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, please measure and note the following parameters:

1) Longitude and latitude of sampling site in decimal degrees
2) Time and data in the format dd:mm:yyyy hh:mm
3) Depth in metres
4) Light intensity in µmol photons m^{-2} s^{-1} both at the surface and at the depth where samples where taken

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

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 time. Attach a 0.45um syringe filter and push a few milliliters 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. (-20oC). 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-labeled 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 build 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.
1. When the 2L 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.
2. 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.
3. Freeze immediately at -80 degrees.
4. 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. http://zoxoro.com.au/Products/Oetiker-14100329-Economy-Tool-Standard-Jaw-Pincers.html?gclid=CKmRkuytrMgCFU0GvAodHewK7w)
5. 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.
6. Extract using the Mobio Powersoil kit, which includes a beating step with the vortex adaptor.

7. The final eluted DNA should be labelled according to the instruction given in the spreadsheet "sampling_schedule" and stored at -80ºC.

Preliminary protocol for water sampling for metatranscriptomics

6. Use the same filtering set-up as in step 5 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.
8. When the 2L 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.
9. Ensure that the sterivex is labelled according to the instruction given in the spreadsheet "sampling_schedule" and append an "RNA" at the end.
10. 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.
11. Freeze immediately at -80 degrees.

Sampling for DIC and TA

12. Use (250 mL)- borosilicate glass bottles with stopper http://www.labdirect.com.au/glassware/bottles/storage-bottles/ or with screw cap https://www.thermofisher.com.au/search.aspx?search=borosilicate+glass+vials+with+screw+caps#gsc.tab=0&gsc.q=borosilicate%20glass%20vials%20with%20screw%20caps to collect samples. All bottles should be acid washed and well rinsed with seawater.
13. 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.
14. Collect n=3 per site. Labelled bottles according to the instruction given in the spreadsheet "sampling_schedule".
15. Leave 1-2cm head-space in sample bottles.
16. Add 100 uL for 250 mL bottle (adjust for volume of container) of $HgCl_2$ using a pipette. Make sure to use a pipette dedicated for toxics. Do not submerge pipette tip into sample.
17. 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 $HgCl_2$ solutions are 0.02-0.05% of the total sample volume.
18. Invert the sample several times to mix the mercuric chloride thoroughly.
19. Seal the sample bottle with silicon grease around the stopper and a rubber band. If using screw cap seal with parafilm.
20. Store bottles at cold/ambient temperature in the dark.

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2) SOP for sediment sampling and processing for amplicon and metatranscriptome sequencing

Sampling equipment:

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

Processing and extractions equipment:
MoBio Power Soil RNA Isolation Kit (25 preps)
MoBio Power Soil DNA Isolation Kit (100 preps)
Molecular grade ethanol (for RNA/DNA clean)
DEPC water (for RNA clean)
Nuclease free water (for DNA clean)
PCR snap tubes (for RNA/DNA clean)
SuperMagnet
Axygen 1.5mL collection tubes
Various size pipettes and pipette tips
Mini centrifuge
Vortex and vortex adaptor (6 place for RNA and 24 place for DNA)
Vacuum pump and Sartorius filter holder with sump
2mm and 63um mesh

Sampling:

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 1.8ml 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 ~150ml 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 freezer and RNA should be extracted within 1 week. Glass jars (TOC) and plastic vials (grain sizes) to be stored in -20 freezer and analysed within 2 weeks.

Sediment grain size (% fines)

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

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

Sediment TOC

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

RNA extraction

Extract RNA from frozen sediment samples (2g) with MoBio Power Soil RNA Isolation Kit (http://www.mobio.com) following the modified extraction protocol below.

1. Prep a 15mL **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 3.5mL 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 1.5mL 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 15mL Collection Tube with 5mL 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 2.2mL **collection tubes from the kit**. Add 1mL 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 **1.5mL 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 aliquotting 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).

1. Using one of the 1.5mL sterile tubes, make a DNase mastermix:
a) FOR 12 SAMPLES: To 52uL of TurboDNase buffer, gently add 13uL of TurboDNase and very gently mix with your pipette tip.
b) Transfer 5uL of this DNase mastermix to each tube (there should be a minimum of 5uL in each tube).
2. Incubate samples for 20min at 37°C

**Take DNase inactivation reagent out of freezer to thaw.*

3. Add 4.5uL of resuspended DNase Inactivation Reagent and mix well.

4. 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.*

5. 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 100uL of RNAclean solution. Gently shake before to resuspend beads.
2. Add ~100uL 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 µL 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 10uL RNA to 3 x 1.5mL tubes. With care, a final drop of sample containing magnet beads can be separated and removed.
14. Place 3 x 10uL RNA aliquots into -80C freezer. Keep 1 x 10uL "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^{o}C.

DNA extractions

Extract DNA from frozen sediment samples (1.5g) with MoBio Power Soil DNA Isolation Kit (http://www.mobio.com) following extraction protocol.

DNA clean

1. In PCR plate, pipette from aliquots 100uL of AMPure solution. Gently shake before to resuspend beads.
2. Add ~100uL 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 100uL nuclease free water for DNA 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 being aliquoting 50uL DNA to remaining 1.5mL tube. With care, a final drop of sample containing magnet beads can be separated and removed.
14. Place 1 x 50uL DNA aliquot at -20C. Keep 1 x 50uL 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^{o}C.

3) SOP for sampling and processing seagrass transcriptome and sediment for metatranscriptome sequencing

Equipment

- Van Veen grab
- Liquid nitrogen dewar (to be used both, on site and in the lab)
- 1.8 mL Nunc Cryotube vials
- Cryolabels
- Aluminum foil
- Gloves
- Scissors
- 250 mL glass jar
- 100 mL specimen jars
- Esky & ice
- Black bag
- Plastic resealable bags
- MoBio Power Soil RNA Isolation Kit
- PureLink RNA Mini Kit and PureLink DNase
- Instrumentation for nutrients analysis of porewater and TOC analysis of sediment (AutoAnalyzer and standard spectrophotometric analysis)
- Vacuum pump and Sartorius filter holder with sump
- 2mm and 63mm sieves

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

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 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 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.

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 aluminum foil envelopes previously labeled 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 aluminum envelops in a labeled box in -80 freezer for further analysis.
7. RNA should be extracted from leaves within 2 weeks.

Sediment nutrients

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).

Sediment grain size (% fines)

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 um mesh) and fines (collected in sump).
2. Transfer sediments into pre-weighed and labeled 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.

Sediment TOC

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

Sediment DNA and RNA extraction

1. Extract frozen sediment samples (0.25g) with MoBio Power Soil DNA Isolation Kit (http://www.mobio.com) following extraction protocol.
2. Extract frozen sediment samples (up to 2g) with MoBio Power Soil RNA Isolation Kit (http://www.mobio.com) following extraction protocol.

Seagrass RNA extraction

1. 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 ~50uL 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 μL 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 1.5uL 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 1.5uL tubes. With care, a final drop of sample containing magnet beads can be separated and removed.
14. Place 3x 10uL RNA aliquots into -80C 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).

4) SOP for the sampling of *Ecklonia radiata* and processing for amplicon and metagenome sequencing

General note for the collection:

1. First time the site is visited, abundance of *E. radiata* will be measured over 10 haphazardly thrown 50cmx50cm quadrats. This will be repeated as required during the 14 month observation period.
2. Select haphazardly 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%)

Sampling:

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 >=30cm^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 um 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^{o}C for long-term).

DNA extraction

1. Extract each replicate swab (n=3) separately with the MoBio Power Soil Isolation kit (http://www.mobio.com/soil-dna-isolation/powersoil-dna-isolation-kit.html).
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.

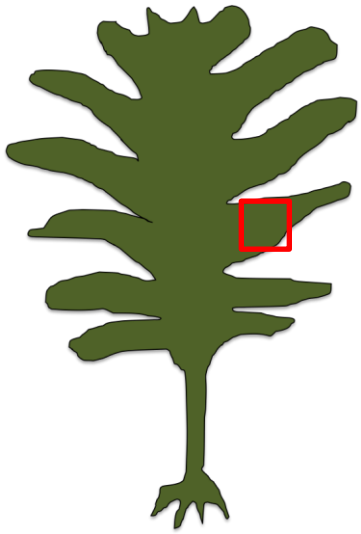
   - Step #18. Make sure filter is completely dry after this step. If not, repeat.

   - Steps #20-21. Split into 2 steps using 50 µl (rather than 100 ul once). Heat up solution C6 to 50^{o}C (when you start the whole lot), add 50 µl and leave it for 5 min, centrifuge at room temperature for 30 sec at 10,000g, and repeat (final volume = 100 ul).
3. The final eluted DNA should be labelled according to the instruction given in the spreadsheet "sampling_schedule" and stored at -20^{o}C.

Additional information:

PAM fluorometry measurements <u>*in situ*</u>: After bringing the algal tissue to the surface and swabbing ~20cm^2 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^{-2} s^{-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^{-2} s^{-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 (F_M') will be determined as the fluorescence value during the saturating flash. The difference between F_M' and F is the variable fluorescence, ΔF. Effective quantum yield (EQY, $\Delta F/F_M'$) 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.



5) SOP for the sampling of sponges and processing for amplicon and metatranscriptome sequencing

General notes on collection:

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)

Sampling:

1. Cut sponge samples underwater into small pieces (~5g) 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).

DNA extraction for amplicon sequencing

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 (~0.5g) for each replicate (n=3) separately with MoBio Power Soil Isolation kit (http://www.mobio.com/soil-dna-isolation/powersoil-dna-isolation-kit.html).
3. The final eluted DNA should be labelled according to the instruction given in the spreadsheet "sampling_schedule" and stored at -80^{o}C.

RNA extraction, host-mRNA removal and rRNA depletion

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 30s 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 100ul. (This usually results in 700 – 1000 ng/ul 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 ug 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 2ul Glycogen, 1/10 volume 3M NaOAc, 2.5 volumes of ethanol and incubate overnight at -20C).
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^{o}C.

6) SOP for the sampling of coral and processing for 16S rRNA gene amplicon sequencing

General note for the collection:

1. Relative abundance of chosen coral species at each site should be contextualised on each visit by 10 haphazardly thrown 50cmx50cm 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)

Sampling:

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 0.5M 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 -80ºC (or -20ºC).

DNA extraction:

1. Extracting DNA with the MoBio PowerPlant Pro DNA Isolation kit using Standard Protocols (http:// 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 -80ºC until required.
3. The final eluted DNA should be labelled according to the instruction given in the spreadsheet "sampling_schedule" and stored at -80ºC.