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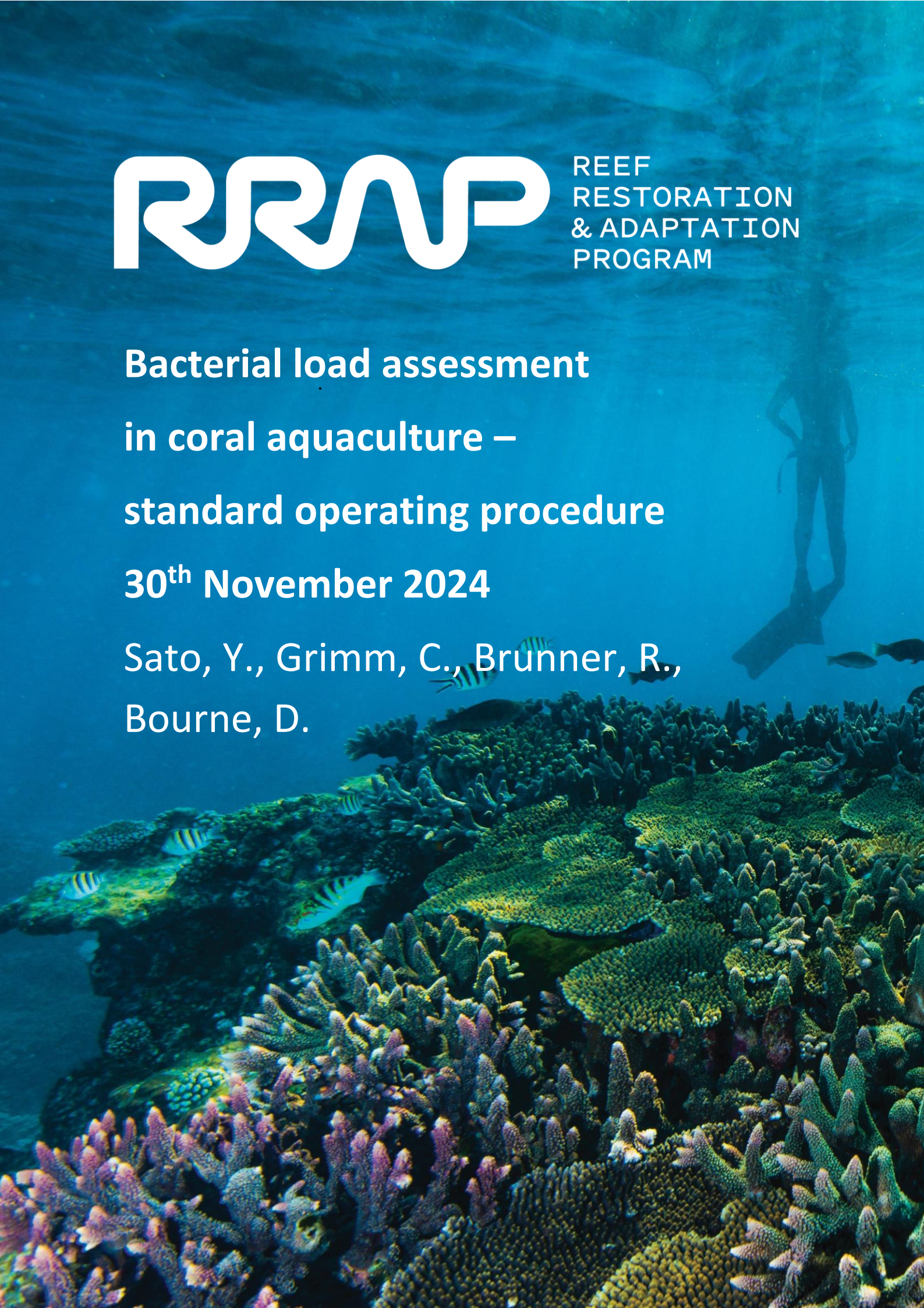
Bacterial load assessment in coral aquaculture – standard operating procedure

30th November 2024

Sato, Y., Grimm, C., Brunner, R., Bourne, D.



Bacterial load assessment in coral aquaculture – standard operating procedure

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Cover Images: Coral Reef, Credit: Gary Cranitch, Queensland Museum

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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
James Cook University, Townsville	Bindal and Wulgurukaba

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1 Executive Summary/Abstract

Coral aquaculture is currently in high demand to mitigate declining coral cover with reef restoration. To efficiently outplant coral recruits, millions of coral larvae must be raised in aquaculture in a cost-effective way. Consequently, there is the urge to scale up coral aquaculture by increasing the stocking density of larvae and reducing water resources without compromising survival of the different life stages.

Coral health is interlinked with microorganisms that surround the animal. It is hypothesised that bacterial increase in culture tanks potentially lead to coral mortality, especially if pathogenic (disease-causing) bacteria such as *Vibrio* species increase. To understand this risk, it is beneficial to establish a method to monitor the abundance of total bacteria and potentially pathogenic bacteria in aquaculture. Such assays can provide an early warning sign that coral mortality is likely to occur.

Here, we provide a microbial load assessment tool based on a duplexed droplet digital polymerase chain reaction (ddPCR) assay to estimate abundance of total bacteria and *Vibrio* species simultaneously. This method consists of the steps of filtering water samples from a target aquaculture facility, extracting bacterial DNA, and quantifying copies of bacterial 16S rRNA sequences as proxies for the abundances.

We also provide recommendations on bacterial monitoring to improve coral aquaculture, based on four case studies where we investigated bacterial load in tanks containing coral larvae, recruits and broodstock parents. Microbial loading data generated by this method from aquaculture setting and natural reef environments will help to identify bacterial abundances below which effective larval production and ecologically safe release of corals to the reef are likely.

2 Background

Coral aquaculture that efficiently produce offspring by sexual reproduction are increasingly gaining traction for reef restoration (McLeod et al., 2022; Randall et al., 2020). Aiming to mitigate declining coral cover, millions of larvae need to be produced by artificial fertilisation in purpose-built facilities (Severati et al., 2024). Coral larvae are settled onto concrete tiles where they transform into juvenile recruits, and the tiles are fitted to deployment devices before being out-planted to the reef (Whitman et al., 2024). To address these needs, the upscaling of coral aquaculture is one of major investigations in the Reef Restoration and Adaptation Program (RRAP), aiming to make coral aquaculture more cost-efficient. Options to maximise the number of produced coral offspring include (1) reducing the seawater requirements per culture tank to be resource-efficient, (2) increasing densities of corals in tanks for rearing larva and growing recruits, and (3) minimising mortality of all life stages.

Larval cultures and recruits occasionally experience high mortality events where the causes are difficult to pinpoint. Communities of microorganisms around coral animals, including bacteria, archaea, viruses, microalgae and fungi, immensely contribute to the health of corals (Voolstra et al., 2024). However, the role of these microbiota communities in corals under aquaculture environment, especially for corals in early life stages, are not well understood. It can be hypothesised that pathogenic bacteria could cause the die-off because several bacteria such as *Vibrio* spp. (e.g. (Kushmaro et al., 1996; Ben-Haim and Rosenberg, 2002), *Thalassomonas loyana* (e.g. Thompson et al., 2006) and *Aurantimonas coralicida* (e.g. Denner et al., 2003) associated with coral diseases. However, the causality is often not established, as the observed bacteria community could cause the disease, while they can be a consequence of increased organic input from coral mortality due to other causes. Other causes of coral death at early life stages may include microbial dysbiosis (i.e., unbalance of microbiota leading to deteriorated healthy without specific pathogens; MacKnight et al., 2021) and genetic incompatibility among parental corals that result in maldevelopment of fertilised embryos (Miller et al., 2018).

Routine monitoring of bacteria is not yet a common practice in coral aquaculture, but it would be beneficial to establish their baseline dynamics and investigate if an increase in potentially pathogenic bacteria above a certain threshold could be used as early warning sign that coral mortality is likely to increase. Here, we provide a ddPCR-based duplex assay to monitor microbial loads in water samples across coral aquaculture, targeting total bacteria and *Vibrio* spp. as presentative taxa associated with disease of aquatic organisms. This standard operation procedure consists of the steps of filtering water samples onto membrane filters, extraction of bacterial DNA from the filter samples, and quantification of bacterial abundances by using droplet digital polymerase chain reaction (ddPCR) assays. In ddPCR, the extracted DNA is encapsulated into minute droplets with PCR reagents, and bacterial marker genes (16S ribosomal RNA gene; 16S rRNA) are amplified with two sets of primers, targeting the broad range of bacteria and the *Vibrio* genus, respectively. Finally, the fluorescence is measured in each droplet, and based on the intensity and frequency of template-present droplet, the number of 16S rRNA copies can be estimated which is a proxy for the bacteria abundance.

3 Objectives and Scope

This standard operating procedure is developed by the Quality Assurance and Quality Control (QAQC) team of the RRAP program. The microbial load assessment procedure is operational and ready to accompany coral aquaculture activities. It is designed for researchers and aquaculture facilities with access to a molecular laboratory to gain insights into microbial properties in aquaculture facilities. It is aiming to guide monitoring of bacterial loads at several time points (see Section 7.4) to identify dynamics in microbial abundances including potentially pathogenic bacteria such as *Vibrio* that may be associated with coral mortality.

We applied this method in four case studies that investigated bacterial load in tanks of broodstock, larvae settlement and recruits grow-out (**Error! Reference source not found.**) to showcase how operational insights can be gained by bacterial load monitoring (see Annexture). Based on these case studies, we make recommendations on how bacterial monitoring is implemented to guide decision-making on aquaculture optimisations such as larval socking densities, the establishment of aquaria systems, and evaluations as to the biosecurity upon out-planting corals generated in aquaculture to the reef (see Section 7.4).

This SOP will cover the required materials (see Section 6) and detailed protocols to take water samples (see Section 7.1), extract bacterial DNA (see Section 7.2**Error! Reference source not found.**) and run ddPCR to estimate total bacteria numbers and *Vibrio* abundances (see Section 7.3). The current turnaround time from sampling to data is 4 days, and its effective usage and potentials to develop more time-effective assays are discussed in this document (see Section 7.6 Outlook).

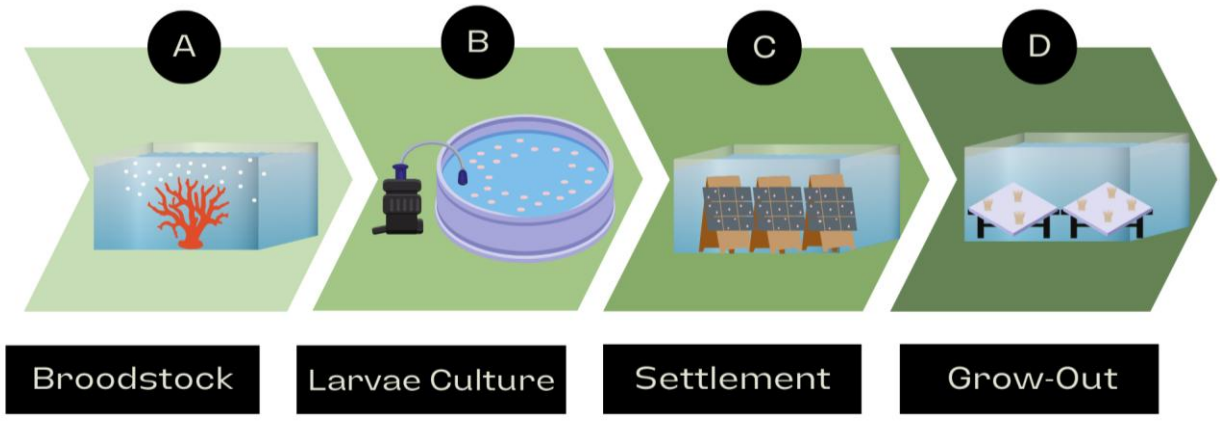


Figure 1: Operational Stages of coral spawning activities where water sampling was conducted for bacterial load testing. A) Broodstock holding tanks, B) Larvae culture tanks, C) Settlement tanks after fertilization with newly settled corals and D) grow out tanks where newly settled corals were housed for up to 42 days post settlement.

4 Pre-requisites

Best practice when working with bacteria

- Ensure personal protective equipment (PPE) is always worn to avoid contamination.
- Indoor, molecular laboratory facility. Although not necessary, a physical containment laboratory (e.g. PC2 lab) would be ideal.
- Proper disposal of laboratory waste (including guanidine hydrochloride) to be established.
- Acid-wash sampling equipment to reuse and prevent contamination, including vacuum filtration cock valves, adapters, Swinnex filter holders (top, bottom, gasket), syringes, 50mL centrifuge falcon tubes, tweezers.
- UV sterilize all tubes, racks & lids, bench foil, and pipette reservoirs prior to DNA extraction and PCR.
- Do not re-use lab consumables for DNA extraction and PCR to avoid contamination.

Pre-preparation of reagents:

Critical to prepare reagents prior to initiation of protocol. Read all Safety Data Sheets (SDS) provided by the suppliers prior to working with reagents.

Sample tubes (1.5 ml screw top) need to be pre-filled with 288µl of the below 1.25x lysis buffer prior to sample collection.

- Lysis buffer 1.25× stock for sample preservation (in 500 ml):
    - 25mM Tris-Cl pH8.0
    - 2.5mM sodium EDTA
    - 1.5% Triton X-100

  ➔ pH adjusted to 8.0 using NaOH; and adjust the total volume with Milli-Q water.

  ➔ Filter-sterilise it with a 0.22 µm filter.

- 100mg/ml Lysozyme stock (for six 96-well plate runs)
    - 4.5g lysozyme powder
    - 45 mL Milli-Q H_2O

  ➔ Dissolve lysozyme thoroughly, and filter through 0.22um syringe filter

  ➔ Aliquot 900µl into sterile 1.5mL tubes (approx. 48 aliquot tubes)

For lysis of sampled materials on filters, the 1.25X lysis buffer and the lysozyme are freshly combined at a 4:1 ratio. Final concentrations of the compounds are as follows:

- 20mM Tris-Cl pH8.0
- 2mM sodium EDTA
- 1.2% Triton X-100
- 20 mg/ml lysozyme

Pre-order extraction kit and primers

- Qiagen DNeasy 96 Blood & Tissue Kit.
- Bio-Rad ddPCR reagents and consumables (listed below).

- Order primer pairs:
    - Primer set 16S1406F/1525R (Woodcroft et al., 2018) to amplify the 16S rRNA genes:
        - F – 5'-GYACWCACCGCCCGT-3'
        - R – 5'AAGGAGGTGWTCCARCC-3'
    - *Vibrio*-specific primer set Vib1-f (567F)/Vib2-r (680) (Thompson et al., 2004) to amplify the 16S rRNA genes:
        - F – 5'-GGCGTAAAGCGCATGCAGGT-3'
        - R – 5'-GAAATTCTACCCCCCTCTACAG-3'

Technology and software required:

- Bio-Rad ddPCR system (e.g. QX200 Droplet Digital PCR System)
    - Droplet generator
    - Plate sealer
    - Thermocycler
    - Droplet reader
- Bio-Rad Quantasoft Analysis Pro Software (v1.0.596~)
- Microsoft Excel

Use the Appendix (Excel worksheet) entitled "ddPCR worksheet Bacteria-*Vibrio* Assay Template" to design ddPCR experiment.

This spreadsheet facilitates to calculate master mix volumes specific to ddPCR assay.

5 Identified Risks and Hazards

Risks and hazards involved with sampling of seawater from coral aquaculture facilities (e.g. outdoor), electric pump-driven filtration of samples, extraction of DNA (in laboratory), and digital PCR quantifications are summarised below with control measures:

Scope of Work		**Bacterial load sample collection, DNA extraction, ddPCR**				
Special Equipment / PPE		gloves, safety glasses, lab coat, closed toe shoes Read SDS for all hazardous chemicals.				
Task	**Hazards**	**Consequence**	**Control Measures**	**Risk Rating**		
				Consequence	Frequency	Risk
Working Outdoors while sampling	Exposure, temperature, UV exposure	Heat exhaustion, skin damage	• Assess daily the expected temperatures and consider the ambient temperature of the actual work environment and plan tasks accordingly •Adequate PPE • Sun protection • Regular hydration • Regular rest periods	Minor	Possible	Low
Vacuum Filtration for sampling	Electrical Shock	Electric shock when touching the water or pump	• Check all electrical equipment has a valid test tag and is safe and undamaged prior to being used each time. • Ensure competency of all users • Ensure hands are not wet when switching outlets or inserting/ removing plugs	Moderate	Rare	Low
	Misuse of portable pump, Leaving the pump on when not in use	Overheating/damaging the portable pump	Ensure that the portable pump is only on when in use. Do not walk away from the lab bench when a pump is on. Ensure the power is switched off when done with use.	Moderate	Unlikely	Low
	Failing to use a liquid trap	Damaging the pump.	Always set up the pump and filtration system and have it checked before use. Do not attempt to set up if you have not been trained to do so. Ensure you are using the correct type of trap to contain your liquid or solvent. Immediately turn the pump off if you notice liquid in the tap hose.	Moderate	Unlikely	Low
Lysozyme stock preparation	Inhalation of Lysozyme powder	May cause allergy or asthma symptoms or breathing difficulties if inhaled.	• Avoid inhalation of dusts. Change gloves after working with substance. Wear safety glasses. Respiratory protection • Required when dusts are generated. Do not let product enter drains.	Moderate	Unlikely	Low

Proteinase K digestion	Inhalation	May cause allergy or asthma symptoms or breathing difficulties if inhaled.	• Avoid breathing dust/ fume/ gas/ mist/ vapours/ spray. • Wear protective gloves/ protective clothing/ eye protection/face protection. • In case of inadequate ventilation wear respiratory protection. Dispose of contents/ container to an approved waste disposal plant. Enzyme is already dissolved and included in kit.	Moderate	Unlikely	Low
Use of Buffer AL in the kit containing guanidine hydrochloride (>= 30 - < 50) and maleic acid (>= 0.1 - < 1)	Contact with chemical	Causes skin irritation. May cause an allergic skin reaction. Causes serious eye irritation.	• Wear protective gloves/ protective clothing/ eye protection/ face protection. • Response: If skin irritation or rash occurs: Get medical advice/attention. Take off contaminated clothing and wash before reuse. • Disposal: Dispose of contents/ container to an approved waste disposal plant.	Moderate	Unlikely	Low
Use of AW1 Buffer in the kit containing guanidine hydrochloride (>= 50 - < 70)	Contact with chemical	Harmful if swallowed or if inhaled. Causes skin irritation. Causes serious eye irritation.	• Wear protective gloves/ protective clothing/ eye protection/ face protection. • Response: Take off contaminated clothing and wash before reuse. • Disposal: Dispose of contents/ container to an approved waste disposal plant.	Moderate	Unlikely	Low
Use of ethanol (EtOH) in extraction protocol and to disinfect surfaces	Fire and/or exposure to harmful solvent	Burns, fire, and/or chemical irritation	• Personnel are trained and experienced in the use of ethanol for disinfection • No open flames used in or around where ethanol is being used. • Use of PPE (lab coat, enclosed shoes, gloves, and safety glasses) • Presence of smoke and fire detectors and alarm systems. • Presence of fire blankets and extinguishers within the PC2 laboratory and within the surrounding corridors. • Building evacuation, emergency, and warden system in place.	Moderate	Unlikely	Low
Use of laboratory equipment with rapidly moving parts (e.g., centrifuge, tissue lyser, rotating oven, vortexer) and ddPCR	Pinch points, misuse of instrument	Potential for injury or equipment damage in case of contact with moving parts.	• Refer to Equipment manual. • Check equipment for damage • Ensure samples are balanced with loaded. Clean apparatus when finished. Workers with long hair (below shoulders) to keep it tied up. Loose jewellery or loose clothing which may become tangled in moving parts to be	Moderate	Possible	Medium

machines with electronic sliding drawers			removed before use of equipment. Workers ensure centrifuge chamber is free of obstructions or loose items, rotors and rotor lids are secure before operation. Equipment with faulty fail-safe to be immediately turned off, power cord removed and labelled as out of order. Tech staff to be notified immediately. Repair by qualified service tech only.			

RISK RANKING WITH EXISTING CONTROLS IN PLACE					
	Negligible	Minor	Moderate	Major	Severe
Certain	Medium	Medium	High	Extreme	Extreme
Very Likely	Low	Medium	High	High	Extreme
Possible	Low	Low	Medium	High	High
Unlikely	Low	Low	Low	Medium	High
Rare	Low	Low	Low	Medium	Medium

6 Equipment and Materials

6.1 Bacterial Load Sample Collection

6.1.1 Procedure equipment

- Pump (Safety note: beware of electrical shock hazard while working near water)
- Filtration manifold + trap + tubing
- Filtration system cock valves
- Adapters to join the filtration unit and filter holders
- Swinnex filter holders (top + bottom + gasket)
- Individually wrapped sterile PES membrane filters (0.2 um; 25 mm diameter)
- Syringes (50mL)
- Plastic bottles (1L, per sampling point)
- PVC pipe with a meshed bottom (100 µm mesh, per sampling tank)
- 50 ml centrifuge falcon tubes
- 1.5ml screw top tubes pre-filled with 288µl 1.25x lysis buffer (see above in pre-requisites, pre-preparation of reagents)
- 80% Ethanol spray for cleaning
- Tweezers to handle filter membranes (per sampling point)
- Tube boxes to freeze samples
- Cooler box with ice
- Tissue paper for cleaning
- Waste bags
- Sampling design sheet (samples to be in a randomised order)
- Pencil and pen

6.1.2 Personal protective equipment (PPE) and other safety equipment

- Gloves
- Closed toe shoes
- Sun protective clothing if sampling outside

6.2 Bacterial Load DNA Extraction from Preserved Filter Paper

6.2.1 Procedure equipment

- Qiagen DNeasy 96 Blood & Tissue Kit
- Aliquots of 100mg/ml lysozyme (see above in pre-requisites, pre-preparation of reagents)
- Pipet reservoirs (5 per plate)
- 1.5mL tubes for Proteinase K aliquots (8 per plate)
- Aluminium foil
- Tube racks and lids
- 8 Channel 50-1250µl VOYAGER Automatic Multichannel Pipette
    - *Note: This isn't required but this protocol is written for use with this specific pipette*
- Rotating oven
- Tube centrifuge
- Plate centrifuge (e.g. Beckman Allegra Centrifuge)
- UV sterilization station

- 80% ethanol for cleaning
- DNeasy spray for de-contaminating workstation

6.2.2 Personal protective equipment (PPE) and other safety equipment

- Gloves
- Microbial lab coat
- Safey glasses
- Closed toe shoes
- Hair tied back

6.3 Bacterial Load Digital Droplet PCR (ddPCR)

6.3.1 Procedure equipment

Equipment and material needed for the measurement of one DNA extract plate (96 reactions) are listed.

- Completed and printed assay sheet for calculating reagent volumes for master mix
    - See the Appendix "ddPCR worksheet Bacteria-*Vibrio* Assay Template" (Excel spreadsheet).
- 2 x Biorad 96-well assay plates
- Pipette reservoir
- Tube(s) for master mix
    - Full 96-well plate will need >2mL master mix, so need to mix in 5mL tube or 2 x 1.5mL tubes.
- 12 Channel VOYAGER automatic multichannel pipettes (0.5-12.5ul, and 5-125µl)
    - *Note: This isn't required but this protocol is written for their use.*
- 125µl Master mix reagents
    - Primer set 16S1406F/1525R (Woodcroft et al., 2018) to amplify the 16S rRNA genes:
        - F – 5'-GYACWCACCGCCCGT-3'
        - R – 5'AAGGAGGTGWTCCARCC-3'
    - *Vibrio*-specific primer set Vib1-f (567F)/Vib2-r (680) (Thompson et al., 2004) to amplify the 16S rRNA genes:
        - F – 5'-GGCGTAAAGCGCATGCAGGT-3'
        - R – 5'-GAAATTCTACCCCCCTCTACAG-3'
    - BioRad QX200 EvaGreen Supermix
    - Nuclease free PCR water
- 1/100 dilution of 10ng/µl *Vibrio* DNA for positive control *(Any DNA extracts from Vibrio culture or environmental samples enriched in Vibrio will suffice the purpose).*
- PCR tubes for *Vibrio* positive control aliquots for multichannel pipetting
- Extra microtubes for dilutions, primers, or PCR water

6.3.2 Personal protective equipment (PPE) and other safety equipment

- Gloves
- Microbial lab coat
- Safey glasses
- Closed toe shoes
- Hair tied back

7 Steps for Implementation

7.1 Bacterial Load Sample Collection

1) Collect the water sample from tank using the collection bottle labelled with the corresponding tank.
    a. Open the drain tap to the tank and let run for 10 seconds to flush seawater from the drain system.
    b. Use mesh PCV cup to filter water into collection bottle to avoid larvae contaminating sample.
    c. Rinse the collection bottle 3 times with the sample water before collecting sample (e.g., 500 ml).

2) Set up filtration system consisting of vacuum pump + trap + manifold.

3) Add valve to filtration system and add sterile adaptor (handle with sterile tweezers).

4) Assemble Swinnex filter top and bottom with membrane filter and gasket and add to adaptor on manifold.

5) Attach 50 mL syringe to the Swinnex unit without the plunger as a funnel.

6) Turn on vacuum pump.

7) Keep vacuum pump running in between samples. Use the valve on the individual filtration system to control flow.

8) First flush the Swinnex filter system with ~50 mL of the water sample.

    Note: This is because the same Swinnex filter is used for all 4 replicates, and this insures the first replicate has the same baseline contamination as the following replicates.

9) Measure 50ml of sample water using a Falcon tube and pour into syringe funnel (Figure 2).

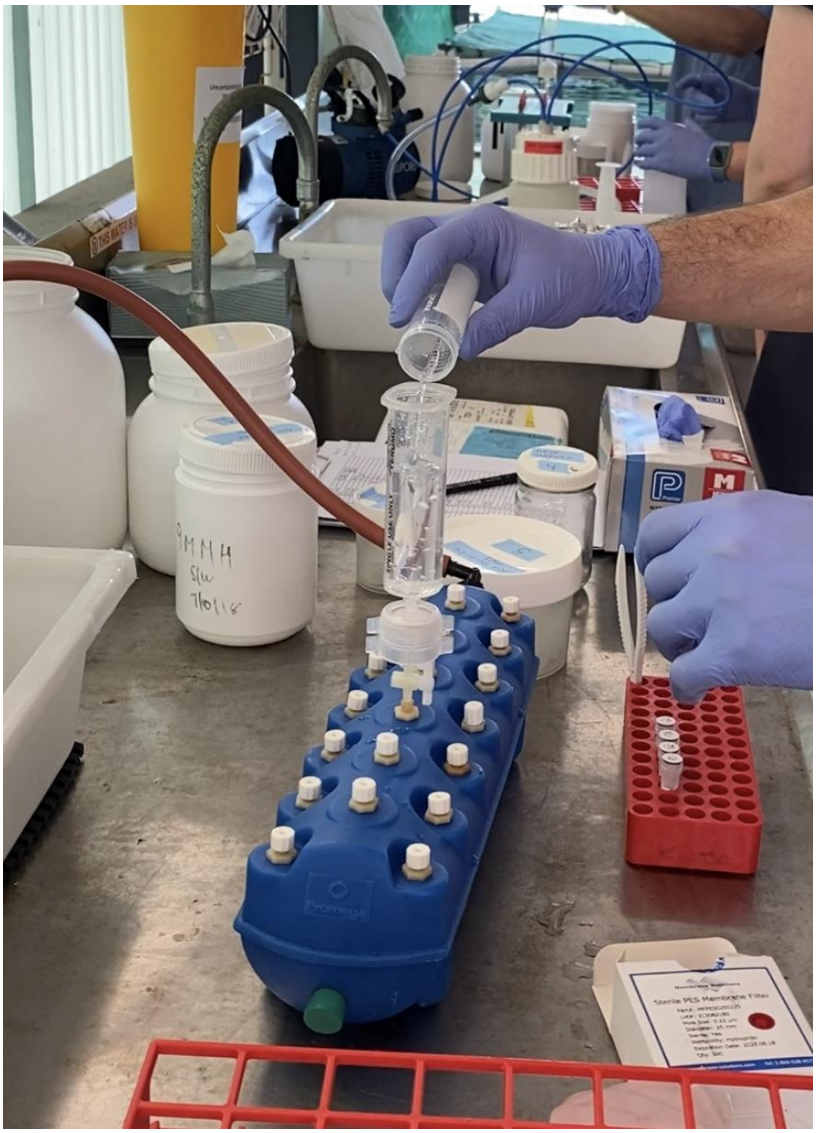


Figure 2: Overview of the filtration station for sampling of seawater-borne bacteria using a filter membrane and vacuum pump.

10) Ensure the pre-labelled tube is filled with 288µl buffer, and check for the correct labelling.

11) Remove Swinnex filter top and use tweezers to add filter membrane into 1.5ml tube (Figure 3).

    Hint: Use two tweezers to roll filter and place filter into tube so that the filter rolls around the tube edge and you can see the bottom of the tube – this is important for downstream DNA extraction.

Figure 3: Removal of the filter membrane for microbial sampling from the filtration unit.

12) Invert closed sample tube to saturate filter with lysis buffer.

13) Store tubes in box on ice until stored in the freezer at -20°C.

14) Exchange filter membranes and tweezers in between each sample replicate (n=4 per sample).

15) Exchange Swinnex filter housing between each sample (e.g. coral rearing tank).
Note: No need to change adaptor and valves unless seen necessary.

16) Collect all used parts of the filtration units, filter holders and tweezers for sterilization soak with Biosan, thorough rinsing with tap water.

17) Acid-wash the above parts with 10% HCl bath and thoroughly rinse with Milli-Q water before dried in the drying oven for reuse.

7.2 Bacterial Load DNA Extraction

This process is modified from the Qiagen DNeasy 96 Blood & Tissue Kit protocol according to the manufacturer. Information and documentation regarding the kit are also to be carefully inspected:

7.2.1 Notes before starting:

- Filters should be preserved in 288µl of lysis buffer.

- All volumes (from original bacterial load extraction protocol) were doubled to use the 8-count multichannel pipette, which has a minimum volume of 50ul.
- Perform all centrifugation steps at room temperature.
- If necessary, redissolve any precipitates in Buffer AL by gentle warming.
- Add ethanol to Buffer AW1 and Buffer AW2 concentrates before the first use. (cf. **not** in Buffer AL)
- Overnight lysis in rotating oven is optional after addition of Prot. K. and Buffer AL (step 15).
- It may be easier to organize the 1.5mL sample tubes split between two 96-well tube racks, alternating empty rows. This makes it easier to unscrew and re-screw caps.
- This protocol works best with 2 people, one to remove and replace screw cap lids and the other to add reagents.
- ***Important****: It is required to have a control sample per DNA extraction run that is a blank filter processed without seawater sampling. This will be used to control the background noise in microbial quantification, including potential laboratory-introduced contamination across samples.*

7.2.2 DNA Extraction Preparation

- Preparation of 100mg/ml lysozyme (see above in "pre-preparation of reagents")
    - Pre-aliquots of lysozyme stored in freeze – thaw prior to extraction.
- UV treat the following:
    - Five pipet reservoirs (for reagents)
    - Lids for tube racks
    - Large piece of aluminium foil to place sample lids on and minimize contamination
    - Eight 1.5mL tubes for Proteinase K aliquots
- Set rotating oven to 37^{o}C degrees.

7.2.3 DNA Extraction Protocol

Note: Green highlighted steps indicate the addition of reagents. The yellow highlighted indicates critical pipetting step with the modified volume.

1) Centrifuge/quick spin 1.5mL sample tubes to avoid contamination through liquid from lids.
    a) *Minimum speed to reduce filter crushing.* E.g., 30 sec. @ <1000 RCF.

2) Carefully remove lids from sample tubes.
    a) Place the lids face down on the UV-treated aluminium foil sheet to avoid contamination from dust in the air or bench surfaces.
    b) Hint: place the lids in the sample order as the sample tubes in the rack.

3) Add 72µl lysozyme.
    a) Place eight 1.5mL lysozyme aliquots in a row and used the multichannel pipet to disperse this reagent across all sample tubes.

4) Place lids back on sample tubes.
    a) Be cautious to place correct lid on sample tube. Make sure the serial number on the lids and the tubes match.
    b) Lids need to be tight for incubation.
    c) Re-sanitize aluminium foil for future use (either UV treat or DNeasy spray).

5) Place sample tubes in a box with a lid suitable for rotating oven.

6) Incubate samples in rotating oven at 37^{o}C for >30 min.

7) While samples are in oven, aliquot Proteinase K solution for multichannel pipette.
   a) Need 50µl for each sample.
   b) 50µl x 12 rows = 600µl (+10% for pipette error = 660µl)
   c) 660µl in 8 1.5mL tubes.

8) After incubation completion, set oven to 56°C.

9) Remove samples from oven and centrifuge/quick spin 1.5mL sample tubes to avoid contamination through liquid from lids.
   a) Minimum speed to reduce filter crushing. E.g., 30 sec. @ <1000 RCF

10) Carefully remove lids from sample tubes.
   a) Place the lids face down on the UV-treated aluminium foil sheet to avoid contamination.

11) Add 50µl Proteinase K solution using aliquots and multichannel pipette.

12) Add 400µl Buffer AL (no ethanol added)
   a) Fill a new pipette reservoir with Buffer AL for multichannel pipette.
   b) Approximately 38.4mL Buffer AL is needed for 1 plate

13) Place lids back on sample tubes.
   a) Be cautious to place correct lid on sample tube. Make sure the serial number on the lids and the tubes match.
   b) Lids need to be tight for incubation.
   c) Re-sanitize aluminium foil for future use (either UV treat or DNeasy spray).

14) Place sample tubes in a box with a lid suitable for rotating oven.

15) Incubate samples in rotating oven at 56°C for >60 min (optionally, up to overnight).

16) Remove samples from oven and centrifuge/quick spin 1.5mL sample tubes to remove liquid from lids.
   a) Minimum speed to reduce filter crushing. E.g., 30 sec. @ <1000 RCF

17) Prepare the DNeasy 96-well spin column plates on top of S-block.

18) Using the "pipette-mix" function on the multichannel pipette, add 400µl ethanol to the sample tubes.
   a) Fill a new pipette reservoir with ethanol for multichannel pipette.
      i) Approximately 38.4mL ethanol for 1 plate
   b) Hint: pipette mixing should occur inside the rolled filters. Try to avoid bubbles to form.
   c) Pipette settings:
      i) Mix cycles = >5
      ii) Mix speed = <4 *(not too quick to form bubbles)*
      iii) Mix volume = 600µl *(any more could overflow tubes)*

19) Using the same pipette tips on the same row of samples, change pipette settings to "pipette only" and change volume to 900ul. Carefully transfer 900µl of sample lysate into wells of the DNeasy 96 spin column plate.
   a) Hint: The filter papers should be rolled around the internal wall of the tubes to facilitate pipetting. Position the multichannel pipette tips inside the filter rolls. This can be difficult to do if the filters are crumpled. Use a sterile tool to fix filters if needed.
   b) There should be ~1.2mL of lysate in the sample tubes. Be careful not to overflow the and cross contaminate the samples when lowering the pipette tips in lysate.

c) 900µl is the max volume that can be added to the S-block.
d) Record the final volume of lysate transferred to the spin column in the lab notebook (in case <900µl) – this is critical for calculations of microbial concentration per sample volume (DNA copies/ml).
e) Take care not to wet the rims of the well to avoid aerosols during centrifugation.

20) Once all lysate is added into the wells, seal the plate with an AirPore Tape Sheet.

21) Centrifuge plate for 10 min at 5,495 x g (5700 rpm).
a) If doing 1 extraction plate – balance with old S-block and spin column.
b) Remember: change the Allegra centrifuge settings to 96-well plate.
i) 2.5-96

22) Remove the tape. Add 500µl Buffer AW1 to each well.

23) Seal plate with a new AirPore Tape sheet. Centrifuge for 5 min at 5788 x g.

24) Remove the tape. Add 500µl Buffer AW2 to each well.

25) Do not seal the plate. Centrifuge for 15 min at 5,495 x g.
a) The heat generated during centrifugation ensures evaporation of residual ethanol in the sample that might otherwise inhibit downstream reactions.

26) Place the DNeasy 96 plate on a new rack of Elution Microtubes RS.
a) Remember to align the well numbers correctly.

27) To elute the DNA, add 50µl Buffer AE to each sample. Seal with new AirPore Tape Sheets. Incubate for 1 min at room temperature. Centrifuge for 2 min at 5,495 x g.
a) Fill a UV-treated pipette reservoir with Buffer AE for multichannel pipette.
b) Approximately 9.6mL Buffer AE for 1 plate

28) Repeat the above step with another 50µl Buffer AE elution for a total elution volume of 100ul. Seal with new AirPore Tape Sheets. Incubate for 1 min at room temperature. Centrifuge for 2 min at 5,495 x g.

29) Seal the Elution Microtubes RS with new caps to store the eluted DNA. Store DNA in 4^{o}C cold room.

7.3 Bacterial Load Digital Droplet PCR (ddPCR)

Use the Appendix Excel worksheet entitled "ddPCR worksheet Bacteria-Vibrio Assay Template" to design your experiment and calculate master mix volumes based on required primer concentrations.

7.3.1 ddPCR preparation

- UV treat the following:
    - 2 x Biorad 96-well assay plates (2 plates are needed per assay. The first is used to set up the assay containing a total volume of 25ul. The second plate is used to transfer generated droplets into).
    - Pipette reservoir
    - PCR tubes for positive control samples
    - 5mL tube for master mix OR 2 x 1.5mL tubes for master mix
    - Extra tubes for dilutions, primers, or PCR water
    - The nuclease free PCR water should be UV treated

- Prepare the ddPCR master mix:
    - Use the ddPCR worksheet template for reagent volumes
    - Reagents needed for the multiplex assay:
        - Bacteria AND *Vibrio* (F & R) primer pairs stored in -20°C storage.
            - *4 primers in total (F & B bacterial AND F & B Vibrio)*
        - BioRad EvaGreen Master Mix stored in -20°C storage.
        - PCR water (UV sterilized)
    - Add the volume of each reagent based on the calculation of in the ddPCR worksheet template.
    - A full 96-well plate will need >2mL master mix, so need to mix in 5mL tube or 2 x 1.5mL tubes.
    - Vortex shake and quick spin – try to remove/avoid bubbles

Critical to follow measurements from assay worksheet to achieve correct concentrations.

7.3.2 ddPCR plate preparation protocol

1) Carefully pipette out the ddPCR master mix into a reagent reservoir. If you are using more than one tube of ddPCR master mix, gently mix the contents of each tube in the reagent reservoir by tipping the reservoir side to side a few times.
    a) Note: The EvaGreen solution is easy to form bubbles – try to avoid while pipetting.

2) Prepare a 25µl master mix reaction (24µl master mix + 1µl DNA template) for each sample. This can be done using the 12-channel 12.5 and 125µl electronic pipette (Integra).
    a) Using the 125µl Integra pipette and the pipette reservoir filled with master mix, pipette 24µl of master mix into all wells in 1 of the Bio-Rad 96-well assay plates.

3) Remove your DNA extracts and controls (positive and NTC, all of which should already be stored in PCR strip tubes) from storage.
    a) If samples are not in PCR tubes and/or in the correct order for the ddPCR assay, pipet samples into PCR tubes to multichannel pipet into the 96-well plate. Once in PCR tubes, centrifuge samples so liquid is at the bottom.
    b) If samples are already in PCR tubes in the correct format, ensure you vortex and briefly centrifuge everything to make sure its mixed and that any liquid within the tubes is located at the bottom of the tube, away from the lid.
    *c) **Important:** Vibrio positive controls should be used in every well in column 12. This is critical for setting the positive thresholding in downstream analysis.*

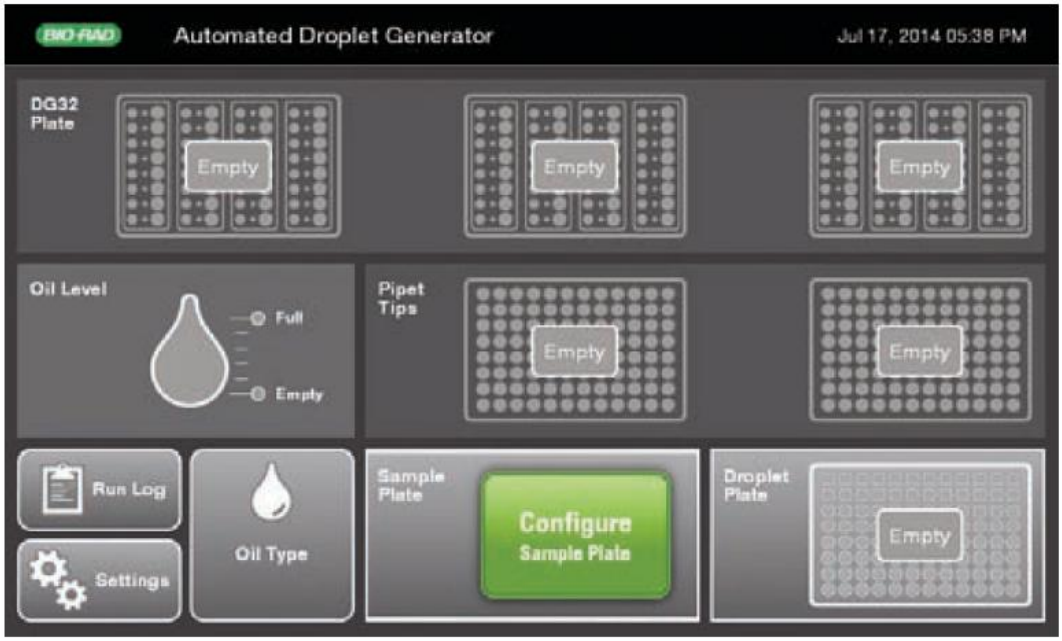
4) Use the pipette and mix function of the 12-channel 12.5µl Integra electronic pipette to aspirate 1µl of your DNA and dispense and mix it into each row of your assay plate.
    a) Set the pipet setting: aspirate 1ul; mix 10ul; mix cycles 5; mix speed 8-10

5) Centrifuge the completed assay plate in the Beckman Allegra Centrifuge at 3000 x g for 3 minutes. This is to remove any bubbles that may have formed in the assay plate wells from the mixing step prior.

7.3.3 Automatic Droplet Generation (AutoDG) protocol

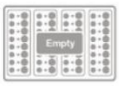
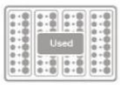
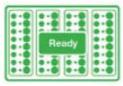
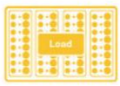
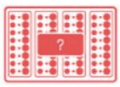
1) Prepare the automatic droplet generator (AutoDG) for the process as follows:
    a) Touch the screen of the AutoDG to bring the instrument out of the idle mode
    b) Open the door of the instrument by lifting up the handle at the front.

c) On the screen at the front of the AutoDG there is an “Oil Type” icon (see below diagram). Check that this matches the chemistry you need. If so, proceed with step ‘2’; if not follow steps below (i to vi) to change the bottle.



i) Select the type of automated droplet generation oil (Probes or EvaGreen) by touching the “oil type” icon (Figure 1). The droplet you select will turn blue. Touch OK to set the oil type.
ii) Bacteria-*Vibrio* assay uses EvaGreen Oil
iii) Once you have selected the type of oil, you will be prompted to remove the bottle of oil in the instrument and replace it with the new bottle of oil.
iv) Then you can remove the oil bottle from the delivery system at the front left corner of the instrument.
v) Put away the bottle you just removed (there should be a box on the shelf with the cap for the bottle), cap the bottle and place inside the box.
vi) Remove the cap from the bottle of Automated Droplet Generation Oil you want to use (EvaGreen). Fasten the bottle into the delivery system (where you removed the previous bottle) by turning the bottle until it stops moving, the label on the bottle should face outwards. Touch “ok” to indicate you finish the exchange. The equipment will flush the lines with the new oil. This process may take a few minutes.
vii) Store the cap of the bottle in the box and put away until needed.
viii) You will receive a message saying “oil change successful” and the system will display the oil type at the bottom left of the screen.
ix) Although the equipment has a display showing the level of oil. This may be inaccurate. Check visually on the bottle the actual level of oil. Exchange the bottle if it has less than 10ml.

2) If the instrument deck is empty, the indicator lights on the deck of the AutoDG should be off, the corresponding areas of the touch screen will be grey (see below chart).

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

3) To configure sample plate, touch the “Configure Sample Plate” button.

4) Touch or swipe across the screen to select columns in which your samples are located. Touching a selected column will deselect it. Any combination of columns can be selected. **Rows cannot be selected.** You don’t need to label the plate, but this can be done if desired, along with adding notes. Touch OK when done.
   a) Note: If there are empty wells in the plate configuration and they need to be selected, fill them with 25µl of water so the robot has something to pipette.

5) The consumable icons will blink yellow to indicate where new consumables need to be loaded into the instrument. If the icon remains grey, that consumable is not needed for the run.
   a) There is no icon to indicate that the pipette tip bin is in place, nor whether it is full or empty. Please check that the bin is in place and empty before starting each run. The bin should be located to the left, in the middle row of the equipment deck, just behind the Droplet Generation Oil bottle.

6) Load consumables from the back to the front to avoid contamination.

7) Remove the plastic wrapping off the DG32 AutoDG cartridges and load them along the back row of the instrument. Orient the cartridges with the green gaskets to the right. If the cartridges are loaded correctly a green light will replace the yellow light. If this is not the case reposition the cartridge until the green light is lit.

8) Remove the plastic wrapping and the lid of the tip boxes and place in the middle row of the instrument. There is no front or back. If the boxes are loaded correctly a green light will replace the yellow light. If this is not the case reposition the box until the green light is lit.

9) Place the assay plate you just centrifuged in the front left plate holder of the instrument, labelled in the screen as “Sample Plate”. The holder is designed to hold the plate in the right orientation (first row “A” to the back of the instrument and last row “H” to the front). If the plate is loaded correctly a green light will replace the yellow light. If this is not the case reposition the plate until the green light is lit.

10) To load the droplet plate assembly, remove the cooling block from freezer 3 (across from the walk-in freezers). Place it into the front right plate holder, labelled on the screen as “Droplet Plate”. If the cooling block is loaded correctly a green light will replace the yellow light. If this is not the case reposition the cooling block until the green light is lit.

11) Place a clean ddPCR 96-well plate for droplet collection into the cooling block. The cooling block should provide the right orientation for the plate.
    a) There is no light to indicate that the plate has been loaded into the instrument, so be mindful to do this and check that the plate is sitting firmly on the cooling block.

12) Once all the rows of the instruments are loaded with the necessary consumables, the icon of the “droplet plate” will turn blue and display a “START Droplet Generation” message. Touch the icon, you’ll be prompted to confirm the run. At this point, please check that the oil you need is the one specified and if you named the run and added notes, these are correct. Touch “confirm”

13) The lid of the instrument will close automatically, and the run will start. After a few moments a message will be displayed with the length of time remaining for completion of the run.
    a) Approximately 40 minutes per full plate.

14) Once completed, take the “droplet plate” and continue to the next step ‘Sealing the plate’.

15) After the run is finished, make sure that all the used consumables are removed from the equipment and disposed of.
    a) Remove the cartridges and the assay plate and place in bin
    b) Remove the tips bin, empty in the bin, wipe with DNA erase and place back in the equipment. After two or three uses, this should be replaced by a new bin.
    c) Remove the tip boxes and take them to the recycling plastic bin in the autoclave room.
    d) Remove the cooling block and place it upside down in the freezer under the bench.
    e) The equipment doesn’t require a cleanup unless a spill is noticed.
    f) Close the lid of the AutoDG, it will return to idle mode on its own.

7.3.4 Plate sealing protocol

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

2) Place the heating block inside the plate sealer.

3) Place the completed assay plate containing your generated droplets on the heating block.

4) Place foil seal on the PCR plate. Ensure the red line faces upwards. Foil seals sometimes stick together so ensure you only have a single foil seal.

5) Press green “seal” button.

6) Plate will be taken into the sealer and heat sealed. This takes about 5 seconds.

7) Remove plate and continue to PCR.

7.3.5 PCR amplification in BioRad C1000 Thermocycler

Standard ddPCR cycling protocol for **bacteria-*Vibrio* multiplex assay** is as follows:

Cycling Step	**Temperature (ºC)**	**Time**	**Ramp rate**	**Cycles**
Enzyme activation	95	10 min	2ºC/sec	1
Denaturation	95	30 sec		40
Annealing/extension	**56**	1 min		
Enzyme deactivation	98	10 min		1
Hold (optional)	4	infinite		1

1) Place plate inside thermocycler

2) Close lid and screw tightening knob until it just becomes tight, then turn another half turn. You may hear a rachet click on the half turn, this is normal.

3) Start run by choosing a PCR profile as above.:
   a) Saved files
   b) Saved folder
   c) press run

4) Ensure that the volume is set to 40µl and 105°C temperature

5) Press OK to start cycling

7.3.6 Quantification in the BioRad Droplet Reader

1) Open droplet reading software (Quantasoft)

2) Select new template

3) Double click the first two cells in column 1
   a) Enter sample name
   b) Check experiment – 'ABS' for absolute quantification.
   c) Check supermix – EvaGreen Supermix
   d) Check target 1 – Bac-Vib
   e) Check target 2 – Bac-Vib2
   f) Click type – unknown (for samples, positive for + control and NTC for – control)

4) Click 'Apply' after each step above - step 3b to 3e will be carried over to the next samples so only sample name needs to be changed.

5) Click 'OK'

6) Click 'Save As' to save the template

7) Click ‘Run’
   a) Note: the droplet reader must be primed if not used for 3 or more days

8) Run in ROWS and set dye set to FAM/VIC.

9) A full plate takes approximately 2.5 hours to read.

7.3.7 Analysing data from the droplet reader protocol

1) Open up Quantasoft Analysis Pro software.
   a) Note: Can download on personal Windows computer from Bio-Rad website.

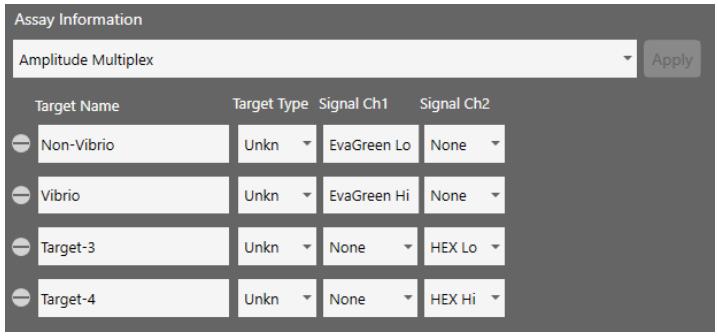
2) Using the “Browse” button – find where data from plate reader was saved.
   a) New file type should be “Quantasoft Plate”

3) Once data is loaded into software, open “Plate Editor” tab at the top.

4) Select/highlight all the wells by clicking on the square in the top left corner (between A and 1)

5) On the righthand side under “Edit Tools”, click the drop-down bar under “Assay Information” and change the setting to “Amplitude Multiplex”.

6) Change the “Target Name” to the following:
   a) “Non-*Vibrio*” = EvaGreen Lo
   b) “*Vibrio*” = EvaGreen Hi
   c) Leave Targets 3 and 4 as defaults (see below)



7) Click Apply

8) While the wells are still highlighted, click on the “Droplets” tab at the top of the page.

9) Check that all sample’s droplet counts are >10,000. *If not, omit the sample from analysis as this may lead to inaccurate quantification.*

10) Click on the “2D Amplitude” tab
    a) This is the best viewing option to see the droplet separation between amplicons.

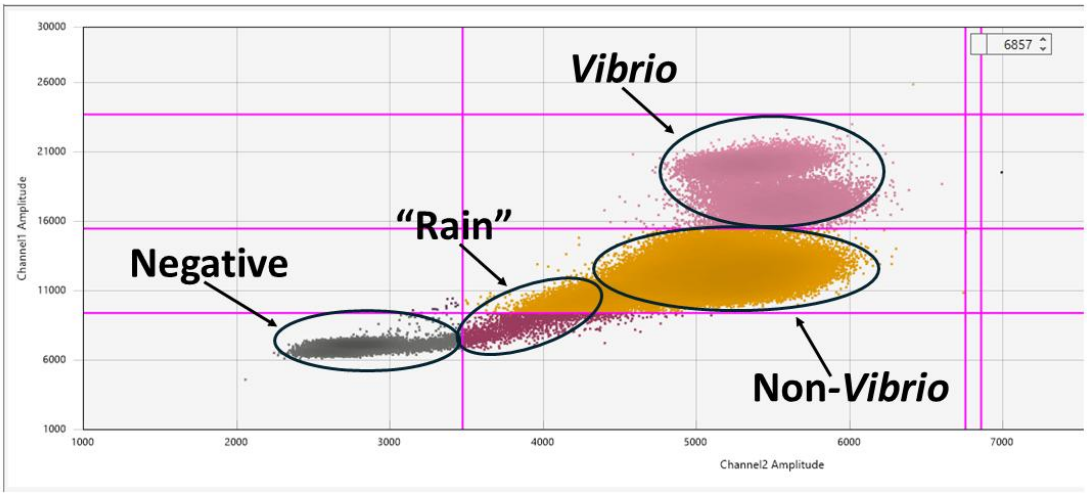
    Note: As a result of Vibrio being amplified to a greater degree due to higher primer concentration, the EvaGreen dye florescence incorporated within the DNA of the Vibrio positive droplets is of a greater amplitude. Thus, the two amplicon targets should be spatially differentiable from one another and the off-target amplification (i.e. non-specific amplification such as primer dimers) and can be quantified within the software. Thresholds are set between the spatially different groups of positive droplets and

negative droplets based on the use of Vibrio positive control. Set the thresholds by row and use the Vibrio positive controls in column 12 to guide setting the upper threshold.

11) In the "2D Amplitude" tab, highlight each row of 12 samples. Hover the mouse over the Vib+ sample to visualize the amplitude of *Vibrio* amplicons.

12) Click on the pink cross in the "Graph Tools" and click the cross icon over the top of the droplets.

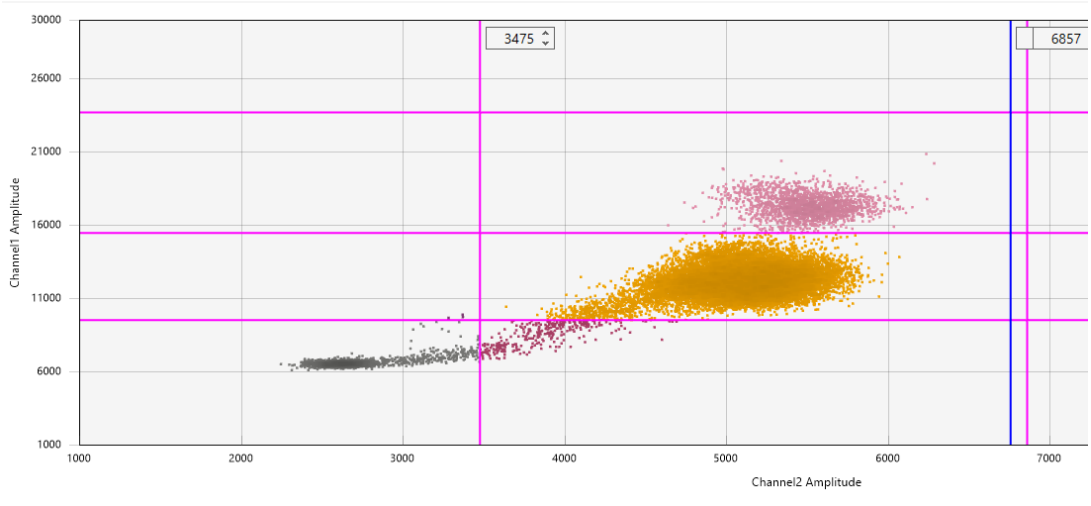
13) Arrange the horizontal pink threshold lines to boarder the spatially different groups of droplets. See the example plot below.



Note: A row of samples (12 samples) are selected, and their profiles are overlaid together. The Vibrio positive control is likely showing higher amplitudes than the Vibrio found in the other samples. If this is the case, draw the pink threshold below both Vibrio droplet clouds (shown in pink. The top pink droplet cloud is the Vib+ control and the bottom pink droplet cloud is from the water samples). Drawn the bottom pink threshold lines and bottom of the non-Vibrio bacterial positive droplets (orange droplet cloud). There will be "non-specific rain" amplification. Since there is no feasible way to avoid the raining droplets being included in the non-Vibrio quantification (orange), this is included in the non-Vibrio quantification though it is typically a minor proportion compared to the dense cloud of the orange cloud. The grey droplets are negative amplification – these are critical for quantification statistics to fit in a numerical model, therefore they need to be present within each profile.

14) Once the pink threshold lines are drawn for the row, move on to the next row (B, C, D…H) until the whole plate has thresholds done.

15) Once the whole plate is threshold, go through individual wells and quality check the thresholding. See the example plot below.



Note: The separation between Vibrio (pink) and non-Vibrio (orange) droplets should be clearer when looking at the individual wells. If the separation isn't clear, don't change the thresholding and use the thresholding based on all data in the same row as done above.

16) **Important:** Ensure every sample has a negative droplet cloud (grey). If not, quantification will be inaccurate. Note the sample ID# and exclude this replicate/sample from analysis until it is repeated. Other unexpected cloud patterns should be also noted and repeated the assay.

17) Once all the samples are individually checked and all the thresholding is complete, highlight all the wells by clicking in the top left corner or the plate (between A and 1).

18) With all the wells highlighted, click on the horizontal dashed lines in the right corner of the "Well Data" frame.

19) Click "Export to Excel…" and save the spreadsheet to the same folder as the data.

7.3.8 Analysing data from the droplet reader in Excel

1) Open the ddPCR worksheet and the exported Excel file with data.

2) Copy and paste all droplet reader result data into the "Raw Well Data" tab in the ddPCR worksheet. Make sure you check the run numbers match.

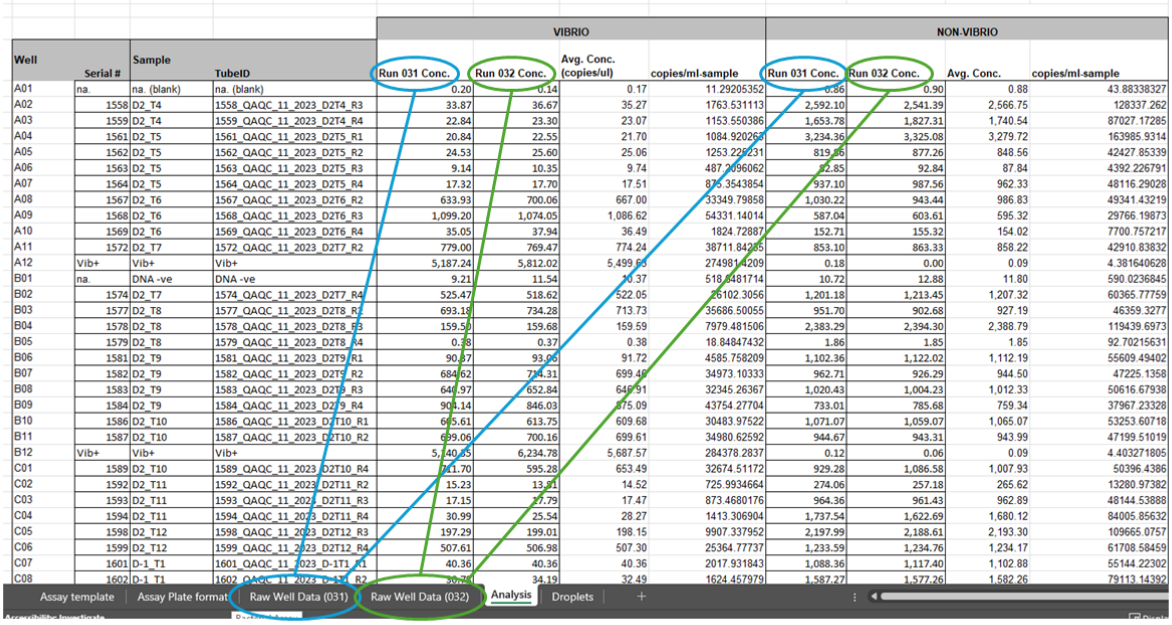
3) Do the same for both replicate runs of the plate (sample batch).

4) In the "Raw Well Data" tab, turn on the Filter function.
   a) This is important to select correct data.

5) Filter the "Target" in column C to display only *Vibrio*.

6) Copy and paste the "Conc(copies/µL)" values into the "Analysis" tab under the "*Vibrio* Run X Conc." column. Figure X below.

7) Go back to the "Raw Well Data" tab and change the "Target" filter to display only non-*Vibrio*. Copy and paste "Conc(copies/µL)" values into the "Analysis" tab under the "Non-*Vibrio* Run X Conc." column. Repeat these steps for the replicate run. (See below).



Well	Serial #	Sample	TubeID	VIBRIO Run 031 Conc.	VIBRIO Run 032 Conc.	VIBRIO Avg. Conc. (copies/ul)	VIBRIO copies/ml-sample	NON-VIBRIO Run 031 Conc.	NON-VIBRIO Run 032 Conc.	NON-VIBRIO Avg. Conc.	NON-VIBRIO copies/ml-sample
A01	na.	na. (blank)	na. (blank)	0.20	0.14	0.17	11.29205352	0.86	0.90	0.88	43.88338327
A02	1558	D2_T4	1558_QAQC_11_2023_D2T4_R3	33.87	36.67	35.27	1763.531113	2,592.10	2,541.39	2,566.75	128337.262
A03	1559	D2_T4	1559_QAQC_11_2023_D2T4_R4	22.84	23.30	23.07	1153.550386	1,653.78	1,827.31	1,740.54	87027.17285
A04	1561	D2_T5	1561_QAQC_11_2023_D2T5_R1	20.84	22.55	21.70	1084.920263	3,234.36	3,325.08	3,279.72	163985.9314
A05	1562	D2_T5	1562_QAQC_11_2023_D2T5_R2	24.53	25.60	25.06	1253.225231	819.86	877.26	848.56	42427.85339
A06	1563	D2_T5	1563_QAQC_11_2023_D2T5_R3	9.14	10.35	9.74	487.2096062	92.85	92.84	87.84	4392.226791
A07	1564	D2_T5	1564_QAQC_11_2023_D2T5_R4	17.32	17.70	17.51	875.3543854	937.10	987.56	962.33	48116.29028
A08	1567	D2_T6	1567_QAQC_11_2023_D2T6_R2	633.93	700.06	667.00	33349.79858	1,030.22	943.44	986.83	49341.43219
A09	1568	D2_T6	1568_QAQC_11_2023_D2T6_R3	1,099.20	1,074.05	1,086.62	54331.14014	587.04	603.61	595.32	29766.19873
A10	1569	D2_T6	1569_QAQC_11_2023_D2T6_R4	35.05	37.94	36.49	1824.72887	152.71	155.32	154.02	7700.757217
A11	1572	D2_T7	1572_QAQC_11_2023_D2T7_R2	779.00	769.47	774.24	38711.84265	853.10	863.33	858.22	42910.83832
A12	Vib+	Vib+	Vib+	5,187.24	5,812.02	5,499.63	274981.4209	0.18	0.00	0.09	4.381640628
B01	na.	DNA -ve	DNA -ve	9.21	11.54	10.37	518.6481714	10.72	12.88	11.80	590.0236845
B02	1574	D2_T7	1574_QAQC_11_2023_D2T7_R4	525.47	518.62	522.05	26102.3056	1,201.18	1,213.45	1,207.32	60365.77759
B03	1577	D2_T8	1577_QAQC_11_2023_D2T8_R2	693.18	734.28	713.73	35686.50055	951.70	902.68	927.19	46359.3277
B04	1578	D2_T8	1578_QAQC_11_2023_D2T8_R3	159.50	159.68	159.59	7979.481506	2,383.29	2,394.30	2,388.79	119439.6973
B05	1579	D2_T8	1579_QAQC_11_2023_D2T8_R4	0.38	0.37	0.38	18.84847432	1.86	1.85	1.85	92.70215631
B06	1581	D2_T9	1581_QAQC_11_2023_D2T9_R1	90.37	93.06	91.72	4585.758209	1,102.36	1,122.02	1,112.19	55609.49402
B07	1582	D2_T9	1582_QAQC_11_2023_D2T9_R2	684.62	714.31	699.46	34973.10333	962.71	926.29	944.50	47225.1358
B08	1583	D2_T9	1583_QAQC_11_2023_D2T9_R3	646.97	652.84	649.91	32345.26367	1,020.43	1,004.23	1,012.33	50616.67938
B09	1584	D2_T9	1584_QAQC_11_2023_D2T9_R4	904.14	846.03	875.09	43754.27704	733.01	785.68	759.34	37967.23328
B10	1586	D2_T10	1586_QAQC_11_2023_D2T10_R1	605.61	613.75	609.68	30483.97522	1,071.07	1,059.07	1,065.07	53253.60718
B11	1587	D2_T10	1587_QAQC_11_2023_D2T10_R2	699.06	700.16	699.61	34980.62592	944.67	943.31	943.99	47199.51019
B12	Vib+	Vib+	Vib+	5,140.85	6,234.78	5,687.57	284378.2837	0.12	0.06	0.09	4.403271805
C01	1589	D2_T10	1589_QAQC_11_2023_D2T10_R4	711.70	595.28	653.49	32674.51172	929.28	1,086.58	1,007.93	50396.4386
C02	1592	D2_T11	1592_QAQC_11_2023_D2T11_R2	15.23	13.81	14.52	725.9934664	274.06	257.18	265.62	13280.97382
C03	1593	D2_T11	1593_QAQC_11_2023_D2T11_R3	17.15	17.79	17.47	873.4680176	964.36	961.43	962.89	48144.53888
C04	1594	D2_T11	1594_QAQC_11_2023_D2T11_R4	30.99	25.54	28.27	1413.306904	1,737.54	1,622.69	1,680.12	84005.85632
C05	1598	D2_T12	1598_QAQC_11_2023_D2T12_R3	197.29	199.01	198.15	9907.337952	2,197.99	2,188.61	2,193.30	109665.0757
C06	1599	D2_T12	1599_QAQC_11_2023_D2T12_R4	507.61	506.98	507.30	25364.77737	1,233.59	1,234.76	1,234.17	61708.58459
C07	1601	D-1_T1	1601_QAQC_11_2023_D-1T1_R1	40.36	40.36	40.36	2017.931843	1,088.36	1,117.40	1,102.88	55144.22302
C08	1602	D-1_T1	1602_QAQC_11_2023_D-1T1_R2	30.78	34.19	32.49	1624.457979	1,587.27	1,577.26	1,582.26	79113.14392

Assay template | Assay Plate format | Raw Well Data (031) | Raw Well Data (032) | Analysis | Droplets

8) **Important:** Make sure you are copying and pasting into the correct rows and columns. Double check the samples match, and the runs are the same.

9) The average calculations and the total bacteria calculations will be output into the spreadsheet.

7.4 Recommendations on implementing across coral aquaculture operations

For the operation of coral aquaculture, aiming to rear larvae and grow recruits for the deployment into reef, it is advisable to minimise the risks of pathogenic bacteria outbreaks. The following are our recommendations on how to apply this Standard Operating Procedure and future real-time bacterial load assays to monitor *Vibrio* numbers and total bacteria abundances based on our case studies at several coral life stages (see Annexture):

7.4.1 Ensure tank environment is suitable before stocking larvae or recruits

Measuring the bacterial load is recommended before adding corals to ensure the tank environment is suitable for growing corals, with relatively low *Vibrio* and high total bacteria abundances (see Annexure Figure 2 D). We recommend starting the waterflow in the aquaculture tanks at least one day before adding corals of different life stages (e.g. embryo, competent larvae, adult) since the first 24 h are usually accompanied with variations in bacteria abundances.

Extremely low total bacteria estimates could indicate potential suppression of bacterial growth, which can also be detrimental to coral survival. This microbial suppression is potentially caused by insufficient rinse of disinfectants (see Case study 1: Coral larvae mortality observed in high *Vibrio* abundance despite low total bacterial loadAnnexure Figure 2 D & E), which can be avoided by belonging the initial rinse period using flowthrough seawater. In contrast, extremely high counts of total bacteria could indicate microbial contamination of the setting. This may occur due to the

carryover of bacteria derived from previously hosted corals or potentially due to insufficient cleaning (see Case study 1: Coral larvae mortality observed in high *Vibrio* abundance despite low total bacterial loadAnnexure Figure 2 D). However, it is noted that high numbers of total bacteria after adding corals did not necessarily have negative impacts for the survivorship of corals such as *A. kenti* (see Case study 1: Coral larvae mortality observed in high *Vibrio* abundance despite low total bacterial load). The increase of total bacteria upon introduction of fertilized corals are expected due to their early mortality and unwashed sperm carryover.

In tanks that contained concrete tiles for settlement, total bacteria numbers and *Vibrio* abundances increased compared to the input water controls, likely due to the decomposition of algae and biofilms that colonized the tiles during conditioning but died during freezing of the tiles for storage (Annexure Figure 6 A & B). This bacterial bloom typically settles over one day of flowthrough seawater conditions as competent coral larvae are added.

If coral spawning of multiple species were expected over a period of time and real-time monitoring of total bacteria available, tanks with unusually low or high values of bacterial load would be best skipped for larval stocking until more suitable values are acquired. Establishing expected microbial load in the tank can reduce the risks of compromising the health of larvae or recruits. In these circumstances, refilling the tanks or increasing the water turnover could help to get bacterial load to similar levels than the control water sources.

7.4.2 Collect microbial load data after observing mortality or diseased corals to refine thresholds

Data of *Vibrio* and total bacteria abundances of larval tanks with decreasing larval densities and their control cultures has proven beneficial to be able to estimate bacteria thresholds associated with increased probability that mortality occurs (Case study 2: Bacterial load associated with reported mortality in larval cultures, recruits and broodstock colonies). These thresholds are important information to develop more user-friendly diagnostic tools based on semi-quantitative and real-time quantification measures in the future. The thresholds appeared species-specific and different for different life stages including larvae, recruits and adults.

To be able to monitor if larval cultures or recruits are facing increased mortality, it is helpful to record larval density estimates and quantify the survival of recruits, since observational records of mortality can be subjective. Real-time larval density and recruitment survival is currently developed by the RRAP partners.

Obtaining directly comparable control samples is also highly recommended (see Case study 2: Bacterial load associated with reported mortality in larval cultures, recruits and broodstock colonies). The controls for systems that experienced mortality should ideally be culture thanks of the same volume and same tank parameters that are stocked with coral life stages of the same batch. This will facilitate ruling out coral genetic aspects, such as parental incompatibility and outbreeding depression, from the maintenance of microbial environment suitable for corals.

7.4.3 Monitor for bacterial load increases during long-term grow-out of recruits

Gradual increase in *Vibrio* load was indicated during a long-term grow-out of coral recruits for several weeks (Case study 4). It is recommended to test microbial load regularly to keep it in check, so that the potential increase of *Vibrio* abundances does not lead to coral mortality (Case study 4: Long-term grow-out of coral recruits). If a decline in recruit survival linked to the increase in *Vibrio* abundances is observed, interventions are possible such as a large-scale water exchange or even the transfer to a clean tank.

Thresholds of microbial load affecting the health of corals could be estimated by either correlating *Vibrio* abundances with total bacteria numbers while highlighting samples with observed mortality (see Case study 2: Bacterial load associated with reported mortality in larval cultures, recruits and broodstock colonies) or by predicting the threshold via Generalized Boosted Regression Models. The data generated in long-term-grow out could be a suitable starting point to establish recruit thresholds if survival data are available to score the presence and absence of recruit mortality between two time points.

7.4.4 Compare microbial loads for improvements of aquaculture tank systems

Studying the bacterial load provides insights into microbial environment of aquaculture systems that is valuable when different aquaculture systems are tested for operational improvement. For example, the Case study 1: Coral larvae mortality observed in high *Vibrio* abundance despite low total bacterial load and cCase study 4: Long-term grow-out of coral recruits demonstrated that recirculation and flow-through systems were equally successful in producing larvae and recruits while harbouring comparable loads of bacteria.

An essential component of the recirculation system is an effective filtration system which performance can be accessed by sampling the filter-treated water in comparison to unfiltered water of the aquaculture tanks (see Case study 1: Coral larvae mortality observed in high *Vibrio* abundance despite low total bacterial load). Microbial load assessment can provide guidance in alternative tank settings with (or even without) corals added, such that tank development between coral spawning seasons can leverage the microbial quantification data.

7.4.5 Establish ecologically relevant guidance for deployment of restoration devices and broodstock

Introduction of high numbers of pathogens into the wild upon coral deployment should be avoided. Comparisons with controls obtained from natural reef water samples provides guidance as to what levels of problematic microorganisms, such as *Vibrio* species, should be treated as a threat (Case study 2: Bacterial load associated with reported mortality in larval cultures, recruits and broodstock colonies). Our assay offers the quantification of total bacteria and *Vibrio* spp. Which is a taxon often associated with a range of aquaculture pathogens (de Souza Valente and Wan, 2021; Sanches-Fernandes et al., 2022; Vandeputte et al., 2024). If an assessment of one specific coral pathogen is required in the future, a similar process can be used to develop a specific assay and understand the natural abundance and the abundance level above which the organism could cause detrimental effects on coral health.

7.5 Limitations

Scalable microbial assays with high resource-efficiency will be key to make positive impacts on decision making during the coral aquaculture operation. The current method has established important baseline data and understanding on microbial loading and effective coral offspring production, based on highly sensitive digital PCR approach. However, the method requires four days for 96 samples, from sampling to the acquisition of the data, which causes a delay in decision making based on bacterial load data. The limitation of this method also comes from the costs of initially acquiring the Bio-Rad ddPCR system (Droplet generator, plate sealer, thermocycler and Droplet reader), as well as the ddPCR reagents and the DNA extraction kits. Potentially, alternative methods with faster and cheaper may be possible to gain sufficient operational insights.

7.6 Outlook

In the future, the time-efficiency of bacterial load monitoring could be improved in several ways. One option is to implement automated water samplers (e.g. Formel et al., 2021) and use automated DNA extraction instruments (e.g. McGaughey et al., 2018). Furthermore, the efficiency could be increased by using water samples and the resulting DNA extracts as input material for the two assays of (1) estimating the bacterial load (this SOP) and (2) eDNA sampling to detect coral pests such as coral-eating flatworms (Doyle et al., 2024);RRAP report CAD02-14.7).

Extracted bacterial DNA could be studied further by microbial taxonomic profiling using marker gene sequencing (e.g. 16S rRNA gene), characterizing bacterial community composition to gain more insights in microorganisms other than *Vibrio* that may be associated with disease symptoms (Case study 2: Bacterial load associated with reported mortality in larval cultures, recruits and broodstock colonies). An assay could be developed to other coral pathogens by replacing the primers to target them.

Finally, building upon results by the present sensitive method, a simplified semi-quantitative assays may be developed to monitor the bacterial load and pathogen abundances with a shorter turnaround time. These rapid assay options include nucleic acid lateral flow devices similar to a COVID-19 test (Plaon et al., 2015; Thongkao et al., 2015; Ying et al., 2021), implementing simpler DNA extraction and visualization of PCR products using differential amplification thermal cycles (Doyle and Uthicke, 2021)

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9 Acronyms

SOP	Standard Operating Procedure
RAS	Recirculation
FT	Flow-through
RRAP	Restoration and Adaptation Program
GBR	Great Barrier Reef
RTN	Rapid tissue necrosis
ddPCR	droplet digital polymerase chain reaction
SDS	Safety Data Sheet
Milli-Q	Milli-Q® ultrapure water
PPE	Personal protective equipment
QAQC	Quality Assurance and Quality Control
PCR	polymerase chain reaction
AutoDG	Automatic Droplet Generation
SE	Standard Error
AIMS	Australian Institute of Marine Science
GPA	General Purpose seawater with Ambient temperature
MIS	Mesocosm Indoor Systems

Annexure

Aiming to investigate the link between the survival of different coral life stages and bacteria load, the above-described Standard Operating Procedure was applied to aquaculture tanks of larvae, recruits and adults at the Australian Institute of Marine Science within the RRAP in 2022 and 2023.

Case study 1: Coral larvae mortality observed in high *Vibrio* abundance despite low total bacterial load

Monitoring the bacterial load in aquaculture tanks of coral larvae was applied to acquire a bacteria load profile of rearing systems and identify unsuitable and unfavourable conditions for coral larvae. By following the described Standard Operating Procedure, we observed mortality in larval tanks with high *Vibrio* abundance despite low total bacteria (i.e. high *Vibrio* proportion). The culture tanks monitored in this case study are the same than in the studies of Abdul Wahab et al. (2024, Chapter 2 & 3), which tested different larval densities and water turnover rates in 2022 (Annexure Figure 1) and compared flow-through systems (FT) with recirculation systems (RAS) in 2023 (Annexure Figure 2).

Examples of suitable aquaculture systems are the culture tanks of *A. kenti* in November 2022 (Annexure Figure 1) and *A. anthocercis* in November 2023 (Annexure Figure 2) showing sustained densities of coral larvae throughout the rearing.

In November 2022, the tanks with a turnover of 0.2 that were stocked at the density of 1 larva per mL had similar survival rates than tanks stocked at lower density (0.3 larvae per mL) (Annexure Figure 1 A, solid lines). The abundance of total bacteria in these culture tanks was high in the first two days and then declined to levels comparable to the control water on day 6 (Annexure Figure 1 C). The *Vibrio* abundances in these tanks were very low with a maximum of 231 ± 17 copies/ml (mean ± SE) for tanks with 0.2 larvae per mL and 392 ± 115 copies/ml (mean ± SE) for 1 larva per mL within the first week of development (Annexure Figure 1 D).

The *A. anthocercis* tanks in November 2023 sustained larval densities in both flow-through (FT) and recirculation (RAS) systems (Annexure Figure 2 B). In the RAS and FT systems, the controls (GPA and sump output) had significantly lower total bacteria abundances compared to the tanks harbouring larvae. Within the first 56 h, the RAS system was still operated as flow-through mode resulting in similar estimates for total bacteria on day 1 and 2. After switching to recirculation, the number of total bacteria increased in the RAS at day 4 and 6 compared to the FT systems, but the significantly lower abundances in total bacteria for the sump output indicated that the ultrafiltration system was suitable to remove bacteria from the system. The associated *Vibrio* abundances for the flowthrough and recirculation tanks were low and ranged on average between 0 and 1441 ± 199 copies/ml (mean ± SE) for the FT, and 15 ± 11 copies/ml and 2188 ± 431 copies/ml for the RAS (Annexure Figure 2 E), while the larval density stayed around 0.6 larvae/ mL over the 7 days (Annexure Figure 2 B) (Abdul Wahab et al., 2024). The ratios of *Vibrio*/total bacteria are comparable between the RAS and FT systems except day 6 (Annexure Figure 2 F).

In comparison to the healthy larval tanks of *A. anthocercis*, other experiments showed that coral mortality in culture tanks can occur when unusual low total bacteria abundances and high *Vibrio* estimates were observed.

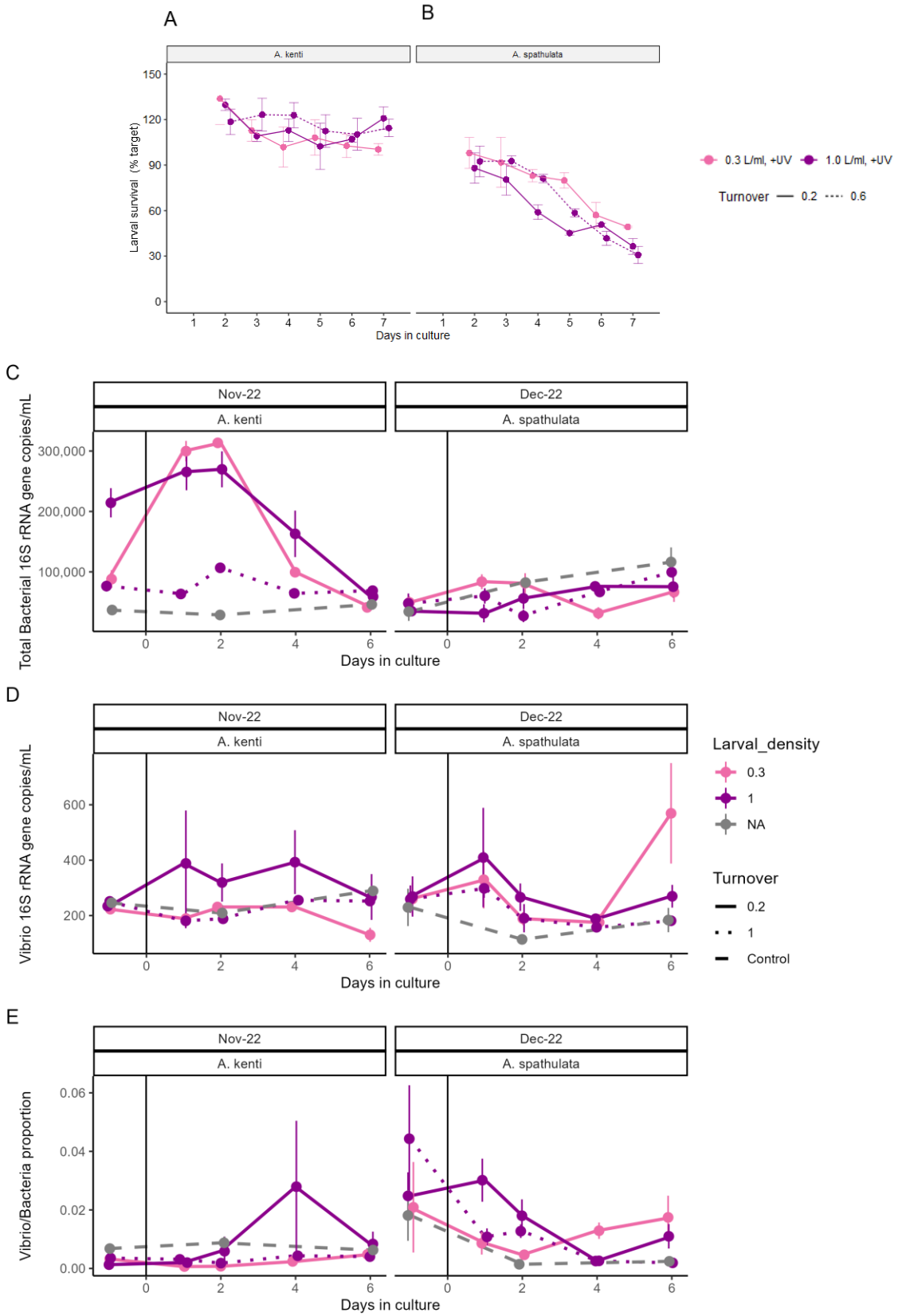
In November 2023, larvae of *Acropora millepora* rapidly died-off in the recirculation systems (Annexure Figure 2 A) which was accompanied with an increase in total bacteria and *Vibrio* estimates (Annexure Figure 2 D & E). In December 2023, larval densities of *Acropora spathulata* declined continuously between day four and six (Annexure Figure 2 C), which was reflected in RAS and FT systems at day four when *Vibrio* 16S gene copy numbers per mL increased (Annexure Figure 2 E), while total bacteria abundances declined (Annexure Figure 2 D). Currently it is unclear whether the mortality of larvae make *Vibrio* strains thrive or

whether high numbers of *Vibrio* cause the mortality in larvae. The combination of both explanations is also possible.

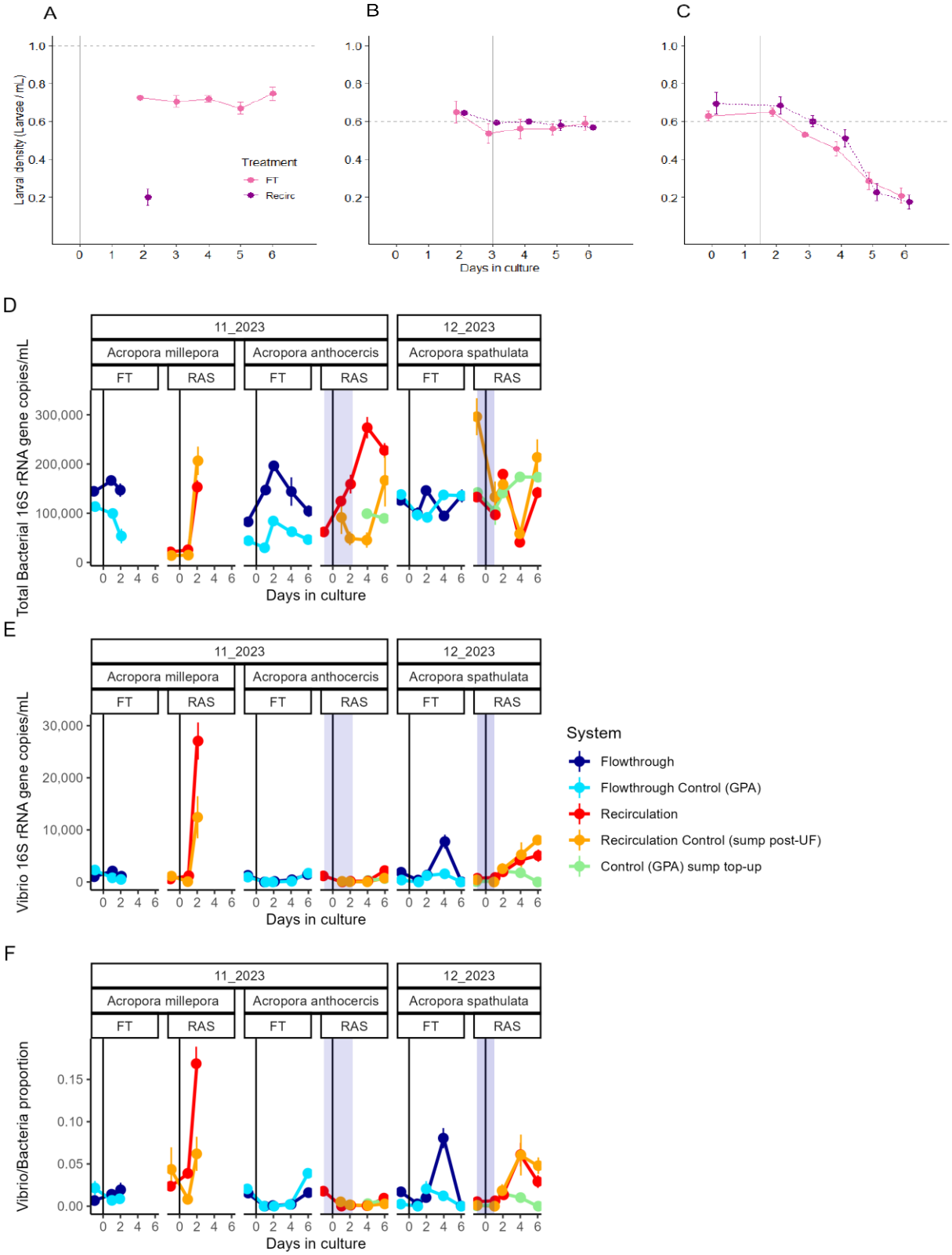
In November 2023, newly assembled RAS tanks had a *A. millepora* larval crash in all replicated tanks one day after stocking (Annexure Figure 2 A). Extremely low numbers of total bacteria compared to the control water source of the flow-through system were recorded at the pre-stocking time point (Annexure Figure 2 D). A potential explanation for the extremely low total bacteria abundances in these RAS tanks could be the disinfectant solutions that were routinely used to sterilize culture tanks before filling them with seawater. Remaining residuals of the cleaning agent (such as bleach and hydroperoxide), could possibly suppressed bacterial abundances and negatively impacted coral larvae health.

In both December 2022 and 2023, cultures of *A. spathulata* had lower levels of total bacteria compared to water source controls and these cultures experienced larval mortality (Annexure Figure 1 B & C). In December 2022, the culture tanks with 1 larvae per mL had significantly lower numbers of total bacteria at day two and six compared to water source controls (Annexure Figure 1 C) which correlated with an approximately 60% decline of larval survival (Annexure Figure 1 B). In December 2023, four days after stocking the larval tanks, the FT, RAS and RAS sump water had lower total bacteria estimates than the water source control of the FT system and the sump top-up source water (Annexure Figure 2 D). These observations could indicate a species-specific effect of *A. spathulata* larvae on total bacteria abundances. However, in this instance, a link between low bacterial abundance and coral larval decline remains unclear. The mortality of *A. spathulata* could be unrelated to bacteria and possibilities include genetic incompatibility of cryptic species during fertilisation or environmental factors influencing gamete quality (Willis et al., 1997; Abdul Wahab et al., 2024).

On the contrary, unusually high bacteria estimates before stocking coral larvae suggest that the culture tank system could be contaminated by residual bacteria from previous operations (Annexure Figure 2 D; *A. spathulata*). However, it is important to note that high number of total bacteria do not necessarily have negative effects on coral larvae. For example, in November 2022, the number of total bacteria were increased in the first two days after adding the *A. kenti* cultures to the low turnover treatment, but survival rates remained above 90 % during the 7 days (Annexure Figure 1 A).



Annexure Figure 1: Survival of Acropora kenti (A) and Acropora spathulata (B) under different larval densities and culture tank turnover rates during the first six days of coral larvae development. In the same aquaculture tanks, bacterial load was monitored in the form of averaged estimates (mean ± SE) of total bacteria (C), Vibrio (D) and Vibrio/total bacteria. The black vertical line highlights the timepoint when fertilised zygotes were added to the culture tank.



Annexure Figure 2: Larval density (mean ± SE) of Acropora millepora (A), Acropora anthocercis (B) and Acropora spathulata (C) in flow-through (FT) and recirculation (RAS) tank systems during the first six days of coral larvae development (Abdul Wahab et al., 2024). In the same aquaculture tanks, bacterial load was monitored in the form of averaged estimates (mean ± SE) of total bacteria (C), Vibrio (D) and Vibrio/total bacteria. Vertical black line highlights the timepoint when fertilised zygotes were added to the culture tank. Blue shading in recirculation system indicates the time in which tanks were still run as flow-through system to flush out organic material.

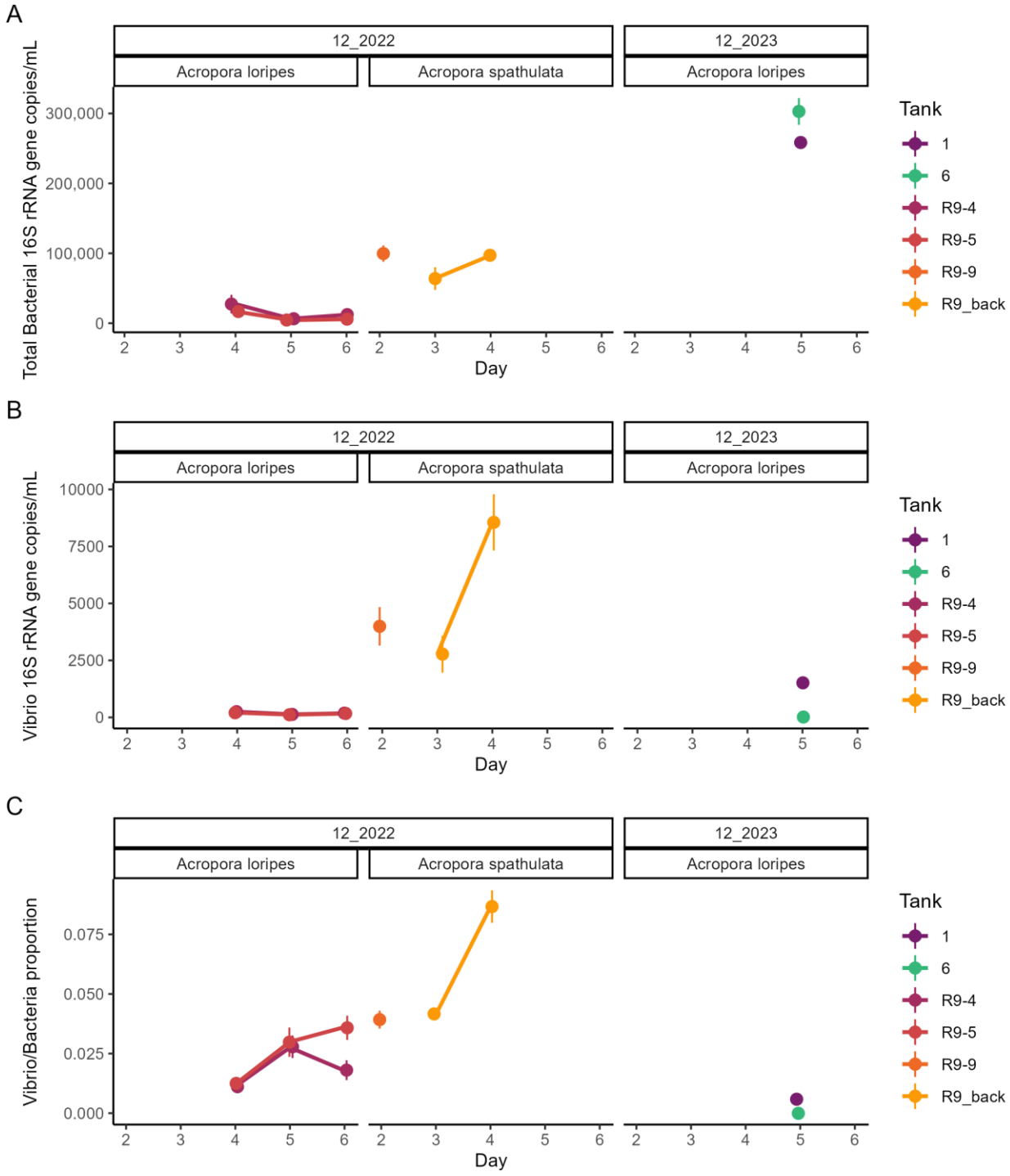
Case study 2: Bacterial load associated with reported mortality in larval cultures, recruits and broodstock colonies

To predict at which bacterial abundances the probability of coral mortality likely increases, we collected samples of culture tanks of larvae, recruits and broodstock with signs of mortality or disease with regard to control tanks.

In 2022 and 2023, signs of increased larval mortality was anecdotally reported in five tanks (Annexure Figure 3, purple - orange), three of which were monitored for 2-3 days to obtain temporal trends.

In 2023, the sampling of *A. loripes* (tank 1) was complemented with a control tank of the same larval batch that did not show signs of mortality (Annexure Figure 3, green colour). The healthy tank had significantly higher number of total bacteria and significantly lower *Vibrio* estimates compared to the tank with signs of mortality (Annexure Figure 3 A & B). In December 2022, total bacteria abundances were relatively low in the two *A. loripes* culture tanks between day 4 and day 6 (Annexure Figure 3 A), compared to the healthy *A. loripes* tank from December 2023, while *Vibrio* abundances were at a similar level with the 2023 healthy control. The *A. spathulata* tank stocked in December 2022 with reported signs of mortality, significantly increased in *Vibrio* abundances from 2774 ± 809 copies/ml (mean ± SE) at day 3 to 8553 ± 1234 copies/ml at day 4.

The association of bacterial load of culture tanks with reported mortality remains unclear for two reasons. Firstly, the classification of mortality was not supported by recorded larval density data over time resulting in an isolated observation. Secondly, the samples with mortality signs from December 2022 were also not paired with a healthy tank control, so interpretation is not feasible. Future samples of larval mortality should be supported by a decline in larval density over time and also paired with a culture tank of the same spawning batch (fertilized at same night from the same parental colonies) without mortality signs.

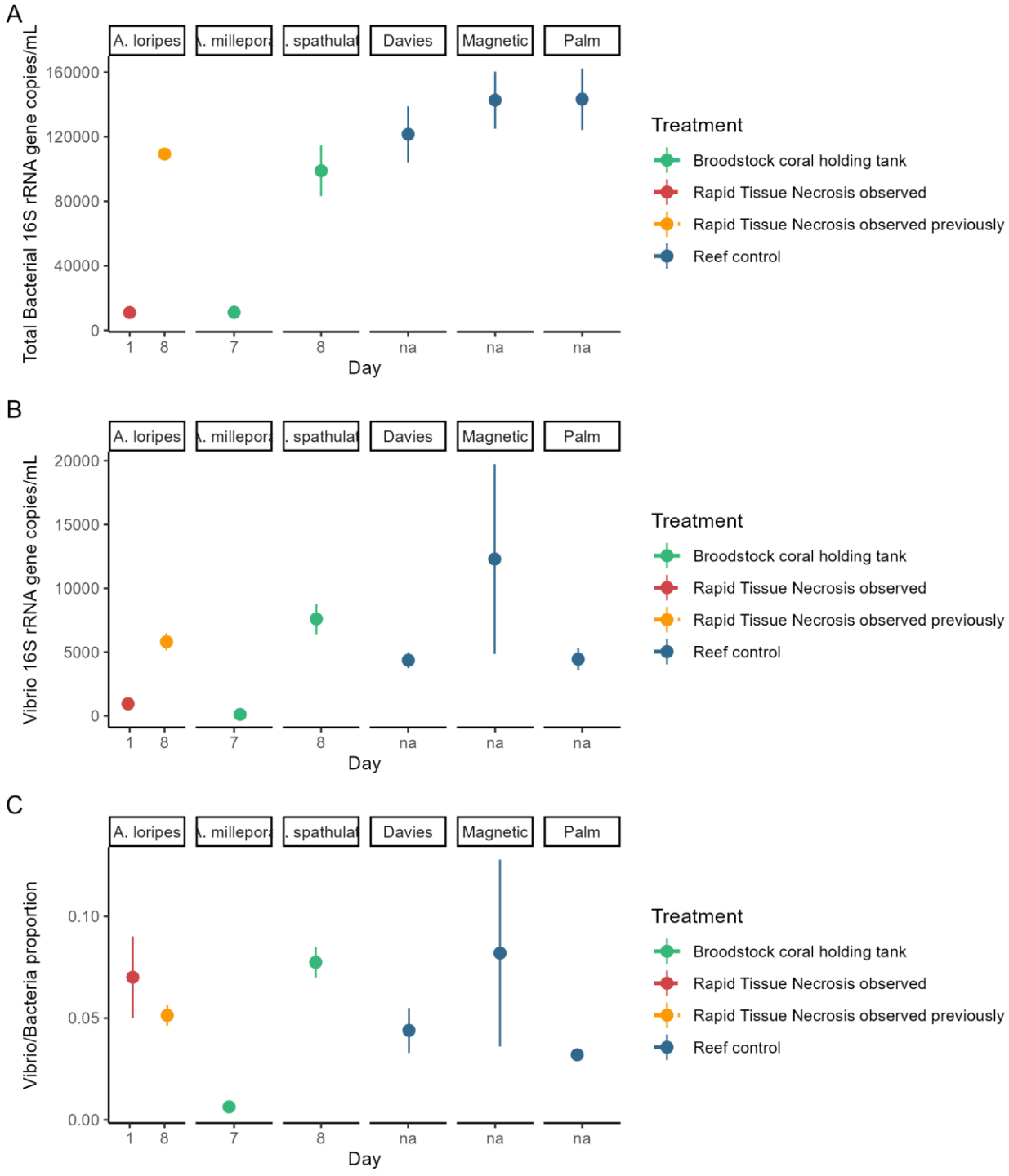


Annexure Figure 3: Bacterial load monitored in culture tanks with observed larval mortality (red-orange) in the form of averaged estimates (mean ± SE) of total bacteria (C), Vibrio (D) and Vibrio/total bacteria. A control of a culture tanks without mortality signs was included in December 2023 (green).

Characterizing a bacterial threshold for adult broodstock is valuable, because it can guide decision-making on their ecologically safe re-outplanting. These thresholds are based on what level of *Vibrio* or total bacteria abundances can minimize the risk that aquaculture-derived pathogens may impact natural coral communities.

To establish baseline data required for obtaining these thresholds of bacterial loads, the bacterial load was monitored in tanks holding broodstock corals including those that showed signs of rapid tissue necrosis (RTN), which were compared to bacterial loads occurring in the natural reefs. The water samples from tanks harbouring *A. loripes* colonies with RTN had low levels of total bacteria, which were however comparable to asymptomatic colonies of *A. millepora* (Annexure Figure 4 A). One week later, the total bacteria abundances of the tank, that previously contained *A. loripes* colonies with RTN, increased to values comparable to culture tanks of asymptomatic *A. spathulata*. In comparison, water samples from Davies Reef, Magnetic Island and Palm Island were associated with highest values of total bacteria (Annexure Figure 4 A). The *Vibrio* abundances in the culture tank with RTN colonies were low at day 1 but showed higher values at day 8 that were similar to those in the sampled water from Davies reef and Palm Island (Annexure Figure 4 B).

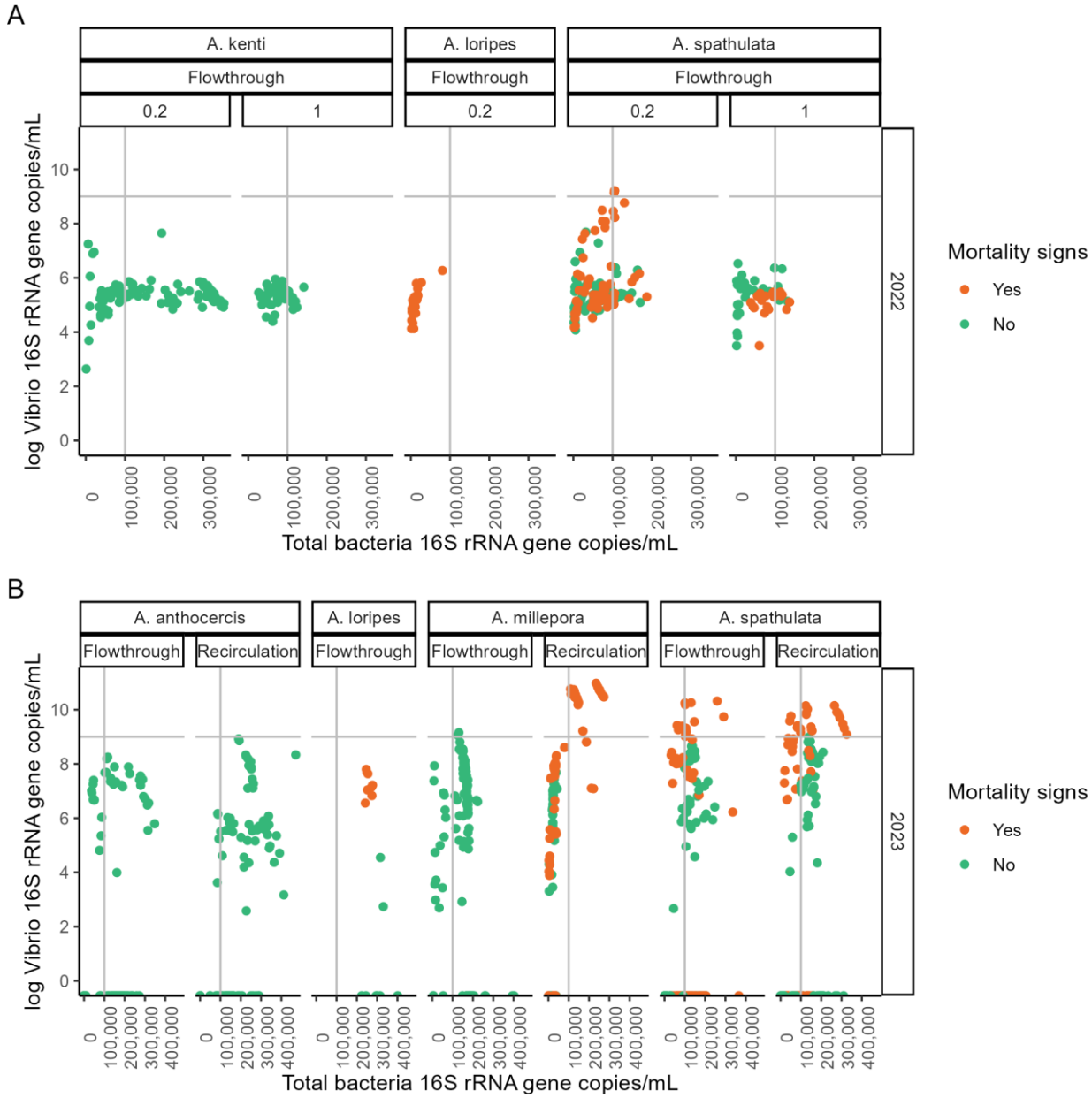
The observation that RTN samples were recorded with low total bacteria and low *Vibrio* numbers could suggest that the tissue loss is a symptom of a microorganism infection other than *Vibrio* (e.g. microbial dysbiosis). Besides *Vibrio*, *Algicola*, *Cohaesibacter* and *Thalassobius* were previously associated with the stony coral tissue loss disease (Becker et al., 2022). Additionally, the bacterial load is coral species-specific since the broodstock tanks of asymptomatic *A. millepora* had similar values of total bacteria and *Vibrio* numbers than the tanks with RTN.



Annexure Figure 4: Bacterial load monitored in the form of averaged estimates (mean ± SE) of total bacteria (C), Vibrio (D) and Vibrio/total bacteria ratio in tanks harbouring adult broodstock. Acropora loripes colonies on day 1 showed signs of rapid tissue necrosis that disappeared on day 8. Samples for Acropora millepora and Acropora spathulata originated from tanks with asymptomatic and healthy colonies and water samples were also monitored from three reefs to have a control.

This type of bacterial load data originating from culture tanks with signs of coral mortality and their symptom-free control tanks will be essential to identify the threshold at which the likelihood increases that coral mortality could occur. To predict these thresholds using a metadata analysis approach, the larval experiment data from case study 1 were annotated with the presence and absence of larval mortality by observing the rate of decline in larval density and larval survival data (Annexure Figure 5).

To incorporate *Vibrio* concentrations with total bacterial abundance, the *Vibrio* abundance was correlated against the total bacteria according to 16S rRNA gene copies per mL. The resulting correlation plot suggested that thresholds of increased likelihood for a decline in larval survival are species-specific and depend on the culture tank parameters such as turnover rates. In the culture tanks of 2023, values above 8100 *Vibrio* gene copies/mL (grey horizontal line at log(9)) occurred with a decline in larval numbers of *A. millepora* and *A. spathulata* (grey horizontal line in Annexure Figure 5). Coral mortality signs in larval tanks were also recorded below this value when the abundance of total bacteria was below 100,000. This was the case for *A. millepora* in recirculation systems in December 2023 and *A. loripes* in flowthrough systems in 2022. The threshold of *Vibrio* abundances linked to larval mortality also appeared species-specific since comparable *Vibrio* numbers were estimated in culture tanks of *A. kenti* and *A. loripes* in 2022, but signs of larval mortality were only recorded in the latter (Annexure Figure 5). *Acropora anthocercis* and *A. kenti* did not show any signs of larval mortality (Annexure Figure 5).



Annexure Figure 5: Vibrio abundances correlated against the total bacteria estimates aiming to identifying the threshold of bacterial load that increases the likelihood that mortality signs are observed. Data of case study 1 were scored for the presence and absence of mortality based on a decline in larval survival. To improve the visualization, the Vibrio abundances were logged on the y-axis. The grey lines were added to visually separate samples with mortality signs from the sample points without.

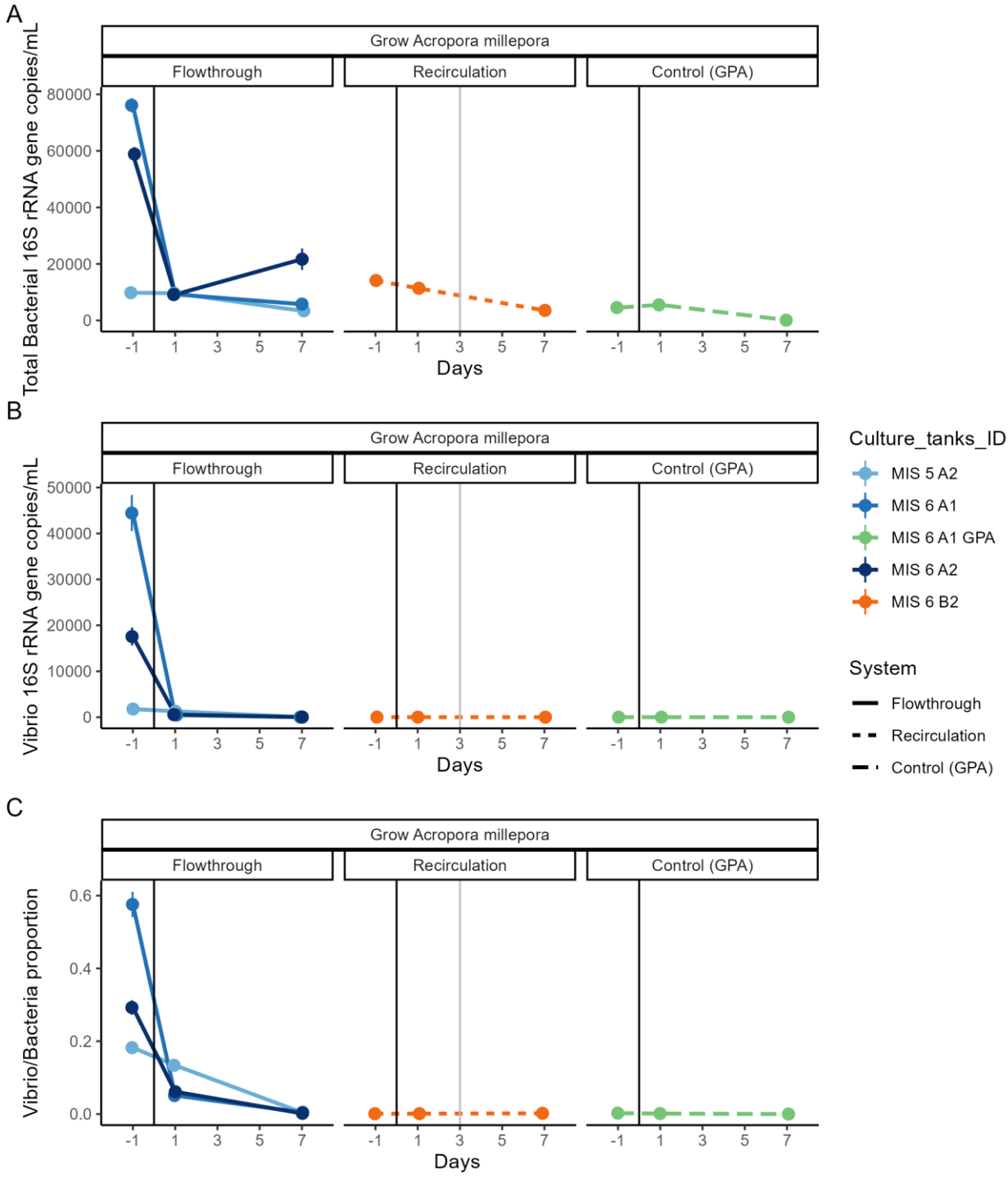
Case study 3: Settlement of coral larvae and symbiont inoculation

In November 2023, *A. millepora* larvae were settled onto conditioned concrete tiles and kept in three shallow aquaculture tanks for seven days to test whether a RAS system would be as suitable to keep recruits short-term as the flow-through tanks (Details to be reported in RRAP CAD Report 14.3). We implemented the bacterial load assays to monitor microbial aspects of the tank settings.

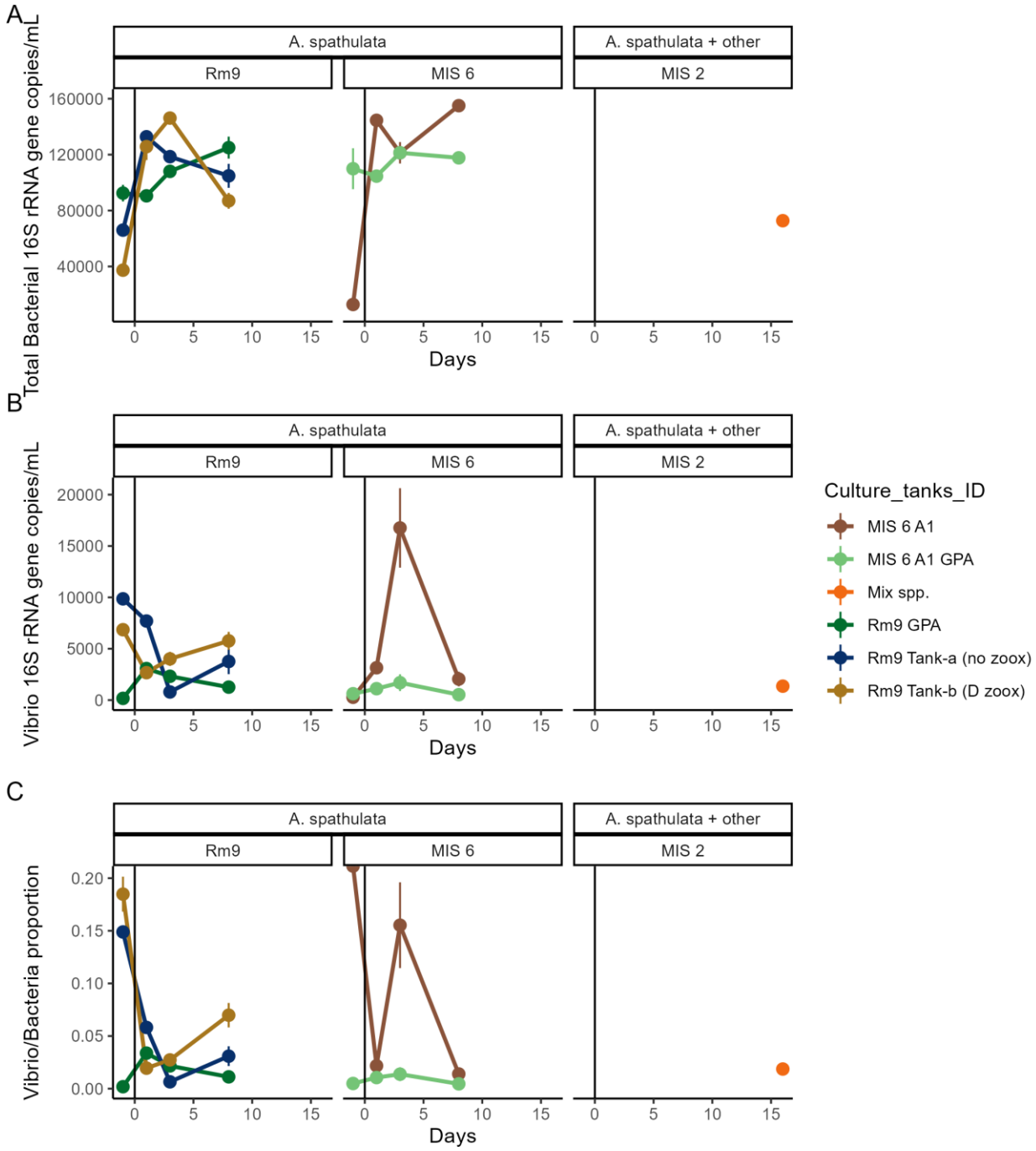
Two of the three flow-through tanks had higher levels of total bacteria and *Vibrio* counts before adding larvae for settlement (Annexure Figure 6 A & B). These high levels decreased to comparable levels in the control water source within 24 hours. A potential explanation for this initially high bacterial load could be derived from the concrete tiles which were conditioned for several months and then frozen. Although tiles were rinsed to remove frozen algae, this degrading organic matter could be a source to make *Vibrio* estimates spike.

On day three, the tiles with settled recruits from the MIS 5 flow-through tank (light blue) were moved to the RAS tank MIS 6B (orange) (Annexure Figure 6 grey vertical line). The bacterial load sampling on day seven indicated that RAS were equal to FT systems in maintaining low *Vibrio* abundances over four days. Survival data for these tiles would be beneficial to be able to compare if FT and RAS were equally suitable to grow recruits short-term.

In summary, the *Vibrio* abundances are low in both systems so that there is no concern to deploy the tiles onto the reef. However, it is recommended to let the systems run for at least one day with the concrete tiles before stocking with larvae to make sure bacteria abundances can adjust within the first 24h.



Annexure Figure 6: Bacterial load monitored in the form of averaged estimates (mean ± SE) of total bacteria (C), Vibrio (D) and Vibrio/total bacteria ratio during settlement and short-term grow-out of Acropora millepora. The black vertical line highlights the timepoint when larvae were added to the settlement tank to attach and undergo metamorphosis into sessile recruits. This developmental process was induced by concrete tiles that were conditioned for 8 weeks in seawater to be colonised by a settlement-inducing crustose coralline algae and biofilm. At day 3, concrete tiles with recruits settled in tank MIS 5 A2 were transferred into the recirculation system of MIS 6 B2.

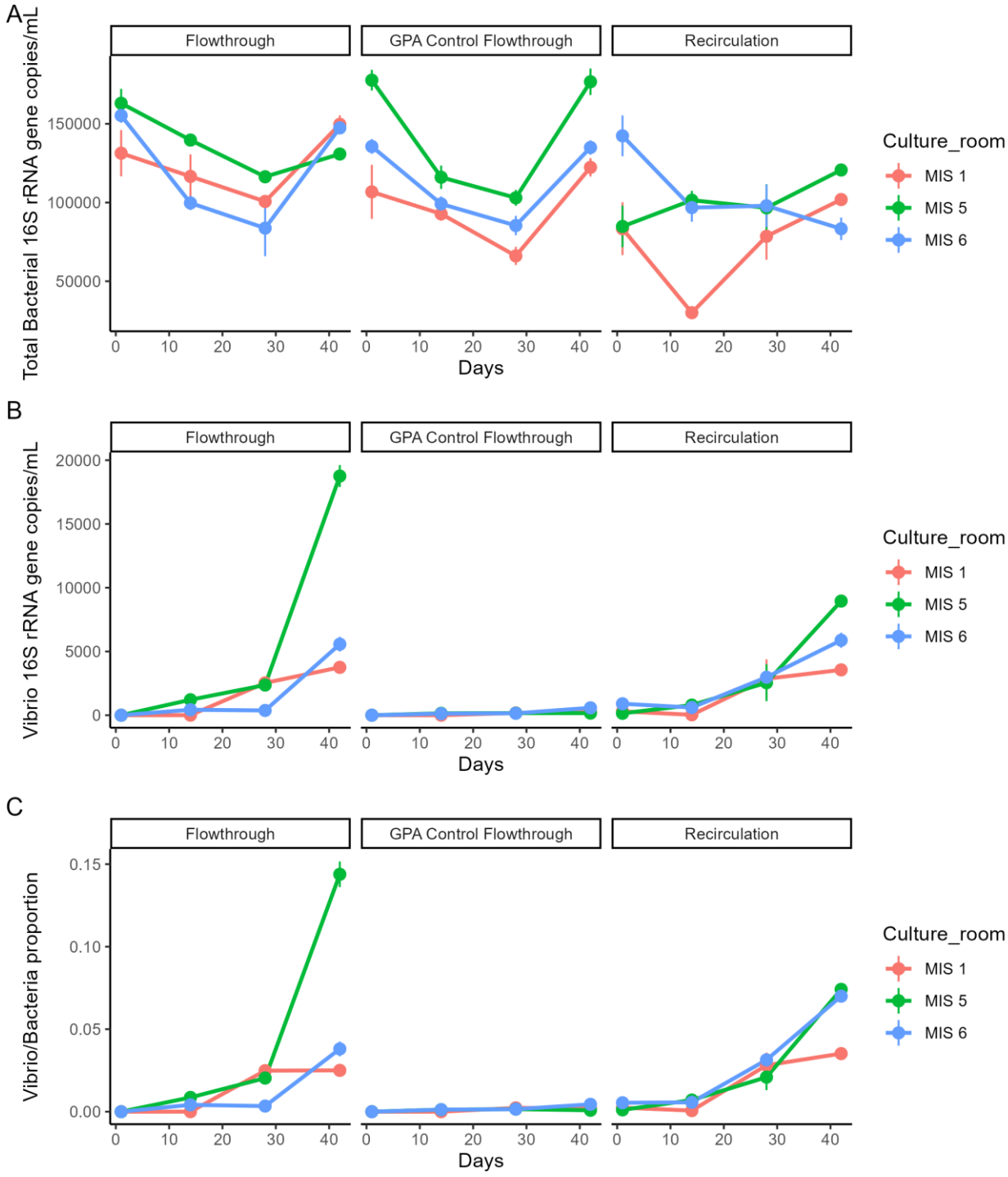


Annexure Figure 7: Bacterial load monitored in the form of averaged estimates (mean ± SE) of total bacteria (C), Vibrio (D) and Vibrio/total bacteria ratio during settlement and short-term grow-out of Acropora spathulata. The black vertical line highlights the timepoint when larvae were added to the settlement tank to attach and undergo metamorphosis into sessile recruits. This developmental process was induced by concrete tiles that were conditioned for 8 weeks in seawater to be colonised by a settlement-inducing crustose coralline algae and biofilm. At day 3, recruits were inoculated with the symbiont of the genus Durusdinium. A different batch of A. spathulata larvae was settled together with other species and the concrete tile was broken into smaller squares to be inserted into the restoration devices which were kept in tank MIS 2.

In December 2023, the bacterial load was also monitored during larval settlement, symbiont inoculation and short-term grow-out for deployment on assembled devices. Adding larvae to settle on concrete tiles increased total bacteria in 50 L tanks Rm9 as well as in 100 L tank (MIS 6). *Vibrio* concentrations increased in the MIS6 tank, most likely due to the stop in water inflow to inoculate the symbionts. This increase was not so extreme in the Rm9 tanks although *Vibrio* abundances were comparable in both tanks at day one. Similar to the November experiment above, the samples taken before corals were added (from day -1) were most variable compared to water source controls, with lower total bacteria numbers and higher *Vibrio* abundances compared to the controls. Fifteen days after spawning, recruits settled on concrete tiles were added into the devices for reef restoration. The tanks harbouring the assembled devices had 1356 ± 60 *Vibrio* 16S rRNA copies per mL (mean ± SE), which was below the threshold for *Vibrio* numbers cooccurring with larval mortality (Case study 2: Bacterial load associated with reported mortality in larval cultures, recruits and broodstock colonies).

Case study 4: Long-term grow-out of coral recruits

In a long-term grow-out experiment with recruits of different coral species (ongoing RRAP CAD study, Nordborg et al.), the flow-through system was compared to the recirculation system by sampling of bacterial load every two weeks. Overall, there was an increase in *Vibrio* abundances after 14 days in both FT and RAS systems, while the water source to these tanks maintained low averages of maximal 579 ± 90 copies/ml (mean ± SE) over the 42 days of the experiment (Annexure Figure 8 B). The steepest increase in *Vibrio* numbers was in one FT tanks suggested that there were tank-specific effects. Recruit survival was monitored in this experiment using a photo time series and image analysis is currently underway. Coral survivorship data will be compared with the bacterial abundances to provide further insights in whether the RAS system is as efficient as the FT. The described method to monitor the bacterial load can also help to assess if interventions to decrease bacteria numbers by exchanging water or increasing water inflow are successful.



Annexure Figure 8: Bacterial load monitored in the form of averaged estimates (mean ± SE) of total bacteria (C), Vibrio (D) and Vibrio/total bacteria ratio during long-term grow-out of recruits in flow-through and recirculation tanks.

Appendix (Excel Worksheet)

REEF RESTORATION & ADAPTATION PROGRAM

Australian Government | Reef Trust

Great Barrier Reef Foundation

Australian Government | Australian Institute of Marine Science | CSIRO | James Cook University Australia | QUT | Southern Cross University | The University of Queensland Australia