

Bryozoan calcification and kelp photosynthesis protocol

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Species: *Laminaria digitata*, *Laminaria hyperborea*, *Laminaria ochroleuca*, *Saccharina latissima* and *Saccorhiza polyschides* (ask Andy for identification)

Sites: West Hoe (50.363629°N, 4.144978°W), Firestone Bay (50.360977°N, 4.160152°W) and/or Rame Head (50.318627°N, 4.227871°W)

Aim: simultaneously measure photosynthesis, respiration, and calcification of the kelp-bryozoan symbiosis to understand inorganic and organic carbon cycling

Methods: light and dark O₂ and total alkalinity evolution and image analysis

Oxygen metre calibration

1. Make up 2 L of 1% (w/v) sodium sulfite (Na₂SO₃) solution (20 g Na₂SO₃ in 2 L ultrapure water) and leave to react for ~30 min so all O₂ is removed
2. In the meantime, let air (not O₂!) bubble through a tub of deionised water from the tap for ~30 min to ensure 100% air saturation
3. Fill each of the 12 sensor spot jars to the brim with O₂-free Na₂SO₃ solution
4. Fill the 13th jar without the sensor spot with the remaining solution
5. Attach custom-made Velcro adapters and adjust position of the opening (red dowel) so it aligns exactly with the sensor spot (best done by looking through)
6. Pair the 12 fibre-optic cables of the oxygen metres with their matching jars (both numbered 1.1 to 3.4) by gently inserting the tip into the red dowel up to the glass
7. Place them on the magnetic stirrer, turn it on and let the solution mix for ~30 s
8. Place the single dipping temperature probe connected to channel 1.1 into the 13th jar to the left of the magnetic stirrer
9. Calibration is less variable under little to no light, so turn off the bright top light
10. Click the “Calibrate” button in the “Live View” tab of the menu bar in PreSens Measurement Studio 2
11. The measurements for all channels, given in °, will be coming through live
12. After letting the values settle for ~1 min, click “Apply” in the first column and then “Save” in the bottom right of the pop-up window to save the 0% air saturation calibration
13. Empty the Na₂SO₃ solution down the sink, thoroughly rinse all jars with deionised water and fill with 100% air-saturated water
14. Repeat steps 4 to 8
15. After letting the values settle for ~1 min, click “Apply” in the second column and then “Save” in the bottom right of the pop-up window to save the 100% air saturation calibration
16. This calibration should be done weekly to ensure reliable measurement
17. If in doubt, read up on manuals “Measurement Studio 2 Instruction Manual” and “Oxygen Sensor Spots PSt3 / PSt6 Instruction Manual”

Sampling

1. Plan with the tides since sampling at low spring tide will make it a lot easier
2. Collect the whole lamina of one at least partially bryozoan-covered sporophyte of any study species above the meristem (sampling regulation to allow regrowth)
3. Return sample to the lab in ziplock bags filled with water in a bagpack (lighter than cool box) within 30 min to ensure there is no O₂ deficit inside the ziplock
4. Place kelp in a large, aerated tub in the 13°C controlled-temperature (CT) room
5. Fill a small tub with fresh seawater from the large 15°C CT room, place an airstone inside it in the 13°C CT room and leave to cool down
6. Place 12 sensor spot jars in the small plastic tray (they should fit exactly)
7. Fill jars with fresh seawater from the large 15°C CT room
8. Place the tray on the workbench by the window in the large 15°C CT room together with a clean scalpel/razorblade and cutting board
9. Fetch the kelp lamina from the 13°C CT room with a small tub filled with seawater from the large tub and place opposite the tray next to the cutting board
10. Cut the lamina into 11 pieces (~5 × 5 cm) with varying bryozoan cover
11. Lamina subsamples should span 0–100% bryozoan cover (in case there is no tissue covered by bryozoans on both sides, 100%, just sample maximal cover)
12. Randomly place the 11 subsamples into the jars as you're cutting, leaving the 12th, randomly assigned, jar empty for the seawater blank
13. Make sure not to accidentally include *Patella pellucida* or *Steromphala cineraria*
14. Carry the tray and tub back to the 13°C CT room and return the remaining lamina to the large tub



Bryozoan calcification on *Laminaria hyperborea* at Rame Head.

Incubation and O₂ measurement

1. Prepare oxygen metres and launch Measurement Studio 2, ensuring that the laptop is also plugged into a power source and doesn't die during measurement
2. Prepare 11 labelled ziplock bags according to the following labelling system:

species initial	no space	sporophyte no.	period	subsample no.
examples	d1.7	=	<i>L. digitata</i> , sporophyte 1, subsample 7	
	p15.11	=	<i>S. polyschides</i> , sporophyte 15, subsample 11	
	o5.5	=	<i>L. ochroleuca</i> , sporophyte 5, subsample 5	
	l8.9	=	<i>S. latissima</i> , sporophyte 8, subsample 9	
	h3.2	=	<i>L. hyperborea</i> , sporophyte 3, subsample 2	

3. Prepare 50-mL syringes with screw-on filter tips, ensuring that they contain fresh 0.45-µm filter paper (this will likely get blocked during sampling so have spares)
4. Label 24 test tubes (50-mL) to store seawater samples for subsequent total alkalinity measurement according to the following labelling system:

sample label as above	no space	light or dark initial
examples	d7.10l	= <i>L. digitata</i> , sporophyte 7, subsample 10, light
	h2.3d	= <i>L. hyperborea</i> , sporophyte 2, subsample 3, dark

or for seawater blanks

blank initial	no space	measurement round	light/dark initial
examples	b6d	= blank, measurement round 6, dark	
	b10l	= blank, measurement round 10, light	

where “measurement round” is defined as one set of light and dark measurements

5. These labels are for seawater samples after light and dark incubations
6. Label two additional 50-mL tubes for baseline seawater samples as follows:

sample label without subsample	slash	blank label with light/dark initial
examples	p8/b3l	= <i>S. polyschides</i> , sporophyte 8 and blank, measurement round 3, light
	l20/b15d	= <i>S. latissima</i> , sporophyte 20 and blank measurement round 15, dark

7. Measure the small tub water salinity using the digital refractometer and its pipette (the refractometer should be calibrated every day using ultrapure water)
 8. If the measurement is far from 35‰, the digital refractometer is either broken or not calibrated (when cross-validating with a handheld refractometer, make sure to do this at room temperature since temperature correction is set at 20°C)
 9. Enter the salinity value and set the measurement interval to 3 s in the last and fifth columns respectively of the “Live View” tab in Measurement Studio 2
 10. Create 12 new measurement files in the “Measurements” tab of Measurement Studio 2 and name them according to the following labelling system:

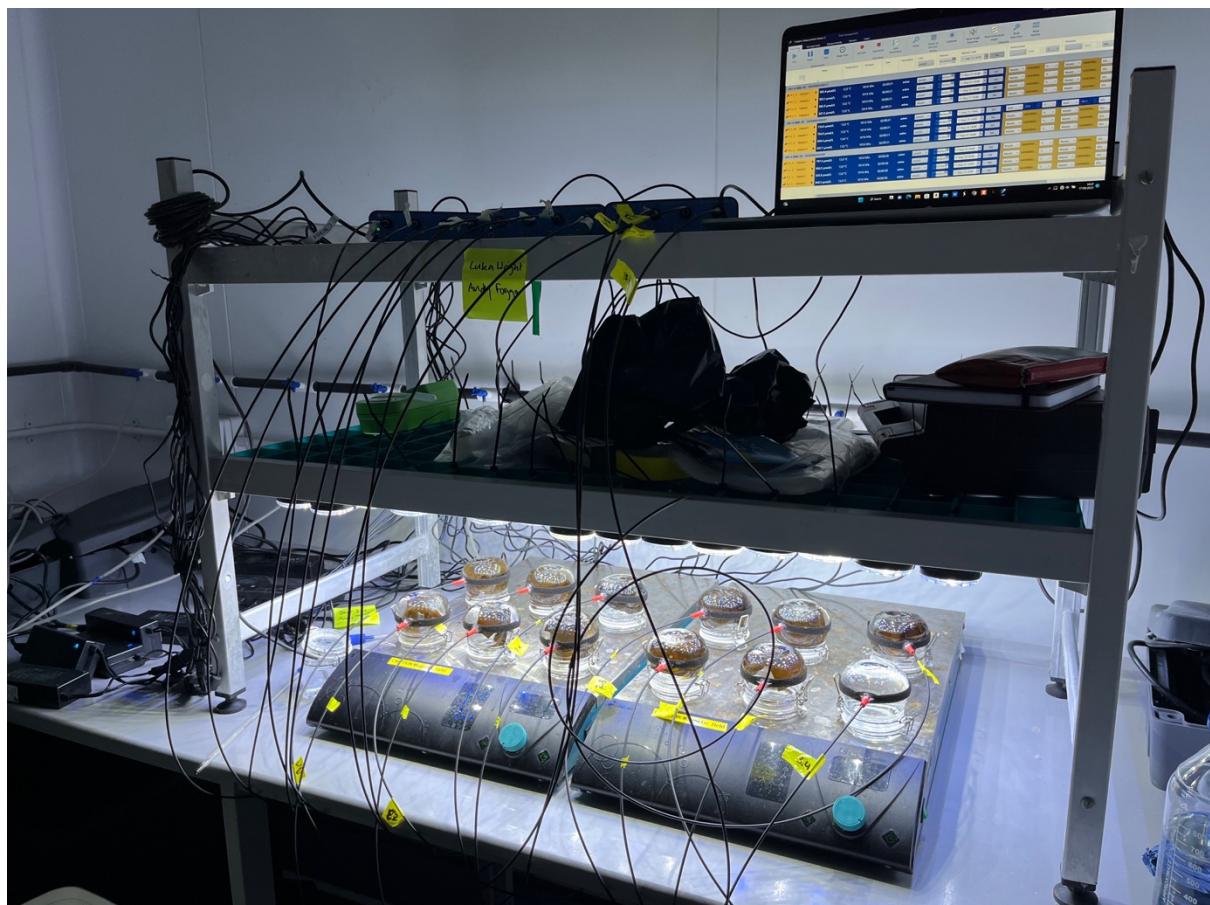
Date as YYMMDD underscore full sample/blank label as above

11. Assign measurements to channels in the “Management” tab of Measurement Studio 2, making sure all channels are deactivated so they don’t start recording
 12. Make sure channels 1.2 to 3.4 (marked S for “slave”) are subordinate to 1.1 (marked M for “master”) and inherit its temperature and pressure measurements
 13. Remove the air stone from the small tub
 14. One at a time, empty each jar, fill it with aerated seawater from the small tub by submerging and locking the seal underwater, making sure to include the tissue sample and magnetic stir bar and exclude air bubbles (check before emersing)
 15. Attach custom-made Velcro adapters and adjust position of the opening (red dowel) so it aligns exactly with the sensor spot (best done by looking through)
 16. Remove any water stuck in the red dowel by blowing into it
 17. Turn the jar upside down so the flat lid faces down, shake to make sure the magnetic stir bar is in the lid and place on stirrer according to this layout:

	Magnetic stirrer 1			Magnetic stirrer 2		
	1	2	3	1	2	3
A						
B						
C	1.1	1.3	2.1	2.3	3.1	3.3
D						
E	1.2	1.4	2.2	2.4	3.2	3.4

18. Fill the spare jar with seawater from the same tub and place the dipping temperature probe connected to channel 1.1 into it to the left of stirrer 1

19. Pair the 12 fibre-optic cables of the oxygen metres with their matching jars (both numbered 1.1 to 3.4) by gently inserting the tip into the red dowel up to the glass
20. Take a 50-mL water sample from the small tub with the syringe by gently aspirating without the filter tip and then gently dispensing into the baseline light sample tube with the filter tip, leaving no airspace (use surface tension of water)
21. Turn magnetic stirrer on at 400 rpm (maximum where magnet stays in place?)
22. Replace the air stone in the small tub
23. Turn off all lights (ceiling light switch is outside CT room)



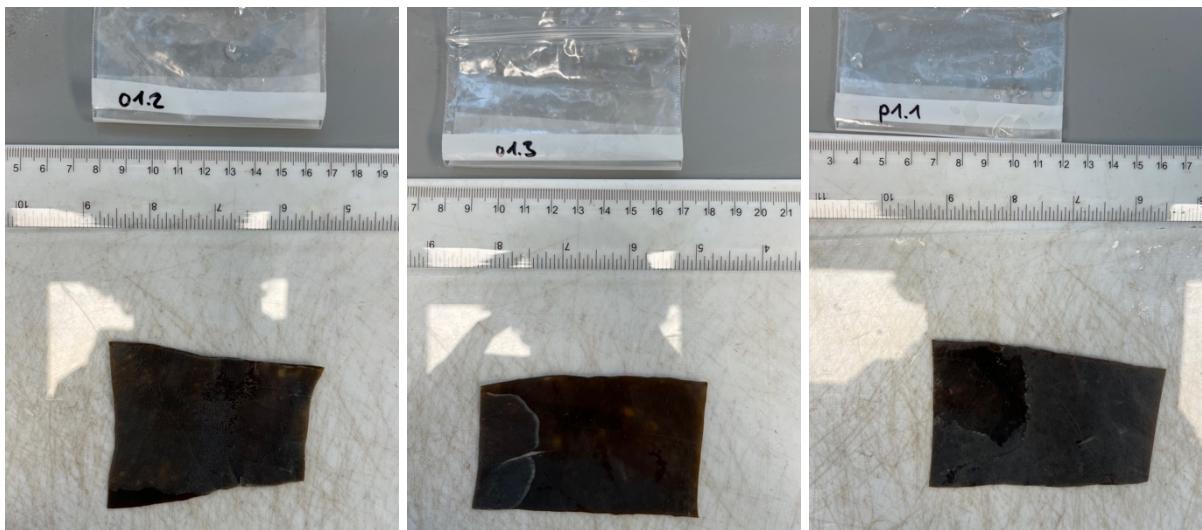
Light incubation setup in 13°C CT room. Note that blank isn't always in the bottom right position but should instead vary position between replicate incubations.

24. With all channels selected and deactivated, click “Start” in the “Live View” tab to ensure there are no errors (marked by red warning triangles in the left column)
25. If measurement runs smoothly for ~12 s, click “Stop” in the “Live View” tab
26. Click “Activate” and then immediately “Start” to activate live view and recording
27. Check that the number of measurements for every channel are increasing every 3 s in the “Measurement points” column in the “Measurements” tab
28. Click “Show Graph Overview” in the “Live View” tab to visually track measurements in all 12 channels simultaneously for ~1 min

29. If all looks fine, set a timer on your phone to 1 h 50 min and leave CT room without allowing light intrusion (got to be quick!) and put a post-it on the door
30. Return when timer goes off (this should leave you with a few spare minutes)
31. Click “Deactivate” and “Stop” when the “Time” column in the “Live View” tab reads exactly 2 h, corresponding to 2401 records in the “Measurements” tab
32. Quickly disconnect the 12 sensor spot jars and, opening one at a time, gently aspirate 50 mL and dispense through the filter tip into the correspondingly labelled tube, leaving as little airspace as possible, and immediately refrigerate
33. Repeat steps 10 to 32 on the same tissue samples, but this time turn on all top lights to their maximum level, while keeping the ceiling light off
34. Carry tray with 12 sensor spot jars to the workbench in the 15°C CT room
35. If in doubt about using Measurement Studio 2, read up on “Measurement Studio 2 Instruction Manual”

Bryozoan cover

1. One at a time, gently blot tissue samples until they are dry to the touch
2. After blotting each sample, sandwich between cutting board and transparent plexiglass sheet
3. Photograph from both sides using a good phone camera or digital camera with ruler (placed on board) and corresponding ziplock bag label in the same frame
4. Double-check photograph to ensure bryozoan colony is clearly visible
5. As a backup, semi-quantitatively estimate proportional bryozoan cover on a scale from 0 to 1 in increments of 0.25 and note down
6. Pack each tissue sample into its correspondingly labelled ziplock bag
7. Fetch the remaining, unused lamina from the large tub in the 13°C CT room
8. Randomly (resist temptation to sample bryozoans) cut it into 19 subsamples, as similar as possible to the previous ones in terms of size (~5 x 5 cm)
9. Repeat steps 1 to 4 (since there aren't corresponding ziplock bags, label a piece of paper with one species initial and sporophyte number for all subsamples)
10. Save 60 photographs to computer as TIFF and name each file according to the ziplock bag or paper label with an additional index (.1 or .2) for which each side
11. This estimation procedure will yield a good estimate of proportional cover but is necessarily biased towards sporophytes with substantial bryozoan cover
12. To get a more accurate understanding of forest-scale proportional bryozoan cover, also collect laminae randomly
13. Take 30 random subsamples (~5 x 5 cm) from these laminae, none of which are used in incubation
14. Repeat steps 1 to 4



Example images o1.2.1 (~100% cover), o1.3.1 (~25% cover) and p1.1.1 (~75% cover). Note that the total cover estimate needs to factor in both sides.

PAR measurement

1. Set PAR metre to middle accuracy setting using the switch on the right side
2. Ensure the light level and magnetic stirrer positions are the same as in the last (light) incubation
3. Place the black sensor head inside a jar equipped with a Velcro strap and with lid facing down, close as much as possible but without damaging the cable
4. Ensure the sensor head is vertical and the white sensor thus perfectly horizontal
5. Move the jar from position 1.1 through to 3.4, recording and noting PAR for each
6. Make sure to consistently record PAR in $\mu\text{mol photons m}^{-2} \text{ s}^{-1}$

Lyophilisation and weighing

1. Transfer the 11 ziplock bags to freeze-drier room and start lyophilisation
2. Once completely dry (usually after 48 h), turn off and immediately close bags
3. Transfer to the precision balance room and weigh samples to 0.01-mg accuracy using the Sartorius balance (if this keeps acting up, use less accurate balance)
4. If possible, separate dry bryozoan colony from lamina using a scalpel
5. For each subsample, weigh bryozoans (essentially CaCO_3) and kelp separately
6. Hence, there should be three mass estimates (total, bryozoans only, kelp only) for each sporophyte subsample

Total alkalinity measurement

1. 50 L of seawater standard, collected from the 15°C CT room, should be stored at room temperature next to the autotitrator in a plastic container with a tap
2. Calibrate the pH probe of the autotitrator (this should be done weekly)
3. Run several seawater standard samples to make sure the autotitrator is precise (total alkalinity and pH values are close to one another) and establish a baseline

4. After every incubation, fetch the 26 tubes from the fridge and let them reach room temperature (total alkalinity is usually reported at 20 or 25°C)
5. Run 1–3 seawater standards to make sure they are within the expected range
6. Analyse samples as 25-mL technical replicates (52 measurements in total) following the instructions for the autotitrator
7. While precision is readily checked using the custom seawater standard, accuracy will be determined after the experimental series using standard solution of known total alkalinity purchased from Scripps, which I already have

Image analysis

1. Install ImageJ in the form of FIJI on your computer (<https://fiji.sc>)
2. Open the TIFF file via File > Open...
3. Select the “Straight” tool and draw a 1-cm long line, using the ruler as a guide
4. Navigate to Analyze > Set Scale...
5. Enter “1” in the “Known distance” box and “cm” in the “Unit of length” box, then click “OK” in the bottom right
6. Navigate to Process > Binary > Make Binary
7. Set “Method” to “Default” and “Background” to “Dark” (this will counter-intuitively make the tissue subsample black and the background white)
8. Select the “Wand” tool and click on the black area that is the subsample
9. Check that the yellow border which appears correctly delimits the area
10. Navigate to Analyze > Measure
11. A popup table will appear and the number in the “Area” column is the measured surface area in cm²
12. Copy and paste this number to the metadata sheet (see Data management)
13. Close FIJI without saving and open the same TIFF file again
14. Repeat steps 3 to 5
15. Select the “Polygon” tool and manually trace the edge of the bryozoan colony
16. Repeat steps 9 to 12

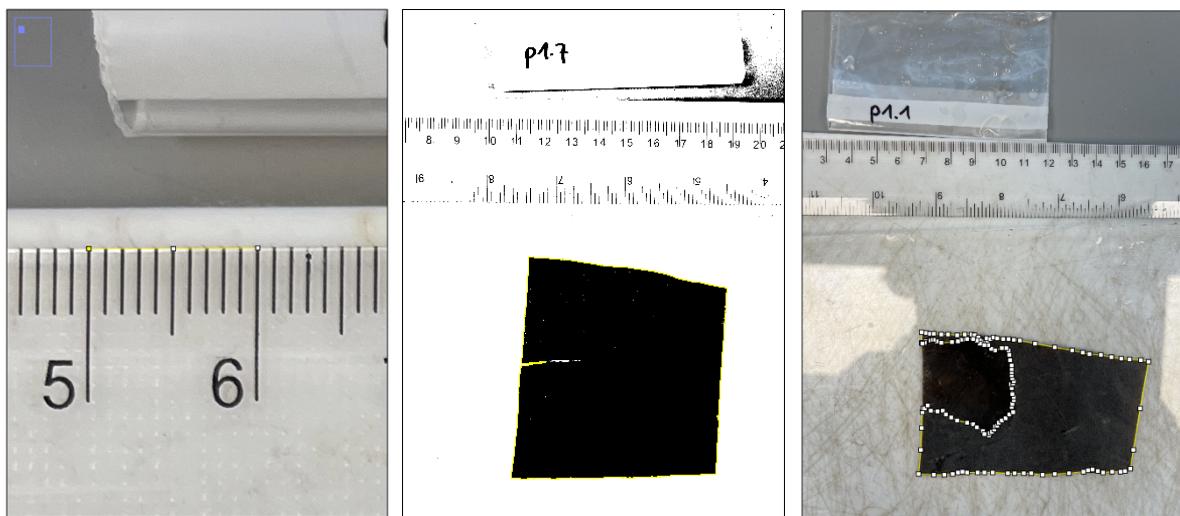


Image analysis process from setting the scale (left) over automatically measuring the total surface area (centre) to manually tracing the encrusted surface area (right).

Data management

1. Data in this experiment are structured into two levels and I refer to the upper level as metadata and the lower level as data
2. The metadata should contain (1) the site, (2) the species, (3) the subsample label or ID, (4) the collection and measurement date (should be the same day on early spring low tides and next day on late spring low tides), (5) the position on the magnetic stirrer, (6) PAR, (7) PAR for the blank in the same measurement round, (8) total, kelp and bryozoan sample mass in grams, (9) semi-quantitative bryozoan cover, (10) total surface area (sum of both sides), (11) bryozoan surface area (sum of both sides), (12) additional fauna which accidentally ended up in incubation (e.g. *P. pellucida*, hydroids etc.) or other notes and (14) your initials
3. The metadata are all contained in a single dataframe
4. The data underlying this are (1) the O₂ measurements output by Measurement Studio 2, (2) the total alkalinity measurements output by the autotitrator, (3) image analysis measurements output by FIJI for incubation and other samples and (4) the raw and TIFF images
5. The data are spread across several dataframes, in the extreme case of O₂ measurements two per subsample, one for light and one for dark incubation
6. Hence, the data folder should be structured into three folders (PreSens, Autotitrator, Images) and two datasheets (Meta, Cover)
7. It is absolutely important to maintain consistency and regularly update