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Technical Report – Assay development and approach to detect pest species in coral aquaculture: a case study with the Acropora eating flatworm, *Prosthiostomum acroporae*.

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The RRAP partners acknowledge Aboriginal and Torres Strait Islander Peoples as the first marine scientists and carers of Country. We acknowledge the Traditional Owners of the places where RRAP works, both on land and in sea Country. We pay our respects to elders; past, present, and future; and their continuing culture, knowledge, beliefs, and spiritual connections to land and sea Country.

We specifically acknowledge and thank the following Traditional Owners of sea Country that this report relates to:

Location	**Traditional Owner Group**
Australian Institute of Marine Science, Townsville	Bindal

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1 Executive Summary

- This report presents an assay development approach using environmental DNA methods with molecular diagnostics for pest detection inside coral aquaculture operations.
- Specifically, this report utilises the development of an environmental DNA method for the molecular detection of Acropora Eating Flatworms (AEFW) as a case study using digital droplet PCR.
- We report a sensitive, specific molecular diagnostic tool for AEFW that does not require visual inspection of each coral colony.
- Water samples from tanks are tested for the presence of AEFW DNA with limits of detection being less than 3 DNA copies per test.
- Testing was conducted across the operations of coral aquaculture including broodstock, settlement and long term grow out.
- Results indicate broodstock holding tanks are susceptible to AEFW outbreaks with several positive detections made, while settlement and long-term grow out operations had no detectable levels of AEFW.
- We recommend the integration of environmental DNA testing within coral aquaculture systems as an efficient tool to test for the presence of pest species such as AEFW.
- Future considerations include the streamlining of sample acquisition with bacterial load testing, the incorporation of semi-autonomous sampling devices and development of rapid ‘point-of-need’ testing capability.

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2 Introduction

Coral aquaculture for large scale restoration activities remains a viable intervention option to address declining reef cover. Coral aquaculture practices face challenges that plague other aquaculture ventures, with some of the most significant considerations being the control of pests and infectious diseases that can undermine coral production, especially under high-density holding conditions (MacAulay et al., 2022).

Coral pathogens and pests are often microscopic, difficult to detect and/or elusive. As such, clinical signs of disease and infections within aquaculture systems are often observed in late stages where delayed treatment options may become inadequate (Bass et al., 2015). Traditional aquaculture pest and pathogen diagnostics, such as histopathology, culture work, genetic screening, and morphological identification, are often time and labour intensive, lack detection sensitivity or only detect the parasite when the culture has grown to an epizootic outbreak (Eble et al., 2020; Gomes et al., 2017). Therefore, good husbandry methods, system management, and biosecurity protocols to prevent the pest- and disease-induced issues are essential in an aquaculture system.

The Acropora-eating flatworm (AEFW), *Prosthiostomum acroporae*, feeds exclusively on several coral species of the genus *Acropora* Oken, 1815 and can persist as a prolific pest in coral aquaria (Litvaitis et al., 2019; Rawlinson et al., 2011). Infestations of these cryptic polyclad flatworms on captive Acropora corals were first observed by coral hobbyists (Barton et al., 2019; Nosratpour, 2008), with further biological traits including, high fecundity, cryptic camouflage, and complex life history (e.g., intracapsular larva) identified (Barton et al., 2020; Barton et al., 2019). Consequently, predation by *P. acroporae* is considered a serious problem to large-scale coral aquaculture facilities as it undermines the health of captive corals leading to irreversible tissue damage of *Acropora* hosts and ultimately colony mortality if left unchecked (Hume et al., 2014; Rawlinson et al., 2011). Developing innovative, robust and sensitive diagnostic assays to detect cryptic coral pest species, such as *P. acroporae*, is therefore critical for developing successful coral aquaculture systems.

Environmental DNA (eDNA) is defined as sloughed or excreted genetic materials within environmental samples (i.e. water, sediment, soil), which can be detected without visual identification of the biological source (Goldberg et al., 2016; Thomsen & Willerslev, 2015). Extracted eDNA can then be analysed with molecular approaches such as polymerase chain reaction (PCR) and next generation sequencing (NGS) (Goldberg et al., 2016; Thomsen & Willerslev, 2015). This non-invasive sampling technique became popular to monitor introduced species in freshwater (Ficetola et al., 2008) and quickly evolved into a powerful diagnostic tool that can be utilized to address questions related to microbial communities, biodiversity, ecology, evolution, invasive species, and even interactions between hosts and pathogens (Bass et al., 2015; Bohmann et al., 2014; Clarke et al., 2021; Klymus et al., 2015; Uthicke et al., 2024). The general applicability across a wide range of taxa and power of detection for elusive species makes eDNA assays a promising detection tool for monitoring pests and pathogens in coral aquaculture systems.

Visual identification and physical removal form the largest component of current pest protection strategies in coral aquaculture, however there is a need to improve protection of cultured corals from pests that evade visual identification. As the corallivorous *P. acroporae* species are often elusive and/or small, the application of eDNA methodologies has the potential to be an early diagnostic tool for identifying pre-infestations of these pest species within coral aquaculture systems. Development of eDNA methodologies for pest detection in general is transferable to any target species. As such, this report focuses on the development of a diagnostic method for *P. acroporae* as a specific case study, but importantly detailing the general establishment of workflows irrespective of the pest target. To achieve this, we (1) designed and validated specific primers targeting a mitochondrial marker gene *in P. acroporae*, (2) developed a digital droplet PCR assay and evaluated the assay for sensitivity and specificity, and (3) tested the assay's applicability to real-life aquaculture operation within various coral propagation systems at the Australian Institute of Marine Science National Sea Simulator facility. Through the workflow described, we developed a sensitive, specific molecular test for *P. acroporae* and demonstrate the application of this test to detect *P. acroporae* in coral aquaculture systems. Further, we outline our recommendations for implementation to address pest infestation issues in coral breeding.

3 Materials and Methods

3.1 Pest Sample Collection and Primer Design

The first step to producing a molecular assay for a target species is to obtain genetic material. *Prosthiostomum acroporae* (Acropora eating flatworms, AEFW) individuals were collected in 2021 and 2022 from Acropora millepora coral hosts originated from Falcon and Martin reef within the central region of the Great Barrier Reefs, Australia. DNA from AEFW was extracted using a Qiagen Blood and Tissue kit, following the manufacturer's protocols with a minor modification; we added the specimen to Qiagen buffer ATL (180 µL) and proteinase K (20 µL) and incubated overnight at 56°C with constant rotation to ensure thorough cell lysis. Once the genetic material is obtained, general primers are used to isolated large regions of DNA from which specific primers can be designed. To obtain these large regions of DNA sequences for designing flatworm specific PCR primers, we amplified and sequenced the mitochondrial cytochrome oxidase subunit 1 (COI) gene from each sample using the metazoan-generic mitochondrial COI primers LCO1490 and HCO2198 (Folmer et al., 1994) and Sanger sequenced by Macrogen Inc, South Korea. Forward and reverse sequences of the COI gene from AEFW samples were edited to remove low-quality and primer-associated bases and assembled to a sequence contig per sample. Contig sequences were then aligned to obtain an AEFW consensus sequence. Forward and reverse primers are then designed that target shorter (60-300 bp), specific AEFW sequence regions suitable for digital droplet PCR We also included the design of a specific hydrolysis probe that binds to the target DNA between the forward and reverse primers. The hydrolysis probe allows for increasing specificity and sensitivity of the molecular assay.

The specific AEFW forward and reverse primers and a hydrolysis probe were designed with the following constraints, 1) melting temperature (Tm): 55-65°C with < 5°C difference between paired primers, 2) G/C content: 40-80%, 3) primer length: 15-25 bp., 4) amplicon size: 60-300 bp., and 5) non target sequences must have six or more mismatches to primer sequence including at least three mis-matches within five base pairs of the 3’ end of the primer. All sequence manipulations and primer design were conducted in Geneious Prime v2022.1.1 (https://www.geneious.com/). Candidate primers and probes were assessed for possible secondary structure or duplex formation in silico using the IDT OligoAnalyzer™ Tool (https://sg.idtdna.com/calc/analyzer) and for potential binding against all non-target metazoan species to avoid off-target PCR amplification using PrimerBLAST (Ye et al. 2012). Primer pairs that successfully met all of the in-silico criteria were synthesized by Merck with HPLC purification and shipped at concentrations of 100 µM in TE buffer.

3.2 Digital Droplet PCR

Digital droplet PCR (ddPCR) is a 3rd generation molecular amplification and quantification method that partitions a PCR reaction into thousands of small encapsulated individual PCR reactions, or droplets. After thermal cycling, each droplet passes through a micro-fluidic chamber where the fluorescence intensity is measured, thereby dividing the individual droplets into those that contain amplified the genetic target (positive, high fluorescence), and those that did not (negative, low fluorescence). This ‘digital’ distinction forms the basis of molecular counting with a high sensitivity.

All ddPCR was performed using the Bio-Rad QX200 ddPCR system, comprising an automated droplet generator, thermal cycler and droplet fluorescence reader. Thermal cycling of the PCR followed a two-step protocol whereby each thermal cycle had single temperature, combined annealing and extension that followed a DNA denaturation step. The ddPCR assay was optimized for annealing/extension temperature, primer concentration and probe concentration. Annealing/extension temperature test range was 57°C – 62°C, primer concentration was tested at 400 nM, 600 nM and 900 nM, and probe concentration was tested at 100 nM and 250 nM. Optimal conditions were selected based on the signal:noise ratio as determined by the mean fluorescence amplitude of positive droplets divided by the mean fluorescent amplitude of negative droplets. The threshold between positive and negative droplets was determined by comparing samples to control ddPCR reactions performed at the same time. Typically, this was approximately half-way between the positive and negative droplet clouds. In addition, we utilised the coefficient of variation (%CV) to numerically describe the variation of the positive droplet cloud fluorescence amplitude to determine optimal assay parameters. The final assay conditions for each 25 μl ddPCR reaction comprised of 12.5 μL Bio-Rad ddPCR Supermix for Probes (no dUTP), 5 μL DNA template or negative control, forward primer/reverse primer/probe at test concentrations with the balance of each reaction made with UV treated MilliQ water. Reactions were prepared in 96-well skirted plates from which droplets were generated with a BioRad AutoDG droplet generator into a second 96-well plate. The plate containing PCR droplets was then sealed with pierceable foil (Bio-Rad) and placed in a Bio-Rad C1000 Thermal Cycler. Cycling conditions were, 95°C for 10 min; 40 cycles of 94°C for 30 sec and the testing temperature (57 - 62°C) for 1 min, 98°C for 10 min, and held at 4°C until signal reading (<24 hours). Droplet fluorescence was read on a QX200 droplet reader (Bio-Rad), raw data analysed using Bio-Rad QuantaSoft™ Analysis Pro software version 1.0.596.0525.

3.3 Sensitivity and Specificity

Sensitivity of the ddPCR assay to determine the limit of detection (LOD) utilised a 5-fold dilution series of an AEFW genomic DNA extract, with test concentrations ranging from 19 pg to 0.0095 pg per reaction. Assay dynamic range and precision was evaluated with linear regression using log10 transformed data. A total of eight replicates was conducted for each test concentration along with four no-template controls. The LOD is defined as the lowest test concentration at which >95% of technical replicates produce a positive amplification of the target DNA (Klymus et al. 2020).

Assay specificity was tested with DNA extracted from non-target marine invertebrate species found within the AIMS Sea Simulator aquarium facility. DNA was extracted from non-target species (Table 3.1) using a Qiagen Blood and Tissue kit with overnight lysis as described above. DNA was quantified using a Qubit HS dsDNA kit (Thermo) and sample concentrations were adjusted 0.1-0.5 ng μL^{-1} for testing on the conservative assumption that the contribution of a single species would be no more than 10% of typical eDNA samples in aquarium water, with total DNA concentrations from eDNA samples averaging between 1 – 5 ng μL^{-1}. Each sample was tested in duplicate with the optimised ddPCR assay established above.

Table 3.1 Non target species within the AMS Sea Simulator aquarium used for testing the specificity of the AEFW ddPCR assay.

Sample ID	Organism	Family	Phylum
G1.2	*Tripneustes gratilla*	Toxopneustidae	Echinodermata
G2.2	*Echinometra mathaei*	Echinometridae	Echinodermata
G3	*Waminoa sp.*	Convolutriloba	Xenacoelomorpha
G4	*Convolutriloba sp.*	Convolutriloba	Xenacoelomorpha
G5	*Turbo sp.*	Trochidae	Mollusca
G7	*Stomatella sp.*	Trochidae	Mollusca
G8	*Dendropoma sp.*	Vermetidae	Mollusca
G9	*Common Vermetid*	Vermetidae	Mollusca
G10	*Common brittle star*	Ophiuroidea	Echinodermata
G11	*Calthalotia strigata*	Trochidae	Mollusca
G12	*Common Chitin*	Polyplacophora	Mollusca
G13	*Keyhole limpet*	Fissurellidae	Mollusca
G14	*Tridacna sp.*	Cardiidae	Mollusca
G15	*Mysid Shrimp*	Mysidae	Arthropoda
G16	*Amphipods*	Amphipoda	Arthropoda
G17	*Tetralia sp.*	Tetraliidae	Arthropoda
G18	*Small free-living isopod*	Sphaeromatidae	Arthropoda
G19	*Aiptasia pallida*	Aiptasiidae	Cnidaria
G20	*Boloceroides sp.*	Boloceroididae	Cnidaria
G21	*Palythoa*	Sphenopidae	Cnidaria
G22	*Asterina sp.*	Asterinidae	Echinodermata
G23	*Brittle Star*	Ophiuroidea	Echinodermata

3.4 Application to Coral Aquaculture

We conducted eDNA sampling in aquarium facilities within the National Sea Simulator (SeaSim) at the Australian Institute of Marine Science (AIMS) to test the applicability of the assay in coral aquaculture settings. The SeaSim is a purpose-built state of the art experimental aquarium facility with central water quality control and routine visual pest-screening on corals, providing an ideal testing ground for the AEFW-targeted ddPCR assay. The assay was tested during the seasonal spawning period from November 2023 to

February 2024 as part of the Reef Restoration and Adaptation Program. This included mass spawning periods for both inshore corals (November 2023) and offshore corals (December 2023). Sampling was conducted at multiple time points to cover a range of checkpoints including pre-settlement, post-settlement and pre-deployment throughout the coral aquaculture rearing trials across operational stages of A) holding of parental (broodstock) corals, B) settlement of larvae onto substrata, and C) a long-term grow-out experiment of coral recruits throughout January and February 2024 (Figure 1, Table 3.2).

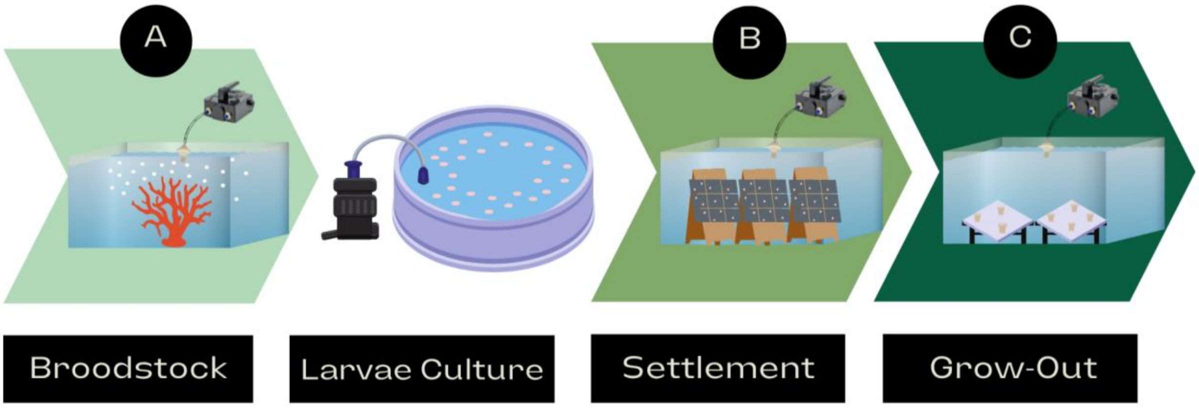


Figure 1 Operational stages of coral spawning activities where eDNA sampling was conducted for AEFW testing. A) Broodstock housing tanks, B) Settlement tanks after fertilization with newly settled corals and C) grow out tanks where newly settled corals were housed for up to 77 days post settlement. eDNA testing for pest was not conducted at the larval culture stage as this was deemed unnecessary due to the negligible likelihood of flatworm infestation in coral larva.

A. Broodstock sampling: For each coral broodstock/spawning period combination, we conducted eDNA sampling on two occasions two investigate the possibility that an AEFW infestation may have been undetectable at the first time point. Details of tank holding including temperature, flow rates, stocking density, etc. can be found in Severati *et al* (2024).

A. millepora broodstock arrived at AIMS on 30th Oct 2023 (November 2023 inshore spawning) and placed into a single holding tank (termed MOS4A) in the AIMS SeaSim facility. *A. millepora* broodstock coral holding tanks were sampled twice during November spawning: once shortly after the spawning event on the 7th November and again five days later, representing 8 and 13 days since captivity respectively. *A. millepora* broodstock was maintained from the November 2023 spawning activities in a different holding tank (MOS4C) from late November 2023. Further eDNA sampling was conducted on the *A. millepora* broodstock on the 5th and 14th of December 2023prior to relocation back to the reef (36 and 45 days of captivity, respectively; Figure 2).

A. spathulata and *A. loripes* broodstock arrived at AIMS on 28th November 2023 and placed into the holding tank MOS4A and a larger holding tank (OH2A) respectively. *A. spathulata* and *A. loripes*, were sampled post-spawning (5th and 8th December, representing 7 and 10 days of captivity, respectively) and just prior to their return to the reef on the 14th of December (16 days holding). On this final day of sampling, it was noted that several of the *A. loripes* colonies had developed rapid tissue necrosis.

The movement of broodstock provided an opportunity to test connected systems to investigate the localisation of any AEFW eDNA. Specifically, *A. millepora* and *A. spathulata* (Figure 2) shared a common sump which returned filtered seawater back to both holding tanks (partial recirculation). This connected system was established for at least 1 week prior to eDNA sampling of these holding tanks.

Figure 2 Broodstock holding tanks for A. spathulata (left, MOS4A) and A. millepora (right, MOS4C) during the December 2023 spawning time. Photo credit J. Doyle.

B. Settlement (pre-deployment) sampling: Coral settlement tanks were established for *A. millepora* after the November 2023 spawning and for *A. spathulata* after the December 2023 spawning.

A. millepora was settled on pre-conditioned concrete tiles which were placed into shallow race-way tanks hosted within a larger flowthrough aquaria as heat sinks (termed MIS5 and MIS6) within the SeaSim (Details to be reported in RRAP CAD Report 14.3). Each MIS tank held four race-way tanks (labelled A1, A2, B1 and B2), such that a total of 8 tanks were utilised for coral settlement (Figure 3a). For testing of the AEFW eDNA ddPCR assay, four of the eight tanks were sampled (namely, MIS5A2, MIS6A1, MIS6A2 and MIS6B2), together with the input seawater control. To pre-screen AEFW eDNA in the settlement tanks and tiles, eDNA water samples were collected before coral larvae were added, one day after the settlement tanks and tiles were set up with flowthrough water (on the 7th November; 'pre-settlement'). To investigate if AEFW infestation may develop over the duration of the coral settlement, eDNA water sampling was conducted one day after coral larvae were added (the 8th November; 'post-settlement'), and one more sampling 5 days after corals were settled but before deployed for out-planting (the 12th November; 'pre-deployment').

A. spathulata settlement was conducted in two different tank setting (a shallow raceway tank as above (MIS6-A1), and 50L flowthrough aquaria Figure 3b), which were given different strains of algal symbionts to test the effect of symbiotic algae on early survival of coral recruits (Details to be reported in RRAP CAD Report 14.3). Specifically, the Symbiodiniaceae clade C symbiont (*Cladocooium sp*.) was inoculated to corals in MIS6, the clade D symbiont (*Durusdinium sp*.) in a Room 9 tank and no symbiont in the other Room 9 tank. Similar to the above experiment for *A. millepora*, pre-settlement eDNA water samples were collected one day after the tank settings were established (the 5th December) from each coral settlement tank and input control seawater samples ('pre-settlement'). Corals were settled on tiles on the 6th December in each tank, with algal symbiont inoculation conducted on the 7th December (within 24 hours). eDNA water samples collected on the 8th December (2 days post settled; 'post-settlement') and the 14th December (8 days post settled, 'pre-deployment').

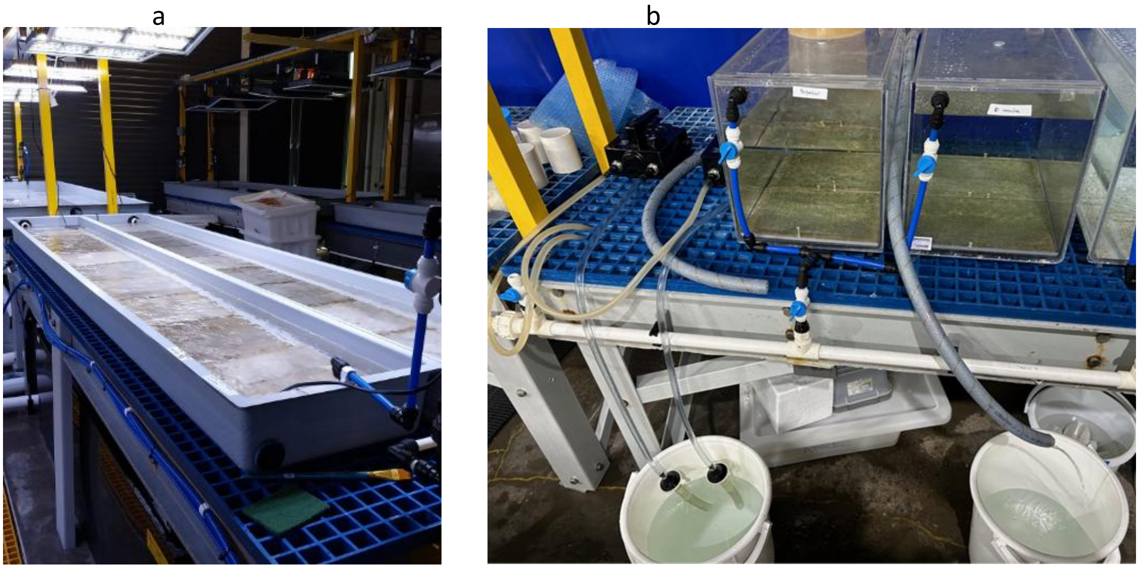


Figure 3 Tank set up for A. spathulata settlement in AIMS SeaSim. (a) Shallow raceway tanks for coral settlement. (b) Aquaria used for separate coral settlement and algal symbiont inoculation trials. The tank on the left is without symbiont and the tank on the right is inoculated with Clade D symbiont. Buckets were used to collect flow through water and eDNA sampling conducted from these buckets (see lower left). Photo credit J. Doyle.

C. Long-term grow out sampling: To evaluate the effect of long-term captive husbandry on post-deployment coral survival, a mixture of tiles settled with two coral species *A. spathulata* and *A. loripes* larvae were placed in MIS tanks under flow-through and partial recirculation conditions for approximately two months (ongoing RRAP CAD study, Nordborg et al.). Details of tank set up will be described in RRAP CAD Report 14.3. Briefly, tanks were 135 L capacity with a turn over rate of 135 L hr^{-1}. Flow through was 100% flow through of seawater and partial recirculation tanks had a water top up rate of three times (i.e. 3 x 135 L) every 24 hrs, with top-up water originating from filtered discharged water from two connected tanks (Figure 4). The eDNA testing of AEFW was incorporated with other QA/QC metrics (e.g. bacterial loading) to gauge the efficacy of a QA/QC monitoring regime in large scale deployment projects. To screen AEFW over the experimental period, three sets of flow through and partial recirculating tanks and input seawater controls were sampled on three occasions: 11th Jan 2024 (37 days post settled), 6th Feb 2024 (63 days post settled) and 20th Feb 2024 (77 days post settled).

Details of eDNA sampling are as follows with tabulated details below (Table 3.2) and further details the companion excel file (23_24 eDNA testing_CAD02_14.7_companion spreadsheet.xlsx).

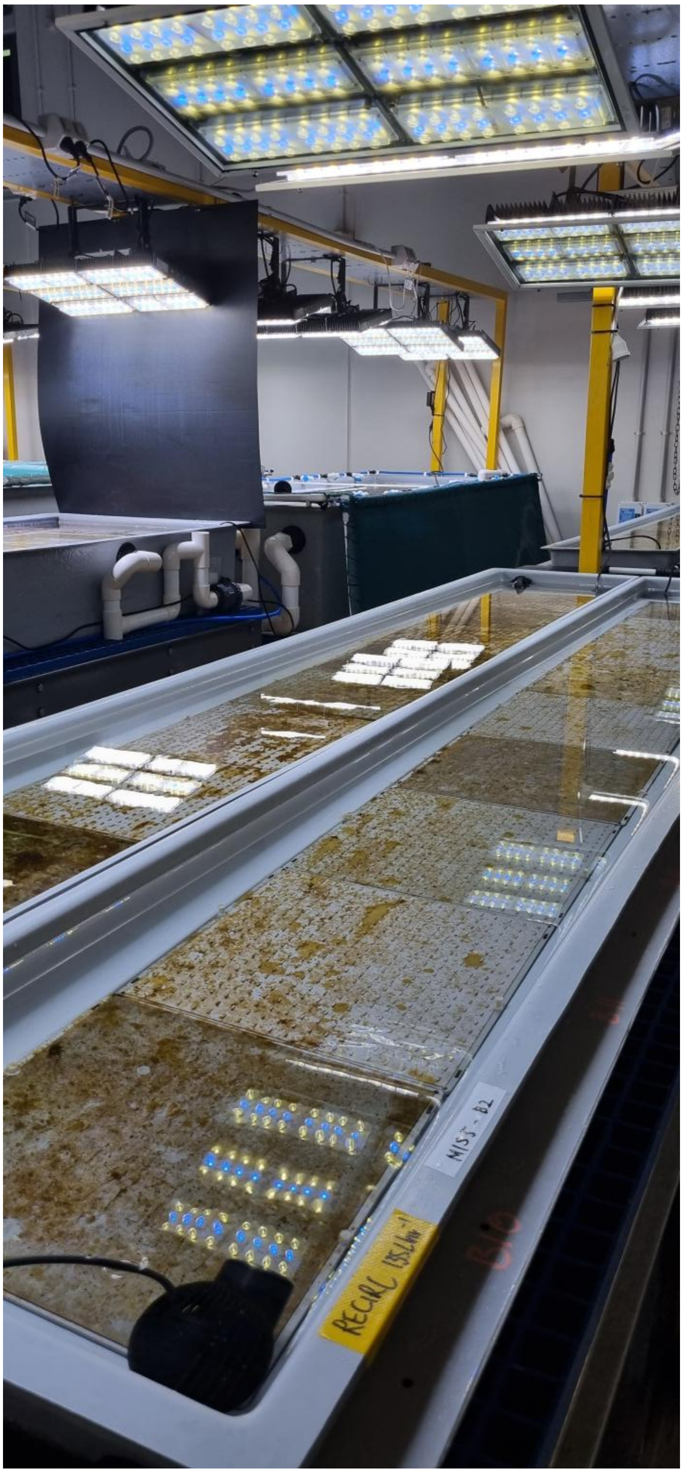


Figure 4 Grow out tanks for large field trials Photo credit M. Nordborg.

Environmental DNA at each point was sampled by filtering 10 L test tank water directly through an eDNA filter 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 were removed from the housings within 2 hours of collection, folded carefully into eighths using bleach cleaned plastic forceps and placed in a 1.5ml screw cap tube containing 540 µl of Qiagen buffer ATL as a preservative (Majaneva et al., 2018). Aquarium filter samples were extracted using a Qiagen DNeasy kit on an automated Qiacube robot according to the manufactures guidelines with the following exceptions: samples were incubated overnight (56°C) with shaking in Qiagen buffer ATL/proteinase K (540 µl/60 µl respectively); a 200 µl aliquot of this initial lysis was transferred to a new tube and 200 µl Qiagen buffer AL added. This ATL/proteinase K/AL mixture was incubated at 56°C for 30 min with shaking; and the final elution was in 50 µl 10 mM Tris pH 8.0. DNA extracts from broodstock tank samples were also tested at 2x, 5x and 10x dilutions to gauge the any effects of ddPCR inhibition due to high DNA concentrations from densely populated tanks. Each collection event was conducted in quadruplicate (4 x eDNA samples), with each eDNA sample analysed in duplicate via ddPCR. The mean copy number from all replicates was calculated and compared to the LOD to determine a positive detection. Negative controls were conducted at each level of sample processing, 1) Collection control. Filtration of tank inlet water prior the water entering the test tank. Filters processed as per samples, 2) Extraction control. A clean membrane filter was added to a 1.5ml screw cap tube containing 540 µl of Qiagen buffer ATL in the lab and extracted as per samples, and, 3) ddPCR no template control. Water was added to separate ddPCR reactions instead of extracted DNA.

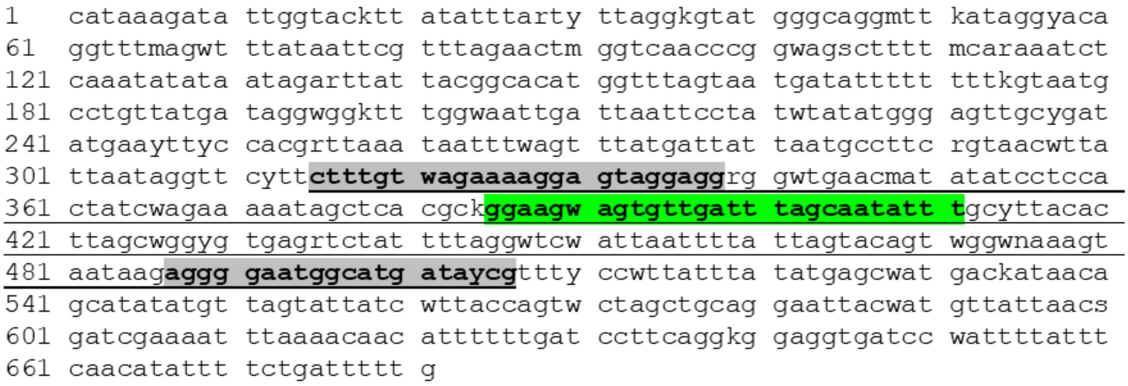
Table 3.2 An overview of QC checkpoints across 2023 summer spawning and 2024 long-term grow-out experiment. Left column indicates different QC checkpoints that were sampled throughout coral aquaculture operations. Information in the table for each QC checkpoint is organized by month (spawning period). Each cell indicates sampling efforts with information such as species (purple), how many tanks/controls were sampled for each effort (blue), the timeline of sampling (shown as date sampled), and other sampling information (italicized). At each timepoint and for every tank/control, there was four replicate eDNA samples taken with individual filters. N/A = not applicable. Further details can be found in the companion excel spreadsheet.

AEFW QC checkpoint	**November 2023**	**December 2023**	**January/February 2024**
A. Broodstock holding tanks	*A. millepora* 1 Tank (MOS4A) + 1 Control 07/11/23, *post-spawning* 12/11/23, *pre-relocation*	*A. spathulata* 1 Tank (MOS4A) 05/12/23, *post-spawning* 14/12/2023, *pre-relocation*	N/A
		A. millepora 1 Tank (MOS4C) 14/12/23, *pre-relocation*	
		A. loripes 1 Tank (OH2A) 08/12/23, *post-spawning* 14/12/23, *pre-relocation*	
B. Settlement tanks for deployment	*A. millepora* 4 Tanks + 1 Control 07/11/23, *pre-settlement* 08/11/23, *post-settlement* 12/11/23, *pre-deployment*	*A. spathulata* 3 Tanks + 2 Controls 05/12/23, *pre-settlement* 08/12/23, *post-settlement* 14/12/23, *pre-deployment*	N/A
C. Long-term grow out experimental tanks	N/A	N/A	*A. spathulate & A. loripes* 6 Tanks + 3 Controls 11/01/24; *37-days* 06/02/24; *63-days* 20/02/24; *77-days*

4 Results

4.1 Primer design

Samples that successfully sequenced in both the forward (LCO1490) and reverse (HCO2198) direction using the metazoan generic primers were aligned and combined after trimming primer sequences and low-quality sequences from the ends. This resulted in large regions (600 – 750bp) of the cytochrome oxidase subunit 1 (CO1) gene for further primer design. The large CO1 gene regions from multiple samples were aligned and a 681bp consensus sequence produced (Figure 5). Primer design and subsequent in-silico analysis yielded a primer set targeting a 192 bp region of the CO1 gene from *Prosthiostomum acroporae.* The finalized primer pair consisted of the forward primer AEFW-315F (5'-TTTGTWAGAAAAGGAGTAGGAGG) and reverse primer AEFW-506R (5'-CGRTATCATGCCATTCCCCT), with the addition of a hydrolysis probe AEFW-385P (5'-GGAAGWAGTGTTGATTTAGCAATATTT).



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1   cataaagata ttggtacktt atatttarty ttaggkgtat gggcaggmtt kataggyaca
61  ggtttmagwt ttataattcg tttagaactm ggtcaacccg gwagsctttt mcaraaatct
121 caaatatata atagarttat tacggcacat ggtttagtaa tgatattttt tttkgtaatg
181 cctgttatga taggwggktt tggwaattga ttaattccta twtatatggg agttgcygat
241 atgaayttyc cacgrttaaa taatttwagt ttatgattat taatgccttc rgtaacwtta
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421 ttagcwggyg tgagrtctat tttaggwtcw attaatttta ttagtacagt wggwnaaagt
481 aataagaggg gaatggcatg ataycgttty ccwttattta tatgagcwat gackataaca
541 gcatatatgt tagtattatc wttaccagtw ctagctgcag gaattacwat gttattaacs
601 gatcgaaaat ttaaaacaac atttttgat ccttcaggkg gaggtgatcc wattttattt
661 caacatattt tctgattttt g
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Figure 5 Consensus sequence for Prosthiostomum acroporae (Acropora eating flatworm, AEFW) used for designing specific primers and probe for ddPCR. The underlined region is the specific region amplified for AEFW, with grey highlighted area showing the AEFW-315F forward primer and AEFW-506R reverse primer. The green highlighted shows the location of the hydrolysis probe, AEFW-385P.

4.2 Digital Droplet PCR

To optimise annealing/extension temperature and primer/probe concentrations for the ddPCR assay, a test matrix of different annealing/extension temperatures were tested with varying primer/probe concentrations, using both signal:noise ratio and %CV of the positive droplet cloud fluorescence amplitude distribution. For all annealing/extension temperature settings, the highest signal:noise ratio, and therefore the greatest separation of positive and negative ddPCR droplets was found using 900nM primers and 100nM probe (Table 4.1, Figure 8-appendix).

Table 4.1 Digital droplet PCR signal:noise ratio defined as the mean amplitude of the positive droplet cloud divided by the mean amplitude of the negative droplet cloud. Signal:noise was calculated for the ddPCR test matrix including difference primer and probe concentrations as well as different annealing/extension temperatures ranging from 57°C to 62°C. . Asterisks denote the optimised combination of conditions.

	Primer/Probe concentrations (nM)					
Annealing/Extension Temperature (°C)	**900/250**	**900/100***	**600/250**	**600/100**	**400/250**	**400/100**
62	1.98	3.23	1.56	2.54	1.36	2.27
61	1.97	3.13	1.58	2.54	1.42	2.38
60	1.96	2.99	1.58	2.41	1.45	2.34
59	2.02	2.90	1.64	2.33	1.52	2.27
58*	2.17	3.04	1.77	2.46	1.64	2.38
57	2.29	3.18	1.90	2.61	1.75	2.53

When we examined the %CV of the positive droplet fluorescence of this primer probe combination, we found the lowest %CV in annealing/extension temperatures of 58°C and 59°C (Table 4.2, Figure 8-appendix), indicating that these temperatures provide the smallest variation in positive amplification fluorescence intensity. At 58°C, the signal:noise ratio was 3.04 while at 59°C the signal:noise ratio was 2.90. Therefore, we concluded that the optimal assay conditions were primers concentrations of 900nM, a probe concentration of 100nM and an annealing/extension temperature of 58°C.

The limit of detection was found to be 2.3 ± 1.2 (SD) copies (equivalent to 0.076 pg genomic DNA) per ddPCR reaction (Table 4.3). At this concentration, all replicate samples were found to be positive.

Table 4.2 Positive droplet mean fluorescent amplitude, standard deviation and %CV for annealing/extension temperatures ranging from 57°C to 62°C. Asterisks denote the optimised combination of conditions.

Annealing/Extension Temperature (°C)	**mean amplitude**	**SD**	**%CV**
62	939.5	75.7	8.1
61	914.8	71.3	7.8
60	878.6	61.1	7.0
59	876.5	55.8	6.4
58*	908.8	58.1	6.4
57	957.5	61.8	6.5

4.3 Sensitivity and Specificity

Sensitivity testing of the above AEFW assay was undertaken to determine the limit of detection for an individual ddPCR reaction. The assay demonstrated a linear response (Figure 6) with decreasing amount of target DNA with linear regression highly correlated (r2 = 0.9982). This linear response covered a large dynamic range of assay detection of approximately 3 orders of magnitude (19 pg to 0.0095 pg per reaction).

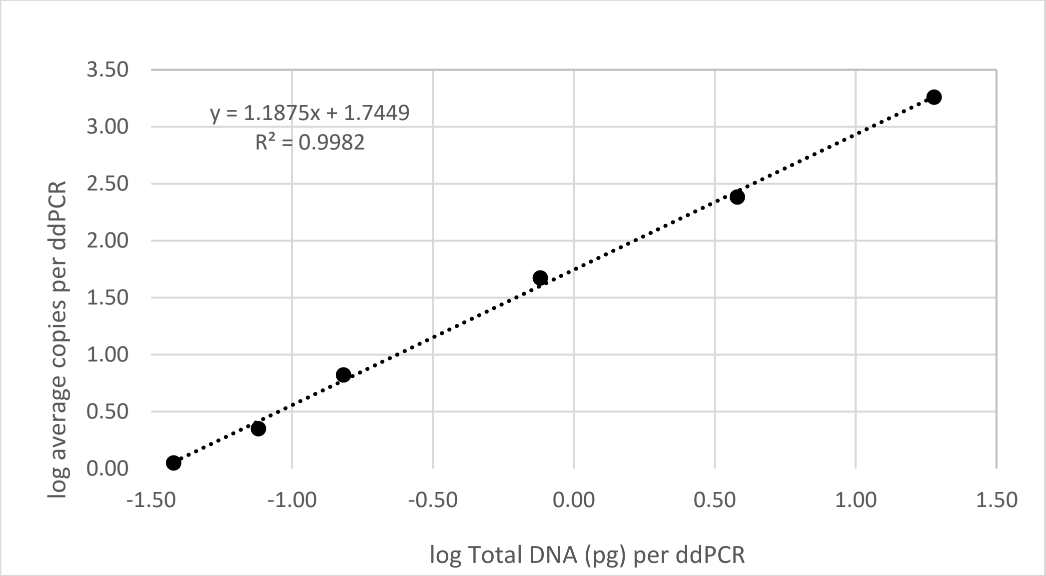

Figure 6 Linear regression of a dilution of AEFW DNA and copy number determination via ddPCR. The high correlation demonstrates a liner response over 3 orders of magnitude.

Table 4.3 ddPCR assay response to a 5-fold dilutions series of AEFW DNA. All replicates were positive down to 0.076 pg per ddPCR reaction, with the mean copy number of 2.3 ± 1.2 copies per ddPCR reaction.

Total DNA per ddPCR reaction (pg)	Mean copies per ddPCR reaction (SD)	No. positive out of 8 replicates
19	1828.5 (48.7)	8
3.8	242.1 (13.7)	8
0.76	47.1 (11.4)	8
0.152	6.7 (3.1)	8
0.076*	2.3 (1.2)	8
0.038	1.12 (1.6)	4
0.019	0.00	0
0	0.00	0

Specificity was then determined using a variety of non-target marine invertebrates detailed in Table 3.1. No cross reactivity was observed (Figure 7) confirming specificity of the AEFW assay.

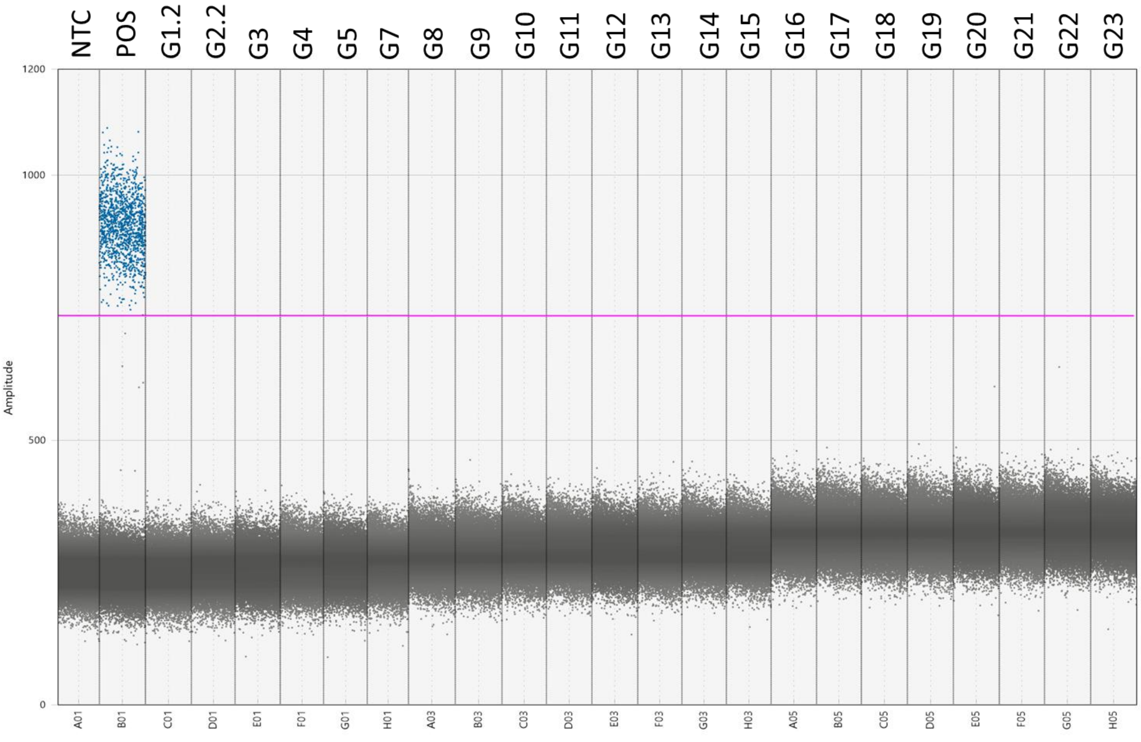

Figure 7 Testing the specificity of the AEFW eDNA ddPCR assay against a variety of species found within the AIMS aquaculture systems. The pink line is the threshold set where droplets above are considered positive amplification and droplets below are considered negative amplification. NTC = no template control (negative control), POS = positive control, Gxx = non-target sample identifier as described in Table 3.1.

4.4 Application of the AEFW assay to Coral Aquaculture

Environmental DNA samples from broodstock, settlement and grow-out tanks were tested for the presence of AEFW pests throughout the coral aquaculture pipeline. AEFW eDNA was not detected in settlement tanks or long-term grow-out tanks providing an assurance that corals settled in these tanks were not infected with AEFW prior to deployment/return to the reef. We did however detect AEFW eDNA in water collected in December 2023 from both *A. millepora* and *A. loripes* broodstock tanks. Throughout testing, inlet seawater collected prior to entering tanks, was collected and analysed as a negative control. In all instances, these negative controls were negative for AEFW eDNA.

The first sampling point for the *A. millepora* broodstock tank in November resulted in a detection of AEFW DNA, with a mean copy number of 1.5 ± 0.6 copies per ddPCR reaction. This is less than the detection limit of the assay (2.3 ± 1.2 copies per ddPCR reaction) but not statistically different ($p = 0.276$), therefore can be considered a 'mild' positive detection of flatworms in this broodstock tank (Table 4.4). The second sampling point for the *A. millepora* broodstock tank resulted in a low-grade detection of AEFW DNA, with an average copy number of 0.5 ± 0.5 copies per ddPCR reaction. This was less than the detection limit of the assay and thus considered below detection limit. *A. millepora* broodstock was maintained in the tank MOS4A from the November 2023 spawning, then relocated to a different holding tank (MOS4C) in December 2023. eDNA samples collected from the *A. millepora* tank in December 2023 showed positive detection of AEFW eDNA at both sampling times on the 5th and 14th December 2023 (Table 4.4). eDNA samples from the adjacent tank connected via a shared sump and partial recirculation of sump water (MOS4A) containing *A. spathulata* broodstock indicated negative for AEFW eDNA.

Additionally, positive detection of AEFW eDNA was made from the *A. loripes* broodstock tank on the 14th December 2023, whereas tests of this same tank six days prior were negative, likely indicating that AEFW biomass increased over the time that *A. loripes* broodstock was held. This positive detection was corroborated with verbal notification from SeaSim aquaculture technicians visually observed AEFW eggs within the *A. loripes* holding tank on the 14th December 2023 (Table 4.4).

Table 4.4 Results from AEFW eDNA testing in broodstock tanks. Mean copies of AEFW DNA per ddPCR reaction presented along with standard deviation (SD). A simplified detection metric (yes/no) based on the limit of detection is also provided. No AEFW eDNA detection were made in any other coral aquaculture operations.

Species	Date	Sample Notes	Mean copies per ddPCR	SD	AEFW detection[1]
A. millepora	07-Nov-23	Control inlet water	0	0	No
	07-Nov-23	Tank MOS4A	1.5	0.6	Mild (below LOD)
	12-Nov-23	Tank MOS4A	0.5	0.5	No
	5-Dec-23	Moved to tank MOS4C	5.1	5.2	Yes
	14-Dec-23	Moved to tank MOS4C	3.0	0.6	Yes
A. spathulata	5-Dec-23	Tank MOS4A	0.7	0.7	No
	14-Dec-23	Tank MOS4A	0	0	No
A. loripes	8-Dec-23	OH2A autospawner	0	0	No
	14-Dec-23	OH2A autospawner[2]	3.2	4.9	Yes

[1] Limit of Detection (LOD) = 2.3 copies per ddPCR; [2] AEFW eggs and rapid tissue necrosis identified in this tank.

5 Discussion

Pest detection within coral aquaculture is needed to ensure deployment of new corals produced or re-location of broodstock corals does not come with an increase pest load that risks infestation in natural coral communities. As in all aquaculture ventures, high density stocking situations carry increased risks of disease and pest outbreak, and therefore a reliable and sensitive detection of coral pests is desired for upscaling coral aquaculture with cost and resource efficiency. Molecular detection of pest eDNA can provide an enhanced tool for detection of pest incursion above the sensitivity and reliability achieved by labour-intensive visual inspection.

This report provides a template workflow for developing a pest detection method in coral aquaculture. We exemplify this workflow utilising eDNA detection methods for a major coral pest, the *Acropora* eating flatworm (AEFW), which can cause substantial damage on the production of popular branching corals of the genus *Acropora*.

Primer design is an essential phase of the assay development as it sets the specificity of the molecular detection. Optimising assay parameters affecting sensitivity and specificity are also critical to establish early in the protocol development. We demonstrated that the analytical sensitivity using digital droplet PCR (ddPCR) is effective for detection of AEFW at low densities. The AEFW ddPCR eDNA assay developed achieved a sensitivity of 2.3 ± 1.2 copies per ddPCR, equivalent to equivalent to 0.076 pg genomic DNA. Additionally, the assay generated no cross amplification with other molluscs and marine invertebrates found within the coral aquaculture systems at AIMS SeaSim, demonstrating high assay specificity.

We tested for AEFW eDNA across of the operational stages required for the coral production, included broodstock holding, coral settlement activities, and long-term grow out prior to deployment. Importantly, taking a system-wide implementation of pest testing allowed this QA/QC activity on pest detection to focus on key areas of more relevance within coral production.

Unsurprisingly, no AEFW eDNA was detected from any of the coral settlement related activities, nor from long term grow out trials. These activities originated from clean stocks of coral gametes/larvae rinsed with filtered seawater without exposure to adult coral colonies, and there is an extremely low likelihood that AEFW would carry through from larval production processes. We elected not to test for AEFW eDNA within the larval culture operational stage (Figure 3.1) as this stage was A) brief in time such that result turnaround would have been longer than larval culture, B) the likelihood of AEFW infestation would have been negligible, especially considering the life cycle of AEFW (Barton et al., 2020) extends beyond the larval culture time frame, and C) primary QA/QC metric at the larval culture phase is bacterial load which is thought to contribute to larval crashes.

In contrast, testing on the broodstock tanks revealed that AEFW eDNA was present, particularly in *A. millepora* and *A. loripes* broodstock holding tanks. An interesting result was the difference between the connected broodstock tanks containing *A. spathulata* (tank MOS-4A) and *A. millepora* (tank MOS-4C). These tanks shared a common sump and included partial recirculation of sump water (see Figure 3.2 above). Although this system was connected, we detected the presence of AEFW eDNA in the *A. millepora* tank only. Results clearly demonstrated that there is potential to differentiate between holding tanks despite sharing a common sump and partial recirculation of sump water to both holding tanks, highlighting the sensitivity of the assay required to identify sources and potentially isolate them.

While our high-sensitivity, target-specific ddPCR assay developed provides confident positive AEFW eDNA detections, the high variability between eDNA sample replicates was observed when the AEFW eDNA was detected near the limit of detection. This variability is also indicative of low densities of target pest species as the ddPCR-based quantification is more influenced by stochastic processes at lower target concentrations (Furlan et al., 2016; Yates et al., 2023). Nevertheless, highly sensitive detection of AEFW eDNA provides a suitable early warning method for the presence of AEFW in broodstock collections. Such early detection can complement detection of pests where visual inspection is labour intensive and requires expert eyes.

6 Outlook

One aspect that is informative in eDNA studies is the nature of eDNA shedding from the target organism and the subsequent decay of the eDNA once it is released (e.g. Kwong et al., 2021). This helps to understand the residence time of the target eDNA within a system, leading to more informed result interpretation. Our aim is to complete such studies whereby we investigate the shedding and degradation of AEFW eDNA within a controlled system, in order to apply the information to coral aquaculture.

We see that there is room to improve workflows, particularly within the sample acquisition phase. Specifically, seawater filter samples for bacterial loading and pest detection samples are often collected at similar times throughout the coral aquaculture life cycle. As part of quality control and quality assurance in coral aquaculture, assessment of bacterial loading also provides information on microbial environmental conditions linked to coral health (Report CAD02-14.8). It stands to reason that we look to develop sampling protocols that allow shared sampling schemes for both bacterial quantification and pest detection. Recent preliminary experimental work has identified improvements that may help streamline sample processing, such that bacterial and pest DNA can be isolated from the one sample by identifying a DNA-extraction method suitable for both assays. Finally, there is also scope to develop, semi-autonomous water sampling instruments which align with the vision of standardizing water sampling and improving the transferability of methods to industry partners.

Currently, we are able to compress the turnaround time from sample collection to analysis to between 3-4 days. However, we recognise that we have both the aquaculture facility and a molecular testing lab adjacent to each other which allows for the samples collected to enter the processing workflow within the same day. We understand that consideration of scalable and portable approaches are required to take pest detection to a level outside of the current research. For example, borrowing from point of care diagnostic field (Wang et al., 2021), .aspects of simplified DNA extraction (Amorim et al., 2012; Steiner et al., 1995), amplification (de Paula et al., 2023) and detection (Doyle & Uthicke, 2020) could be investigated to improve workflow efficiencies.

7 Recommendations

Pest detection remains an important element of quality control as the rate of coral production in aquaculture settings grows with high coral holding density. The work herein, though specific for AEFW, showcases a standard development pathway for any target pest species.

With respect to AEFW, we recommend that AEFW eDNA testing continue for Acropora spp. broodstock colonies, especially broodstock that is maintained in aquariums for long periods and traveling between captive facilities and natural habitats. Intervention options are possible including pest removal (Barton et al., 2020) and a decision not to re-deploy infested corals to the nature. Our testing for AEFW eDNA in settlement and long term grow out facilities confirmed expectations that pest detection during this phase is not viewed as a priority.

Continued development that aligns with priorities of bacterial load testing will streamline sampling and laboratory processing workflows. This includes unified sampling procedures and improvements in sampling automation for industry partner uptake.

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9 Appendix

Figure 8 AEFW ddPCR assay optimisation. The combined annealing/extension temperature cycle for each primer/probe combination (e.g. 900/100) was tested in 1°C increments from 57 – 62°C (left to right). The image below shows an example from duplicate reactions.