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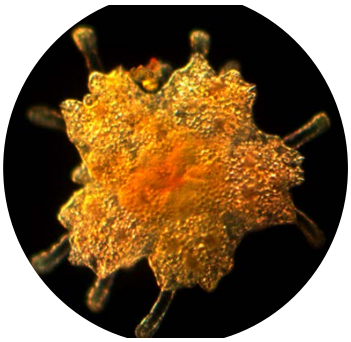

Refining Genetic Markers to Detect and Quantify Crown-of-Thorns Starfish on the Great Barrier Reef

Final Report

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PREPARED FOR GREAT BARRIER REEF MARINE PARK AUTHORITY

AIMS: Australia's tropical marine research agency

Townsville
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	Comments addressed	Jason Doyle, Sven Uthicke	30-05-2016	

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EXECUTIVE SUMMARY

- The Australian Institute of Marine Science (AIMS) has developed and field tested a method for qualitative detection of crown-of-thorns starfish (COTS) larvae and this study aims to extend this to a quantitative tool.
- COTS larvae were not detected in three samples during winter at sites that previously tested positive for COTS larvae during the 2014–15 spawning season.
- Following on from the qualitative assay, a quantitative genetic assay (Quantitative Polymerase Chain Reaction [qPCR]) for COTS larvae in plankton samples has been developed and validated.
- The quantitative genetic assay was successfully applied to field plankton samples and results presented. In the spawning season of 2014-15, the highest larval densities (> 50 x m^{-3}) were observed in the area between Rudder Reef and Tongue Reef; north of Port Douglas.
- This method now opens completely new opportunities for management relevant studies on causes of and controls for COTS outbreaks.
- Preliminary evaluation of eDNA as method to detect benthic juveniles or adults has shown encouraging results. Preliminary field trials at Rib Reef detected up to 0.52ng of COTS DNA x L^{-1} of water from the back reef. Further testing is underway focusing initially on sampling methodology.
- In the long term, AIMS plans to develop automated sampling of COTS eDNA as an early warning signal of COTS outbreaks in the potential primary outbreak area.

INTRODUCTION

In 2016, a fourth COTS outbreak established in the northern section of the Great Barrier Reef and it is feared that, following the pattern of the previous outbreaks, this outbreak will rapidly spread south. For modelling most efficient ways to reduce COTS spread (e.g., by reducing land runoff in targeted regions, or using targeted control to protect ecologically and economically important reefs in the outbreak area) the spread potential of COTS larvae needs to be known, but currently information on larval ecology is based on few aquarium experiments. Morphological identification of COTS larvae in the field is near impossible because of their similarity to other echinoderm species (see photographs in: Uthicke et al. 2015).

AIMS has developed genetic markers which were tested in the field and successfully identified COTS larvae. In an analysis of plankton data collected in December 2014, COTS larvae were detected in nearly every plankton sample obtained in the 'COTS Box', an outbreak initiation area between Cairns and Cooktown (Uthicke et al. 2015).

These new genetic methods can be used to identify the spread of larvae by collecting plankton samples over several spatial and temporal scales. The data are important to describe the distribution of COTS larvae in more spatial and temporal detail. For example, to date it is not even known where larvae are in the water column which has important consequences for their dispersal modelling and for the food available to the larvae (i.e. high chlorophyll concentrations are often near the bottom).

Further development of these genetic markers into a quantitative assay that can determine not only presence, but density is essential to populate outbreak and dispersal models. Works completed under this project describe the development and validation of a quantitative genetic assay for the detection of COTS larvae in plankton samples and the application of this method to field samples. Further exploration of the same genetic markers to detect juvenile or adult COTS DNA in the water column ('e-DNA' approach) is described that would potentially allow early detection of the spread of current outbreaks or the source of the next outbreak allowing early intervention.

APPROACH

The work reported on in this project is a further development and confirmation of the qualitative genetic method to detect COTS. Firstly, plankton samples need to be collected and genetically analysed to verify that the methods applied in summer identified COTS larvae, but not smaller sized cell fragments or dissolved DNA. Secondly, AIMS will investigate the possibility to quantify larval abundance using quantitative DNA techniques. Lastly, AIMS will employ other sampling methods (larger water volumes, different filtration techniques, different DNA extraction protocols) to test if we can find methods to detect e-DNA of COTS in water samples around reefs know to have COTS outbreaks.

PLANNED PROJECT OUTCOMES

(a) Build upon existing COTS genetic marker research to:

i) Develop methodology to quantify COTS larvae in water samples in order to provide information on COTS larval abundance.

ii) Test genetic markers for detecting adult COTS in the water column in order to provide information on adult COTS distribution

MILESTONES ASSOCIATED WITH THIS REPORT

Deliver to the GBRMPA Project Manager a final report on the methodology and preliminary results of:

a) Further refining AIMS genetic markers to quantify COTS larvae from the plankton; and
b) Testing AIMS genetic markers for detecting adult COTS in the water column.

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RESULTS

Standard PCR test of 'field negative' winter samples

Plankton sampling was conducted during winter of 2015 at three locations which we found to be positive for COTS larvae during December 2014 (see Uthicke *et al.* 2015). These locations were Ellison Reef adjacent to the Tully River mouth, Fitzroy Island and Green Island. Replicate samples from each location were spiked with three cultured COTS larvae and tested in parallel. DNA was extracted from all samples and evaluated for the presence of COTS using specific COTS genetic markers. No COTS DNA was detected from plankton samples collected from these sites during June 2015. (Figure 1).

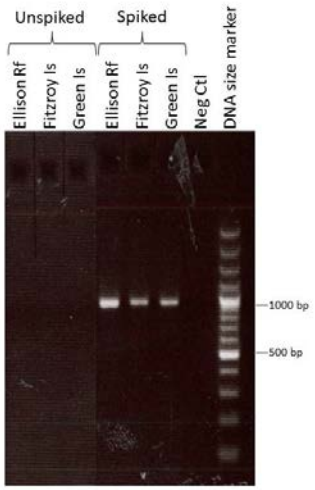

Figure 1. Plankton samples collected in June 2015 (winter) from three locations found positive for COTS larvae in Dec 2014. Spiked samples included three cultured COTS larvae.

Quantitative detection of COTS larval density

Quantitative DNA Extraction

Dry biomass in the order of 100 – 500 mg is typical in plankton samples collected. This biomass represents well in excess of 10 000 individual plankton organisms (mainly copepods, chaetognaths, and other zooplankton) filtered from several cubic metres of water (depending on depth). The quantitative determination of COTS larvae hinges on a quantitative DNA extraction method. AIMS had previously found that a Qiagen DNeasy

Blood and Tissue kit, which utilises spin columns for DNA purification, effectively extracts high quality DNA from plankton samples.

In order to determine if all DNA applied to the spin column was bound; the filtrate collected from spin columns from test samples was applied to new spin columns. In all cases, less than 1% of the DNA remained in the filtrate indicating that at the volumes tested, the DNA extract can be passed through the Qiagen spin column with >99% of the DNA binding to the column. This step is usually not taken for standard, qualitative PCR, but crucial for quantification.

DNA recovery was examined with different amounts of extract applied to spin column as well as multiple elution steps. Table 1 details the amount of DNA recovered from consecutive elution steps. Applying 600 μl or 1200 μl of lysate to spin columns would guarantee a 94% recovery of DNA after two elution steps. Application of 2400 μl lysate to spin columns results in recovery dropping to 90% after two elutions. In all cases however, at least 97% of DNA can be recovered after three elution steps. Future work will continue with the combination of 3 separate elution steps. DNA recovery was also linear with increasing amounts of extract applied to the spin column (Figure 2).

Table 1. Percent recovery from each elution step from each volume of lysate applied to spin columns.

Sample/vol	1st elution	2nd elution	3rd elution	4th elution
1 – 600ul	78%	16%	4%	2%
1 – 1200ul	71%	23%	4%	2%
1 – 2400ul	58%	32%	7%	3%

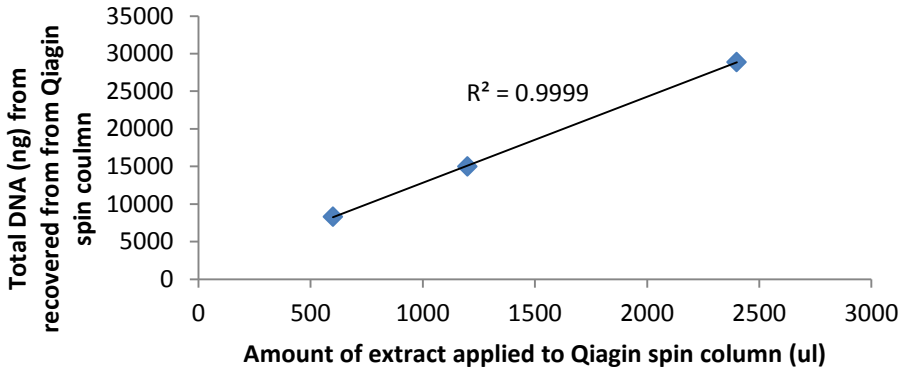

Figure 2. DNA recovery from Qiagen spin column

Development of Quantitative Polymerase Chain Reaction (qPCR) assay

In our initial qualitative study, we developed specific COTS primers amplifying a 919 base pair (bp) fragment. Quantitative PCR (qPCR) requires shorter fragment length (typically 50 – 150 bp). As such new primers needed to be developed to suit qPCR analysis. We designed primers which amplified a 126bp of the *Acanthaster planci* mitochondrial cytochrome oxidase subunit 1 (COI) gene with PrimerBlast. Primer design used the complete *A. planci* COI (1553 bp, accession number: AB116377.2) against all other Animalia. Specificity of the primers was tested against an in house tissue library (Figure 3) which included all four recognised lineages of *A. planci* (Vogler et al. 2008). A TaqMan probe was also designed to attach to the region spanning these primers.

A standard was produced using cloned plasmid containing the 126 bp insert with primer and TaqMan probe optimisation conducted at a fixed plasmid DNA concentration. Efficiency curves were generated with a range of plasmid DNA concentrations (Figure 4) demonstrating assay linearity over 7 orders of magnitude (R^2 >0.99)

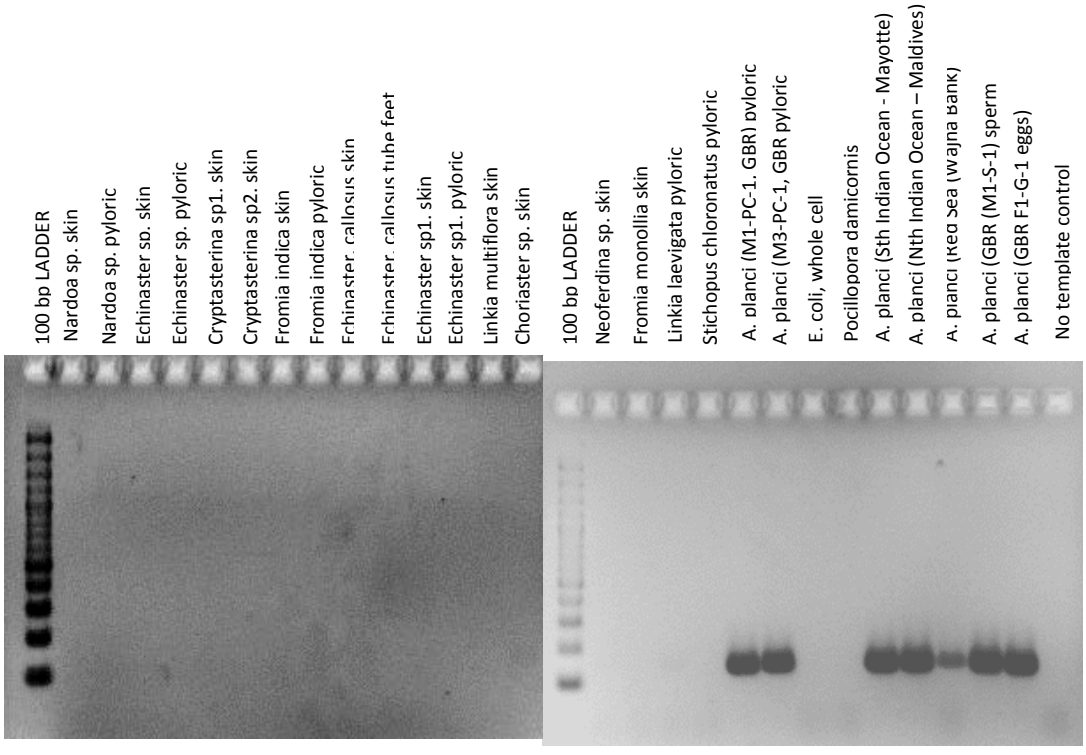

Figure 3. Primer specificity testing using in-house tissue collection. The primer pair amplified all four lineages of *A. planci*, but none of the other species tested.

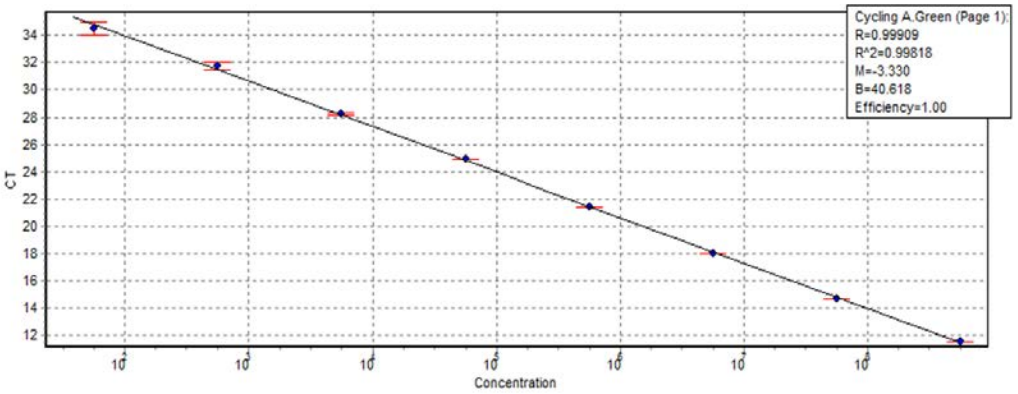

Figure 4. TaqMan qPCR standard curve. Concentration is given as number of copies per reaction. CT: Cycle threshold.

Determining COTS larvae COI gene copy number

In order to translate a measurement from this standard curve back to the number of COTS larvae, the copy number of the COI gene in individual COTS developmental stages needs to be known. Cultured eggs, embryos and larvae representing the variety of pre-settlement life stages were produced in the SeaSim facility at AIMS (following Uthicke et al. 2015b) and were utilised to determine copy number within an individual larvae. A threefold variation in copy number across all larval life stages was found, with copy numbers ranging from 4.8 x 10^6 to 1.4 x 10^7 (Figure 5).

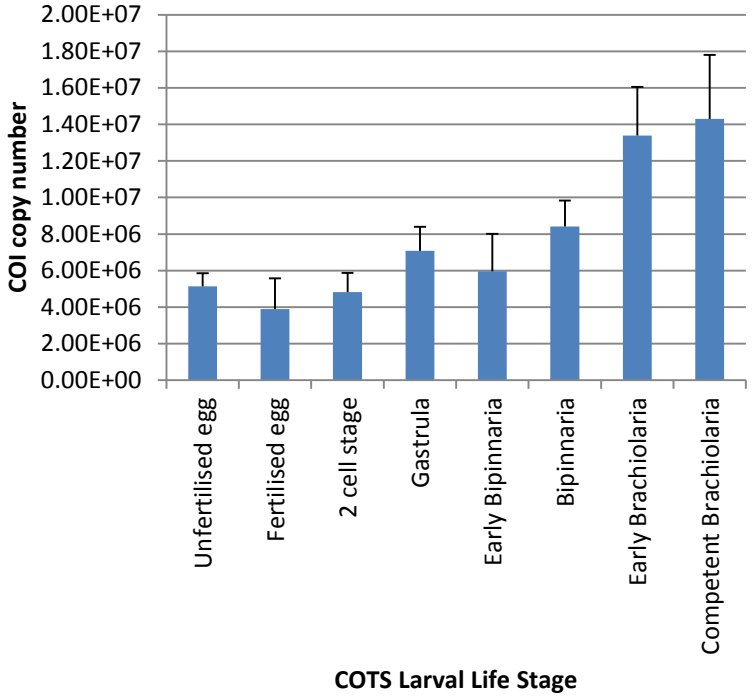

Figure 5. COI gene copy number variation across COTS larval life stages. Data is the mean and SD of eight replicate measurements.

Quantitative determination of COTS larval density in field samples

Samples collected during December 2014 (Figure 6, Supplementary Table 1) were subjected to the quantitative extraction and qPCR analysis to determine COT larval density. Sampling concentrated in the norther section of the Great Barrier Reef, within the COTS initiation zone and extended east into the Coral Sea and south as far as Magnetic Island off the coast of Townsville. Sampling depths ranged from 15 – 100 m with the volume of water filtered calculated from the area of the plankton net opening and the sampling depth.

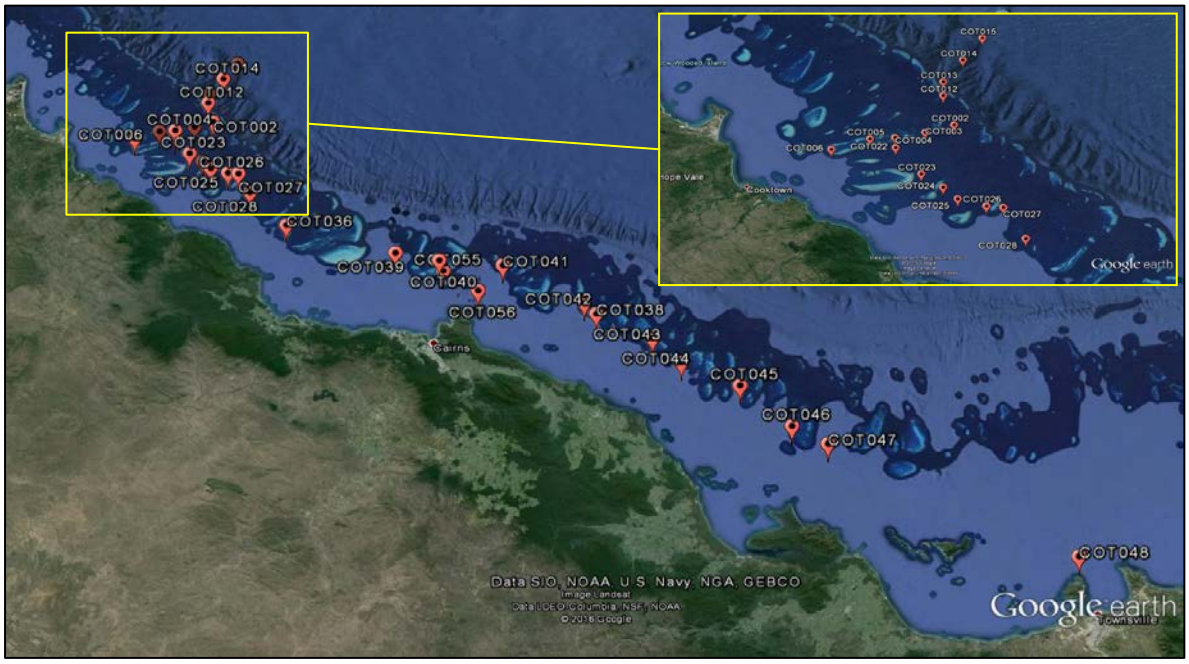

Figure 6. Location of samples subjected to qPCR analysis for determination of COTS larval densities.

As it is impossible to determine which developmental stage is being detected, COTS larval densities are reported as the mean and range based on the copy number of a fertilized egg having the lowest copy number per individual and a competent brachiolaria having the highest copy number per individual. COTS larval density per cubic metre is detailed in Table 2. Two sub-samples from the field negative control, COT055 (spike 1 and spike 2) were spiked with a single early bipinaria larvae and subjected to the same quantitative extraction/qPCR regime as the samples. The spiked samples were normalised to the mean copy number of an early bipinaria and, the quantitative extraction and qPCR analysis revealed a single larvae was detected in each of these spiked samples (Table 2). This is an additional way to confirm extraction and quantification methods give reliable estimates. .

Table 2. COTS larval density (larvae x m^{-3}) in locations from far north Queensland to Magnetic Island and out to the Coral Sea.

Station Name	Mean	Minimum estimate	Maximum estimate	Detected in Uthicke et al 2015
COT002	10	4	16	Y
COT003	11	5	17	Y
COT004	10	4	16	Y
COT005	9	4	14	Y
COT006	16	7	25	Y
COT012	13	6	21	Y
COT013	1	0	1	Y
COT014	0	0	0	N
COT015	0	0	0	N
COT022	0	0	0	Y
COT023	3	1	5	N
COT024	8	4	13	Y
COT025	0	0	0	Y
COT026	15	6	23	Y
COT027	31	13	48	Y
COT028	31	13	49	Y
COT036	55	24	87	Y
COT038	9	4	15	Y
COT039	0	0	0	Y
COT040	4	2	6	Y
COT041	5	2	8	Y
COT042	13	5	20	Y
COT043	1	1	2	Y
COT044	0	0	0	N
COT045	2	1	4	Y
COT046	0	0	0	Y
COT047	3	1	4	N
COT048	0	0	0	N
COT055	0	0	0	N
COT056	0	0	0	N
COT055 spike 1*	1	na	na	na
COT055 spike 2*	1	na	na	na

*COT055 and COT056 were field negative controls collected out of the spawning season from Green Island and Fitzroy Island respectively. Not subjected to minimum/maximum estimates as these samples were used to estimate recovery of a single early bipinaria larvae.

The mean COTS larvae x m^{-3} ranged from 0 to 55. Most reefs between Cooktown and Townsville contained COTS larvae in reasonable numbers with relatively high concentrations found at Pickersgill, Evening, Mackay, Rudder and Tongue Reefs compared to other reefs with mean larval densities ranging from 15 – 55 larvae x m^{-3}. The two Coral Sea samples (COT014 and COT015) were negative for COTS larvae. This observation and other zero larvae measured confirmed our sample collection protocols prevented cross contamination. There was good agreement between samples measured via the qualitative method of Uthicke et al (2015a) and the current quantitative method. However, there were occasions were a negative result was found with the qualitative method while a positive result was found with the quantitative method and vice versa. This may be due to low larval densities in these samples with the process of sub-sampling resulting in the few larvae present in some sub-samples but not others. With this information, it is prudent to pursue an analysis regime that ensures a positive result is determined from multiple sub-samples. These results demonstrate the successful application of the qPCR approach to determine COTS larval densities.

Adult COTS detection through e-DNA

A preliminary experiment was conducted on board the RV Cape Ferguson. Several adult COTS were placed in a 100 L plastic tank filled with seawater (static, no flow through) and acclimated for several hours prior to filtering 400ml of the water through a 25 mm GF/F filter. The filter was extracted using a Qiagen DNeasy Blood and Tissue kit and the extracted DNA tested for the presence of COTS DNA using specific genetic makers (Uthicke et al. 2015). A PCR product of the correct size was observed from these samples indicating that we had extracted adult COTS DNA from this water sample. This gave us the preliminary evidence we needed to continue with the development of COTS DNA detection in the water column.

The above described qPCR assay was employed for COTS eDNA detection with slight modifications. The reason being that the eDNA method will not be able to determine the number of adult COTS (cf to COTS larvae), rather it will determine the concentration of the COTS DNA in the water which can then be utilised as a relative comparison between locations. Standard curves using adult COTS DNA resulted in a detection limit of 2 pg COTS DNA per qPCR reaction.

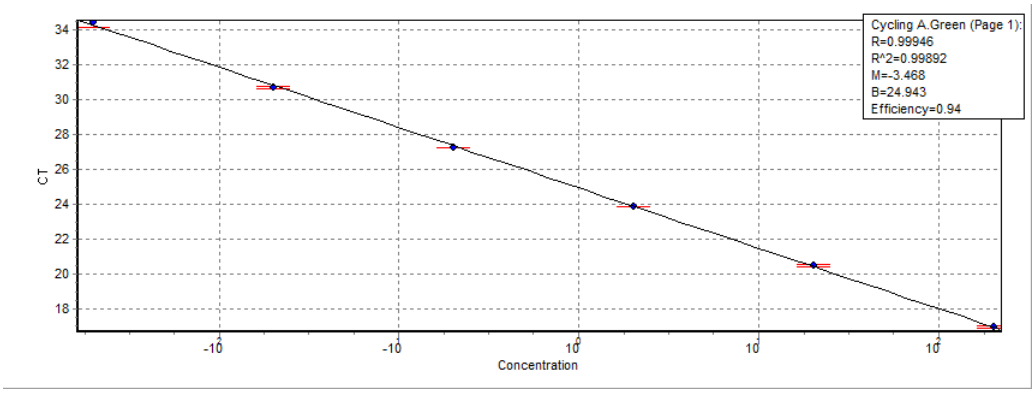

Figure 7. TaqMan qPCR standard curve with COTS DNA. Concentration is given as ng COTS DNA per reaction. CT: Cycle threshold.

After demonstrating that adult COTS DNA was detectable in water from the preliminary experiment, a 1000lt flow though (approx. 10 L x min^{-1}) tank was set up at AIMS SeaSim Facility in August 2015 containing 10 adults COTS as part of a step wise approach to determine the assay sensitivity. No spawning was evident from these adult COTS during the sampling. The adult COTS were acclimated in this tank for several weeks prior to taking 1 lt samples of the water and filtering through a 0.2 [illegible]. Blanks were 1 L of MilliQ water filtered through a 0.2 [illegible]. DNA was extracted using a Qiagen DNeasy Blood and Tissue kit with the final process representing a 10 000 fold concentration of the water (ie. 1 L water extracted and concentrated to 100 [illegible]). For these test samples no COTS DNA was detected in the blank samples whereas the qPCR assay detected 20 ng x L^{-1} of COTS DNA in the water samples collected from the 1000 L flow through tank. Further refinement of the limits of detection of COTS DNA in water is progressing with larger aquaria.

Parallel to aquarium studies, water samples were collected from Rib Reef in October 2015 to establish if COTS DNA could be detected in field samples using the methods described. Although Rib Reef was not undergoing a COTS outbreak at the time of collection, adult COTS were present at the reef (Uthicke, unpublished data). Surface water (2 L) was collected from four sites on the back reef of Rib Rf. (samples: Rib 1-4) with a further 2 samples (Rib 5 and 6; 2 litres each) collected directly (~10-20 cm) over a single adult COTS at 11 m depth nearby (Table 3). All water samples were pre-filtered through a 63 um mesh prior to filtering on a 0.2 um Sterivex filter.

Table 3. Field samples collected for COTS eDNA detection. nd = not detected

	Lat (S)	Long (E)	COTS DNA (ng/L)
Rib 1	18.4800	146.8710	0.13
Rib 2	18.4800	146.8681	nd
Rib 3	18.4959	146.8676	nd
Rib 4	18.4788	146.8658	0.08
Rib 5	18.4831	146.8639	0.52
Rib 6	18.4831	146.8639	0.24

COTS DNA was detected in 2 of the 4 surface water samples with higher amounts of COTS The highest concentration of COTS DNA was detected in water samples collected directly over the top of an adult COTS (Table 3). These data identify that using the sensitive qPCR method developed, we are able to detect COTS DNA from water samples. Refinement of the eDNA sampling technique is now underway includes both aquarium and field trials.

Future work:

1. Continue to monitor plankton from Moore Reef and Agincourt No.3 Reef to develop a comprehensive time series of COTS spawning cycles in the northern sector of the Great Barrier Reef. This may be useful for management because the COTS spawning season is currently only known broadly from field observations. Understanding triggers for outbreak mechanisms rely on knowing when larvae are in the plankton (e.g. to verify if they could benefit from nutrients through flooding).

2. Establish further sampling stations (e.g. on Lizard Island), and develop methods for fine scale sampling. Investigating fine scale distribution (i.e. vertical profiles) will allow a better understanding of larval distribution to investigate for example small scale patchiness in food supply.

3. Continue COTS eDNA method validation including sample optimising, sample collection, filtering and storage methods.

4. Collect and test COTS eDNA samples from both tank and field to further test and validate the possibility of detecting adult COTS eDNA in field samples.

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LITERATURE

Uthicke S, Doyle J, Duggan S, Yasuda N, McKinnon AD (2015a) Outbreak of coral-eating Crown-of-Thorns creates continuous cloud of larvae over 320 km of the Great Barrier Reef. Scientific Reports 5:16885 doi:10.1038/srep16885, http://www.nature.com/articles/srep16885#supplementary-information

Uthicke S, Logan M, Liddy M, Francis D, Hardy N, Lamare M (2015b) Climate change as an unexpected co-factor promoting coral eating seastar (*Acanthaster planci*) outbreaks. Scientific Reports 5 doi:Artn 8402, 10.1038/Srep08402

Vogler C, Benzie J, Lessios H, Barber PH, Woerheide G (2008) A threat to coral reefs multiplied? Four species of crown-of-thorns starfish. Biol Lett 4:696-699 doi:DOI 10.1098/rsbl.2008.0454

OUTPUTS

Publication plan

An initial publication on the qualitative assay previously developed has been published:

Uthicke S, Doyle J, Duggan S, Yasuda N, McKinnon AD (2015) Outbreak of coral-eating Crown-of-Thorns creates continuous cloud of larvae over 320 km of the Great Barrier Reef. Scientific Reports 5:16885

Mid 2016. Planned submission on manuscript - Doyle, McKinnon, Uthicke." Quantitative determination of COTS larvae on the Great Barrier Reef: Implications for modelling future outbreaks."

PROJECT TEAM

Dr Sven Uthicke

Dr Sven Uthicke is Team Leader for COTS research at AIMS and has worked on echinoderms for more than 20 years.

Mr Jason Doyle

Mr Jason Doyle has been responsible for the development and implementation of the genetic assay for the detection of COTS larvae as well as the development of eDNA monitoring method.

Supplementary Table 1 Detailed list of sample locations.

Station Name	Latitude (S)	Longitude (E)	Date	Water Depth (m)	Location
COT002	15.6958	145.7290	18/12/2014	52	S of Osterlund Rf.
COT003	15.6638	145.6607	18/12/2014	40	S of Osterlund Rf.
COT004	15.6250	145.5950	18/12/2014	35	S of Osterlund Rf.
COT005	15.5867	145.5447	18/12/2014	35	S of Osterlund Rf.
COT006	15.5455	145.4560	18/12/2014	32	S of Osterlund Rf.
COT012	15.6155	145.7700	19/12/2014	48	Irene Rf./Ribbon Rf. 1
COT013	15.5862	145.7993	19/12/2014	44	Ribbon Rf. 1/2
COT014	15.5712	145.8838	19/12/2014	1400*	Coral Sea
COT015	15.5558	145.9700	19/12/2014	2000*	Coral Sea
COT022	15.6470	145.5763	20/12/2014	38	E of Ribbon Rf. 1
COT023	15.7422	145.5710	20/12/2014	31	Cairns Rf./Endeavour Rf.
COT024	15.8035	145.5847	20/12/2014	33	Endeavour Rf./Pickersgill Rf.
COT025	15.8496	145.5883	20/12/2014	34	N of Pickersgill Rf.
COT026	15.9092	145.5750	20/12/2014	32	S of Pickersgill Rf.
COT027	15.9385	145.6527	20/12/2014	39	Evening Rf.
COT028	16.0328	145.6317	20/12/2014	33	Mackay Rf.
COT036	16.2325	145.6430	21/12/2014	42	Rudder Rf./Tongue Rf.
COT038	16.4878	145.8273	21/12/2014	39	S of Batt Rf.
COT039	16.5973	145.8703	21/12/2014	39	E of Oyster Rf.
COT040	16.7303	145.9717	21/12/2014	43	Arlington Rf./Green Isl.
COT041	16.9042	146.1355	22/12/2014	39	E of Sudbury Rf.
COT042	17.2202	146.2545	22/12/2014	38	S of Flora Rf.
COT043	17.4918	146.3413	22/12/2014	38	E of Peart Rf.
COT044	17.6320	146.3516	22/12/2014	42	N of Ellison Rf.
COT045	17.9402	146.4468	22/12/2014	36	S of Beaver Rf.
COT046	18.0801	146.4744	22/12/2014	32	E of Otter Rf.
COT047	18.2140	146.5213	22/12/2014	35	E of Britomart Rf.
COT048	19.1010	146.8630	23/12/2014	15	Magnetic Isl.
COT055	16.7716	145.9540	17/06/2015	38	Green Isl.
COT056	16.9207	145.9967	17/06/2015	18	Fitzroy Isl.

*: Two deep stations in the Coral Sea only towed to 100 m.