

Metadata Field Codes

1. Sample Collection Procedures	4
1.1. Soil Sampling (as per BASE)	4
1.2. Soil Sampling (as per UTAS Urban Soil & Aerial Microbiome Study)	5
1.3. Marine Sediment Sampling (as per MM Coastal)	11
1.4. Pelagic Water Sampling for IMOS National Reference Stations	11
1.5. Coastal Seawater Sampling for Australian Coastal Microbial Observatory Network (ACMON; IMOS)	12
1.5.1. Botany Bay, NSW, and Port Phillip Bay, VIC, ACMON Sites	12
1.5.2. Great Barrier Reef, QLD, ACMON Sites	13
1.6. Pelagic Water Sampling for Voyages (provided by CSIRO Environmental Genomics team)	14
1.7. Coastal Seawater Sampling for Downstream DNA Analyses (as per MM Coastal ORIGINAL)	16
1.8. Coastal Seawater Sampling for Downstream DNA Analyses (as per MM Coastal UPDATED SEPT 2019 TT)	17
1.9. Coastal Seawater Sampling for Downstream RNA Analyses (as per MM Coastal)	18
1.10. Coastal Seawater Sampling for Downstream DIC & TA Analyses (as per MM Coastal)	18
1.11. Seagrass Sampling (as per MM Coastal)	18
1.11.1. Surficial Sediment Sampling	19
1.11.2. Seagrass Sampling	19
1.12. Sponge Sampling (as per MM Coastal)	19
1.13. Seaweed (<i>Ecklonia radiata</i>) Sampling (as per MM Coastal)	20
1.14. Coral Sampling (as per MM Coastal)	20
1.15. Water sampling from acidic saline lakes (supplied by Elizabeth Watkin/Katelyn Boase)	21
1.16. Water sampling from Brisbane river estuary (supplied by Christian Rinke/Apoorva Prabhu)	22
1.17 Plant Sampling	26
2. Contextual Metadata Analytical Procedures	27
2.1. CSBP Laboratory Procedures for Soils (as per BASE)	27
2.2. IMOS National Reference Stations Biogeochemical Operations Manual (as per IMOS NRS & ACMON)	27
2.3. CSIRO RV <i>Southern Surveyor</i> Laboratory Procedures SS2010_V09	27
2.4. CSIRO RV <i>Southern Surveyor</i> Laboratory Procedures SS2012_T06	27
2.5. CSIRO RV <i>Southern Surveyor</i> Laboratory Procedures SS2012_T07	27
2.6. CSIRO RV <i>Southern Surveyor</i> Laboratory Procedures SS2012_V04	27
2.7. CSIRO RV <i>Southern Surveyor</i> Laboratory Procedures SS2013_T03	28
2.8. CSIRO RV <i>Investigator</i> Laboratory Procedures IN2014_E03	28
2.9. CSIRO RV <i>Investigator</i> Laboratory Procedures IN2015_C02	28
2.10. CSIRO RV <i>Investigator</i> Laboratory Procedures IN2015_V03	28
2.11. CSIRO RV <i>Investigator</i> Laboratory Procedures IN2016_T01	28
2.12. CSIRO RV <i>Investigator</i> Laboratory Procedures IN2016_V03	28

2.13.	CSIRO RV <i>Investigator</i> Laboratory Procedures IN2016_V04	28
2.14.	Sediment Grain Size Fractionation (% fines) (marine sediment as per MM Coastal)	28
2.15.	Sediment Grain Size Fractionation (% fines) (marine seagrass sediment as per MM Coastal)	29
2.16.	TOC (marine sediment as per MM Coastal)	29
2.17.	TOC (marine seagrass sediment as per MM Coastal)	29
2.18.	Marine sediment porewater nutrients (as per MM Coastal)	29
2.19.	PAM Fluorometry in situ measurements (seagrass as per MM Coastal)	29
2.20.	Analytical Services Tasmania (AST) Laboratory Procedures (as per Derwent Estuary Program, Ambient Water Quality Program)	30
2.21.	YSI details (as per Derwent Estuary Program, Ambient Water Quality Program)	30
2.22.	CSBP Laboratory Procedures for water (Watkin/Boase, BushBlitz)	30
3.	OMICS Sample Extraction & Purification Procedures	31
3.1.	DNA Protocols	31
3.1.1.	Qiagen DNeasy® PowerLyzer PowerSoil® Kit (soil, as per BASE @ AGRF)	31
3.1.2.	Qiagen DNeasy® PowerSoil® Kit (soil, marine sediments, sponge)	31
3.1.3.	Qiagen DNeasy® PowerSoil® (modified for marine sediments as per MM Coastal)	31
3.1.4.	Modified Qiagen DNeasy® PowerSoil® (modified for coastal seawater as per MM Coastal)	32
3.1.5.	Modified Qiagen DNeasy® PowerSoil® (modified for seagrass as per MM Coastal)	32
3.1.6.	MoBio PowerPlant® Pro DNA Isolation Kit (coral as per MM Coastal)	32
3.1.7.	Qiagen DNeasy® PowerWater® Sterivex™ Kit	32
3.1.8.	Modified Qiagen DNeasy® PowerWater® Sterivex™ Kit (as per some open ocean voyage samples)	32
3.1.9.	Modified Qiagen DNeasy® PowerWater® Sterivex™ Kit (modified for pelagic water from IMOS NRS, ACMON & voyage samples extracted at CSIRO Hobart Genetics Laboratories)	33
3.1.10	Modified MP Biomedicals FastDNA® Spin Kit for Soil (modified for soil, crust and filtered water samples from acidic saline lakes; supplied by Elizabeth Watkin)	34
3.1.11	DNA Quantification at AGRF	34
3.1.12	Plant Leaf DNA Extraction	35
3.1.13	Plant Root DNA Extraction	35
3.1.14	Plant Rhizosphere DNA Extraction	35
3.2.	RNA Protocols	36
3.2.1.	Qiagen RNeasy® PowerSoil® Total RNA Kit (marine sediments as per MM Coastal)	36
3.2.2.	Qiagen RNeasy® PowerSoil® Total RNA Kit (modified for marine sediments as per MM Coastal)	36
3.2.3.	Modified PureLink RNA Mini Kit (modified for seagrass as per MM Coastal)	38
3.2.4	Modified PureLink RNA Mini Kit (modified for sponge as per MM Coastal)	38
4	OMICS Sequencing Procedures	40
4.1	Library Preparation (incl. amplicon generation where appropriate)	40
4.1.1	Bacterial 16S rRNA gene (27F-519R) 1-step using Immolase™ DNA Polymerase (Bioline)	40
4.1.2	Bacterial 16S rRNA gene (27F-519R) 1-step using KAPA HiFi HotStart ReadyMix (Roche)	41
4.1.3	Archaeal 16S rRNA gene (A2F-519R*) 2-step using Immolase™ DNA Polymerase (Bioline)	42

4.1.4	Archaeal 16S rRNA gene (A2F-519R*) 2-step using KAPA HiFi HotStart ReadyMix (Roche)	43
4.1.5	Eukaryotic 18S v4 rRNA gene (primers) 1-step	45
4.1.6	Eukaryotic 18S v9 rRNA gene (1391F and EukBR) 1-step	46
4.1.7	Fungal ITS gene (ITS1F and ITS4) 1-step	48
4.1.8	Illumina TruSeqNano Library Preparation for Metagenomics (BASE samples)	50
4.1.9	Illumina Nextera XT Library Preparation for Metagenomics (as per MM 2015-2017)	51
4.1.10	Metagenomics Library Preparation (AM new)	51
4.1.11	Illumina Ribo-Zero rRNA Removal Kit for Metatranscriptomics (as per MM 2015-2017)	51
4.1.12	Illumina TruSeq Stranded mRNA Library Preparation for Metatranscriptomics (as per MM 2015-2017) 52	
4.1.13	rRNA removal step for Metatranscriptomics Library Preparation (AM new)	52
4.1.14	Library Preparation for Metatranscriptomics Library Preparation (AM new)	52
4.2	Next Generation Sequencing	53
4.2.1	Illumina MiSeq 2 x 300 bp paired reads	53
4.2.2	Illumina MiSeq 2 x 250 bp paired reads A	53
4.2.3	Illumina MiSeq 2 x 250 bp paired reads B	53
4.2.4	Illumina HiSeq 2500 Rapid v2 (as per BASE metagenomics sequencing methods date?)	54
4.2.5	Illumina HiSeq 2500 Rapid SBS 500-cycle v2 (as per MM metagenomics sequencing methods 2015-2017)	54
4.2.6	Illumina HiSeq 2500 Rapid SBS v4 (as per MM transcriptomics sequencing methods 2015-2017)	54
4.2.7	Illumina NovaSeq 6000	54
5	Bioinformatics	55
5.1	Bacterial & Archaeal 16S v1-3 Amplicon Analysis	55
5.2	Eukaryote 18S v4 Amplicon Analysis	55
5.3	Fungal ITS Amplicon Analysis	55
6	Known GAPS	56

1. Sample Collection Procedures

1.1. Soil Sampling (as per BASE)

Method:

1. Select a 25 x25 m plot at the sample site in a reasonably homogenous environment that reflects the characteristics of the site (based on soil, vegetation and land use).
2. Collect soil (comprising between 9-30 samples) from the plot in a manner that adequately samples the whole plot and ensures biological integrity of the sample. These can be sampled regularly on a grid if you think there is not much microgeographic variation, or the sample points could be stratified to take account of anything you think might be important.
3. Collect the samples as two depths (1) top 10 cm; (2) 20 cm and below (define). Homogenise all within plot sub-samples to make a pooled sample for each of the two depths. Samples are best mixed in the field in a large ziplock plastic bags from which aliquots can easily be drawn. Sieve if needed.
4. Ensure collection of adequate soil for nucleic acid extraction and contextual data generation from each sampling unit (e.g., 1 kg from each depth).
 - For DNA extraction: fill 50 mL Falcon tube with soil for DNA analysis (leaving 1-2 cm of space at top of tube) and freeze as soon as possible. (see DNA analysis section)
 - Samples must be gently air dried for all remaining workflows
 - For chemical analysis, sample 250 g dry weight of soil (see chemical analysis section)
 - For particle size, 180 g dry weight of soil. (see particle size analysis section)
5. Assign unique sample identifiers to each sample using the last digits after the "/" i.e. 102.100.100/8202 would be called **8202**. Each depth is to have a separate identifier.
6. Collect and record all other local contextual data listed in the 'Australian Microbiome contextual data template spreadsheet'.
7. Take photos of plot – soil and surrounding environment (example below).
8. Send each sample for DNA analysis, chemical analysis as described in sections 1.1.3 and 1.1.4 (contact the program manager for initiative specific information).
9. Submit **contextual data** into the excel sheet (<https://www.australianmicrobiome.com/protocols/>) to the Project Manager (Sophie Mazard, smazard@bioplatforms.com)

1.2. Soil Sampling (as per UTAS Urban Soil & Aerial Microbiome Study)

Soil sampling protocol 2019

Penelope Jones: Penelope.Jones@utas.edu.au

Emily Flies: Emily.Flies@utas.edu.au

1) Residential soil sampling

Materials

- Clipboard with:
 - Information and consent form (x 2 per participant)
 - Housing questionnaire
 - Soil field data sheet
- Pens
- Gloves
- Sharpee for labelling
- 2 x Large zip-lock plastic bags
- Soil corer & mallet
- Small trowel
- Scrubbing brush for corer
- Spray bottle of bleach
- Measuring tape
- Tent pegs
- Digital camera
- Small digital scale for weighing soil samples
- Rubbish bag
- Hand-held GPS
- Photographic ID on lanyards
- Plastic box for carrying samples and kit
- Falcon tubes

1. **Before you visit** Check all visits have been confirmed.
2. Check you have the spreadsheet with names, addresses, contact details and visit dates with you and that you are wearing photographic ID.
3. Pre-label the soil sample bag and dust swab transport tubes, writing directly onto the bags/tubes.
4. Pre-fill the study-IDs & AMI codes on the questionnaires and soil data sheets.

When you visit

1. Introduce yourselves and the purpose of your visit.
2. Provide the participant with the information sheet and consent form and **review it with them**. They will already have been sent the information sheet via email before signing up, so acknowledge this but explain that you need to run through it once more and have them sign the consent form while you are there. Get them to sign **two copies**: leave one signed copy with them and take the other with you for filing. *Key points to check re consent: OK to take photos, OK for soils data to be contributed to the Australian Microbiome Initiative**.
3. Fill in the housing questionnaire with the participant. Ask if you can read the questions to them and record the response (preferable), but if they wish they can fill it in themselves.

4. Take the soil sample:
 - a. Put on a fresh pair of gloves.
 - b. Scrub any chunks of dirt off the soil corer (inside/out) and then spray with bleach. Allow bleach to sit for 30 seconds – 1 minute, then take a “cleansing” soil core (to remove any bleach). Replace this core back into the yard and proceed to collecting actual soil samples.
 - c. Use the measuring tape to set up a grid across the **grassy areas** only of the backyard. This can be a 3 x 3 OR a 5 x 2 grid depending on the space but **make the grid as large as possible** (up to 25 x 25 m square). Mark out the grid corners with the tent pegs and record the grid size on the soils metadata sheet.
 - d. Use soil corer to take **12-15 sub-samples at 0-10 cm** depth across the grid. Each time, turn the corer upside-down and dump the soil into the bag (keep your hands outside on the bag). Take the actual bits of grass off before putting it in the bag. Use the trowel (cleaned with bleach) to help if the ground is very hard. Stomp down the area around the hole to minimize the hole’s visibility. *Homogenise as you go if you can.*
 - e. In addition to the grid, take some additional samples if there is ‘habitat variation’ (e.g. slope differences), that you need extra samples not on the grid to capture.
 - f. **Make sure you have at least 1 kg of soil.**
 - g. Homogenise all sub-samples into the same bag to make a pooled sample; with the bag zipped closed and with gloves on, squish the cores of soil so that they mix together. Be careful not to rip the bag. Double bag the soil bag if you are concerned it may rip.
 - h. Record all other local contextual data listed on the soils metadata field sheet.
 - i. Photograph the label of the bag. Then take photos of plot – soil and surrounding environment (*only if the participant has consented to photography*). Make sure that there is nothing identifiable in the photos: e.g. the house, a letterbox, the streetscape.
5. Thank the participant. Let them know that we hope to have results early next year and will provide them with an individual report as soon as we can.

After you have completed the day’s sampling run:

1. Return to the lab.
2. Fill in the sample visit logsheet, noting whether all samples collected successfully.
3. **File ALL paperwork in the locked drawer.**
4. Label and fill a 50 mL Falcon tube with a sub-sample of the soil for DNA analysis (make sure it was adequately homogenised before you do this). Leave 1-2 cm space at top of tube.
5. Place the falcon tubes of soil AND the dust swabs in the -80 degree freezer. The falcon tubes should go into Freezer X in freezer boxes. The swabs should go into the upright freezer, laying down in a freezer box which should be labelled on the side (they won’t fit with the lid).
6. Leave the bags of soil to air dry on the benches with the bag top open.
7. Fill in the sample storage logsheet.
8. Check on the air-drying bags of soil. If needed, shake it around to make sure the bottom bits of soil are open to the air. Once the soil is dry (this might take a few days), close the bag.

****Important note – soil samples and the Australian Microbiome Initiative***

Note that the consent form explains that if they agree, the soils data will be contributed to the Australian Microbiome Initiative. This is a national project that is trying to gather as many soil samples as possible from a diversity of Australian environments, to better understand the diversity of soil microbiomes across the continent. If the participant agrees to this, their data will provide part of this national database.

The potential concern for participants is that we have to provide the location of the soil sample (a GPS latitude and longitude), so theoretically someone could work out where the soil sample came from. We would also submit

the photographs of the soils and vegetation (taking care that the photos are as non-identifiable). We would not attach any other personal information to the sample – e.g. address, their name or anything from the housing questionnaire. It would simply be the genetic sequences, the GPS location and the contextual data you take on the AMI spreadsheet.

If they are not comfortable with the location of the sample being public, we can still take the sample but we won't submit it to the Australian Microbiome Initiative (we will process it ourselves).

2) Public green space (playground) soil sampling

Materials

- As for the residential soil sampling
- Printed and/or email copy of the council permissions

Methods

1. Identify your “high and low use areas” to sample (ideally ~25 m x 25 m each):
 - a. High use areas will be around benches/picnic tables or between pathway/gate and playground equipment
 - b. Low use areas will generally be in the centre of the grassy area but not below/too close to trees/other vegetation that would alter the microbial composition
2. Write a label on the 2 x ziplock bags in permanent marker (sample location, high intensity or low intensity, and date **& the AMI code**)

FOR THE HIGH INTENSITY AREA:

3. Put on a fresh pair of gloves.
4. Scrub any chunks of dirt off of the soil corer (inside/out) and then spray with bleach
5. Take a “cleansing” soil core (to remove any bleach). Replace this core back into the ground and proceed to collecting actual soil samples.
6. Use the soil corer provided to take **ten sub-samples at 0-10 cm** depth from the **grassy areas only**. Take the core sample, then turn the corer upside-down and dump the soil into the bag (keep your hands outside on the bag). Stomp down the area around the hole to minimize the visibility of the hole.
7. Distribute the samples across the grassy area in a way that best captures the whole ‘plot’ (preferably sample on a grid if you think there isn't much microgeographic variation, or the sample points could be stratified to take account of anything you think might be important). **Make sure you have at least 1 kg of soil.**
8. Homogenise all ten sub-samples into the same bag to make a pooled sample; with the bag zipped closed and with gloves on, squish the cores of soil so that they can mix together. Be careful not to rip the bag. Double bag the soil bag if you are concerned it may rip.
9. Collect and record all other local contextual data listed in the ‘soil field datasheet’
10. Take photos of plot – soil and surrounding environment (make sure not to include people).

REPEAT FOR THE LOW INTENSITY LOCATION

11. Take photos of high and low use locations

After you have completed the day's sampling run:

1. Return to the lab.
2. Fill in the sample visit log sheet.
3. Label and fill a 50 mL Falcon tube with a sub-sample of the soil for DNA analysis (make sure it was adequately homogenised before you do this). Leave 1-2 cm space at top of tube.

4. Place the falcon tubes of soil in the -80 degree freezer.
5. Leave the bags of soil to air dry on the benches with the bag top open.
6. Fill in the sample storage log sheet.

UTAS asthma, allergies and the microbiome pilot study

Field data-sheet – soil collection

UTAS Study ID: _____

AMI code: _____

Date sampled (YYYY-MM-DD)	
Time sampled (hh:mm)	
Latitude (decimal degrees)	
Longitude (decimal degrees)	
Sample site description*	
Vegetation total cover %*	
Vegetation dom grasses %*	
Horizon (O and/or A)*	
Elevation (m)	
Temperature (deg C)	
Grid size & sampling pattern* (draw below if necessary)	

*** See notes overleaf**

At the end of the day ALL soil metadata needs to be entered into the spreadsheet.

Notes for specific fields

Sample site

- For backyards: Backyard + population density band (e.g. 0-500)
- For playgrounds: Playground + pop density band

Soil Horizons

- O: The organic or litter layer mainly over the soil surface composed of fresh and decaying plant residue. Typically composed of >25% organic soil materials. This layer can usually be easily brushed away by hand.
- A: This is the top layer of soil (often called top soil) that typically has the highest microbial activity. Composed of a mixture of mineral material and accumulated humified organic matter. Also, any plowed or disturbed surface layer

Vegetation total cover % and dom grasses %

- In most cases should be 100% for both

Grid size and sampling pattern

- Not in AMI metadata but for our purposes
- What size grid did you lay out? What pattern?
- Did you sample regularly on the grid and/or vary by topography etc?

1.3. Marine Sediment Sampling (as per MM Coastal)

Equipment:

- Liquid nitrogen dewar
- 1.8 ml Nunc Cryotube vials
- Cryolabels
- 250 ml glass jars
- 60 ml specimen jars
- Esky & ice
- Black bag

Method:

1. Sample sediment from a depth of 5-10 m.
2. If you use a Van Veen grab, empty the sediment into clean tray without disturbing the surface layer. Use sterile gloves to collect 1.8 ml of surface layer sediment (top 1 cm) with a microspatula into a pre-labelled cryovial. Label vial according to the instruction given in the spreadsheet "sampling_schedule". Microspatula should be sterilised with ethanol wipes between grabs and gloves changed between samples.
3. If you collect while diving, use a 50 ml Flacon tubes to scoop up the top 1 cm of sediments. Transfer sediment into pre-labelled cryovial.
4. Drop cryovial into liquid nitrogen.
5. Homogenise remaining sediments in the tray thoroughly using gloved hands.
6. Collect ~60ml sediment in a plastic jar (for grain size analyses). Label jar according to the instruction given in the spreadsheet "sampling_schedule".
7. Collect ~150 ml sediment in a glass jar and store in black bag in esky on ice (for TOC analyses). Label jar according to the instruction given in the spreadsheet "sampling_schedule".
8. Cryovials to be stored in -80 °C freezer and RNA should be extracted within 1 week. Glass jars (TOC) and plastic vials (grain sizes) to be stored in -20 °C freezer and analysed within 2 weeks.

1.4. Pelagic Water Sampling for IMOS National Reference Stations

The pelagic seawater sampling carried out as part of the IMOS National Reference Station (NRS) microbial sampling is conducted as detailed in the NRS Biogeochemical operations manual (https://s3-ap-southeast-2.amazonaws.com/content.aodn.org.au/Documents/IMOS/Facilities/national_moorings/IMOS_NRS_BGCManual_LATEST.pdf) (according to the previous AMMBI project SOP). The protocol for the various sampling aspects has been provided in a series of methodological videos that can be found at to following link: <http://imos.org.au/facilities/nationalmooringnetwork/moorings-documentation/bgcwatersamplingvideos/>. This manual is maintained by the IMOS NRS BGC working group.

Reference

Davies, C. and Sommerville, E. (Eds.) (2017), National Reference Stations Biogeochemical Operations Manual Version 3.2.1. Integrated Marine Observing System.
DOI: 10.26198/5c4a56f2a8ae3 (<http://dx.doi.org/10.26198/5c4a56f2a8ae3>)

1.5. Coastal Seawater Sampling for Australian Coastal Microbial Observatory Network (ACMON; IMOS)

Standard operating procedures (SOP) for coastal sampling

These SOPs were produced to describe the step involved in a) sampling microbial communities b) obtaining relevant metadata and c) processing sample for subsequent sequence analysis as part of the marine microbiology project support by Bioplatforms Australia.

General comments:

For each sampling event request three unique BPA IDs.

For each sampling event, please measure and note the following parameters:

- 1) Longitude and latitude of sampling site in decimal degrees
- 2) Time and date in the format dd:mm:yyyy hh:mm
- 3) Depth in metres

Equipment list

- 1) Water sampler or 20 L bucket
- 2) 50 ml syringes
- 3) 0.45µm syringe filter (e.g. Millex HV Syringe Filter Unit; for nutrients)\
- 4) 50 ml Falcon tubes (for nutrient samples)
- 5) Syringe filter holder with 25 mm Whatmann GF/F filter (for chlorophyll sampling)
- 6) Cryovials
- 7) Cryovial with paraformaldehyde (for cell counts)
- 8) Pipette and tips (for 1 ml of seawater for cell counts)
- 9) Dry shipper for liquid N₂ (to freeze samples for cell counts)
- 10) Container with screw lid for disposal of pipette tips
- 11) Sterivex filters (for microbial sampling)
- 12) Peristaltic pump system with pre-filter (a 15 Falcon tube with a 100 micron mesh over the threaded part of the lid)
- 13) Data sheets
- 14) Labelling markers
- 15) Ziplock bags

SOP for sampling of seawater for DNA extraction and nutrient analysis.

1.5.1. Botany Bay, NSW, and Port Phillip Bay, VIC, ACMON Sites

1. Wash a ~20 L bucket or water sampler (e.g. Niskin system) at least five times with seawater from the site. Drop bucket/water sampler attached to a rope to ~2 m depth and retrieve. If possible, this is best done from a boat. Alternatively, collect water from a depth of ~2 m using a foldable, acid-washed canister. Take three independent buckets and note at which time each sample was taken. Assign each bucket to one of the unique BPA IDs. Perform the following steps for each bucket separately.
2. **Nutrient samples:** Suck 50 ml of seawater from bucket or canister into a syringe. Wash syringe a few times. Attach a 0.45µm syringe filter and push a few milliliters into two 50mL Falcon tubes. Wash tubes with filtrate three times. Then fill the two tubes with 40 ml of filtrate, close tube and keep on ice. Samples are then frozen

once returned to the laboratory. (-20°C). These two tubes are for nutrient analysis and please label them with the appropriate BPA ID and the word “nutrients”.

3. **Chlorophyll samples:** Use the 50 ml syringe from step 2 to 5 times filter 50 ml (250 ml in total) of seawater (from the container/bucket of step 1) through a syringe filter holder containing a 25 mm Whatman GF/F filter. If the filter does not show color, then filter more water through it. Take note of the amount of water filtered, if it is not 250 ml. At the end, press air with the syringe through the filter to dry it. Place GF/F filter into pre-labelled (BPA ID and word “chlorophyll”) cryovial and snap-freeze in liquid N₂.
4. **Flow cytometry samples:** Pipette 1 ml of seawater (from the container/bucket of step 1) in a pre-labeled cryovial (BPA ID and word “flow”) with paraformaldehyde (1% v/v final concentration). Invert tube a few times, incubate for 15 min on ice and then snap-freeze in liquid N₂. Use gloves and dispose of pipette tip into a sealed container for safe disposal back in the lab.
5. **Microbial samples:**
 - a. For microbe sampling, attach a prefilter to the intake side of a peristaltic pump tubing. The filter can be simply built by cutting a hole (0.5-1 cm) in the cap of a 15 Falcon tube and the placing a 100 micron mesh over the threaded part of the lid. Put the lid back on to jam the mesh between the lid and the tube. Cut the bottom of the tube and attach it to the intake side of the peristaltic pump line. Filter 2 litres of seawater using a peristaltic pump onto a 0.22 micron sterivex filter. Use speeds of up to ~120 rpm and platinum cured silicone pump tubing (8.0 mm dia. Internal diameter 3.2 mm e.g. Masterflex L/S 16) or similar. If the filter gets blocked before 2 litres are filtered, then note the volume filtered.
 - b. When filtration is completed, disconnect the inlet and pump air through the filter for 1-2 min to remove any remaining liquid.
 - c. Disconnect filter and label with the BPA ID. Cap both ends of the sterivex filter [alternatively mould some clean blutak around the ends to seal if you have the version with a luer lok on one side only]. Place the filter in a ziplock bag.
 - d. Freeze immediately at -80 degrees.

1.5.2. Great Barrier Reef, QLD, ACMON Sites

1. Wash a 5 L Niskin with seawater from the site. Collect water sample from surface and bottom at each site. Take three independent samples and note at which time each sample was taken. Assign each replicate to a unique BPA ID. Perform the following steps for each niskin separately.
2. **Flow cytometry samples:** Wearing nitrile gloves, fill a 2L acid-washed bottles to the brim with seawater from each replicate niskin collection using acid washed funnel. Pipette 1 ml of this seawater into a pre-labeled cryovial (BPA ID and word “flow”) and add 63ul 16% paraformaldehyde (1% v/v final concentration) in fume hood. Invert tube a few times, incubate for 15 min in the fridge and then snap-freeze in liquid N₂. Dispose of pipette tip into a sealed container (Formaldehyde waste) for safe disposal back in the lab. Repeat for each replicate water sample.
3. **Microbial samples:**
 - a. Push a 10ml serological pipette (filter removed with sterile tweezers) into each inlet tubing end and place in the 2L bottle. Run ~200 mL seawater through tubing (with pump flow controller set at midpoint) then pause the pump.
 - b. For each water sample at a site, attach a 0.22um sterivex filter to the luer lock outlet of the peristaltic pump tubing. Filter 2 litres of seawater using a peristaltic pump using speeds of up to ~120 rpm (Pharmed pump tubing 8.0 mm dia. Internal diameter 3.2 mm e.g. Masterflex L/S 16 or similar). If the filter gets blocked before 2 litres are filtered, please note the volume filtered.
 - c. When all water is filtered, disconnect the inlet and pump air through the filter for 1-2 min to remove any remaining liquid.

- d. Disconnect filter and label with the BPA ID. Cap both ends of the sterivex filter. Wrap filters in foil and place in a LN2 resistant whirlpak bag.
 - e. Snap freeze in LN2 by dipping with a canister, and then place in -20 freezer until transfer to -80 degree freezer.
 - f. To clean the pump tubing after use, run 200 mL 5-10% bleach solution through tubing, followed by 200 mL milliQ or distilled water, and lastly 200 mL 70% EtOH (to dry the tubing and prevent biofilm growth between uses).
4. **Nutrient Samples as per AIMS WQ Program:** A separate niskin cast was done at each site/depth and this single cast was used to do:
- 1 x salinity (refrigerated) – 250ml seawater into acid-washed bottle.
 - 1 x CDOM (refrigerated) – 50ml filtered through 0.2um Pall Acropak supor filter into amber glass bottle. Keep additional filtrate (>200ml) for wet filter blanks - 10ml for each duplicate GFF sample below (total 8 blanks) and 60ml for each duplicate TSS sample (2 blanks).
 - **GFFs** (using vacuum manifold) plus 2 wet filter blanks for each:
 - 2 x Chl-a (frozen at -20) – 100ml filtered through 25mm Whatman GF/F. Filters folded in half (sample side inwards) and wrapped in foil.
 - 2 x Particulate C (frozen at -20) – 500ml filtered through 25mm Whatman GF/F. Filters folded in half (sample side inwards) and wrapped in foil.
 - 2 x Particulate N (frozen at -20) – 500ml filtered through 25mm Whatman GF/F. Filters folded in half (sample side inwards) and wrapped in foil.
 - 2 x Particulate P (frozen at -20) – 250ml filtered through 25mm Whatman GF/F. Filters folded in half (sample side inwards) and wrapped in foil.
 - 2 x TSS (pre weighed filters in 20ml scint vials) – 1L filtered through 47mm polycarbonate 0.4um filter. Filters folded in half and half again (sample side inwards) placed in a Scint vial and stored frozen at -20. Also 2 x wet filter blanks as described above with 60ml 0.22um filtered seawater passing through each.
 - Dissolved nutrients:
 - 2 x DIN (frozen at -20) – 10ml 0.4um filtered seawater into acid-washed sample tubes
 - 2 x TDN (frozen at -20) – 10ml 0.4um filtered seawater into acid-washed sample tubes
 - 2 x Silica (refrigerated) – 10ml 0.4um filtered seawater into acid-washed sample tubes
 - 2 x DOC (refrigerated, HCl added) - 10ml 0.4um filtered seawater into acid-washed sample tubes with 100ul hydrochloric acid added. Do not freeze.
 - 1 x alkalinity/DIC – 250ml seawater into acid-washed Schott bottle filled to the brim. 125ul mercuric chloride added. Store at RT in cool environment.

1.6. Pelagic Water Sampling for Voyages (provided by CSIRO Environmental Genomics team)

Equipment:

1. Seawater sampling bottles (plastic) with screw cap lids × 8
2. Bottle carriers × 2 (each carrier will hold 4 bottles)
3. Peristaltic pump, attached to a (preferably) 8-head pump head
4. Tubing with luer-lock attachment, attached to pump heads
5. 0.2 µm Sterivex™ filter units with female Luer-lock inlet and male luer lock outlet
6. Small snap-lock bags for storage of individual filter units
7. Larger snap-lock bags for storage of all filter units from a single sampling station
8. Laboratory single-use gloves
9. Sterivex inlet and outlet caps OR Parafilm
10. 50 mL Luer-lock disposable syringes
11. Sampling log sheet (see attached appendix)
12. 5-10% bleach
13. 70% EtOH

Method:

Pre-sampling preparation

1. **Sterivex filter labelling:** The preferred Sterivex filter labelling convention would be “CCC-RR-depth” with CCC corresponding to the CTD cast number and RR corresponding to the CTD rosette/Niskin bottle number the sample was taken from. For example, if the sample was taken from CTD cast 1, and niskin bottle number 36, at 100 m depth, the label on the Sterivex filter would be “001-36-100 m”
2. If Sterivex inlet caps were not provided with the sampling box for the voyage, Parafilm should be pre-cut to an appropriate size to seal both ends of the Sterivex filter after filtering.
3. The sampling bottles should be rinsed with MilliQ water before sampling each cast.
4. The tubing (with luer lock attachment) should be flushed with 70% ethanol at least once a day before sampling and flushed with MilliQ water after every filtering procedure before use in subsequent rounds of filtering.

At Sampling Station:

5. Conduct CTD-Niskin bottle casts to pre-determined depths.

Samples are usually collected at 6-8 depth points but this is dependant on full depth of water column and CTD cast regime/voyage priorities etc.

We are only interested in samples from the same depth that hydrochemistry are conducting analyses.

Surface, deep chlorophyll maximum, mixed layer depth, and max cast depth (bottom depth where possible) are the most important; other samples through the water column we usually aim for are; 500 m, 1000 m, 2000 m, etc. that is, 1000 m intervals till bottom). Ideally these depths will be consistent at all stations but understand there are variations depending on depth at individual stations.

6. While collecting seawater samples from the Niskin bottles, rinse bottle and bottle cap at least once with seawater sample, discard, and fill the bottle with 2 L of seawater. If possible, overfill to the brim before capping and transporting to filtering station.

If filtering isn't occurring immediately, please place bottles (for a maximum of 4 hours) in a cold and dark location (such as refrigerator) to minimize changes.

At Filtering Station:

7. Wearing gloves, place one end of tubing in the bottles and pass tubing through pump head ensuring barb/luer lock fitting is attached to the end of the tubing (but without filter attached).
8. Run ~200 mL (or until the seawater level reaches the 2 L mark on the sampling bottle) seawater to flush the tubing, without filter attached (with pump flow controller set at midpoint).
9. Attached labelled Sterivex filter (labelled according to the guide mentioned in 1. above) to the luer lock fitting, connected to the tubing of the respective bottle of sample.
10. Filter the sample with the pump controller set to midpoint.

*If filter blocks before 2 L has passed through, stop pump and record volume of water that was filtered in the **Comments** section of logsheet. Generally, this should not happen with seawater samples collected from open ocean locations.*

11. After 2 L has passed through filter, let the pump continue for ~2 min to assist in drying filter.
12. Disconnect each filter from tubing, connect to a 50 mL syringe and purge out as much residual water from inside the filter housing as possible.
13. Cap both ends of filter with the respective inlet/outlet caps place in an individual snap-lock bag

If small snap-lock bags are not available, save the wrappers the Sterivex filters came in and use this to store the filters individually after filtering.

If inlet/outlet caps are not available, seal the ends of the filter with pre-cut Parafilm mentioned above.

14. Place individually wrapped filters from one sampling station into a larger ziplock bag. Label the large ziplock bag with
 - the CTD cast/sampling station number,
 - date
 - total number of Sterivexes/depths at which samples were collected from that station.
15. Store samples at -80 °C as soon as possible after filtering.
16. Record sample details in the sampling log sheet.
17. To clean the pump tubing after use, run 200 mL 5-10% bleach solution through tubing, followed by 200 mL milliQ or distilled water, and lastly 200 mL 70% EtOH (to dry the tubing and prevent biofilm growth between uses).

1.7. Coastal Seawater Sampling for Downstream DNA Analyses (as per MM Coastal ORIGINAL)

Method:

1. Wash a ~20 L bucket at least five times with seawater from the site. Drop bucket attached to a rope to ~2 m depth and retrieve. If possible, this is best done from a boat. Alternatively, collect water from a depth of ~2 m using a foldable, acid-washed canister.
2. Suck 50 ml of seawater from bucket or canister into a syringe. Wash syringe a few times. Attach a 0.45 µm syringe filter and push a few millilitres into three 50mL Falcon tubes. Wash tubes with filtrate three times. Then fill tubes with 40 ml of filtrate, close tube and keep on ice. Samples are then frozen once returned to the laboratory. (-20 °C). These three tubes are for nutrient analysis and please label them according to the instruction given in the spreadsheet "sampling_schedule".
3. Use the 50 ml syringe from step 2 to 5 times filter 50 ml of seawater (from the container/bucket of step 1) through a syringe filter holder containing a 25 mm Whatman GF/F filter. If the filter does not show colour, then filter more water through it. At the end, press air through it to dry filter. Place GF/F filter into pre-labelled cryovial and snap-freeze in liquid N on site. Repeat three times. These filters will be used for chlorophyll analysis.
4. Pipette 1 ml of seawater (from the container/bucket of step 1) in a pre-labelled cryovial with paraformaldehyde (1% v/v final concentration). Invert tube a few times and then snap-freeze in liquid N on site. Repeat three times. Use gloves and dispose of pipette tip into a sealed container for safe disposal back in the lab. These samples will be used for cell counts.
5. For microbe sampling, attach a prefilter to the intake side of a peristaltic pump tubing. The filter can be simply built by cutting a hole in the cap of a 15 Falcon tube and the placing a 100 micron mesh of the threaded part of the lid. Put the lid back on to jam the mesh between the lid and the tube. Cut the bottom of the tube and attach it to the intake side of the peristaltic pump line. Filter ~2 L of seawater separately three times within 2 hours using a peristaltic pump onto three separate 0.22 micron sterivex filter. Use speeds of up to ~120 rpm and platinum cured silicone pump tubing (8.0 mm dia. Internal diameter 3.2 mm e.g. Masterflex L/S 16) or similar.

6. When the 2 L has been filtered continue to run the pump for 1-2min to remove all of the liquid. If the filter gets blocked before, then note the approximate volume filter, disconnect the inlet and try to pump air through the filter for 1-2 min to remove remaining liquid.
7. Ensure that the sterivex is labelled according to the instruction given in the spreadsheet "sampling_schedule". Cap both ends of the sterivex filter [alternatively mould some clean blutak around the ends to seal if you have the version with a luer lok on one side only]. Place the filter in a ziplock bag.
8. Freeze immediately at -80 °C.

1.8. Coastal Seawater Sampling for Downstream DNA Analyses (as per MM Coastal UPDATED SEPT 2019 TT)

Method:

1. Wash three 3-4 L containers at least five times with seawater from the site and label with numbers 1-3. Drop containers separately attached to a rope to ~2 m depth and retrieve. Take note of the time you took the three individual samples. If possible, this is best done from a boat. Alternatively, collect water from a depth of ~2 m using a foldable, acid-washed containers. Again, take note on when you took each sample. These three sample represent replicates and should be handled separately in the following steps.
2. Suck 50 ml of seawater from the container into a syringe. Wash syringe a few times. Attach a 0.45 µm syringe filter and push a few millilitres into three separate 50mL Falcon tubes. Wash tubes with filtrate three times. Then fill each tube with 40 ml of filtrate, close tube and keep on ice. You should have three 50 mL tubes per sample replicate. Samples are then frozen once returned to the laboratory. (-20 °C). These tubes are for nutrient analysis and please label them according to the instruction given in the spreadsheet "sampling_schedule".
3. Use the 50 ml syringe from step 2 to 5 times filter 50 ml (i.e. total of 250 mL) of seawater (from the container of step 1) through a syringe filter holder containing a 25 mm Whatman GF/F filter. If the filter does not show colour, then filter more water through it. Take note of the volume of water filtered. At the end, press air through it to dry filter. Place GF/F filter into pre-labelled cryovial and snap-freeze in liquid N on site. These filters will be used for chlorophyll analysis.
4. Pipette 1 ml of seawater (from the container of step 1) in a pre-labelled cryovial with paraformaldehyde (1% v/v final concentration). Invert tube a few times and then snap-freeze in liquid N on site. Repeat three times. Use gloves and dispose of pipette tip into a sealed container for safe disposal back in the lab. These samples will be used for cell counts.
5. For microbe sampling, attach a prefilter to the intake side of a peristaltic pump tubing. The filter can be simply built by cutting a hole in the cap of a 15 Falcon tube and the placing a 100 micron mesh of the threaded part of the lid. Put the lid back on to jam the mesh between the lid and the tube. Cut the bottom of the tube and attach it to the intake side of the peristaltic pump line. Filter ~2 L of seawater within 2 hours using a peristaltic pump onto 0.22 micron sterivex filter. Use speeds of up to ~120 rpm and platinum cured silicone pump tubing (8.0 mm dia. Internal diameter 3.2 mm e.g. Masterflex L/S 16) or similar.
6. When the 2 L has been filtered continue to run the pump for 1-2min to remove all of the liquid. If the filter gets blocked before, then note the approximate volume filter, disconnect the inlet and try to pump air through the filter for 1-2 min to remove remaining liquid.
7. Ensure that the sterivex is labelled according to the instruction given in the spreadsheet "sampling_schedule". Cap both ends of the sterivex filter [alternatively mould some clean blutak around the ends to seal if you have the version with a luer lok on one side only]. Place the filter in a ziplock bag.
8. Freeze immediately at -80 °C.

1.9. Coastal Seawater Sampling for Downstream RNA Analyses (as per MM Coastal)

1. Use the same filtering set-up as in step 5 above (1.5.1) to filter ~2 L of seawater separately three times onto three separate 0.22 micron sterivex filter. The filtering should be completed within 20 min of the water sample being collected.
2. When the 2 L has been filtered continue to run the pump for 1-2 min to remove all of the liquid. If the filter gets blocked before, then note the approximate volume filter, disconnect the inlet and try to pump air through the filter for 1-2 min to remove remaining liquid.
3. Ensure that the sterivex is labelled according to the instruction given in the spreadsheet "sampling_schedule" and append an "RNA" at the end.
4. Cap the sterivex filter at one end and add 1.6 ml of RNAlater (Sigma). Cap the other end and shake well to distribute solution. Place the filter in a ziplock bag.
5. Freeze immediately at -80 °C.

1.10. Coastal Seawater Sampling for Downstream DIC & TA Analyses (as per MM Coastal)

1. Use (250 mL)- borosilicate glass bottles with stopper or with screw cap to collect samples. All bottles should be acid washed and well rinsed with seawater.
2. Collect at depth of 2 m. Either fill bottles underwater and seal underwater to avoid excess gas exchange. Alternatively, lower bottle in bucket (see above) and then close under water. If sampling using a Niskin bottles, then attach tubing (make sure there are no gas bubbles in tubing) to tap of Niskin bottle, insert the tubing into the bottom of the sample bottle and begin water flow. Allow the bottle to fill completely and to overflow 3 full volumes.
3. Collect n=3 per site. Labelled bottles according to the instruction given in the spreadsheet "sampling_schedule".
4. Leave 1-2cm head-space in sample bottles.
5. Add 100 µl for 250 mL bottle (adjust for volume of container) of HgCl₂ using a pipette. Make sure to use a pipette dedicated for toxics. Do not submerge pipette tip into sample.
6. Note: Prepare a saturated solution of mercuric chloride: 1:10 ratio for a saturated HgCl₂ solution; e.g. 10 g mercuric chloride salt per 100 mL DIW. Standard volumes used for saturated HgCl₂ solutions are 0.02-0.05% of the total sample volume.
7. Invert the sample several times to mix the mercuric chloride thoroughly.
8. Seal the sample bottle with silicon grease around the stopper and a rubber band. If using screw cap seal with parafilm.
9. Store bottles at cold/ambient temperature in the dark.

1.11. Seagrass Sampling (as per MM Coastal)

Equipment:

- Van Veen grab
- Liquid nitrogen dewar (to be used both, on site and in the lab)
- 1.8 mL Nunc Cryotube vials
- Cryolabels
- Aluminium foil
- Gloves
- Scissors
- 250 mL glass jar
- 100 mL specimen jars
- Esky and ice
- Black bag
- Plastic resealable bags

General Notes:

First time the site is visited, abundance of *Zostera muelleri* will be measured over 10 randomly thrown 50cmx50cm quadrats.

1.11.1. Surficial Sediment Sampling

NOTE: samples to be collected *immediately before* seagrass samples (for each plant, both surficial sediment and plant tissue will be collected).

1. Pull out a seagrass plant and, out of water, collect surficial sediment, using sterile gloves onto a clean tray.
2. Homogenize sediments with gloved hands and avoid collecting roots/rhizome.
3. Fill a 1.8 mL Nunc cryotube vial up to the top (collect up to 3 cryotube vials/sample). Label tubes according to the scheme outlined in the spreadsheet "sample_schedule".
4. Flash freeze cryotube vials in liquid nitrogen, using the dewar brought to the field, and keep the samples on ice for transport to the laboratory.
5. Store samples in -80 °C freezer for further analysis.
6. RNA and DNA should be extracted from sediment within 2 weeks.
7. Collect ~200 mL sediment in a standard glass jar and store in black bag in esky on ice (for nutrients and TOC analyses). Label according to the scheme outlined in the spreadsheet "sample_schedule".
8. Store glass jars in -20 °C freezer to be analysed within 2 weeks.
9. Collect ~60 mL sediment in a standard plastic jar (for grain size analyses). Label according to the scheme outlined in the spreadsheet "sample_schedule".
10. Store plastic jars at room temperature for further analysis.

1.11.2 Seagrass Sampling

NOTE: samples to be collected *immediately after* surficial sediment samples (for each plant, both surficial sediment and plant tissue will be collected).

1. Collect seagrass healthy plants, using sterile gloves. Select *only* green, no-damaged plants.
2. Separate *only* the leaves (above ground biomass) from the rest of plant tissues by using sterile scissors (section at the interface between leaves and roots).
3. Discard the seagrass roots/rhizome tissue (below ground biomass), making sure that necrotic/dark plant tissue is *not* collected.
4. Wash seagrass leaves with seawater *directly in the ocean* until all sediment and littler is detached. Be careful not to damage the leaves.
5. Pack the seagrass leaves without sediment into aluminium foil envelopes previously labelled according to the scheme outlined in the spreadsheet "sample_schedule", and flash freeze these envelopes in liquid nitrogen (or place in dewar).
6. After return to the laboratory, store aluminium envelopes in a labelled box in -80 °C freezer for further analysis.
7. RNA should be extracted from leaves within 2 weeks.

1.12. Sponge Sampling (as per MM Coastal)

General Notes:

1. First time the site is visited, sponge species abundance will be measured over 10 random quadrants. This will be repeated as required during the 14 months observation period.
2. Select only sponge individuals that appear healthy and document specimen with a photo.
3. Three different individuals will be collected at each sampling event.
4. Record the following parameter for each individual: size (approximate in cm); covered with detritus (yes/no); any contact with other benthic, sessile organisms (yes, no; if yes, specify); morphological anomalies (discolouration; outgrowth etc.); epiphytic growth (type and coverage)

Method:

1. Cut sponge samples underwater into small pieces (~5 g) using sterile blades. For each sponge specimen (n=3) collect six pieces each covering the entire body of the sponge
2. Transfer pieces into 50 ml Falcon tube. Label containers according to the instruction given in the spreadsheet "sampling_schedule".
3. On the surface after the dive, use sterile gloves to squeeze water out of three sponge, pieces per sponge, transfer to a fresh 15 ml Falcon tube and plunge into liquid nitrogen (for RNA extraction). Label tubes according to the instruction given in the spreadsheet "sampling_schedule" and add "R" at the end (for RNA extraction).
4. On the surface, wash three pieces three times in sterile seawater (5 min), transfer to a fresh 15 ml Falcon tube and then plunge into liquid nitrogen (for amplicon sequencing). Label tubes according to the instruction given in the spreadsheet "sampling_schedule" and add "A" at the end (for amplicon analysis).

1.13. Seaweed (*Ecklonia radiata*) Sampling (as per MM Coastal)

General Notes:

1. First time the site is visited, abundance of *E. radiata* will be measured over 10 randomly thrown 50x50 cm quadrats. This will be repeated as required during the 14 months observation period.
2. Select randomly from a kelp bed only those seaweed individuals that appear healthy and document specimen with a photo.
3. Three different healthy specimens are analysed at each sampling event.
4. Record the following parameter for each specimen: length (approximate in cm); any contact with other benthic, sessile organisms (yes, no; if yes, specify); morphological condition (discolouration; outgrowth, spots etc.); epiphytic growth (type and coverage via categories <10, 10-25, 25-50, 50-75, >75%), photosynthetic efficiency *in situ* (quantum yield using a Diving-PAM fluorometer; if available (see Additional Information below), evidence of grazing (presence/ absence and estimated % of thallus affected via categories <10, 10-25, 25-50, 50-75, >75%)

Method:

1. Collect for each of the three replicate specimens the middle section of a secondary lamina located at approximately the same distance from the meristem (see Additional Information below). Make sure the collected tissue has an area $\geq 30 \text{ cm}^2$ (enough for swabbing and PAMing; see below).
2. Enclose samples individually inside press-seal bags *in situ* and bring to the surface. Label the bag according to the instruction given in the spreadsheet "sampling_schedule".
3. On the surface, rinse the algal samples with filtered-sterilised seawater. Use a 50 ml syringe with a 0.22 μm filter to run sterilised seawater for about 10-20 second over thallus to remove parts (e.g. detritus) that are not biofilm.
4. Use a sterile cotton tip (head size about 1 cm) and swab an area of approximately 20 cm^2 of the lamina gently (i.e. without breaking the algal tissue) for 30 seconds
5. Transfer the tips of the swabs into individual sterile cryo-tubes, label the tubes according to the instruction given in the spreadsheet "sampling_schedule" and immediately store in a dry-shipper with liquid nitrogen onsite (or -80 °C for long-term).

1.14. Coral Sampling (as per MM Coastal)

General Notes:

1. Relative abundance of chosen coral species at each site should be contextualised on each visit by 10 randomly thrown 50x50 cm quadrats.
2. Select several colonies that are of dominant and consistent appearance (colour morph, growth pattern etc.) and document each colony with a photo.
3. Three different healthy specimens are analysed at each sampling event.
4. Record the following parameters for each specimen: size (approximate in cm); any contact with other benthic, sessile organisms (yes, no; if yes, specify); morphological condition (discolouration; outgrowth, spots etc.); epiphytic growth (type and coverage (%)), evidence of grazing (presence/ absence and estimated % affected)

Method:

1. Collect coral mucus samples of 3 colonies with autoclaved cotton swabs placed in 2 ml Eppendorf tubes (see picture). Invert those tubes and open underwater next to the coral surface to minimize contamination with microbes from the surrounding seawater. Swabs should be gently rolled over the coral surface and then placed back in the tube. Labelled tubes according to the instruction given in the spreadsheet "sampling_schedule".
2. Once on the surface, remove excess seawater from the tube without touching the swab and snap freeze sample in liquid Nitrogen.
3. Also remove 3 random samples per coral colony of the same 3 colonies (avoid sampling of the swabbed location). Enclose the 3 random samples separated for each colony inside press-seal bags (or Falcon tubes) with surrounding seawater *in situ* and bring to the surface. Each sample should comprise of a fragment 5-10mm in length or a small branch (species dependent). Labelled tubes according to the instruction given in the spreadsheet "sampling_schedule"
4. Water is emptied before sample is snap frozen in liquid nitrogen to preserve the mucus layer onsite (liquid N is transported in dry shipper).
5. In the laboratory, remove mucus and tissue with an air gun and 1X PBSE pH 7.4 (137 mM NaCl (8.07 g/L), 2.7 mM KCl (0.201 g/L), 4.3 mM Na₂HPO₄ (0.611 g/L), 1.4 mM KH₂PO₄ (0.191 g/L), 10 mM EDTA (20 ml 0.5 M EDTA). Liquefy coral tissue by repeatedly drawing into a 5 ml syringe or using a hand-held homogenizer. Divide samples into 1.5 ml cryo-tubes and centrifuge at 13,000 rpm for 10 minutes. Remove top liquid phase and discard keeping the cell pellet. Freeze samples at -80 °C (or -20 °C).



1.15. Water sampling from acidic saline lakes (supplied by Elizabeth Watkin/Katelyn Boase)

As per the Coastal seawater sampling methodology (1.7) with the following modifications:

1. Skim water from shallow pools, taking care not to touch sediment, swirl in the bucket, and dump out to rinse. In the same manner, collect ~ half a bucket of water for sampling.
2. Suck 50 ml of seawater from bucket into a syringe. Wash syringe a few times. Attach a 0.45 µm syringe filter and push a few millilitres into three 50 mL Falcon tubes. Wash tubes with filtrate three times. Then fill tubes with 40 ml of filtrate, close tube and keep on ice. Samples are then frozen once returned to the laboratory (-20 °C). These three tubes are for nutrient analysis and please label them according to the instruction given in the spreadsheet "sampling_schedule".
3. Use the 50 ml syringe from step 2 to 5 times filter 50 ml of seawater (from the bucket of step 1) through a syringe filter holder containing a 25 mm Whatman GF/F filter. If the filter does not show colour, then filter more water through it. At the end, press air through it to dry filter. Place GF/F filter into pre-labelled cryovial and snap-freeze in liquid N on site. Repeat three times. These filters will be used for chlorophyll analysis.
4. Pipette 1 ml of water (from the bucket of step 1) into a pre-labelled cryovial with paraformaldehyde (1% v/v final concentration). Invert tube a few times and then snap-freeze in liquid N on site. Repeat three times. Use gloves and dispose of pipette tip into a sealed container for safe disposal back in the lab. These samples will be used for cell counts.
5. For microbe sampling, we use PES filter towers (0.2 µm and 0.1 µm Nalgene™ Rapid-Flow™ Sterile Disposable Filter Units with PES membrane #catalogue numbers TBC) with a hand vacuum pump and 500 mL – 1L capacity and filter until the salt blocks the filter. Aim to filter 1 L, but stop and record volume filtered if less than this.
6. When the water has been filtered continue to run the pump for 1-2min to remove all of the liquid. If the filter gets blocked before, then note the approximate volume filtered, and try to pump air through the filter for 1-2 min to remove remaining liquid.

7. Filters are placed in sterile Whirlpak bags and labelled appropriately.
8. Freeze immediately at -80 °C.

1.16. Water sampling from Brisbane river estuary (supplied by Christian Rinke/Apoorva Prabhu)

Please note, the below protocol includes all aspects of Brisbane River program, sampling, flowcytometry, DNA extraction.

A. Sampling SOP (every two months)

1. At each of the sampling site (Shorncliffe = SH, Eagle Farm = EF, Indooroopilly = IN), collect ~35L of subsurface water off the deck/pontoon using smaller containers and transfer to the 35L container.
2. Label the container and wheel it back to the car, where it is transported into the vehicle using a wooden plank/crates.
3. Collect 250ml unfiltered sample (in triplicates) from each sampling site and transfer into **green** containers supplied by ALS Environment testing for checking chemical parameters (Nitrates/Nitrates). Label the bottles with name, date and time of sampling.
4. Collect 60ml unfiltered sample (in triplicates) from each sampling sites and into **purple** containers supplied by ALS Environment testing for checking chemical parameters (Ammonium, Phosphates). Label the bottles with name, date and time of sampling.
5. Collect 60ml of field filtered (0.45um) sample in triplicates, from each sampling sites, into **red** containers supplied by ALS Environment testing for checking chemical parameters (Silicates). Label the bottles with name, date and time of sampling
6. Record physical parameters of surface waters (10cm deep) with Horiba probe (temperature, pH, dissolved oxygen, salinity, TDS, GPS for location). Take observations in triplicates, and record them onto table (below) and backup the observations with pictures of the readings.
7. Store the green, purple and red bottles in eskys filled with frozen ice packs. At the end of sampling, carry the quote, sample list and hand over the bottles to ALS Environment Testing Centre, Blyth Street, Stafford, Brisbane. Also email them a list of samples as well as temperature and pH from each sampling site.
8. Transport ~35L of the water sample (SH, EF and IN) to the lab at ACE, Level 5, Building 76, UQ St Lucia using trolleys.

B. Physico-Chemical Parameters

1. Using the Horiba, record the readings (temperature, pH, dissolved oxygen, and GPS for location) in triplicates from each sampling site (SH, EF and IN) on the contextual metadata table.
2. Record the values for dissolved silicates, nitrates, nitrites, ammonium, phosphates as obtained from ALS Environmental Testing onto the contextual metadata.

C. Sample processing by filtration (Filtermaster 2001, designed and fabricated by Dr Rinke, ACE)

1. Prepare a filtration setup for amplicon samples. This includes three PVC lines connected to each of the 35L sample containers to their respective 0.22um Sterivex filters through a peristaltic pump.
2. Keeping rotation less than 100rpm, filter X volume of samples using the 0.22um Sterivex filter.
3. Seal the Sterivex filters using filter caps and store them in UV-ed 50ml falcon tubes and store at -20°C until further processing.
4. For metagenomics samples, the filtration setup is prepared such that water from 2 x 35L containers (SH and EF at first, then IF) is drawn simultaneously into 2 x filtration towers containing 8um pre-filters, with the help of a peristaltic pump set at 100rpm. The 2 x filtration towers are, in turn 6x 0.22um Sterivex lines (3x for each sample)

and the sample is filtered out into 6 x 10L containers. Filter the X L of the sample through EACH of the Sterivex filters. *Note: The filtermaster 2001 will allocate different volumes to each Sterivex filter. Therefore, it is important to watch the volume flowing through each filter. Once the first filter reaches the required sample volume, stop the flow to this filter using a flow control clip tubing roller, this will allocate the flow to the remaining filters and speed up filtration. You can now remove this filter.* 5. Seal the 0.22um Sterivex filters using filter caps, and store the Sterivex and the 8um prefilter for respective samples in UV-ed 50ml falcon tubes

and store at -20°C until further processing. 6. For microbial enumeration, prepare 2% glutaraldehyde stocks of 5x whole water samples, 5x 8um filtered samples, 5x 0.22um filtered samples from each of the sampling site. Flash freeze the stocks in liquid nitrogen and store at -80°C.

Fig: Part of filtermaster 2001. The blue house is connected to the 8um prefilter tower, with the three lines connected to 0.22um Sterivex filters.

D. DNA extraction by Phenol-Chloroform

1. Defrost the filter and seal the bottom of the 0.22um Sterivex filter using a Sterivex cap.
2. Fill the filter with 1.8ml Lysis buffer and add 18ul of lysozyme (100mg/ml) to the Sterivex filter. Cap the top of the filter.
3. Incubate in a beaker of water for 1h in a shaking incubator at 37°C.
4. Add 200ul of Proteinase K (2mg/ml) and incubate in a shaking 100rpm incubator for 1h at 55°C.
5. Recover the lysate into 4 x 2ml tubes such that 600ul was collected into 2 tubes from each Sterivex.
6. Add equal volumes of Phenol:Chloroform:Isoamyl alcohol (25:24:1) and invert to mix.
7. Spin at max 16,000g for 10min, recover aqueous phase to a new tube.
8. Add equal volumes of Chloroform:Isoamyl alcohol (24:1) and invert to mix.
9. Spin at max 16,000g for 10min, recover aqueous phase to a new tube.
10. Precipitate DNA with 600ul of isopropanol and let sit for 15mins at RT.
11. Spin at max 20,000g for 30min at RT, discard supernatant.
12. Add 500ul of 70% EtOH, flick tube to mix.
13. Spin at max 20,000g for 10min at RT, discard supernatant.
14. Air dry till EtOH is evaporated.
15. Resuspend the pellet in 25-50ul of sterile water and incubate overnight at 4C.
16. Quantify DNA with Qubit and store at 20C. Record the results in the contextual metadata.

E. Sample submission for Sequencing

1. Submit x ul of samples from each sampling site (SH, EF & IN) for metagenomics sequencing (~10Gb)
2. Submit y ul of samples from each sampling site for amplicon sequencing
3. Record results in the contextual metadata.

F. Microbial Enumeration of samples (Cytotflex protocol communicated by Dr Martin Ostrowski, UTS)

Note: limit of size for autotrophs is 100 µm and TE buffer is preferred to PBS for clearer cytograms for flow cytometry.

Reagents

Tris-EDTA (TE) Buffer

1. Purchase molecular grade TE buffer – 93283 Sigma Aldrich 500 mL ~\$100
2. Filter through a 0.2 µm syringe filter to ensure a clear solution.

SYBR Green I Nucleic Acid Gel Stain 10,000X concentrate in DMSO - Invitrogen S7563 ~\$429 for 500 µL *Need a 5×10^{-5} final concentration of SYBR Green in sample for staining (Marie et al. 1999, Brussaard et al. 2004, Seymour et al. 2005, Patten et al. 2008).

1. Make a working stock of a 1:500 dilution of SYBR Green in TE Buffer. To do this, add 10 µL to 4990 µL TE Buffer in a 15 mL centrifuge tube. Aliquot 500 µL into Eppendorf tubes and freeze until needed. One 500 µL tube will be enough to analyse 1 plate of samples.

Reagents Preparation

1. Thaw one of the 500 µL aliquots of the 1:500 diluted SYBR Green working stock.
2. Transfer the 500 µL to a 50 mL centrifuge tube and add 15.5 mL TE Buffer. This will further dilute the SYBR Green working stock by 1:32 (which will yield a final dilution of 1:20,000 once added to the plate).

Note: this is enough for one full plate of samples.

Number of Wells	SYBR Green Working Stock	TE Buffer
96	500µL	15.5mL
72	375µL	11.625mL
48	250µL	7.75mL
36	187.5µL	5.815mL
24	125µL	3.875mL
12	62.5µL	1.94mL
6	31.25µL	0.97mL

3. Filter sterilize the SYBR Green working stock using a syringe and disposable 0.2 µm filter
4. If you are simultaneously analysing stained and unstained fractions of the samples (to determine back ground levels), filter sterilize an extra 16 mL TE buffer using a syringe and disposable 0.2 µm filter.

Sample Preparation Procedure

1. Set up flow cytometer.
2. Turn on heat block to 37°C.
3. Collect samples from liquid nitrogen or -80C freezer.
4. Thaw samples at 37°C on heat block for approx. 2-3 mins.
5. Viruses, bacteria and autotrophs need to be analysed on separate plate.
6. Prepare Autotroph plate first and run while you prepare the virus, then prepare the bacteria plate while the virus plate is running.

Autotrophs

1. Prepare size beads by diluting 1 drop beads into 300 uL MQ.
2. Add MQ to wells B1-B2 and B10, B11, B12. 3. Add 200 uL Yellow Green Beads (YB) to well B3.
4. Add size beads to wells B4-B9 in reverse order of size (2.0, 1.0, 0.5, 0.2, 0.1, 0.02 µm).

5. Add 200 uL neat sample to well remaining in the 96 well plate. (e.g. x1 CTD per row, therefore 8 samples per row).
6. Add MQ in two wells at the end of samples in each row as blanks.

Viruses To set up virus unstained plate:

1. Add 160 uL of TE Buffer to each well.
2. Add 40 uL sample to each well.
3. Cover plate with foil.
4. Incubate on the heat block at 80°C for 10 mins.
5. Add TE in two wells at the end of samples in each row as blanks.

To set up virus stained plate:

6. Add 160 uL of prepared SYBR Green I and TE Buffer diluted solution to each well.
7. Add 40 uL sample to each well of a u-bottom 96 well plate.
8. Cover plate with foil.
9. Incubate on the heat block at 80°C for 10 mins.
10. Add TE with SYBR in two wells at the end of samples in each row as blanks.

Bacteria

1. Add 160 uL of prepared SYBR Green I and TE Buffer (1:20,000) diluted solution to each well.
2. Add 40 uL sample to each well of a u-bottom 96 well plate.
3. Cover plate with foil.
4. Add TE with SYBR in two wells at the end of samples in each row as blanks.

Record all the values in contextual metadata file.

1.17 Plant Sampling

TBC

2. Contextual Metadata Analytical Procedures

2.1. CSBP Laboratory Procedures for Soils (as per BASE)

The analytical methods used for soils should be carried out to the same standards and methodology as conducted through the CSBP Laboratories <https://csbplab.com.au/tests/soil>

The methodologies used are described in their available manual <https://csbplab.com.au/media/2020/05/CSBP-Lab-Methods-0520-Web.pdf> (May 2020 version). This manual is maintained by CSBP Laboratories.

2.2. IMOS National Reference Stations Biogeochemical Operations Manual (as per IMOS NRS & ACMON)

The analytical methods used for NRS & CRS pelagic and coastal water samples as detailed in the IMOS Biogeochemical Operations manual https://s3-ap-southeast-2.amazonaws.com/content.aodn.org.au/Documents/IMOS/Facilities/national_mooring/IMOS_NRS_BGCManual_LATEST.pdf This manual is maintained by the IMOS NRS BGC working group.

Reference

Davies, C. and Sommerville, E. (Eds.) (2017), National Reference Stations Biogeochemical Operations Manual Version 3.2.1. Integrated Marine Observing System.

DOI: 10.26198/5c4a56f2a8ae3 (<http://dx.doi.org/10.26198/5c4a56f2a8ae3>)

2.3. CSIRO RV *Southern Surveyor* Laboratory Procedures SS2010_V09

Hydrology Data Processing Report: TBC

*metadata record for this voyage below; requires link to hydrochemistry data processing report

<http://www.marlin.csiro.au/geonetwork/srv/eng/search#!bc1b3741-e355-5039-e044-00144f7bc0f4>

2.4. CSIRO RV *Southern Surveyor* Laboratory Procedures SS2012_T06

Hydrology Data Processing Report: TBC

*survey record for this voyage below; requires link to hydrochemistry data processing report

https://www.cmar.csiro.au/data/trawler/survey_details.cfm?survey=SS2012_T06

2.5. CSIRO RV *Southern Surveyor* Laboratory Procedures SS2012_T07

Hydrology Data Processing Report:

http://www.marine.csiro.au/cgi-bin/marlin-dl/Southern_Surveyor_NF/ss2012_t07/doc/ss2012_t07HYD.pdf

2.6. CSIRO RV *Southern Surveyor* Laboratory Procedures SS2012_V04

Hydrology Data Processing Report: TBC

*metadata record for this voyage below; requires link to hydrochemistry data processing report

<https://www.marlin.csiro.au/geonetwork/srv/eng/search#!ce42691b-b947-4a42-e043-08114f8c0ca4>

2.7. CSIRO RV *Southern Surveyor* Laboratory Procedures SS2013_T03

Hydrology Data Processing Report:

http://www.marine.csiro.au/cgi-bin/marlin-dl/Southern_Surveyor_NF/ss2013_t03/doc/ss2013_t03HYD.pdf

2.8. CSIRO RV *Investigator* Laboratory Procedures IN2014_E03

Hydrology Data Processing Report:

http://www.cmar.csiro.au/datacentre/process/data_files/Investigator_NF/in2014_e03/doc/in2014_e03_HYD_ProcessReport_v1.0.pdf

2.9. CSIRO RV *Investigator* Laboratory Procedures IN2015_C02

Hydrology Data Processing Report: TBC

*metadata record for this voyage below; requires link to hydrochemistry data processing report

<https://www.marlin.csiro.au/geonetwork/srv/eng/search#!6b24d91a-e801-4e1e-b7cd-b50603aaacc4>

2.10. CSIRO RV *Investigator* Laboratory Procedures IN2015_V03

Hydrology Data Processing Report:

http://www.cmar.csiro.au/datacentre/process/data_files/Investigator_NF/in2015_v03/doc/in2015_v03_HYD_ProcessReport_v1.3.pdf

2.11. CSIRO RV *Investigator* Laboratory Procedures IN2016_T01

Hydrology Data Processing Report:

http://www.cmar.csiro.au/datacentre/process/data_files/Investigator_NF/in2016_t01/doc/in2016_t01_HYD_ProcessingReport.pdf

2.12. CSIRO RV *Investigator* Laboratory Procedures IN2016_V03

Hydrology Data Processing Report:

http://www.cmar.csiro.au/datacentre/process/data_files/Investigator_NF/in2016_v03/doc/in2016_v03_HYD_ProcessingReport.pdf

2.13. CSIRO RV *Investigator* Laboratory Procedures IN2016_V04

Hydrology Data Processing Report:

http://www.cmar.csiro.au/datacentre/process/data_files/Investigator_NF/in2016_v04/doc/in2016_v04_HYD_ProcessingReport.pdf

2.14. Sediment Grain Size Fractionation (% fines) (marine sediment as per MM Coastal)

1. Wash sediment (~5 g) from the top 1 cm through filter holder to retain different fractions; gravel (collected on 2 mm mesh), sand (collected on 63um mesh), and fines (collected in sump).

2. Transfer sediments into pre-weighed and labelled specimen jars.
3. Leave for 24 h to allow sediments to settle and then suction off excess water.
4. Transfer to drying oven at 60 °C for 24-48 h or until sediments are completely dry.
5. Re-weigh jars and calculate the weight of each fraction to determine % fines in the sample.

2.15. Sediment Grain Size Fractionation (% fines) (marine seagrass sediment as per MM Coastal)

1. Wash sediment (~60 mL) of top 1 cm through sieves to collect three size fractions; gravel (collected on 2 mm mesh), sand (collected on 63 µm mesh) and fines (collected in sump).
2. Transfer sediments into pre-weighed and labelled specimen jars. Label jars according to the scheme outlined in the spreadsheet "sample_schedule".
3. Leave for 24 h to allow sediments to settle and then suction off excess water.
4. Transfer to drying oven at 60 °C for 24-48 h or until sediments are completely dry.
5. Re-weigh jars and calculate the weight of each fraction to determine % fines in the sample.

2.16. TOC (marine sediment as per MM Coastal)

1. Acidify sediments with 2 ml of 1 M HCl overnight following Hedges & Stern (1984).
2. Analyse sediments with a LECO CN2000 analyser at a combustion temperature of 1050 °C.

2.17. TOC (marine seagrass sediment as per MM Coastal)

1. Acidify sediments left (~140 mL) with 60 mL of 1 M HCl overnight following Hedges & Stern (1984).
2. Analyse sediments with a LECO TOC analyzer at a combustion temperature of 1050°C.

2.18. Marine sediment porewater nutrients (as per MM Coastal)

1. Collect a 20 mL pore water sample by centrifuging sediment for nutrients analysis (phosphorus, ammonia, nitrate + nitrite).
2. Analyse phosphorus, ammonia, nitrate + nitrite in 20 mL of pore water using AutoAnalyzer and standard spectrophotometric methods (Strickland & Parsons, 1972; Grasshoff, 1976; Oudot & Montel, 1988).

2.19. PAM Fluorometry in situ measurements (seagrass as per MM Coastal)

PAM fluorometry measurements in situ: After bringing the algal tissue to the surface and swabbing

~20 cm² to sample surface-associated microorganisms, use a 'leaf clip' provided with a diving Pulse Amplitude Modulated (diving-PAM) fluorometer (Walz, Germany) to first 'dark-adapt' for 15 minutes an area of algal thallus adjacent to where the swabbing was done (i.e. not swabbed) that is not covered by visible epiphytes (note: the leaf clip must be in the 'closed' position). A 0.8 s 'saturating pulse' of light (> 4500 µmol photons m⁻²s⁻¹) is delivered to the algal surface *via* a 1.5 mm diameter optical fibre. The same fibre also delivers the 'measuring light' (which should be set < 0.15 µmol photons m⁻²s⁻¹, below the level required to initiate photosynthesis. Chlorophyll fluorescence (wavelength > 710 nm) will be measured on the Diving-PAM (gain = 4). Minimum dark-adapted fluorescence (F) is determined prior to the saturating flash, while the maximum dark-adapted fluorescence (FM') will be determined as the fluorescence value during the saturating flash. The difference between FM' and F is the variable fluorescence, ΔF. Effective quantum yield (EQY, ΔF/FM') is a measure of photosystem II photochemical efficiency in the dark-adapted algae. Area of thallus appropriate for sampling and PAM measurements: Because *E. radiata* has a basal meristem, the tissues at the top of the thallus (primary lamina) and the ends of each lateral 'branch' are typically older, may be more poorly chemically defended and are thus more likely to show evidence of epibiosis or natural senescence. We wish to sample young, healthy tissue, so avoid those areas. Select a lateral 'branch' that is in the lower half of the alga and sample that lateral 'branch' on the half closest to the primary lamina.

2.20. Analytical Services Tasmania (AST) Laboratory Procedures (as per Derwent Estuary Program, Ambient Water Quality Program)

Analytical Services Tasmania (AST) operates under a Quality Management System in compliance with the requirements of international standard ISO/IEC 17025, accredited in Australia by [NATA](#).

AST website:

<https://analyticalservices.tas.gov.au/>

Standard methods performed at AST:

https://www.nata.com.au/entity_scope/?q1=2e6&str=&&BENName=Analytical

2.21. YSI details (as per Derwent Estuary Program, Ambient Water Quality Program)

TBC

2.22. CSBP Laboratory Procedures for water (Watkin/Boase, BushBlitz)

The analytical methods used for soils should be carried out to the same standards and methodology as conducted through the CSBP Laboratories <https://csbplab.com.au/tests/water>

The methodologies used are described in their available manual <https://www.csbp.com.au/docs/default-source/csbp-lab/csbp-lab-methods-1118.pdf> (April 2019 version). This manual is maintained by CSBP Laboratories.

3. OMICS Sample Extraction & Purification Procedures

3.1. DNA Protocols

3.1.1. Qiagen DNeasy® PowerLyzer PowerSoil® Kit (soil, as per BASE @ AGRF)

1. DNA extraction is carried out in triplicate using the DNeasy PowerLyzer PowerSoil® Kit (Cat No./ID:12855, <https://www.qiagen.com/au/products/discovery-and-translational-research/dna-rna-purification/dna-purification/microbial-dna/dneasy-powerlyzer-powersoil-kit/#orderinginformation>). The DNA is extracted according to Earth Microbiome protocol <http://www.earthmicrobiome.org/emp-standard-protocols/dna-extraction-protocol/>
2. A soil sample input of 250 mg is used for each extraction
3. For consistency if added DNA output is required an increased number of extractions will be performed and pooled and noted in the metadata (rather than increasing the soil input into an extraction)
4. DNA replicate extractions are pooled for further QC and sequencing analysis

3.1.2. Qiagen DNeasy® PowerSoil® Kit (soil, marine sediments, sponge)

Soil/Sediment:

1. Extract frozen sediment samples (0.25g) with Qiagen DNeasy® PowerSoil® Kit (<https://www.qiagen.com/au/products/dneasy-powersoil-kit/#orderinginformation>) following extraction protocol.

Sponge:

1. Cut all three piece of each specimen (n=3) into small pieces under liquid nitrogen. Mix the small pieces for each specimen, but don't mix pieces of the sponge replicates.
2. Extract frozen samples (~0.5 g) for each replicate (n=3) separately with Qiagen DNeasy® PowerSoil® Kit (<https://www.qiagen.com/au/products/dneasy-powersoil-kit/#orderinginformation>).
3. The final eluted DNA should be labelled according to the instruction given in the spreadsheet "sampling_schedule" and stored at -80 °C.

3.1.3. Qiagen DNeasy® PowerSoil® (modified for marine sediments as per MM Coastal)

Extract DNA from frozen sediment samples (1.5 g) with Qiagen DNeasy® PowerSoil® Kit (<https://www.qiagen.com/au/products/dneasy-powersoil-kit/#orderinginformation>) following manufacturer's recommendation for extraction protocol except with additional purification steps:

1. In PCR plate, pipette from aliquots 100 µL of AMPure solution. Gently shake before to resuspend beads.
2. Add ~100 µL sample and pipette to mix. Mix gently as fast pipetting can displace sample from well.
3. Leave to stand for a minimum of 10 min to allow strands to bind to magnet beads.
4. Transfer PCR plate to SuperMagnet. Leave for 10 min for rings to form.
5. Carefully remove solution in wells without disturbing the ring of beads.
6. Add 200 µL 70% ethanol and leave for 30 s.
7. Careful not to disturb the bead ring, pipette mix the ethanol to wash and remove from sample. Repeat for a total of 3 washes.
8. After the last wash, make sure all ethanol is pipetted out from wells.
9. Allow to air dry for 9-10 min. Do NOT allow beads to dry!
10. Remove plate from magnet. Add 100 µL nuclease free water for DNA and pipette to elute beads and strands. Elution is rapid.
11. Place plate back on magnet for 10 min.
12. Carefully pipette out the sample into 1.5 mL sterile tubes labelled earlier and place on ice.
13. If you suspect carryover of magnet beads, place tube back on magnet plate and being aliquoting 50 µL DNA to remaining 1.5 mL tube. With care, a final drop of sample containing magnet beads can be separated and removed.
14. Place 1 x 50 µL DNA aliquot at -20°C. Keep 1 x 50 µL DNA aliquot "dirty" sample on ice to nanodrop.
15. The final DNA should be labelled according to the instruction given in the spreadsheet "sampling_schedule" and stored at -80 °C.

3.1.4. Modified Qiagen DNeasy® PowerSoil® (modified for coastal seawater as per MM Coastal)

1. For DNA extraction, above a clean surface (e.g. petri dish) crack open the sterivex filter at the inlet end using long-handled pincers (e.g. Oetiker-14100329-Economy-Tool-Standard-Jaw-Pincers)
2. The PES membrane can be carefully removed by scoring along the edges with a pair of clean watchmakers forceps and peeling off its support and before placing the membrane into an extraction tube.
3. Extract using the Qiagen DNeasy® PowerSoil® Kit (<https://www.qiagen.com/au/products/dneasy-powersoil-kit/#orderinginformation>), which includes a beating step with the vortex adaptor.
4. The final eluted DNA should be labelled according to the instruction given in the spreadsheet “sampling_schedule” and stored at -80 °C.

3.1.5. Modified Qiagen DNeasy® PowerSoil® (modified for seagrass as per MM Coastal)

1. Extract each replicate swab (n=3) separately with the Qiagen DNeasy® PowerSoil® Kit (<https://www.qiagen.com/au/products/dneasy-powersoil-kit/#orderinginformation>).
2. For this, aseptically transfer swab from cryotube to extraction tube by tipping from one to the other (or where necessary use clean, sterile forceps). Then follow the protocol as outlined in the manual, with the following modifications:
 - Step #5. Instead of using a vortex etc., replace this step by bead beating at full speed (30 Hz) for 10 min.
 - Step #18. Make sure filter is completely dry after this step. If not, repeat.
 - Steps #20-21. Split into 2 steps using 50 µL (rather than 100 µL once). Heat up solution C6 to 50 °C (when you start the whole lot), add 50 µL and leave it for 5 min, centrifuge at room temperature for 30 sec at 10,000 x g, and repeat (final volume = 100 µL).
3. The final eluted DNA should be labelled according to the instruction given in the spreadsheet “sampling_schedule” and stored at -20 °C.

3.1.6. MoBio PowerPlant® Pro DNA Isolation Kit (coral as per MM Coastal)

1. Extracting DNA with the MoBio PowerPlant Pro DNA Isolation kit using Standard Protocols (<http://www.mobio.com/images/custom/file/protocol/13400.pdf>). This kit has the ability to remove many of the inhibitors present in a variety of samples.
2. Store DNA at -80 °C until required.
3. The final eluted DNA should be labelled according to the instruction given in the spreadsheet “sampling_schedule” and stored at -80 °C.

3.1.7. Qiagen DNeasy® PowerWater® Sterivex™ Kit

Extracted DNA from frozen sterivex samples using Qiagen DNeasy® PowerWater® Sterivex™ Kit (<https://www.qiagen.com/au/products/discovery-and-translational-research/dna-rna-purification/dna-purification/microbial-dna/dneasy-powerwater-sterivex-kit/?clear=true#orderinginformation>) following manufacturer’s instructions.

3.1.8. Modified Qiagen DNeasy® PowerWater® Sterivex™ Kit (as per some open ocean voyage samples)

Extracted DNA from frozen sterivex samples using Qiagen DNeasy® PowerWater® Sterivex™ Kit (<https://www.qiagen.com/au/products/discovery-and-translational-research/dna-rna-purification/dna-purification/microbial-dna/dneasy-powerwater-sterivex-kit/?clear=true#orderinginformation>) following manufacturer’s instructions with the following modifications:

1. Sterivex were cracked open aseptically and the filter membrane transferred into the 5 mL PowerBead tubes prior to the addition of any lysis buffers. The extraction procedure was continued from Step 2 (with steps 11 and 12 omitted).
2. DNA was eluted in 30µl of 10 mM TE pH 8.0.

3.1.9. Modified Qiagen DNeasy® PowerWater® Sterivex™ Kit (modified for pelagic water from IMOS NRS, ACMON & voyage samples extracted at CSIRO Hobart Genetics Laboratories)

Equipment:

- Lysis buffer
 - o 200 mM NaH₂PO₄·2H₂O (monobasic)
 - o 200 mM Na₂HPO₄ (dibasic)
 - o To make up 200 mL lysis buffer 39 mL 200 mM Na₂HPO₄
 - o 61 mL 200mM Na₂HPO₄ 17.54 g NaCl
 - o 2 g CTAB
 - o 4 g PVP K30
 - o + ddH₂O to make up to 200 ml
 - o Adjust to pH 7.0 (using NaOH – try couple of mL of 10 M NaOH)
- Lysozyme
- Proteinase K – 20 mg/ml
- MT Buffer (MP Biomedicals)
- From Qiagen DNeasy® PowerWater® Sterivex™ Kit (<https://www.qiagen.com/au/products/discovery-and-translational-research/dna-rna-purification/dna-purification/microbial-dna/dneasy-powerwater-sterivex-kit/?clear=true#orderinginformation>)
 - o Columns and sample recovery tubes, 3 ml and 20 ml syringes, 5 ml tubes Buffer
 - o ST4 (warmed to 65 °C before use)
 - o Buffer ST5 and ST6
- Inlet and outlet caps for Sterivex filters
- Phenol:Chloroform:Isoamyl (25:24:1) (PCI) Chloroform:Isoamyl (24:1) (CI)
- TE buffer

Method:

(Processing limited by capacity of horizontal vortexer, 2 vortexers x 6 filters = 12 filters per extraction set)

1. weigh 125 mg lysozyme into 50 mL falcon tube and add 25 mL Lysis Buffer to dissolve (lysozyme final conc. 5 mg/ml).
2. remove filters from -80 °C, remove inlet cap and using a pipette add 1.875 ml Lysis buffer and 125 µl MT buffer. *(if filters are covered with RNAlater, remove RNAlater with back pressure from 3 ml syringe)*
3. recap the Sterivex filter and attach filter (with inlet end facing out) to the horizontal vortexer, Speed 5-7 for 60 min (turning the filter a couple of times during the h)
4. using 3 ml syringe, draw back plunger and attach to inlet end of filter until pressure builds up – release plunger and buffer in filter should flow into syringe. Divide approx 2 ml of buffer evenly into 2 × 2.0 ml tubes *(do not use the 2 ml collection tubes that come in the PowerWater® Sterivex kit – they don't tolerate PCI) (may need to use syringe several times to get all buffer out of filter, should be about 0.800-1.00 ml per tube)*
5. in fume hood, add 900 µl PCI to each tube, invert several times, spin down 13000/10 min/RT
6. combine the aqueous phases from both tubes into one 2.0 ml tube (which will be between 1.2 – 1.5 ml), add 20 µl Proteinase K, onto heat block for 2 h at 60 °C
7. in fume hood, add 500 ul CI, spin down 13000/10 min/RT – put aqueous phase into new tube
8. in fume hood, add a further 500 ul CI, spin down 13000/5 min/RT – put aqueous phase into new tube
9. after 2nd spin, take out 1 ml of aqueous phase, add to 5 ml tube
10. add 3 ml of warmed ST4 buffer (65 °C), mix by inversion
11. attach column to barrel of 20 ml syringe and attach to vacuum manifold
12. pour contents of 5 ml tube into barrel while still warm
13. using vacuum, pull contents through the column
14. while keeping column attached to the manifold, remove barrel and add 800 ul ST5 to column
15. using vacuum, pull contents through the column
16. add 800 µl ST6 to column

17. using vacuum, pull contents through the column, then keep on vacuum for 2 mins
18. turn vacuum off, put column into new 2.0 ml tube and let air dry on bench for 10 mins
19. add 80 µl 0.1 x TE to column, incubate at 37 °C for 45 min
20. spin down column and tube at 13000/2 min/RT to elute DNA

Reference

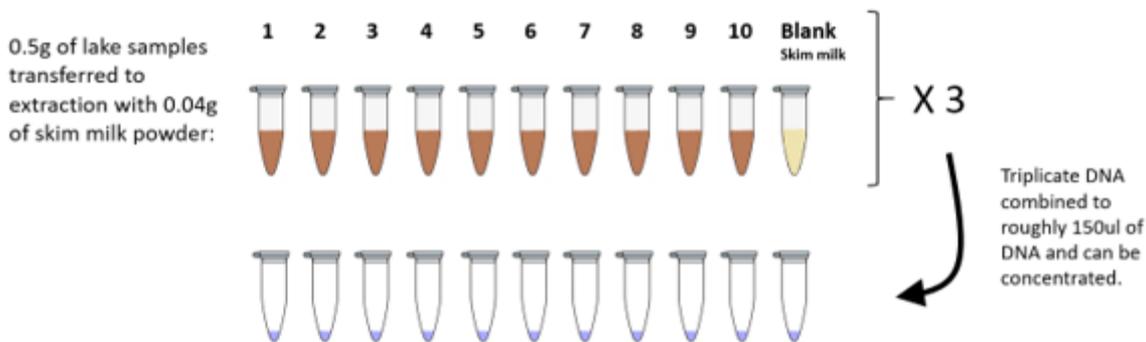
Appleyard, S., Abell, G. & Watson, R. Tackling microbial related issues in cultured shellfish via integrated molecular and water chemistry approaches. Seafood CRC report No. 2011/729 (2011).

3.1.10 Modified MP Biomedicals FastDNA® Spin Kit for Soil (modified for soil, crust and filtered water samples from acidic saline lakes; supplied by Elizabeth Watkin)

Extractions performed using the MP Biomedicals FastDNA® Spin Kit for Soil (<https://www.mpbio.com/116560000-fastdna-spin-kit-for-soil-samp-cf>), following the supplied protocol with the following alterations:

For filter papers: In 8 x separate extractions, use 1/8th of pre-cut 90 mm filters (0.2 µm and 0.1 µm Nalgene™ Rapid-Flow™ Sterile Disposable Filter Units with PES membrane #catalogue numbers TBC) into the bead beating tubes*.

For sediment and salt crusts: Triplicates of lake samples were extracted and combined after elution (see below diagram). 0.5 g of sample was added into each bead beating tube.



NB. Skim control extraction performed with each extraction set to be sequenced.

- Step 1: ~40 mg.mL⁻¹ steam sterilized skim milk powder was added to the Bead beating tubes prior to the addition of the samples.
- After step 3: five rounds of freeze thawing in liquid nitrogen and heating at 95 °C.
- Step 4: three rounds of homogenisation at 6.5 m/s for 45 seconds in a Fast-Prep24™ ribolyser system with 5-minute rests on ice between cycles.

* TBC if using 1/8th of the 0.2 µm and 0.1 µm filter paper into in a single extraction tube or, extract separately and combine the DNA for sequencing (this is to ensure capture of Nanoarchaea).

3.1.11 DNA Quantification at AGRF

Fluorometric nucleic acid quantification on SPECTRAMax Gemini XPS Dual-Scanning Microplate Spectrofluorometer using the Promega QuantiFluor Quantification Kit, following manufacturer’s instructions

(<https://www.promega.com.au/products/rna-analysis/dna-and-rna-quantitation/quantifluor-dsdna-system/?catNum=E2670#protocols>).

3.1.12 Plant Leaf DNA Extraction

TBC

3.1.13 Plant Root DNA Extraction

TBC

3.1.14 Plant Rhizosphere DNA Extraction

TBC

3.2. RNA Protocols

3.2.1. Qiagen RNeasy® PowerSoil® Total RNA Kit (marine sediments as per MM Coastal)

Extract frozen sediment samples (up to 2g) with Qiagen RNeasy® PowerSoil® Total RNA Kit

(<https://www.qiagen.com/au/products/discovery-and-translational-research/dna-rna-purification/rna-purification/microbial-rna/rneasy-powersoil-total-rna-kit/?clear=true#orderinginformation>) following extraction protocol.

3.2.2. Qiagen RNeasy® PowerSoil® Total RNA Kit (modified for marine sediments as per MM Coastal)

Extract RNA from frozen sediment samples (2 g) with Qiagen RNeasy® PowerSoil® Total RNA Kit

(<https://www.qiagen.com/au/products/discovery-and-translational-research/dna-rna-purification/rna-purification/microbial-rna/rneasy-powersoil-total-rna-kit/?clear=true#orderinginformation>) following the modified extraction protocol below.

1. Prep a 15 mL **Bead Tube** with 2.5 ml of Bead Solution, 0.25 ml of Solution SR1 and 0.8 ml of Solution SR2.
2. Add 2 g of sediment
**Weigh 6 samples, then continue.*

Move to FUME HOOD

3. Add 3.5 mL of phenol:chloroform:isoamyl alcohol to the bead tube, cap tightly and vortex to mix until the biphasic layer disappears.
**All waste needs to be kept in fume cupboard and double bagged in a hazardous waste bag.
*Double glove and change gloves any time working outside of the fume cupboard. All pipettes etc. to be ethanol wiped at the end of use.
Once phenol:chloroform has been added for 6 samples, start Step 4: vortexing as this takes 15min.
4. Vortex in vortex adaptor at maximum speed for 15 minutes.
**While these are vortexing, weigh other 6 samples.
Start preparing 2 sets of collection tubes with 1) 1.5 ml of Solution SR3, 2) 5mL of Solution SR4.
5. Remove the Bead Tube from the Vortex Adapter and centrifuge at 2500 x g for 10 minutes at room temperature.
6. Remove the Bead Tube from the centrifuge and carefully transfer the upper aqueous phase (avoiding the interphase and lower phenol layer) to the 15 ml Collection Tube with 1.5 mL SR3, vortex to mix.
**Avoid tilting the tube.
MAKE SURE NO PHENOL:CHLOROFORM IS TRANSFERRED before adding to SR3. If interphase is punctured, centrifuge again for 10 min.
7. Incubate at 4 °C for 10 minutes. Wait until last batch has incubated to catch up.
8. Centrifuge at 2500 x g for 10 minutes at room temperature. Transfer the supernatant, without disturbing the pellet (if there is one), to the 15 mL Collection Tube with 5 mL SR4.
9. Invert or vortex to mix, and incubate at room temperature for 30 minutes (choose a cool place in the lab, as heat is not good for RNA – desk cupboard).
**Time to start thinking about LUNCH :)*
10. Centrifuge at 2500 x g for 30 minutes at room temperature.
**In the last 10 minutes of centrifuging, RNaseAway and ethanol treat biological safety cabinet. Get waste beaker and lay out kimwipes to UV sterilise. Any racks, pipettes, open pipette tips that will be used can also be sterilized in the cupboard.*

FROM NOW ON IN STERILE BSC

11. Decant the supernatant and invert the 15 ml Collection Tube on a paper towel for 5 minutes. Watch that the pellet does not drop onto kimwipe. This may happen if the sample was left to sit for a while after centrifugation.
12. Shake Solution SR5 to mix. Add 1 ml of Solution SR5 to the 15 ml Collection Tube and resuspend the pellet completely by repeatedly pipetting or vortexing to disperse the pellet.
**Depending on the soil type, the pellet may be difficult to resuspend. Resuspension may be aided by placing the tubes in a heat block or water bath at 45 °C for 10 minutes, followed by vortexing. Repeat until the pellet is resuspended.*
13. With new RNase free gloves, prepare one RNA Capture Column for each RNA Isolation Sample:
 - a) Remove the cap of a new 15 ml Collection Tube and place the RNA Capture Column inside the 15 ml

- Collection Tube. The column will hang in the 15 ml Collection Tube.
- b) Add 2 ml of Solution SR5 to the RNA Capture Column and allow it to gravity flow through the column and collect in the 15 ml Collection Tube. Allow Solution SR5 to completely flow through the column.
**DO NOT ALLOW THE COLUMN TO DRY OUT PRIOR TO LOADING THE RNA ISOLATION SAMPLE.*
 14. Add the RNA Isolation Sample from Step 12 onto the RNA Capture Column and allow it to gravity flow through the column. Collect the flow through in the 15 ml Collection Tube.
When there is time, with new RNase free gloves, start carefully capping and labelling 2.2 mL **collection tubes from the kit. Add 1 mL of SR4.*
 15. Wash the column with 1 ml of Solution SR5. Allow it to gravity flow and collect the flow through in the 15 ml Collection Tube.
 16. Transfer the RNA Capture Column to a new 15 ml Collection Tube. Shake Solution SR6 to mix and then add 1 ml of Solution SR6 to the RNA Capture Column to elute the bound RNA into the 15 ml Collection Tube. Allow Solution SR6 to gravity flow into the 15 ml Collection Tube. Do NOT throw out Capture Column.
**PREPARE 15mL DNA COLLECTION TUBES. After the SR6 has completely flowed through the column, transfer the Capture Column to the DNA Collection Tubes.*
 17. Transfer the eluted RNA to the 2.2 ml Collection Tubes with 1ml of Solution SR4. Invert at least once to mix and incubate at -20 °C for a minimum of 10 minutes.
Start capping and labelling **1.5 mL sterile RNase free tubes (from zip-locked Axygen packets). For each sample, label two tube briefly for DNase treatment and then 4 extra tubes for aliquoting final RNA product, i.e. a total of 6 tubes per sample.*
 18. Centrifuge the 2.2 ml Collection Tube at 13,000 x g for 15 minutes at room temperature to pellet the RNA.
**In the last 5 minutes of centrifugation, UV sterilise KimWipes in the BSC.*
 19. Decant the supernatant and invert the 2.2 ml Collection Tube onto a paper towel for 10 minutes to air dry the pellet.
**Take TurboDNase buffer out of freezer to defrost. Prepare esky of ice.*
 20. Resuspend the RNA pellet in 40 µL of Solution SR7 and place samples on ice.

TurboDNase treatment for 12 RNA samples

- *Remove TurboDNase from freezer and thaw on ice.*
- *TurboDNase, buffer and DNase inactivation reagent should all be kept on ice while working with them and in the dark (covered esky).*
6. Using one of the 1.5 mL sterile tubes, make a DNase mastermix:
 - a) FOR 12 SAMPLES: To 52 µL of TurboDNase buffer, gently add 13 µL of TurboDNase and very gently mix with your pipette tip.
 - b) Transfer 5 µL of this DNase mastermix to each tube (there should be a minimum of 5 µL in each tube).
 7. Incubate samples for 20 min at 37 °C
**Take DNase inactivation reagent out of freezer to thaw.*
 8. Add 4.5 µL of resuspended DNase Inactivation Reagent and mix well.
 9. Incubate 5 min at room temperature, mixing occasionally. Flick the tube 2–3 times during the incubation period to redisperse the DNase Inactivation Reagent.
**If room temperature cools below 22–26 °C, move the tubes to a heat block or oven to control the temperature. Cold environments can reduce the inactivation of the TurboDNase, leaving residual DNase in the RNA sample.*
 10. Centrifuge at 10,000 × g for 1.5 min and transfer the RNA to an RNase free PCR plate.
**Avoid pipetting/disturbing the DNase Inactivation Reagent, it is difficult to remove, even with the magnet clean and interferes with downstream applications.*

RNA Clean

1. In PCR plate, pipette from aliquots 100 µL of RNAClean solution. Gently shake before to resuspend beads.
2. Add ~100 µL sample and pipette to mix. Mix gently as fast pipetting can displace sample from well.
3. Leave to stand for a minimum of 10 min to allow strands to bind to magnet beads.
4. Transfer PCR plate to SuperMagnet. Leave for 10 min for rings to form.
5. Carefully remove solution in wells without disturbing the ring of beads.

6. Add 200 μ L 70% ethanol and leave for 30 s.
7. Careful not to disturb the bead ring, pipette mix the ethanol to wash and remove from sample. Repeat for a total of 3 washes.
8. After the last wash, make sure all ethanol is pipetted out from wells.
9. Allow to air dry for 9-10 min. Do NOT allow beads to dry!
10. Remove plate from magnet. Add 40 μ L of DEPC RNase free water for RNA samples and pipette to elute beads and strands. Elution is rapid.
11. Place plate back on magnet for 10min.
12. Carefully pipette out the sample into 1.5mL sterile tubes labelled earlier and place on ice.
13. If you suspect carryover of magnet beads, place tube back on magnet plate and begin aliquoting 10 μ L RNA to 3 x 1.5 mL tubes. With care, a final drop of sample containing magnet beads can be separated and removed.
14. Place 3 x 10 μ L RNA aliquots into -80 °C freezer. Keep 1 x 10 μ L "dirty" sample on ice to nanodrop.
15. The final RNA should be labelled according to the instruction given in the spreadsheet "sampling_schedule" and stored at -80 °C.

3.2.3. Modified PureLink RNA Mini Kit (modified for seagrass as per MM Coastal)

Extract RNA from seagrass leaves using PureLink RNA Mini Kit as per the manufacturer's protocol (Life Technologies) with the addition of On-column PureLink DNase (Life Technologies) to remove contaminating gDNA.

RNA clean

1. In PCR plate, pipette from aliquots 100uL of RNAClean solution. (*Gently shake before to resuspend beads*)
2. Add ~50 μ l sample and pipette to mix *Mix gently as fast pipetting can displace sample from well.
3. Leave to stand for a minimum of 10 min to allow strands to bind to magnet beads.
4. Transfer PCR plate to SuperMagnet. Leave for 10 min for rings to form.
5. Carefully remove solution in wells without disturbing the ring of beads.
6. Add 200uL 70% ethanol and leave for 30 s.
7. Careful not to disturb the bead ring, pipette mix the ethanol to wash and remove from sample. Repeat for a total of 3 washes.
8. After the last wash, make sure all ethanol is pipetted out from wells.
9. Allow to air dry for 9-10 min. Do NOT allow beads to dry!
10. Remove plate from magnet. Add 40 μ L of DEPC RNase free water for RNA samples and pipette to elute beads and strands. Elution is rapid.
11. Place plate back on magnet for 10min.
12. Carefully pipette out the sample into 1.5 μ l sterile tubes prepared earlier and place on ice.
13. If you suspect carryover of magnet beads, place tube back on magnet plate and being aliquoting 10uL RNA to remaining 1.5 μ l tubes. With care, a final drop of sample containing magnet beads can be separated and removed.
14. Place 3x 10 μ l RNA aliquots into -80 °C freezer. Keep one "dirty" sample on ice to nanodrop. Labelled according to the scheme outlined in the spreadsheet "sample_schedule".

RNA quality control, transcriptome and metatranscriptome sequencing

1. Check RNA quantity and quality using Nanochip technology (Agilent 2100 Bioanalyzer) according to manufacturer's instructions (Agilent).
2. Store high-quality RNA samples (integrity number >7) in -80 °C freezer for further sequencing.
3. Send RNA samples by sequencing (5GB per sample).

3.2.4 Modified PureLink RNA Mini Kit (modified for sponge as per MM Coastal)

- 1 Cut all three pieces of each specimen (n=3) into small pieces under liquid nitrogen. Mix the small pieces for each specimen, but don't mix pieces of the sponge replicates.
- 2 Add approximately 0.5 g of material to a tube separately for each sponge replicate.
- 3 Add 1ml of Trizol directly in the tube (still frozen) and let it defrost while cutting the tissue with small scissors

- 4 Transfer the liquid (with the crushed tissue) into a tube with beads and beat for 30 s at 5.5 speed
- 5 Follow the protocol for Trizol extraction (i.e. PureLink RNA Mini Kit) with the DNase I treatment step, elute in 100 μ l. (This usually results in 700 – 1000 ng/ μ l of total RNA by Qubit)
- 6 Check the samples by agarose gel and Bioanalyzer before continuing
- 7 Deplete the polyA + sequences with “Poly(A) purist Kit” following the protocol, but discarding the beads and recover the RNA from the supernatant with the PureLink RNA Mini Kit doing another DNase I treatment step
- 8 Check the samples with universal bacterial 16S rRNA gene primers (to confirm that there is no contaminant DNA)
- 9 Use this RNA with another round of with “Poly(A) purist Kit” as before (DNA I treatment can be omitted, if previous PCR was negative)
- 10 Use 5 μ g of the previous RNA (PolyA-) to deplete in ribosomal RNA using “RiboZero Bacteria Kit” following the protocol.
- 11 Remove the beads and precipitate the supernatant with the ethanol method (adding 2 μ l Glycogen, 1/10 volume 3M NaOAc, 2.5 volumes of ethanol and incubate overnight at -20 °C).
- 12 Resuspend the final pellet in water. The final RNA should be labelled according to the instruction given in the spreadsheet “sampling_schedule” and stored at -80 °C.

4 OMICS Sequencing Procedures

4.1 Library Preparation (incl. amplicon generation where appropriate)

4.1.1 Bacterial 16S rRNA gene (27F-519R) 1-step using Immolase™ DNA Polymerase (Bioline)

The bacterial communities in environmental samples are investigated by amplification of the 16S rRNA gene using the primer set 27F and 519R (single and dual indexed) and paired-end sequencing on the Illumina MiSeq platform.

Primers for amplification: 27F (Lane 1991) and 519R (Lane et al. 1993)

ILM_27F_Uv3 –forward primer

Both un-indexed and indexed versions are used, depending on the need for dual indexing

1. 5' Illumina adapter
2. Barcode (indexed primer only)
3. Forward primer pad
4. Forward primer linker
5. Forward primer (1391f)

Non-indexed: AATGATACGGCGACCACCGAGATCTACAC TATGGCGAGT GA AGAGTTTGATCMTGGCTCAG

Indexed: AATGATACGGCGACCACCGAGATCTACAC XXXXXXXX TATGGCGAGT GA AGAGTTTGATCMTGGCTCAG

ILM_519R_NNNN – reverse primer

- 5 Reverse complement of 3' Illumina adapter
- 6 Golay barcode*
- 7 Reverse primer pad
- 8 Reverse primer linker
- 9 Reverse primer (EukBr)

CAAGCAGAAGACGGCATACGAGAT XXXXXXXXXXXX AGTCAGTCAG GG GWATTACCGCGGCKGCTG

* This primer includes a 12 base Golay barcode as described by Caporaso et al.

Preparation of master mix for amplification (for 1 rxn)

Component	Volume (µl)	Final concentration
10x ImmoBuffer ^(a)	2.50	1 X
10 mM dNTP	0.50	200 nM
50mM MgCl ₂	1.25	2.5 mM
ILM_27F_Uv3 (forward) (5 µM)	2.50	500 nM
ILM_519R_XXXX (5µM)	2.50	500 nM
Immolase DNA Polymerase (5U/µL) ^(a)	0.20	1 Unit
H ₂ O	14.55	---
Template	1.0	---
Total Volume	25	---

a IMMOLASE™ DNA Polymerase (Bioline, #BIO---21047)

Thermocycler conditions for amplification (for 96 well thermocycler)

Reaction Step	Temperature	Time (mm:ss)
Activation	95°C	10:00
Amplification (35 cycles)	94°C	00:30
	55°C	00:10
	72°C	00:45
Final Extension	72°C	10:00

Amplification method

1. Use neat DNA for initial attempt, 1:10 dilution for failed samples (2nd attempt)
2. Amplify samples with conditions outlined above
3. Run amplicons on an agarose gel. Expected band size for 27F/519R is approx. 530 bp.
4. Clean and normalise samples in a one-step process using the SequelPrep Normalisation Plate Kit according to manufacturer instructions (Invitrogen Cat No. A10510-01)

5. Combine equivalent volumes of normalised amplification into a single maximum-recovery tube.
6. Perform a double cleanup of the pool using 0.8x beads
7. Perform library QC on the pool using Qubit (concentration) and Tapestation (size). Calculate final molarity of the pool.

Sequencing of the 27F and 519R fragment

- Sequencing Primers Read 1 Primer: ACACTATGGCGAGTGAAGAGTTTGATCMTGGCTCAG
- Read 2 Primer: AGTCAGTCAGGGGWATTACCGCGGCKGCTG
- Index Primer: CAGCMGCCGCGGTAATWCCCCTGACTGACT

References

Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, Owens SM, Betley J, Fraser L, Bauer M, Gormley N, Gilbert JA, Smith G, Knight R. (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J

Lane DJ. (1991) 16S/23S rRNA sequencing, p 115–175. In Stackebrandt E, Goodfellow M (ed), Nucleic acid techniques in bacterial systematics. Wiley, New York, NY.

Lane DJ, et al. 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. Proc. Natl. Acad. Sci. U. S. A. 82:6955–6959.

4.1.2 Bacterial 16S rRNA gene (27F-519R) 1-step using KAPA HiFi HotStart ReadyMix (Roche)

The bacterial communities in environmental samples are investigated by amplification of the 16S rRNA gene using the primer set 27F and 519R (single and dual indexed) and paired-end sequencing on the Illumina MiSeq platform.

Primers for amplification: 27F (Lane 1991) and 519R (Lane et al. 1993)

ILM_27F_Uv3 –forward primer

Both un-indexed and indexed versions are used, depending on the need for dual indexing

6. 5' Illumina adapter
7. Barcode (indexed primer only)
8. Forward primer pad
9. Forward primer linker
10. Forward primer (1391f)

Non-indexed: AATGATACGGCGACCACCGAGATCTACAC TATGGCGAGT GA AGAGTTTGATCMTGGCTCAG

Indexed: AATGATACGGCGACCACCGAGATCTACAC XXXXXXXX TATGGCGAGT GA AGAGTTTGATCMTGGCTCAG

ILM_519R_NNNN – reverse primer

1. Reverse complement of 3' Illumina adapter
2. Golay barcode*
3. Reverse primer pad
4. Reverse primer linker
5. Reverse primer (EukBr)

CAAGCAGAAGACGGCATACGAGAT XXXXXXXXXXXX AGTCAGTCAG GG GWATTACCGCGGCKGCTG

* This primer includes a 12 base Golay barcode as described by Caporaso et al.

Preparation of master mix for amplification (for 1 rxn)

Component	Volume (µl)
KAPA HiFi Hot Start Readymix (2x) ^(a)	10.0
H ₂ O	12.0
Forward primer ILM_27F_Uv3 (10 µM)	1.0
Reverse primer ILM_519R_XXXX (10 µM)	1.0
Template	1.0
Total Volume	25

(a) Kit code KK2601 or KK2602

Thermocycler conditions for amplification (for 96 well thermocycler)

Reaction Step	Temperature	Time (mm:ss)
Activation	95°C	3:00
Amplification (35 cycles)	95°C	00:30
	55°C	00:10
	72°C	00:45
Final Extension	72°C	5:00

Amplification method

8. Use neat DNA for initial attempt, 1:10 dilution for failed samples (2nd attempt)
9. Amplify samples with conditions outlined above
10. Run amplicons on an agarose gel. Expected band size for 27F/519R is approx. 530 bp.
11. Clean and normalise samples in a one-step process using the SequelPrep Normalisation Plate Kit according to manufacturer instructions (Invitrogen Cat No. A10510-01)
12. Combine equivalent volumes of normalised amplification into a single maximum-recovery tube.
13. Perform a double cleanup of the pool using 0.8x beads
14. Perform library QC on the pool using Qubit (concentration) and TapeStation (size). Calculate final molarity of the pool.

Sequencing of the 27F and 519R fragment

- Sequencing Primers Read 1 Primer: **ACACTATGGCGAGTGAAGAGTTTGATCMTGGCTCAG**
- Read 2 Primer: **AGTCAGTCAGGGWATTACCGCGGCKGCTG**
- Index Primer: **CAGCMGCCGCGGTAATWCCCCTGACTGACT**

References

- Caporaso JG, Lauber CL, Walters WA, Berg-Lyons D, Huntley J, Fierer N, Owens SM, Betley J, Fraser L, Bauer M, Gormley N, Gilbert JA, Smith G, Knight R. (2012) Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J*
- Lane DJ. (1991) 16S/23S rRNA sequencing, p 115–175. In Stackebrandt E, Goodfellow M (ed), *Nucleic acid techniques in bacterial systematics*. Wiley, New York, NY.
- Lane DJ, et al. 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc. Natl. Acad. Sci. U. S. A.* 82:6955–6959.

4.1.3 Archaeal 16S rRNA gene (A2F-519R*) 2-step using Immolase™ DNA Polymerase (Bioline)

The archaeal communities in environmental samples are investigated by amplification of the archaeal 16S rRNA gene (A16S) and paired-end sequencing on the Illumina MiSeq platform. The preparation of archaeal 16S enriched libraries uses a two-stage PCR strategy. The first round of PCR uses locus specific primers with overhang adapters (A2F_Nex and 519R_Nex). The locus specific region of the forward primer was based on the A2F primer from Reysenbach et al (1995), which is specific to archaeal targets. The reverse primer has a locus specific region that is universal for prokaryotes. The second round PCR and subsequent steps of library preparation and sequencing follow the “16S Metagenomic Sequencing Library Preparation” guidelines from Illumina.

Primers for amplification: A2F and based on 519R*

A2F_Nex PCR Primer Sequence – Forward primer

1. Forward overhang
2. Locus specific sequence (A2F primer)

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TTCCGGTTGATCCYGCCGGA

519R_Nex – Reverse primer*

1. Reverse overhang

2. Locus specific sequence (16S universal primer)

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GWATTACCGCGGCKGCT

***PLEASE NOTE THAT THE FINAL BASE (G) ON THE 519R PRIMER IS MISSING. THIS IS CONSISTENT ACROSS A16S AMPLICON DATA GENERATED.**

Preparation of master mix for amplification A2F / 519R* (for 1 rxn)

Component	Volume (µl)
PCR Grade H ₂ O ^(a)	17.55
10x immoBuffer ^(b)	2.50
50 mM MgCl ₂ ^(b)	0.75
10 mM dNTPs	0.50
A2F Nex primer (10 µM) ^(c)	1.25
519R Nex primer (10 µM) ^(c)	1.25
Immolase DNA polymerase ^(b)	0.20
Template DNA	1.0
Total Volume	25

(a) PCR grade water was purchased from MoBio Laboratories (MoBio Labs: Item#170001 11)

(b) IMMOLASE DNA Polymerase Kit (Cat number: BIO-2146)

(c) Final primer concentration in reaction: 0.5 µM

Thermocycler conditions for amplification (for 96 well thermocycler)

Reaction Step	Temperature	Time (mm:ss)
Activation	95°C	10:00
Amplification (35 cycles)	95°C	00:30
	60°C	00:15
	72°C	00:50
	72°C	5:00

Amplification method

1. Use undiluted DNA for first attempt, and 1:10 dilution for second attempt (if failed samples).
2. Amplify samples with conditions outlined above.
3. Run amplicons on an agarose gel. The expected band size is roughly 520 bp
4. If there is no band present, repeat PCR using a 1:10 dilution of the sample. Use the concentration of the DNA extract to determine if the DNA should be further diluted or used at higher volumes.
5. Clean amplicons with Agencourt AMPure XP beads, according to manufacturer's instructions.
6. Perform a second round PCR (Index PCR) following Illumina's 16S Metagenomic Sequencing Library Preparation, section Index PCR (part #15044223). A half reaction (5µl total rxn volume) can be used.
7. Clean and normalise the PCR products equalPrep Normalisation plates (Invitrogen, A1051001) according to manufacturer instructions.
8. Pool equal volumes of each normalised amplicon.
9. Perform QC on pool using Qubit (concentration) and TapeStation (size) and calculate molarity

References

Reysenbach AL, Pace NR. In: Robb, F.T., Place, A.R. (Eds.), *Archaea: A Laboratory Manual Thermophiles*. CSHLP, 101I 107 (1995).

4.1.4 Archaeal 16S rRNA gene (A2F-519R*) 2-step using KAPA HiFi HotStart ReadyMix (Roche)

The archaeal communities in environmental samples are investigated by amplification of the archaeal 16S rRNA gene (A16S) and paired-end sequencing on the Illumina MiSeq platform. The preparation of archaeal 16S enriched libraries uses a two-stage PCR strategy. The first round of PCR uses locus specific primers with overhang

adapters (A2F_Nex and 519R_Nex). The locus specific region of the forward primer was based on the A2F primer from Reysenbach et al (1995), which is specific to archaeal targets. The reverse primer has a locus specific region that is universal for prokaryotes. The second round PCR and subsequent steps of library preparation and sequencing follow the “16S Metagenomic Sequencing Library Preparation” guidelines from Illumina.

Primers for amplification: A2F and 519R*

A2F_Nex PCR Primer Sequence – Forward primer

1.Forward overhang

2.Locus specific sequence (A2F primer)

TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG TTCCGGTTGATCCYGCCGGA

519R_Nex – Reverse primer*

1.Reverse overhang

2.Locus specific sequence (16S universal primer)

GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG GWATTACCGCGGCKGCT

***PLEASE NOTE THAT THE FINAL BASE (G) ON THE 519R PRIMER IS MISSING. THIS IS CONSISTENT ACROSS A16S AMPLICON DATA GENERATED.**

Preparation of master mix for amplification A2F / 519R* (for 1 rxn)

Component	Volume (µl)
KAPA HiFi Hot Start Readymix (2x) ^(a)	10.0
H ₂ O	12.0
Forward primer A2F Nex (10 µM)	1.0
Reverse primer 519R Nex (10 µM)	1.0
Template	1.0
Total Volume	25

(a) Kit code KK2601 or KK2602

Thermocycler conditions for amplification (for 96 well thermocycler)

Reaction Step	Temperature	Time (mm:ss)
Activation	95°C	3:00
Amplification (35 cycles)	95°C	00:30
	60°C	00:15
	72°C	00:50
Final Extension	72°C	5:00

Amplification method

10. Use undiluted DNA for first attempt, and 1:10 dilution for second attempt (if failed samples).
11. Amplify samples with conditions outlined above.
12. Run amplicons on an agarose gel. The expected band size is roughly 520 bp
13. If there is no band present, repeat PCR using a 1:10 dilution of the sample. Use the concentration of the DNA extract to determine if the DNA should be further diluted or used at higher volumes.
14. Clean amplicons with Agencourt AMPure XP beads, according to manufacturer’s instructions.
15. Perform a second round PCR (Index PCR) following Illumina’s 16S Metagenomic Sequencing Library Preparation, section Index PCR (part #15044223). A half reaction (5µl total rxn volume) can be used.
16. Clean and normalise the PCR products equalPrep Normalisation plates (Invitrogen, A1051001) according to manufacturer instructions.
17. Pool equal volumes of each normalised amplicon.
18. Perform QC on pool using Qubit (concentration) and TapeStation (size) and calculate molarity

References

Reysenbach AL, Pace NR. In: Robb, F.T., Place, A.R. (Eds.), *Archaea: A Laboratory Manual Thermophiles*. CSHLP, 1011 107 (1995).

4.1.5 Eukaryotic 18S v4 rRNA gene (primers) 1-step

The protocol detailed here is designed to amplify the V4 region of the 18S rRNA gene for paired-end 18S community sequencing on the Illumina MiSeq platform. This protocol is based on Illumina’s 16S Metagenomic Sequencing Library Preparation guide and the protocol used by Ocean Sampling Day, modified to amplify the target and add indexed adapter sequences in a single PCR step.

Primers for amplification: V4 region

See section 3.2.3.7 Appendix 1 for full list of primer sequences.

Forward primer

1. Field number (space-delimited), description:
2. 5' Illumina adapter
3. Nextera XT i5 index sequence Illumina forward overhang sequence 18S V4 forward

AATGATACGGCGACCACCGAGATCTACAC XXXXXXXX TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG CCAGCASCYCGGTAATTCC

Reverse primer

1. Field number (space-delimited), description:
2. Reverse complement of 3' Illumina adapter Nextera XT i7 index sequence
3. Illumina reverse overhang sequence 18S V4 reverse

CAAGCAGAAGACGGCATAACGAGAT XXXXXXXX GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG ACTTTCGTTCTTGATYRATGA

Preparation of master mix for amplification V4 region (for 1 rxn)

Component	Volume (µl)
KAPA HiFi Hot Start Readymix (2x) ^(a)	12.5
H ₂ O	9.0
Forward primer (10 µM)	1.25
Reverse primer (10 µM)	1.25
Template	1.0
Total Volume	25

(a) Kit code KK2601 or KK2602

Thermocycler conditions for amplification (for 96 well thermocycler)

Reaction Step	Temperature	Time (mm:ss)
Activation	98°C	0:30
Amplification (10 cycles)	98°C	0:10
	44°C	0:30
	72°C	0:15
Amplification (20 cycles)	98°C	0:10
	62°C	0:30
	72°C	0:15
Final Extension	72°C	7:00

Amplification method

1. Use undiluted DNA as a first attempt, and 1:10 diluted for repeats/failed reactions
2. Amplify samples with conditions outlined above.
3. Run amplicons on an agarose gel. Expected band size for 18S-V4 is approximately 536bp.
4. Clean and normalize the PCR products using SequelPrep Normalization plates according to manufacturer’s

instructions (Invitrogen cat no. A10510-01)

5. Pool equal volumes of each normalised amplicon.
6. Perform QC on pool using Qubit (concentration) and Tapestation (size) and calculate molarity of pool.

References

16S Metagenomic Sequencing Library Preparation (Illumina Part # 15044223 Rev. B) available here: http://www.illumina.com/content/dam/illumina-support/documents/documentation/chemistry_documentation/16s/16s-metagenomic-library-prep-guide-15044223-b.pdf

LifeWatch Italy Ocean Sampling Day 2014 Protocol – available here: http://mb3is.megx.net/osd-files/download?path=/2014/protocols&files=OSD2014_protocol_B_18S_V4andV9_Sequencing_LifeWath_MoBiLab_BARI.pdf

4.1.6 Eukaryotic 18S v9 rRNA gene (1391F and EukBR) 1-step

The 18S protocol detailed here is designed to amplify eukaryotes broadly with a focus on microbial eukaryotic lineages. The primers are based on those of Amaral---Zettler et al 2009 and designed to be used with the Illumina platform. The protocol is based on that used by the Earth Microbiome Project (EMP), found here: <http://www.earthmicrobiome.org/emp---standard---protocols/18s/>

Primers for amplification: 18S V9 region (1391F and EukBR)

The primer sequences in this protocol are always listed in the 5' ---> 3' orientation. See Section 4 for more information on ordering, concentration, and resuspension. Primer constructs designed by Laura Wegener Parfrey.

ILM_Euk_1391f PCR Primer Sequence – Forward primer

Field number (space---delimited), description:

1. 5' Illumina adapter
2. Forward primer pad
3. Forward primer linker
4. Forward primer (1391f)

AATGATACGGCGACCACCGAGATCTACAC TATCGCCGTT CG GTACACACCGCCCGTC

ILM_EukBr PCR primer sequence – Reverse primer, barcoded

Each sequence contains different 12 base Golay barcode as described by Caporaso et al.

1. Reverse complement of 3' Illumina adapter
2. Golay barcode
3. Reverse primer pad
4. Reverse primer linker
5. Reverse primer (EukBr)

CAAGCAGAAGACGGCATACGAGAT XXXXXXXXXXXX AGTCAGTCAG CA TGATCCTTCTGCAGGTTACCTAC

Full list of primer sequences is provided in Section 9.1.2.7 Appendix 1.

Preparation of master mix for amplification 18S V9 region (for 1 rxn)

Component	Volume (µl)
PCR Grade H2O ^(a)	13.0
5 Primer Hot MM ^(b)	10.0
Forward primer (10µM) ^(c)	0.5
Reverse primer (10µM) ^(c)	0.5
Template DNA	1.0
Total reaction volume	25.0

(a) PCR grade water was purchased from MoBio Laboratories (MoBio Labs: Item#17000---11)

(b) 5 PRIME HotMasterMix (5 PRIME: Item# 2200410)

(c) Final primer concentration of mastermix: 0.2 µM

Thermocycler conditions for amplification (for 96 well thermocycler)

Reaction step	Temperature	Time (mm:ss)
Activation	94°C	3:00
Amplification (35 cycles)	94°C	00:45
	57°C	01:00
	72°C	01:30
Final Extension	72°C	10:00

Amplification method

- 1 Dilute DNA 1:10.
- 2 Amplify samples with conditions outlined above.
- 3 Run amplicons on an agarose gel. Expected band size for 1391F/EukBR is roughly 200 bp.
- 4 If there is no band present, repeat PCR using either the undiluted DNA or a 1:100 dilution. Use the concentration of the DNA extract to determine if the DNA should be further diluted or used neat.
- 5 Clean and normalise the PCR products. For this step, both Agencourt AMPure XP bead clean-up and SequalPrep Normalisation plates (Invitrogen, A1051001) are acceptable. If using Agencourt AMPure XP beads:
 - a. Perform a bead clean-up following manufacturer's instructions.
 - b. Quantify amplicon yields. Acceptable methods include Picogreen (see manufacturers protocol; Invitrogen Item #P11496) or the high sensitivity Quant-iT™ DNA Assay Kit (Life Technologies, Q-33120).
 - c. Run clean amplicons on Agilent Bioanalyzer, LabChip GX, or 2200 TapeStation to get accurate sizing information.
 - d. Normalise amplicons by diluting to 10nM with variable volume of buffer (EB buffer, or 10mM Tris pH 8.5) and a set volume of the amplicons.
 - e. Pool equal volumes of each normalised amplicon.
 - f. If using SequalPrep Normalisation plates:
 - g. Perform a plate normalisation following manufacturer's instructions.
 - h. Pool equal volumes of each normalised amplicon.
 - i. Run clean amplicon pool on Agilent Bioanalyzer, LabChip GX, or 2200 TapeStation to get accurate sizing information.
- 6 Optional: If spurious bands were present on gel (in step 3), or in the clean amplicons (step 5c) or amplicon pool (step 5h), a portion of the final amplicon pool can go through a gel extraction or a second round of bead clean up. A lower bead to template ratio will get rid of small bands.
- 7 Measure concentration of the final clean pool using Qubit or picogreen.

Sequencing Primers

ILM_Euk_R1: Read 1 Sequencing Primer

Field description (space - delimited):

1. Forward primer pad
2. Forward primer linker
3. Forward primer

TATCGCCGTT CG GTACACACCGCCCGTC

ILM_Euk_R2: Read 2 Sequencing Primer

Field description (space - delimited):

1. Reverse primer pad
2. Reverse primer linker
3. Reverse primer

AGTCAGTCAG CA TGATCCTTCTGCAGGTTACCTAC

ILM_Euk_INDEX: Index Read Sequencing Primer

Field description (space-delimited):

1. Reverse complement of reverse primer

2. Reverse complement of reverse primer linker
3. Reverse complement of reverse primer pad

GTAGGTGAACCTGCAGAAGGATCA TG CTGACTGACT

Primer ordering and resuspension: tips and getting started (From EMP protocol)

Resuspension of primers is a critical step and must be done with Ultra pure water under sterile conditions. It is recommended that resuspension be done in a hood.

- Stock plates are generally 100 μ M and are aliquoted and diluted to 10 μ M for use.
- When making stock plates it is a good idea to make multiple replicate plates so that there are back up plates of primers.
- The primer sequences in this protocol are always listed in the 5' ---> 3' orientation. This is the orientation that should be used for ordering.
- We use the standard desalting cleanup option.
- If you are using a robot the volume in the plates should be 30 μ L per well or greater. 15 μ L per well is the absolute minimum.

Ordering barcoded primers

- Contact IDT or your primer provider to get a quote prior to ordering.
- Barcoded primers are ordered in 96 well plates at the 100 nMole scale.
- Make sure to select the option of receive the same amount per well of each primer (do not get full yield). This is generally the 8 nMole / well option.
- Select the rows option for load scheme: [Rows [A01,A02,A03]]
- If you will be archiving plates of primers for later use consider selecting the option for replicate plates.

References

- Amaral---Zettler, LA, EA McCliment, HW Ducklow, SM Huse. 2009. A method for studying protistan diversity using massively parallel sequencing of V9 hypervariable regions of small---subunit ribosomal RNA genes. PLoS ONE 4:e6372.
- Caporaso JG, Lauber CL, Walters WA, Berg---Lyons D, Huntley J, Fierer N, Owens SM, Betley J, Fraser L, Bauer M, Gormley N, Gilbert JA, Smith G, Knight R. 2012. Ultra---high---throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. ISME J. 2012 Aug; 6(8): 1621–1624.

4.1.7 Fungal ITS gene (ITS1F and ITS4) 1-step

The protocol detailed here is designed to investigate the fungal diversity in environmental samples through amplification of the ITS1F and ITS4 region of the fungal ITS for paired-end sequencing on the Illumina MiSeq platform.

Primers for amplification: ITS1F and ITS4 region

ILM_ITS1F_Uv2 – Forward primer

Field number (space---delimited), description:

1. 5' Illumina adapter
2. Forward primer pad
3. Forward primer linker
4. Forward primer (ITS1F)

AATGATACGGCGACCACCGAGATCTACAC TGTCGGGCTT CG CTTGGTCATTTAGAGGAAGTAA

ILM_ITS4Rv2_00NN – Reverse primer

Each sequence contains different 12 base Golay barcode as described by Caporaso et al.

1. Reverse complement of 3' Illumina adapter
2. Golay barcode

3. Reverse primer pad
4. Reverse primer linker
5. Reverse primer (ITS4)

CAAGCAGAAGACGGC ATACGAGAT XXXXXXXXXXXX AGTCCGTC CG GA TCCTCCGCTTATTGATATGC

Preparation of master mix for amplification ITS1F and ITS4 region (for 1 rxn)

Component	Volume (µL)	Final Conc.
10x ImmoBuffer ^(a)	2.50	1 X
10 mM dNTP	0.50	200 nM
50mM MgCl ₂	1.25	2.5 mM
ILM_ITS1F_Uv2 (5µM)	2.50	500 nM
ILM_ITS4Rv2_XXXX (5µM)	2.50	500 nM
Immolase DNA Polymerase (5U/µL) ^(a)	0.20	1 Unit
H ₂ O	14.55	---
Template	1.0	---
Total Volume	25	---

(a) Immolase DNA Polymerase (Bioline, #BIO---21047)

Thermocycler conditions for amplification (for 96 well thermocycler)

	Temperature	Time (mm:ss)
Activation	94°C	10:00
Amplification (35 cycles)	94°C	00:30
	55°C	01:00
	72°C	01:00
Final Extension	72°C	10:00

Amplification method

6. Optional: Dilute DNA 1:10.
7. Amplify samples with conditions outlined above.
8. Run amplicons on an agarose gel. Expected band size for ITS1F / ITS4 fragment is approximately 850 bp.
9. If there is no band present, repeat PCR using either the undiluted DNA or a 1:100 dilution. Use the concentration of the DNA extract to determine if the DNA should be further diluted or used neat.
10. Clean and normalise the PCR products. For this step, both Agencourt AMPure XP bead clean-up and SequalPrep Normalisation plates (Invitrogen, A1051001) are acceptable. If using Agencourt AMPure XP beads:
 - a. Perform a bead clean---up following manufacturer’s instructions.
 - b. Quantify amplicon yields. Acceptable methods include Picogreen (see manufacturers protocol; Invitrogen Item #P11496) or the high sensitivity Quant-iT™ DNA Assay Kit (Life Technologies, Q-33120).
 - c. Run clean amplicons on Agilent Bioanalyzer, LabChip GX, or 2200 TapeStation to get accurate sizing information.
 - d. Normalise amplicons by diluting to 10 nM with variable volume of buffer (EB buffer, or 10 mM Tris pH 8.5) and a set volume of the amplicons.
 - e. Pool equal volumes of each normalised amplicon.
 - f. If using SequalPrep Normalisation plates:
 - g. Perform a plate normalisation following manufacturer’s instructions.
 - h. Pool equal volumes of each normalized amplicon.
 - i. Run clean amplicon pool on Agilent Bioanalyzer, LabChip GX, or 2200 TapeStation to get accurate sizing information.

Optional: If spurious bands were present on gel (in step 3), or in the clean amplicons (step 5c) or amplicon pool (step 5h), a portion of the final amplicon pool can go through a gel extraction or a second round of bead clean up. A lower bead to template ratio will get rid of small bands.

Measure concentration of the final clean pool using Qubit or picogreen.

Sequencing Primers

Read 1 Primer (ILM_ITS_R1v2)

Field description (space---delimited):

1. Forward primer pad
2. Forward primer linker
3. Forward primer

ACACTGTCCGGCTT CG CTTGGTCATTTAGAGGAAGTAA

Read 2 Primer (ILM_ITS_R2v2)

Field description (space---delimited):

1. Reverse primer pad
2. Reverse primer linker
3. Reverse primer

AGTCCGTC CG GA TCCTCCGCTTATTGATATGC

Index Read Primer (ILM_ITS_INDEXv3)

Field description (space---delimited):

1. Reverse complement of reverse primer
2. Reverse complement of reverse primer linker
3. Reverse complement of reverse primer pad

GCATATCAATAAGCGGAGGA TC CGGACGGACT

References

- Caporaso JG, Lauber CL, Walters WA, Berg---Lyons D, Huntley J, Fierer N, Owens SM, Betley J, Fraser L, Bauer M, Gormley N, Gilbert JA, Smith G, Knight R. 2012. Ultra---high---throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. *ISME J*
- Gardes, M., and T. D. Bruns. 1993. ITS primers with enhanced specificity for basidiomycetes --- application to the identification of mycorrhizae and rusts. *Mol. Ecol.* 2: 113---118
- White, T. J., T. Bruns, S. Lee, and J. W. Taylor. 1990. Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. Pp. 315---322 In: *PCR Protocols: A Guide to Methods and Applications*, eds. Innis, M. A., D. H. Gelfand, J. J. Sninsky, and T. J. White. Academic Press, Inc., New York.

4.1.8 Illumina TruSeqNano Library Preparation for Metagenomics (BASE samples)

This protocol describes the procedure for preparing libraries from metagenomic samples to be sequenced on the Illumina HiSeq 2500 platform. The library preparation follows Illuminas TruSeqNano DNA Sample Preparation Guide (Part # 15041110 Rev. B, November 2013).

Quality check of the input DNA

Verify the quality of the extracted genomic DNA using the Caliper LabChipGX (Perkin Elmer) or the TapeStation genomic chip (Agilent), and the nanodrop (Thermo Scientific) or Xpose (Agilent). The concentration should be verified with the Qubit or picogreen assay (Invitrogen).

Library Preparation

1. Dilute 200ng of DNA in a volume of 52.5ul of RSB or EB.
2. Shear the DNA as per protocol following the 550bp Covaris settings.

3. Perform clean-up of the DNA using AMPure beads following the 550bp insert bead ratio. End-repair, A-tailing and ligation of adapters follow the HT Nano Protocol of the TruSeq Nano DNA Sample Preparation Guide (Part # 15041110 Rev. B, November 2013). Use the DAP plate to index each individual library.
4. Follow the protocol to perform the PCR, using 8 PCR cycles.
5. Clean-up the PCR-amplified DNA following the 550bp insert ratio of AMPure beads.

Quality check of the libraries

1. Verify the size of the libraries on the LabChipGx HS chip, the Bioanalyzer HS or the TapeStation.
2. Measure the concentration of the libraries using Qubit HS or picogreen.
3. Dilute the libraries to 10nM stocks and pool an equal amount of each library. Bring the final 10 nM pool to 2nM.

4.1.9 Illumina Nextera XT Library Preparation for Metagenomics (as per MM 2015-2017)

This protocol describes the procedure for preparing metagenomic libraries from marine samples to be sequenced on the Illumina HiSeq 2500 platform. The library preparation follows Illumina's Nextera XT DNA Library Prep Reference Guide (Document # 15031942 v01, January 2016) except for the bead-based normalisation and pooling steps.

Quality check of the input DNA

Verify the quality of the extracted genomic DNA using the NanoDrop (Thermo Scientific) or DropSense 16 (Trinean, formerly known as Xpose). The concentration should be verified with the Qubit or PicoGreen assay (Invitrogen).

Library preparation

1. Dilute 1ng of DNA to a concentration of 0.2ng/ul.
2. DNA tagmentation follows Illumina's Nextera XT DNA Library Prep Reference Guide (Document # 15031942 v01, January 2016).
3. Library amplification follows the protocol and includes 12 cycles of PCR to enrich for fragments with correctly added indexed adapters.
4. Clean up the amplified libraries using AMPure XP or AxyPrep Mag PCR clean-up beads according to the protocol.

Quality check of the libraries

1. Verify the size of the libraries on the LabChip GXII (Perkin Elmer) or the TapeStation (Agilent).
2. Measure the concentration of the libraries using Qubit, PicoGreen, or qPCR.
3. Normalise libraries to 3 nM and pool the libraries manually, do not follow bead-based normalisation and pooling protocol detailed in the sections of the Prep Guide entitled "Normalize Libraries" and "Pool Libraries".
4. Dilute the final pool to a concentration of 2nM.

4.1.10 Metagenomics Library Preparation (AM new)

4.1.11 Illumina Ribo-Zero rRNA Removal Kit for Metatranscriptomics (as per MM 2015-2017)

This protocol describes the procedure for preparing metatranscriptomic libraries from samples to be sequenced on the Illumina HiSeq 2500 platform. The library preparation follows the Ribo-Zero rRNA Removal Kit Reference

Guide (Document # 15066012 v02, August 2016) and TruSeq Stranded mRNA Sample Preparation Guide (Document # 15031047 Rev. E, October 2013) with some protocol variations.

Quality check of the input DNA

1. Verify the quality of the extracted RNA using the Bioanalyzer RNA 6000 Nano or RNA 6000 Pico Kit.

Library preparation

Note: The Ribo-Zero rRNA Removal kits support rRNA depletion from 1-5 µg of total RNA. Probes from the Bacteria and Pant Ribo-Zero kits can be combined (50:50 ratio) to simultaneously deplete both bacterial and plant rRNA. In this instance the inputs of RNA should be halved (i.e. 500 ng -2.5 ng of total RNA). The inputs for the sediment RNA sample maybe much lower than these recommended inputs.

1. Bring ≤1 µg of total RNA to a final volume of 28 µl with RNase-free water.
2. Perform rRNA depletion following the Ribo-Zero Kit Reference Guide (Document # 15066012 v02, August 2016).
3. Perform clean-up of rRNA depleted supernatant using Agencourt RNAClean XP Kit according to the Ribo-Zero Kit Reference Guide. Elute samples in 8.5 µl of RNase-free water.

4.1.12 Illumina TruSeq Stranded mRNA Library Preparation for Metatranscriptomics (as per MM 2015-2017)

Library preparation

1. Perform TruSeq Stranded mRNA-seq library preparation according the TruSeq Stranded mRNA Sample Preparation Guide (Document # 15031047 Rev. E, October 2013), skipping the purification of poly(A) RNAs.
2. Add 13 µl of Fragment, Prime and Finish Mix to 5 µl of rRNA depleted rRNA sample and follow the library prep protocol from the Incubate RFP step (page 20).
3. At the Enrich DNA Fragments step (page 38-41) perform 13-15 cycles of PCR

For inputs of 1 µg use 13 cycles, for inputs < 200 ng use 15 cycles

Quality check of the libraries

1. Assess the size of the RNA-seq libraries via electrophoresis using the Agilent TapeStation TapeScreen DNA 1000 Assay or similar (Agilent Bioanalyzer, Perkin Elmer LabChip GXII).
2. Quantify libraries using qPCR (KAPA Library Quantification Kits for Illumina or similar).
3. Normalise the libraries to 3 nM and pool libraries for sequence following the library prep protocol (Normalise and Pool Libraries, page 44).

4.1.13 rRNA removal step for Metatranscriptomics Library Preparation (AM new)

Currently there are no new projects in the AM utilising metatranscriptomics. Methods will be updated when/if there is a requirement for metatranscriptomics data.

4.1.14 Library Preparation for Metatranscriptomics Library Preparation (AM new)

Currently there are no new projects in the AM utilising metatranscriptomics. Methods will be updated when/if there is a requirement for metatranscriptomics data.

4.2 Next Generation Sequencing

4.2.1 Illumina MiSeq 2 x 300 bp paired reads

E.g. B16S, A16S, ITS1 and 4

1. Dilute pool to **4nM**.
2. Denature according to Illumina protocol, with increased PhiX control spike as recommended for low diversity libraries. See *Preparing Libraries for Sequencing on the MiSeq* (part #15039740).
3. Prepare MiSeq Reagent Cartridge (v3 600 cycles). see *MiSeq Reagent Kit v3 Reagent Preparation Guide* (part # 15044983).
4. Add custom sequencing primers into reservoirs 12-14. See *Using Custom Primers on the Miseq* (part # 15041638).
5. Using an extra-long pipette tip set to 75 µL, add 3.4 µL of Read1 sequencing primer (100 µM) into well 12 of the MiSeq Reagent Cartridge and mix 10 times. Repeat adding the Index Primer into well 13 and the Read2 sequencing primer into well 14.
6. Load 600 µl of library pool into the MiSeq reagent cartridge in designated reservoir/sample well.
7. Modify sample sheet to include appropriate primer's sequence/indexes
8. Start sequencing run following *MiSeq System User Guide* (part # 15027617).

4.2.2 Illumina MiSeq 2 x 250 bp paired reads A

E.g. 18Sv4

1. Dilute pool to **4nM**.
2. Denature according to Illumina protocol, with increased PhiX control spike as recommended for low diversity libraries. See *Preparing Libraries for Sequencing on the MiSeq* (part #15039740).
3. Prepare MiSeq Reagent Cartridge (v2 500-cycles). See *MiSeq Reagent Kit v2 - Reagent Preparation Guide* (part # 15034097).
4. Using an extra-long pipette tip set to 75 µL, add 3.4 µL of Read1 sequencing primer (100 µM) into well 12 of the MiSeq Reagent Cartridge and mix 10 times. Repeat adding the Index Primer into well 13 and the Read2 sequencing primer into well 14.
5. Load 600 µl of library pool into the MiSeq reagent cartridge in designated r reservoir/sample well.
6. Modify sample sheet to include appropriate primer's sequence/indexes.
7. Start sequencing run following *MiSeq System User Guide* (part # 15027617).

4.2.3 Illumina MiSeq 2 x 250 bp paired reads B

E.g. 18Sv9

1. Dilute pool to **4nM**.
2. Denature according to Illumina protocol, with increased PhiX control spike-in as recommended for low diversity libraries. See *Preparing Libraries for Sequencing on the MiSeq* (part #15039740).
3. Prepare MiSeq Reagent Cartridge (v2 300 - cycles). See *MiSeq Reagent Kit v2 - Reagent Preparation Guide* (part # 15034097).
4. Using an extra-long pipette tip set to 75 µL, add 3.4 µL of Read1 sequencing primer (100 µM) into well 12 of the MiSeq Reagent Cartridge and mix 10 times. Repeat adding the Index Primer into well 13 and the Read2 sequencing primer into well 14.
5. Load 600 µL of library pool into the MiSeq reagent cartridge in designated reservoir/sample well.
6. Modify sample sheet to include the appropriate primer's sequence/indexes.
7. Start sequencing run following *MiSeq System User Guide* (part # 15027617).

4.2.4 Illumina HiSeq 2500 Rapid v2 (as per BASE metagenomics sequencing methods date?)

1. Thaw Illuminas HiSeq2500 Rapid v2 kits. Use one 200-cycle kit and two 50-cycle kits if performing one run, and three 200-cycle kits if performing two runs simultaneously. Follow Illuminas HiSeq Rapid SBS Kit v2 Reference Guide (Part# 15058772, Rev. B, May 2015).
2. Denature the 2nM pool according to the Illumina protocol (Denaturing and Diluting Libraries for the HiSeq and GAllx, Part# 15050107 Rev. C, November 2014).
3. Start the HiSe2500 Rapid sequencing run according to the user guide (HiSeq2500 System User Guide, Part# 15035786 Rev. D, November 2014).
4. After sequencing is complete, demultiplex the data using Casava 1.8.2.

4.2.5 Illumina HiSeq 2500 Rapid SBS 500-cycle v2 (as per MM metagenomics sequencing methods 2015-2017)

1. Following Illumina's HiSeq 2500 System Guide (Document # 15035786 v01, October 2015), thaw one HiSeq 2500 Rapid SBS 500-cycle v2 kit per flowcell.
2. Denature the 2nM pool according to the Illumina's HiSeq and GAllx Systems -Denature and Dilute Libraries Guide (Document # 15050107 v03, November 2016).
3. Start the HiSeq 2500 Rapid sequencing run according to the system guide referenced above.
4. After sequencing is complete, perform the basecalling and demultiplexing using bcl2fastq Conversion Software.

4.2.6 Illumina HiSeq 2500 Rapid SBS v4 (as per MM transcriptomics sequencing methods 2015-2017)

1. Denature the 2nM pool according to the Illumina's HiSeq and GAllx Systems -Denature and Dilute Libraries Guide (Document # 15050107 v03, November 2016).
2. Following Illumina's cBot System Guide (Document # 15006165 v02 January 2016), perform clustering of HiSeq PECluster Kit v4 (PE-401-4001).
3. Following Illumina's HiSeq 2500 System Guide System Guide (Document # 15035786 v01 October 2015), prepare the HiSeq SBS v4 Reagents for a 2x100 bp PE or 2x125 bp PE sequencing run.
4. Start the HiSeq2500 Rapid sequencing run according to the system guide referenced above.
5. After sequencing is complete, perform the basecalling and demultiplexing using bcl2fastq Conversion Software.

4.2.7 Illumina NovaSeq 6000

1. Following Illumina's NovaSeq 6000 System Guide (Document # 1000000019358 v11, February 2019), NovaSeq 6000 SP Reagent Kit (500 cycles) reagent cartridge.
2. Dilute the library pool to 1.75 nM and denature according to the NovaSeq 6000 System Guide.
3. Start the NovaSeq 6000 sequencing run according to the system guide referenced above.
4. After sequencing is complete, perform the base-calling and demultiplexing using bcl2fastq Conversion Software.

5 Bioinformatics

Codes relating to the Australian Microbiome Initiative will be deposited on the initiative GitHub

- <https://github.com/AusMicrobiome>

5.1 Bacterial & Archaeal 16S v1-3 Amplicon Analysis

<https://www.australianmicrobiome.com/protocols/16sanalysisworkflow/>

5.2 Eukaryote 18S v4 Amplicon Analysis

<https://www.australianmicrobiome.com/protocols/18sanalysisworkflow/>

5.3 Fungal ITS Amplicon Analysis

<https://www.australianmicrobiome.com/protocols/itsanalysisworkflow/>

6 Known GAPS

Need to have Elizabeth/Katelyn check the water sampling and extraction methods for saline lakes.

Confirm ACMON sampling protocol

Contextual data methods for voyages on Investigator (CSIRO), a couple of hydrochem processing reports not available on data trawler.

Flow cytometry method for voyage samples e.g. GOSHIP, Martin/Martina?

Omics library prep and sequencing methods all need confirmation, these have been put together from multiple sources, e.g. the new manual, old data portal methods, email etc.

Document Control

VERSION	DATE	AUTHOR(S)	DESCRIPTION
V1.0	05/05/2020	Jodie van de Kamp	A preliminary document detailing the outline of the roadmap draft including software list obtained from researchers.
V1.1	21/05/2020	Sophie Mazard	Comments and edits, formatting
V1.2	01/09/2020	Jodie van de Kamp	Draft ready for distribution to the community.