *A. planci* and *A. mauritiensis* have caused substantial coral loss in the Indian Ocean (summarised in Uthicke et al. 2024).

The Great Barrier Reef (GBR) has been subjected to four distinct waves of outbreaks of *A.* cf. *solaris* since the 1960s (Pratchett et al. 2014, 2017), with early stages of a 5th outbreak recently reported (Chandler et al. 2023) in the northern sections of the GBR. Outbreak dynamics and causes are still poorly understood (Pratchett et al. 2017), partly because outbreaks are detected only when CoTS densities are already at elevated levels (Babcock et al. 2020). The standard method used to monitor densities of CoTS on the GBR is manta tows (Miller et al. 2019), where a snorkeler records the number of CoTS sighted while being towed behind a tender vessel. While useful for detecting the occurrence of established outbreaks (Vanhatalo et al. 2017), manta tows have limited detectability, particularly at low to moderate densities, due to the often cryptic behaviour of juveniles and adults (Fernandes et al. 1990). Effective surveillance methods which are capable of monitoring all stages of an outbreak, in particular the earliest stages of population build-up, are critical for understanding the cause(s) of outbreaks and will allow for early intervention (Pratchett et al. 2017; Chandler et al. 2023).

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Environmental DNA (eDNA) monitoring represents an alternative method for detecting low densities of organisms in aquatic environments (Ficetola et al. 2008), defined as genetic material obtained from environmental samples (i.e. soil, sediment, water column) (Taberlet et al. 2012). Environmental DNA approaches can be divided in two methods. First, metabarcoding using general primers can describe species presence and diversity in different communities. Second, species specific primers can be used to detect and to some extent quantify individual species. In freshwater environments, eDNA was applied to detect introduced species (e.g. Jerde et al. 2013; Rees et al. 2014; Fukumoto et al. 2015; Hunter et al. 2017) or to describe community composition of plants (Espinosa Prieto et al. 2023), fishes (Ito et al. 2023) or invertebrates (Coghlan et al. 2021).

Quantifying eDNA in marine environments is challenging due to dilution factors and environmental conditions, including currents (Uthicke et al. 2022). However, an increasing number of studies have effectively applied eDNA-based methods to the marine realm. Metabarcoding has been used in temperate and arctic seas (Thomsen et al. 2016; Yamamoto et al. 2017; Jeunen et al. 2019), but also in coral reef environments (Dugal et al. 2022, 2023; Marwayana et al. 2022). Species-specific primer approaches in marine environments have also now been used for several marine taxa, including crustaceans (Forsström and Vasemägi 2016), molluscs (Mauvisseau 2017), echinoderms (Uthicke et al. 2018; Ellis et al. 2021), cnidarians (Minamoto et al. 2017) and cubozoans (Morrissey et al. 2022).

CoTS specific PCR primers were initially developed to quantify planktonic larvae (Uthicke et al. 2015; Doyle et al. 2017). The same primers have also been employed to detect and quantify post-settlement CoTS from high to very low densities using digital PCR (Uthicke et al. 2018, 2022; Kwong et al. 2021) or lateral flow assays (Doyle and Uthicke 2020). In addition, these DNA-based methods have recently been tested to detect CoTS recruits in artificial settlement collectors (Doll et al. 2021).

The purpose of this study was to assess the utility of eDNA to detect changes in local densities of CoTS within reef environments, which may provide early warning of the onset of new or renewed population outbreaks. We conducted eDNA sampling over five consecutive years (2019—2023) to test for inter-annual changes in densities of CoTS at Lizard Island in the Northern GBR (14.7°S, 145.5°E). Lizard Island is located within the putative "initiation box" of CoTS outbreaks and has been a key location for previous studies on the population dynamics and impacts of CoTS (Pratchett 2005, 2010; Caballes et al. 2021). To test how changes in eDNA compare to direct density estimates, we also undertook extensive in-water surveys at corresponding sites, using a new method designed to provide high-resolution abundance data, even at low to moderate density levels (Chandler et al. 2023). Here, we directly compare the eDNA data with density estimates from the in-water surveys to corroborate the application of eDNA for effective CoTS population monitoring in a temporal context.

Methods

Field collection

Environmental DNA sampling was undertaken at five sites around Lizard Island between August and September in each year from 2019 until 2023 (Table 1). Sites were selected to allow access in most weather conditions from the Lizard Island Research Station (Fig. 1, Table 1). To account for any possible minor effect of water residence or tide (Uthicke et al. 2022), on most occasions, two collection events separated by 1–5 d were undertaken for each site/year combination. A total of 30 replicates (15 per collection event) were conducted at each site/year combination with the exception being in 2020 when 31 and 29 samples were collected from Big Vicki's Reef and the Lagoon site, respectively. A subset

Table 1 Site details, collection dates (day/month) and times (24h format) for eDNA samples collected at Lizard Island, 2019–2023

Site (latitude, longitude)	2019	2020	2021	2022	2023
Lagoon (-14.6875, 145.455)	26/08, 0930 27/08, 1500	26/08, 1500 27/08, 1530	21/08, 1340 24/08, 1220	21/08, 1245 23/08, 1600	15/09, 1640 17/09, 1520
Big Vicki's Rf. (-14.6838, 145.4394)	27/08, 0900	26/08, 0900 27/08, 0900	20/08, 1630 22/08, 1230	22/08, 1045 23/08, 1020	15/09, 0830 16/09, 1630
Casuarina Beach (-14.6744, 145.4409)	30/08, 0900	30/08, 0930	20/08, 1110 25/08, 0900	21/08, 1640 23/08 0950	15/09, 0900 16/09, 1000
Clam Gardens (-14.6616, 145.4489)	29/08, 0930	28/08, 1000 28/08, 1600	20/08, 0845 23/08, 1000	22/08, 1610 24/08, 1020	15/09, 1030 16/09, 0830
Mermaid Cove (-14.646, 145.4536)	28/08, 090029/08, 0830	28/08, 0930 29/08, 0900	22/08, 0840 25/08, 0830	23/08, 0840 24/08, 1050	15/09, 0945 16/09, 0900

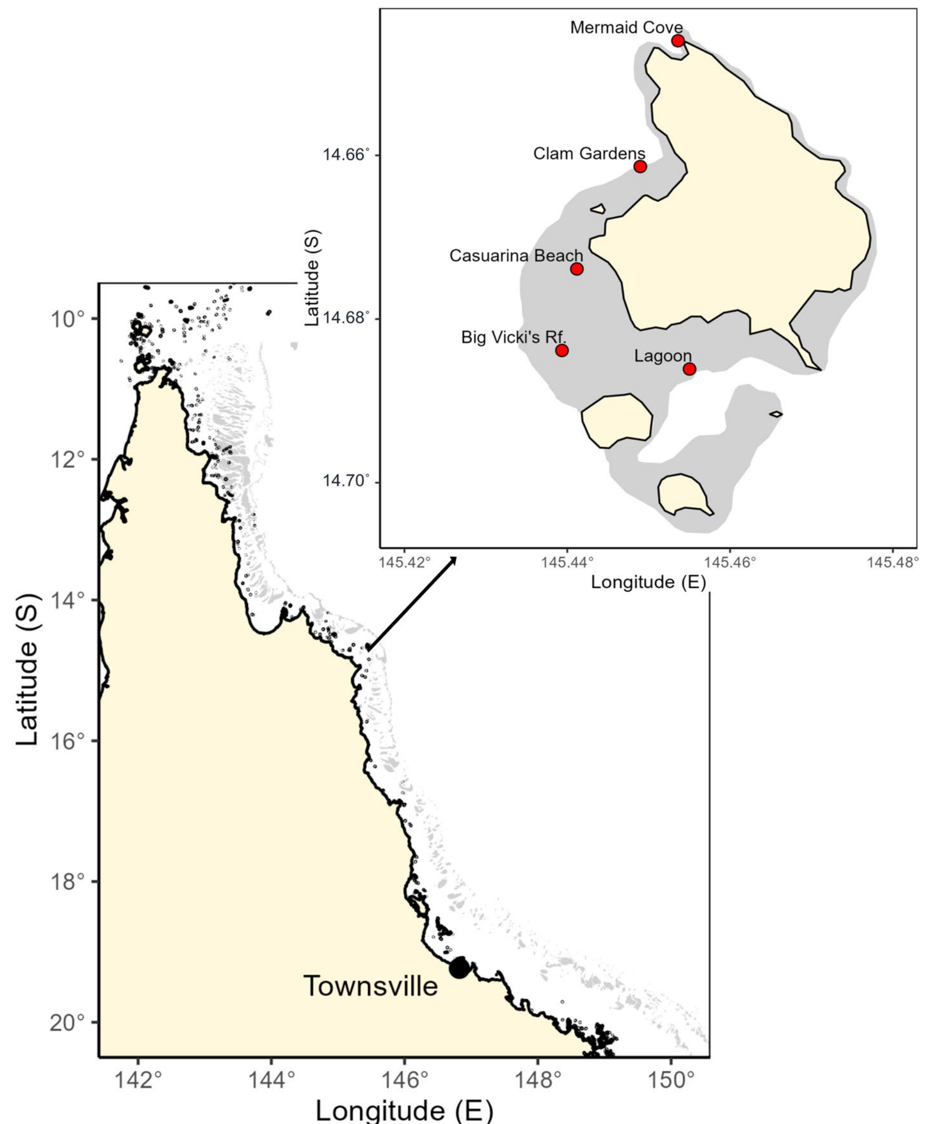

Fig. 1 Map of North-East Australian and the Great Barrier Reef. Insert: Location of eDNA sampling sites (red dots) and complementary SALAD survey areas (red dashed boxes) at Lizard Island, northern Great Barrier Reef

of samples from the first two sampling years were included in a previous publication (Uthicke et al. 2022).

Scooter-Assisted Large Area Diver-based (SALAD) surveys were conducted as described in Chandler et al. (2023). Data presented here were collected over 5 consecutive years (2019–2023) at complementary study sites to allow direct comparison to eDNA data. The mean annual location-wide density was estimated using SALAD data from across the north-western side of Lizard Island (Mermaid Cove to Palfrey Island), while corresponding site-level density estimates were calculated for each sampling year using data from the vicinity of eDNA sites (Big Vicki's Reef, Casuarina Beach, Clam Gardens and Mermaid Cove). No comparative SALAD data exist for the eDNA Lagoon site. SALAD data collected in 2019–2022 were included in Chandler et al. (2023). Data presented here are based on actual observations of CoTS (i.e., recorded densities), as opposed to inferred density estimates that include distinct sets of feeding scars where the CoTS was not detected.

DNA extraction and digital droplet PCR assay

Water samples for eDNA analyses were collected as previously described (Uthicke et al. 2022). Briefly, 2.5lt water was pumped directly from the ocean through an eDNA housing (Smith-Root, USA) containing a 1.2 mm mixed cellulose ester membrane using an eDNA sampling device (Grover-Pro™, Grover Scientific, Townsville Australia). Membrane filters were removed from the housings within 2 h of collection, folded carefully into eighths using sterilised tweezers and placed in a 1.5-ml screw cap tube containing 540 µl of Qiagen buffer ATL as a preservative (Majaneva et al. 2018). All sampling equipment was bleach-cleaned with a concentrated chlorine solution (100 g dichloroisocyanuric acid per

20 L, equivalent to 0.275% w/v available chlorine). Filters were extracted using a Qiagen Blood and Tissue DNeasy kit on a Qiacube automated nucleic extraction instrument following the method described in Doyle and Uthicke (2020). All samples were eluted in 50 µl of a 10 × diluted TE buffer (TE0.1) except for the 2019 samples which were eluted in 2 × 50 µl TE0.1. Digital droplet PCR (ddPCR) was conducted as per Uthicke et al. (2018) in the absence of restriction enzyme. For 2019 and 2020 samples, a manual QX200 droplet generator was used, whereas for subsequent samples, an automated droplet generator was employed (Bio-Rad, QX200 AutoDG). Accurate calling of positive detection in ddPCR includes quality control of each ddPCR reaction. This includes running positive and negative controls (see below) to confirm expected distribution of droplet fluorescence for each microplate run, rejecting a ddPCR reaction if the accepted droplet count is less than 10,000 (https://www.bio-rad.com/webroot/web/pdf/lsr/literature/Bulletin_6407.pdf) to ensure consistent quantification (independent of droplet generation method), and consistent thresholding for determination of positive and negative droplets. Controls were included at all levels of sample handling (Table 2), namely 'No Template Controls' (NTC), laboratory extraction controls (blank filters extracted alongside samples) and field controls (MilliQ water filtered on site). No positive detections were observed in any NTC, or other controls from 2019, 2022 to 2023. A small number of false positive droplets were observed in 2020 and 2021 (Table 2). Although the small proportion of false positives was unlikely to affect the outcome, we randomly removed positive droplets from positive PCR replicates in those years, based on the proportion of false positives in the pooled field and extraction controls.

Statistical analyses

For CoTS eDNA, we previously analysed two separate metrics (Uthicke et al. 2022). The actual DNA concentration (*Acanthaster* cf. *solaris* mitochondrial DNA copy numbers) works at intermediate to high concentrations and was not suitable to track the build-up of low-density populations in the present dataset. Hence, these data were not formally analysed, but a summary graph is presented. The second metric is binomial, scoring each individual sample as positive or negative for the presence of CoTS eDNA. Binomial results in Uthicke et al. (2022) were analysed using an occupancy approach. Here, we simplified this method and analyse these data as the proportion of positive samples to allow for more appropriate accounting of error structure in this larger dataset. Thus, we used a binomial model with 'Year' and 'Site' as fixed factors. The 'Sampling Occasion' (15 filters at 2 occasions per year at each site) was included as a random variable during initial model runs but explained low amounts of variance and models did not converge. This factor was thus excluded from the model. The density data obtained from SALAD surveys were analysed in a linear model including 'Year' and 'Site' as fixed factors. eDNA and SALAD samples were compared using a linear model with 'CoTS density' (SALAD estimates, log transformed) and 'Site' as factors explaining variation in the proportion of samples positive for CoTS eDNA. The interaction term in the latter model was insignificant and removed from the final model. All models fitted (using the lme4 library) complied well with assumptions, as tested in the DHArma library. All analysis and graphs were done in the R environment (R-Core-Team 2018).

Table 2 eDNA control types and numbers for each year. Numbers are individual ddPCRs conducted from respective control samples. NTC = No template control

Control type	2019	2020	2021	2022	2023
NTC	14	16	14	12	12
Extraction	2	22[(1)]	12[(1)]	10	10
Field	32	10	24[(2)]	16	32

[(1), (2)]: One single positive droplet was detected in extraction controls in 2020 and 2021, 2 droplets were detected in one field control in 2021

Results

A total 750 eDNA samples were collected over five years (2019–2023) at five sites around Lizard Island, of which, 364 samples were positive (i.e. above detection limit) for CoTS mtDNA. A binomial linear model of the presence–absence data exhibited significant effects of both year ($\chi^2 = 126.21$, DF = 4, $p < 0.0001$) and sampling site ($\chi^2 = 125.83$, DF = 4, $p < 0.0001$). However, the interaction of both factors was also significant ($\chi^2 = 57.22$, DF = 16, $p < 0.0001$), suggesting that trends were not parallel across all sites.

Averaged across all sites, the proportion of positive samples distinctly increased from year to year (Fig. 2A, factor Year). Low proportions of positive samples ($p < 0.30$) were observed in the first two sampling years, but the proportion of positive samples increased past 0.50 in 2021. Values in the final year were the highest observed and significantly (based on non-overlapping confidence intervals) above those of all other years. Similarly, the average mitochondrial DNA copy numbers (not formerly analysed) for all sites were close to zero within the first 2–3 y of this study, with averages distinctly increasing from 2021 onwards at most sites (Fig. 3).

Overall, there were several distinct differences in the proportion of positive samples over the years among sampling sites (Year x Site interaction). The Lagoon site generally exhibited the lowest values, with the proportion of positive samples only slightly increasing in 2023

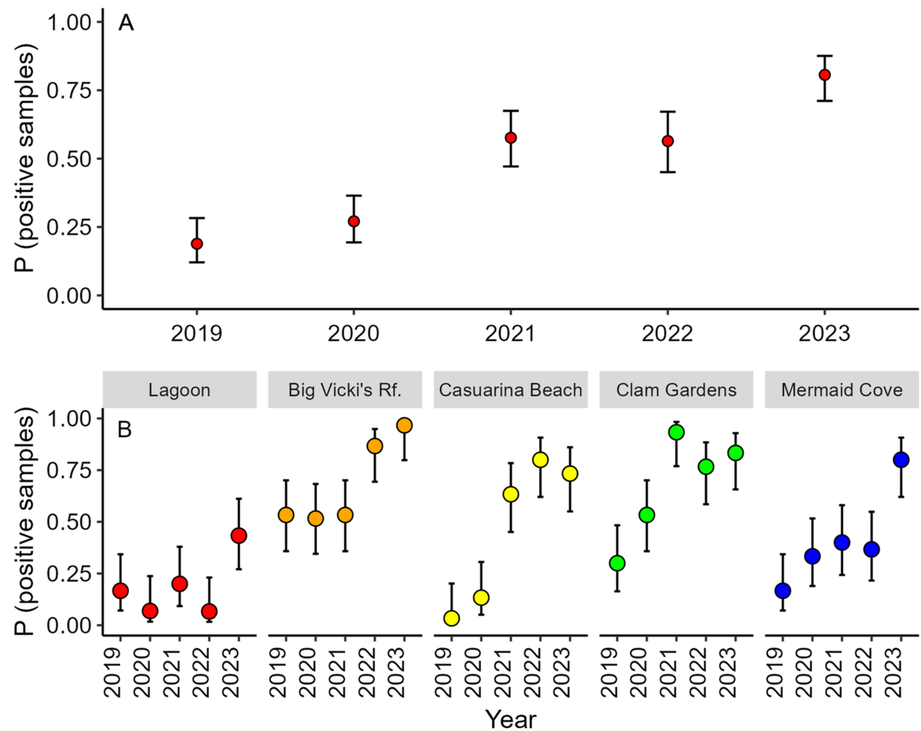

Fig. 2 The proportion of positive samples for *Acanthaster* cf. *solaris* eDNA on Lizard Island, **A** averaged for five years (Factor "Year", $N = 150$) and **B** separated by sampling site for each year (Factor "Year x Site", $N = 30$). Coloured dots represent model fit and vertical bars represent the lower and upper bounds of the 95% confidence interval

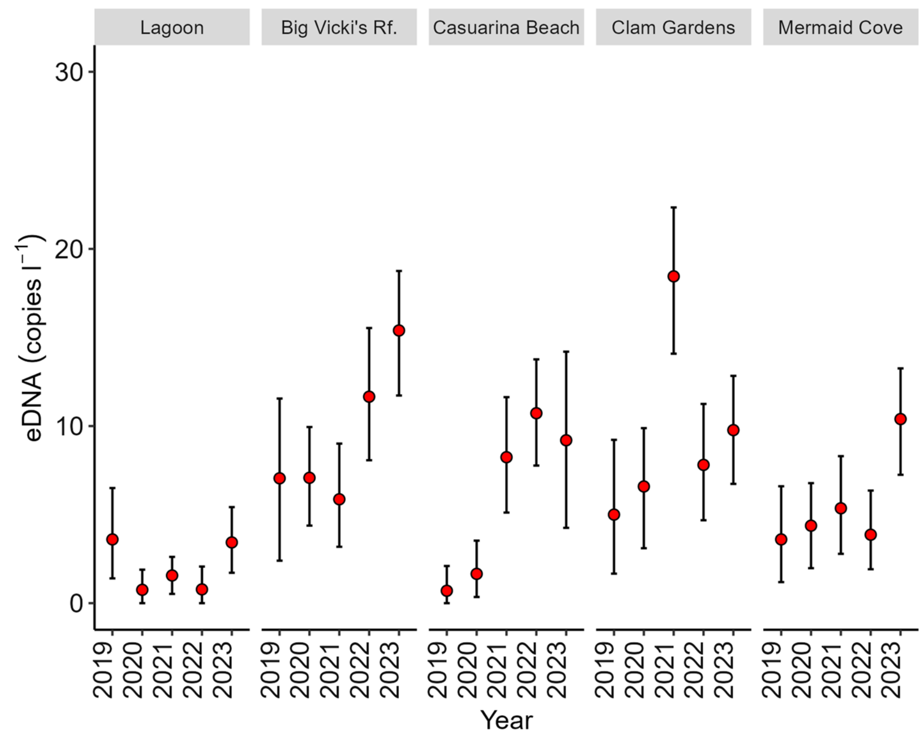

Fig. 3 Average copy number of *Acanthaster* cf. *solaris* eDNA on Lizard Island, separate for five sampling sites. Vertical bars represent the lower and upper bounds of the 95% confidence interval, based on bootstrapping

(Fig. 2B). Casuarina Beach, Clam Garden and Mermaid Cove follow the same trend as the Island-wide averages with continuously increasing proportion of positive samples between 2019 and 2023. The proportion of positive samples at Big Vicki's Reef was already around 0.50 at the beginning of the study and increased further past 2021.

CoTS densities recorded using SALAD surveys, averaged over the North-Western sites (Big Vicki's Reef,

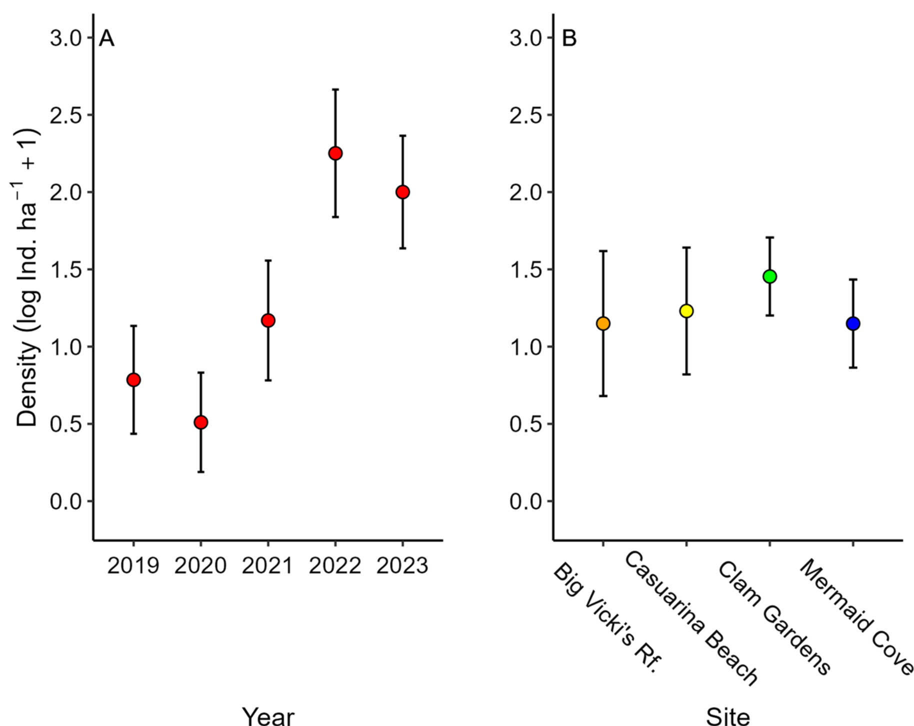

Fig. 4 Densities of *Acanthaster* cf. *solaris* recorded with SALAD surveys **A** at Lizard Island over 5 sampling years (2019–2023) and **B** at four sampling locations, averaged across years. Coloured dots represent model fit (factor 'Year' and factor 'Site') and vertical bars represent the lower and upper bounds of the 95% confidence interval

Table 3 Linear model analysis of the effect of (log transformed) CoTS density on the proportion of positive CoTS eDNA samples, averaged for four sites and five years. Site was added to the model as a co-variate

	Df	MS	F	*P*
Density	1	0.5697	15.07	0.0015
Site	3	0.1101	2.91	0.0688
Residuals	15	0.0378		

Casuarina Beach, Clam Gardens, Mermaid Cove) of Lizard Island, differed among sampling years ($\chi^2 = 64.047$, DF = 4, $p = < 0.0001$; Fig. 4A). Differences in CoTS densities among sites were also significant (linear model, $\chi^2 = 8.020$, DF = 3, $p = 0.0456$), but the interaction term was not significant ($\chi^2 = 15.455$, DF = 12, $p = 0.2175$). Densities recorded were relatively low (< 3 ha^{-1}) in 2019 and 2020. Similar to the eDNA data, average CoTS densities increased substantially from 2020 to 2021 and were distinctly higher again (near 10 Ind. ha^{-1}) in 2022 and 2023 (Fig. 4A). Significant differences between sites were likely driven by somewhat higher overall values at Clam Gardens (Fig. 4B).

Linear model analysis indicated a significant effect of recorded CoTS density on measured eDNA, with the overall model explaining 51% of the variation (Tab. 3). Thus, increasing proportions of positive eDNA samples were well explained by (log transformed) CoTS densities detected using the SALAD surveys (Fig. 5).

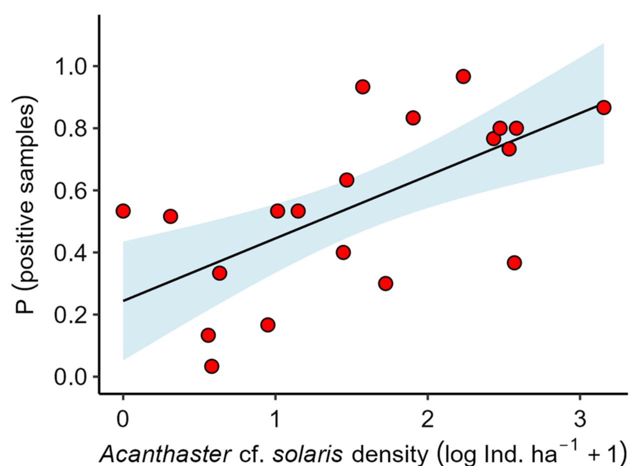

Fig. 5 Linear model fit (Table 3) of the proportion of positive eDNA samples versus recorded densities of *A.* cf. *solaris* using the SALAD method. The blue shaded area depicts the 95% confidence interval, the black line the model fit. Red dots are average observations per site and year

Discussion

The primary objective of this study was to assess whether eDNA sampling can resolve inter-annual changes at low densities of CoTS. This is a critical component for achieving early detection and has previously only been possible with high-resolution in-water surveys (Chandler et al. 2023). The proportion of samples with positive detections

for CoTS eDNA increased across all sites over the 5y study period and corresponds well with variation in CoTS densities recorded using in-water SALAD surveys. Over the same time, manta tow surveys (traditional monitoring method) conducted around Lizard Island detected no such temporal variation and generally reported low CoTS densities, below the relevant threshold of an outbreak, in each of the sampling years ($\leq$0.01 CoTS per tow; AIMS LTMP, available at: https://apps.aims.gov.au/reef-monitoring/reef/Lizard%20Isles/manta). In contrast, both sets of monitoring data presented here report substantial and rapid (densities more than tripled and the proportion of positive eDNA samples more than doubled between 2 years) population growth at this key location, indicating the onset of a 5th CoTS outbreak on the GBR since the 1960s. The results from the present study clearly demonstrate the utility and necessity for novel sampling methods to detect the early onset of population outbreaks of CoTS.

Based on seemingly consistent periodicity of CoTS outbreaks on the GBR since the 1960s, renewed outbreaks were expected in 2025–2027 (Babcock et al. 2020). Our data suggest that these anticipated population outbreaks have already commenced, with clear and consistent increases in densities of CoTS at Lizard Island since 2020 (see also Chandler et al. 2023). However, the methods presented herein provide unprecedented capacity to detect the initiation of population outbreaks and protracted build-up of CoTS densities over several years. The initiation of past outbreaks has been reported based on detection of highly elevated CoTS densities (e.g. Wooldridge and Brodie 2015), which largely fails to recognise or detect the protracted build-up in CoTS populations that may often precede the initial establishment of outbreaks (Pratchett 2005). It appears the build-up of the new outbreak occurs, while the previous outbreak is still underway in parts of the central and southern GBR. Thus, an alternative explanation for the marked temporal patterns observed in this study may also be that elevated densities at Lizard Island represent a flare-up of the 4th outbreak in the northern GBR. At present it is unresolved if current strong anthropogenic intervention (culling) could cause a flare up or create conditions for permanent outbreaks.

The broad size range of CoTS at Lizard Island, presented in Chandler et al. (2023), suggests that increasing densities resulted from the accumulation of seastar over several successive years of successful recruitment. Notably, CoTS densities ($<$3 Ind. ha^{-1}) recorded in the first years of the present study (2019 and 2020) were below those expected to contribute to accelerated reproductive success (Rogers et al. 2017). It is uncertain if these relatively low densities constitute natural baseline levels at this location or were already an indication of CoTS population build-up. In addition, density estimates based solely on the direct observation of CoTS specimens during SALAD surveys are likely still an underestimate of CoTS abundance. Inferred densities, including observations of distinct sets of feeding scars where the CoTS was not detected, are generally higher and present a more realistic estimate of population size (Chandler et al. 2023). It is also unknown to what extent sustained and ongoing recruitment may be driven by continual input of larvae from reefs to the north (Luick et al. 2007) and/or retention of larvae produced by increasing local populations. Nevertheless, considering the substantial increase in CoTS densities documented here from 2021 onwards, the reproductive capacity of this population is rapidly building and may reinforce population growth around Lizard Island and contribute to the establishment and spread of population outbreaks.

The eDNA analysis presented here also detected some spatial differences among sampling sites at Lizard Island. Particularly low proportions of positive eDNA samples were collected from the Lagoon site, which was not included in the SALAD surveys but had reported low CoTS densities during a previous outbreak (Pratchett 2005). Another difference among sites in the eDNA dataset was the relatively high proportion of positive samples from Big Vicki's Reef, even before this population build-up was detected in the SALAD surveys. It is possible that eDNA at that location was influenced by currents and other environmental factors. However, water residence time, sea temperature and water depth explained only a small amount of variance in CoTS eDNA concentration (Uthicke et al. 2022).

eDNA detection corresponded well with SALAD-based estimates along this low to moderate density gradient, with the overall model explaining more than 50% of the total variation. These insights further corroborate and compliment previous demonstrations of eDNA sampling in areas of higher density populations, compared to 200m^2 belt transects (Uthicke et al. 2018, 2022). Similar to Uthicke et al. (2022), we found that a simplified metric (proportion of positive samples) effectively represents changing densities of CoTS better than average mitochondrial DNA copy numbers, although an increase in the latter metric was also detectable in the last years of the present study.

Both SALAD surveys and eDNA detect CoTS at low and moderate densities during the early stages of outbreaks, and given inherent differences in methodological approaches, requirements and scalability, these two methods are complementary tools for early warning and intervention efforts. Key advantages of the eDNA method are that field sampling is fast (e.g. $<$2 h are needed to survey a reef on three sites, Uthicke, Doyle, Gomez Cabrera, unpublished data) and overcomes some of the inherent time, bias, risk and training limitations associated with in-water surveys, underlining its application for wide-sweeping monitoring of potentially hundreds of reefs. In addition, eDNA sampling is particularly useful for sampling in locations where in-water

sampling is not safe (e.g. due to the presence of saltwater crocodiles) or practicable. The laboratory-based processing of numerous samples can take several weeks; however, eDNA samples can be securely stored and subsequently analysed for other species groups or biodiversity assessments. An additional disadvantage is that the sensitivity of ddPCR does come at higher price per sample compared to standard PCR or even real-time PCR. While there may be cost-prohibitive aspects of the current eDNA method, we have demonstrated proof-of-concept alternatives that, with appropriate development, may provide an entry point for less developed nations to adopt a similar monitoring program (Doyle and Uthicke 2020). Although eDNA monitoring will add cost to existing surveys (in the range of 1-2 k A$ per reef), ship time is usually up to 1 order of magnitude more expensive, hence the relative increase of costs is small. In addition, field methods for eDNA collection are easy and rangers and 'citizen scientists' can be trained to collect samples. This will overall reduce costs for eDNA surveys by using ships of opportunity for sampling.

By contrast, SALAD surveys take much longer to complete, but data are instantaneously available. In addition, those surveys also yield fine-scale data on additional variables such behaviour and population structure of CoTS (all recorded CoTS are measured) and provide environmental context by recording benthic data and other disturbances (Chandler et al. 2023). Thus, an effective and efficient use of both methods would be to conduct large-scale eDNA monitoring over many reefs and focus SALAD surveys on ecologically important locations and selected reefs that recently reported high and/or increasing proportions in positive eDNA samples.

Conclusions

The eDNA and SALAD methods utilised here are important, and complementary, new tools enabling early detection of CoTS population increases, thereby allowing for early and effective intervention through targeted population control. Moreover, early detection of population outbreaks and the location of these 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 (Wooldridge and Brodie 2015) play a significant role in the initial population build-up, it is important to pinpoint the start of these outbreaks in time and space. Similarly, to understand the role of predation on juvenile (Balu et al. 2021; Desbiens et al. 2023) 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.

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Declarations

Conflict of interest Morgan Pratchett currently serves as Editor-in-Chief for *Coral Reefs*, but is recused from any role in the peer-review process for this manuscript. The authors declare no other conflict of interest.

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