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Labelling kelps with 13C and 15N for isotope tracing or enrichment experiments V.1

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We use this protocol and it's working

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Abstract

Isotope tracing experiments can be used to trace organic material flow through the ecosystem by artificially adding labelled biomass into a system. The advantage of this process is the direct control of carbon and nitrogen addition to the system for measuring uptake rates by consumers which can substantially reduce the uncertainties associated with food web models. This protocol details the steps involved in successfully culturing and isotopically enriching (^{13}C & ^{15}N) juvenile sporophytes of two common North Atlantic kelp species (Laminariales): *Saccharina latissima* and *Laminaria digitata*. A first-order successful isotopic enrichment study of *S. latissima*, as well as the first inclusion of ^{15}N enrichment for *L. digitata*, is detailed. This protocol provides a comprehensive description of the stable isotope enrichment process in two kelp species, potentially serving as a foundation for its application in other macroalgal taxa.

Attachments

PDF provasoli-enriched-s... 640KB

PDF von-stosch-vs-enrich... 586KB

Materials

Equipment:

- Juvenile kelp sporophytes
- 5 L sterile culture bottles (glass or plexiglass)
- 2 L Kautex bottles with lids or similar
- Dry air and tubes
- Plant/algae grow-lights
- Clean and pasteurised seawater
- Dissecting scissors
- Rotating or tilting shakers
- Deionized (DI) water
- Freeze-dryer
- Aluminium foil
- Tweezers
- Zip-lock bags
- Combusted 14 mL glass vials
- Ball mill
- Aluminium cups
- Analytical laboratory scale
- 96-well plate

Growth media chemicals:

- Von Stosch (VS) medium chemicals
- Provasoli Enriched Seawater (PES) medium chemicals
- Sodium nitrate-15N (CAS# 31432-45-8)
- Sodium bicarbonate-13C (CAS# 87081-58-1)

Protocol materials

Liquid nitrogen

Sodium 2-glycerophosphate pentahydrate

Sodium nitrate **P212121**

Sodium bicarbonate **Merck MilliporeSigma (Sigma-Aldrich)**

Sodium glycerophosphate hydrate

Sodium nitrate-15N **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**364606-5G

Sodium bicarbonate-13C **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**372382-5G

Troubleshooting

Before start

The generation of juvenile sporophyte material for isotopic labelling can be achieved through various methods, such as sampling from the field or growing sporophytes from spores or vegetative stock gametophytes. For details of our experiment see *PLOS ONE* article. Juvenile kelp sporophytes for labelling purposes have to be cultured in a way, which ensures good growth to produce actively growing healthy material. The following steps are tailored to the two species cultivated for this experiment and may require adaptation based on the available equipment and sporophyte physiology.

Culture set-up (pre-labelling)

1 Sterile culture bottles (glass or plexiglass) of sufficient size are used for cultivation. In our case, sporophytes of *Laminaria digitata* and *Saccharina latissima* were cultured in 5 L DURAN glass bottles with the nutrient addition of 100 mL (half concentration) Provasoli Enriched Seawater (PES) in 10 L fresh seawater stored in temperature-controlled laboratories at 10 °C. Medium was changed weekly and continuously bubbled with dry air via tubes. Irradiance should be set to around 40-50 µmol photons m^{-2} s^{-1} in long-day conditions (16 h light : 8 h dark; 16:8 LD). For cultivation of sporophytes, clean and pasteurised seawater is needed, either from natural fully marine sources or artificial seawater at approximately 30-33 PSU. Here, seawater was filtered through a 5" Polypropylene Yarn Water Filter and was pasteurised for 04:00:00 at 99 °C with a combi steamer (PALUX, Germany).

Protocol

NAME

Provasoli Enriched Seawater (PES) medium solution

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Note

During the culturing process full PES (200 mL PES in 10 L seawater) can also be used to induce higher growth rates (Fortes & Lüning, 1980). For optimal results, it is recommended to add the full concentration a few days prior to the labelling start.

Citation

M. D. Fortes & K. Lüning (1980)
. Growth rates of North Sea macroalgae in relation to temperature, irradiance and photoperiod.
Helgoländer Meeresuntersuchungen.

https://doi.org/10.1007/BF01983538 LINK

Equipment

DURAN™ Original Laboratory Bottle, Clear, with DIN 168-1 Thread, Graduated NAME

DURAN glass bottle TYPE

DWK Life Sciences BRAND

Z232122-1EA SKU

5000 mL (narrow neck) SPECIFICATIONS

Equipment

5" Polypropylene Yarn Water Filter	NAME
Water Filter	TYPE
Vyair	BRAND
n.a.	SKU
https://www.vyair.com/en	LINK

Equipment

PALUX Touch 'n' Steam 611QL	NAME
Combi Steamer	TYPE
PALUX	BRAND
E611 QBRN 000000	SKU
https://www.palux.de/en	LINK

Determining growth rates

2 Macroalgal growth rates should be determined in simulated labelling conditions prior to labelling start to ensure the material is growing well. Otherwise, successful incorporation of labelled compounds is not guaranteed. Wet weight (mg) and surface area (cm^2) are the main variables that should be measured at regular intervals (e.g. every 4 days), but blade length can also be used as a variable. In addition, pH measurements can be taken to monitor the acidity of the media.

Note

To ensure uptake of enriched chemicals, the algal biomass should ideally double during the labelling period.

3 **Labelling medium**

The simulated medium for labelling consists of von Stosch (VS) medium (in contrast to PES, which is used during early cultivation – see Note below) modified to exclude phosphate and nitrate. Phosphate ([M] 0.01546 millimolar (mM) Sodium 2-glycerophosphate pentahydrate $C_3H_{17}Na_2O_{11}P$), nitrate ([M] 0.54908 millimolar (mM) Sodium nitrate **P212121** $NaNO_3$) and bicarbonate ([M] 2.32545 millimolar (mM) Sodium bicarbonate **Merck MilliporeSigma (Sigma-Aldrich)** $NaHCO_3$) were added individually in the same concentrations as in PES (table below). The full concentration is 200 mL VS for 10 L fresh seawater.

	A	B	C	D
	Chemical compound	CAS number	Concentration (µmol/L) [as in full PES]	Concentration in millimolar (mM)
	Sodium bicarbonate	144-55-8	2325.45 µmol/L	2.32545
	Sodium nitrate	7631-99-4	549.08 µmol/L	2.32545
	Sodium glycerophosphate – hydrate	55073-41-1	15.46 µmol/L	0.01546

Chemical concentrations added individually to von Stosch (VS) medium used for cultures (pre-labelling) with CAS Registry Number of chemical substances.

Note

Best growth conditions of macroalgae are generally achieved with Provasoli Enriched Seawater (PES), however, given the complexity of the preparation of this medium and the difficulty in leaving out all nitrate sources, the preferred medium for isotopic labelling is von Stosch (VS) (according to Guiry & Cunningham, 1984). Phosphate was added separately because of ongoing separate experiments, however, for the sole purpose of isotopical labelling, phosphate can be left in the VS medium.

Citation

M. D. Guiry & E. M. Cunningham (1984)
. Photoperiodic and temperature responses in the reproduction of north-eastern Atlantic Gigartina acicularis (Rhodophyta: Gigartinales).
Phycologia.

https://doi.org/10.2216/i0031-8884-23-3-357.1 LINK

Protocol

NAME

Von Stosch (VS) enriched seawater medium solution

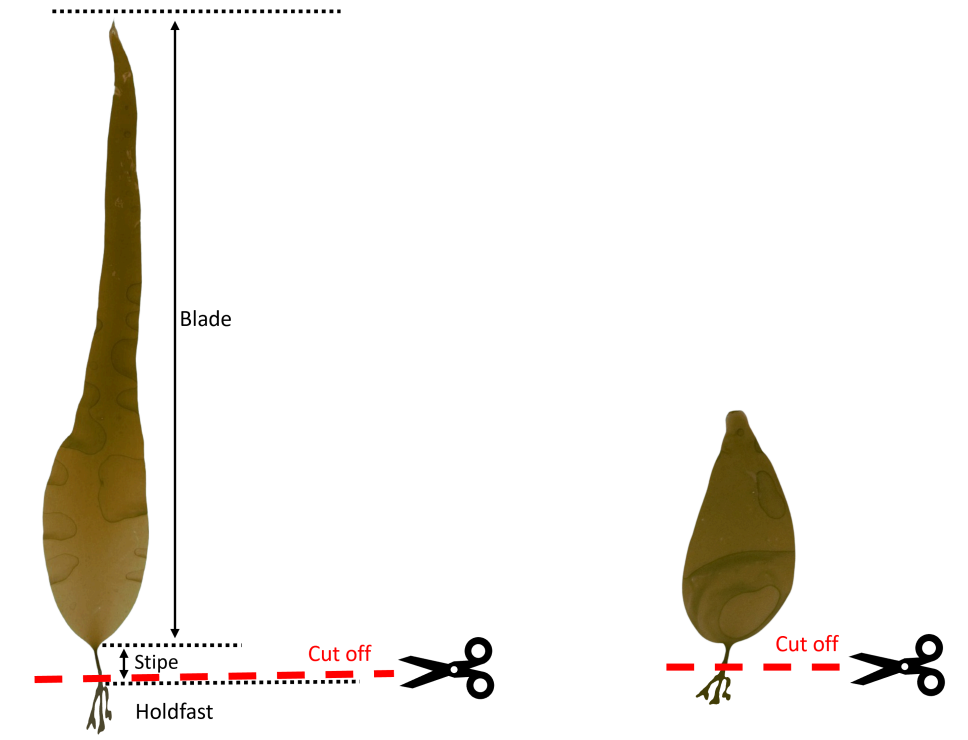
CREATED BY

Anton Kuech

Preview

4 **Sporophyte preparation for labelling**

Separate sporophytes of the two species by cutting them at the stipe just above the holdfast using dissecting scissors. This enables single unidirectional growth as compared to an unequal distribution of growth between blade and holdfast. Select sporophytes of a good growth stage (e.g. widening of blade for *S. latissima*) and avoid individuals with white spots or other noticeable damaged areas. The blade should have an even brown colour.



Picture of *Saccharina latissima* and *Laminaria digitata* including the cut-off point marked by the red line.

Equipment	
Fisherbrand™ Dissecting Scissors	NAME
Scissors	TYPE
Fisherbrand	BRAND
15277168	SKU
https://www.fishersci.co.uk/gb/en/home.html	LINK

5 **Example incubation of sporophytes for determining growth rates**

Either two sporophytes of *Saccharina latissima* or four sporophytes of *Laminaria digitata* were placed into a single 2 L Kautex bottle (ensure sufficient replication). Wet weight (mg), surface area (cm^2) and pH were measured in 4-day intervals for a 14-day period.

Note

The number of sporophytes placed into the 2 L Kautex bottles will depend on (a) the amount of enriched organic matter needed for the incubations (see *PLOS ONE* article for example), (b) the size of the pre-labelling sporophytes and (c) the ratio between sporophyte size and bottles. For this experiment, we used sporophytes of approximately 11 cm and 4 cm length of a single *S. latissima* and *L. digitata* thallus. The average wet weight of a single sporophyte was approximately 104 mg and 24 mg for *S. latissima* and *L. digitata* respectively. Thus, two sporophytes of *S. latissima* and four sporophytes of *L. digitata* were placed into a Kautex bottle each. In case that you need more material or sporophytes are bigger, you should also increase the size of the incubation bottles to ensure good growth.

Note

In case you use new Kautex bottles, which are made from polyethylene terephthalate glycol (PETG), you have to take care to reduce the risk of contamination. To reduce the impact of the new PETG material and polyethylene (PE) foam insert of the closures on the macroalgae, the bottles were filled with tap water, closed and stored Overnight . This was followed by washing the closures and bottles in the dishwasher without detergent at 50 °C and dried in the oven at 40 °C .

Equipment

Wide Necked square container, PETG, 2000mL w/ screw closure, crystal clear	NAME
Wide neck container	TYPE
Kautex	BRAND
225274374	SKU
https://www.analytics-shop.com/gb/	LINK

Labelling of kelps

6 **Pre-labelling start conditions**

A few days prior to labelling start, full PES (200 mL PES in 10 L pasteurized seawater) should be added and irradiance should be set to 50 µmol photons m^{-2} s^{-1} to induce optimum growth rates (16 h light : 8 h dark per 24-hour period).

Note

Depending on species and developmental stage the conditions for inducing optimum growth rates may vary.

7 **Labelling conditions**

The nutrient concentrations used in von Stosch (VS) were adjusted to represent the same concentration that the macroalgae would receive in the full PES mixture (200 mL PES in 10 L pasteurized seawater). This concentration is considered the ideal medium for growth and is also in accordance with the concentrations used by Braeckman et al. (2019) and Rossi et al. (2013). Exact concentrations of chemicals added separately were [M] 0.01546 millimolar (mM) Sodium glycerophosphate hydrate , [M] 0.55457 millimolar (mM) Sodium nitrate-15N **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**364606-5G and [M] 2.35294 millimolar (mM) Sodium bicarbonate-13C **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**372382-5G . Remaining nutrients and vitamins were added according to the von Stosch (VS) enrichment medium modified to exclude phosphate and nitrate go to step #3 .

	A	B	C	D
	Chemical compound	CAS number	Concentration (µmol/L) [as in full PES]	Concentration in millimolar (mM)
	Sodium glycerophosphate – hydrate	55073-41-1	15.46 µmol/L	0.01546
	Sodium nitrate-15N	31432-45-8	554.57 µmol/L	0.55457

	A	B	C	D
	Sodium bicarbonate-13C	87081-58-1	2352.94 µmol/L	2.35294

Concentrations of main chemicals used for labelling with CAS Registry Number of chemical substances.

Protocol

NAME

Von Stosch (VS) enriched seawater medium solution

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Anton Kuech

Preview

Citation

Braeckman U, Pasotti F, Vázquez S, Zacher K, Hoffmann R, Elvert M, Marchant H, Buckner C, Quartino ML, Mác Cormack W, Soetaert K, Wenzhöfer F, Vanreusel A (2019). Degradation of macroalgal detritus in shallow coastal Antarctic sediments..

https://doi.org/10.1002/lno.11125 LINK

Citation

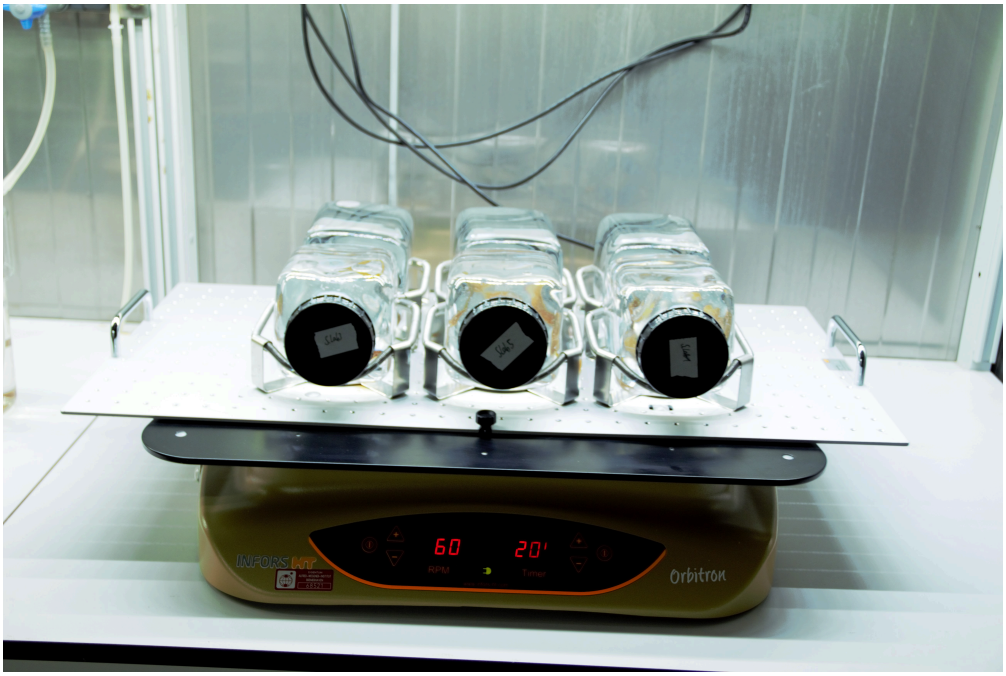
Rossi F, Gribsholt B, Gazeau F, Di Santo V, Middelburg JJ (2013)
. Complex Effects of Ecosystem Engineer Loss on Benthic Ecosystem Response to Detrital Macroalgae..

https://doi.org/10.1371/journal.pone.0066650 LINK

8 Separate sporophytes of the two species by cutting them at the stipe just above the holdfast using nail scissors as detailed in step #4. go to step #4

9 **Incubation of sporophytes for labelling**

Selected kelp sporophytes are stored in Kautex bottles, filled with the pasteurized seawater and labelling solution to the top, tightly closed and placed on rotating or tilting shakers for 9 days. Kautex bottles lie on their sides to allow full illumination. Make sure that there are no air bubbles in the bottle. We used Orbitron rotating shakers at 90 rpm, 10°C. Irradiance set to 50 µmol photons m^{-2} s^{-1} in a 16 : 8 LD cycle.



Picture of labelling setup depicting Kautex bottles containing kelp sporophytes placed on a rotating shaker.

Note

The optimal incubation period for sporophytes in the enriched medium may vary depending on the species selected and their respective growth rates. To ensure uptake of enriched chemicals, the algal biomass should ideally double during the labelling period.

Equipment

Wide Necked square container, PETG, 2000mL w/ screw closure, crystal clear	NAME
Wide neck container	TYPE
Kautex	BRAND
225274374	SKU
https://www.analytics-shop.com/gb/	LINK

Equipment

Orbitron	NAME
Shaker	TYPE
INFORS HT	BRAND
n.a.	SKU
https://www.infors-ht.com/en/	LINK

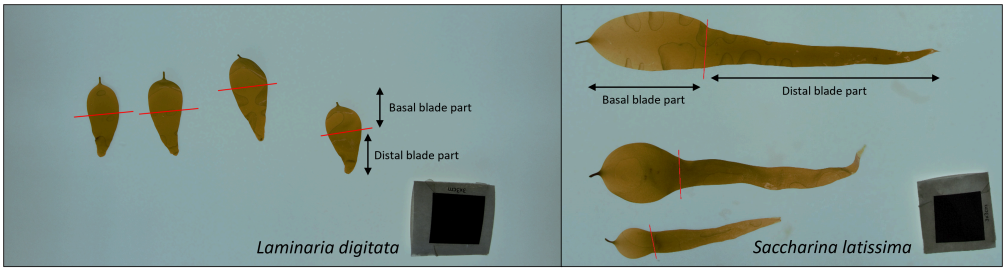
Harvesting biomass

10 Remove sporophytes from a single Kautex bottle.

11 Take picture and wet weight of sporophytes using a macro photography camera or similar.

12 **Separation of growing from non-growing blade parts**

After the incubation period, newly formed blade parts of sporophytes normally become visible by a widening at the basis (see image below). We expected that the basal blade part that was formed during incubation would have a higher labelling result than the distal blade part. This assumption was supported by our results (see *PLOS ONE* article for details). Thus, if you cut sporophytes at the intersection between the estimated new growth (highly labelled) and prior biomass (slightly labelled), you separate material with a differential labelling result.



Examples of cut off points (red lines) for *Laminaria digitata* and *Saccharina latissima*, which approximately separate the newly formed basal blade area during labelling incubation (highly labelled area) from the distal blade part which had been formed during pre-cultivation (slightly labelled part).

Note

When dealing with other kelp species, their behaviours can vary. Separating the blades halfway along their length after termination of the labelling process is generally expected to generate highly and slightly labelled material. Kelps also distribute chemical compounds throughout the blades via translocation and thus, both the new biomass and 'old' biomass should be stored.

Citation

BC Parker
. Translocation in the giant kelp Macrocystis. I. Rates, direction, quantity of C14-labeled products and fluorescein.
Journal of Phycology.

https://doi.org/10.1111/j.1529-8817.1965.tb04554.x LINK

13 Dip sporophytes briefly in deionized (DI) water to remove residual saltwater and dry material with clean tissue paper.

14 **Shock freezing of samples**
Sporophytes wrapped in punctured aluminium foil (highly/slightly labelled separate) and either (a) dipped in Liquid nitrogen until bubbling of liquid stops or (b) stored for a minimum of 24 hours at -80 °C prior to freeze-drying.

15 Freeze-drying of samples for 48 hours.

Equipment	
RVC Alpha 3-4 LSCbasic	NAME
Laboratory freeze-dryer	TYPE
CHRIST	BRAND
n.a.	SKU
https://www.martinchrist.de/en	LINK

16 Storage of freeze-dried biomass in zip lock bags in freezer at -20 °C (or -80 °C). Material can also be stored in a desiccator filled with silica gel for shorter periods.

Determining labelling uptake

17 Freeze-dried material should ideally be made into powder, best with a ball mill. Remove sporophytes from foil and transfer into the metal tubes from the ball mill. Ball mill set to 00:03:00 at 25 Hz. Afterwards, store the powder in combusted glass vials (previously combusted at 500 °C overnight).

Safety information

Handle hot glass vials with care! After combustion glass vials should be left to stand for 02:00:00 to cool down. Use heat resistant gloves when handling.

Equipment

Mixer Mill MM 400	NAME
Retsch	BRAND
MM400	SKU
https://www.retsch.com/products/milling/ball-mills/mixer-mill-mm-400/function-features/	LINK

Equipment

Fisherbrand™ Snap Cap Vial, Clear Glass	NAME
Glass vial	TYPE
Fisherbrand	BRAND
10749644	SKU
https://www.fishersci.co.uk/gb/en/home.html	LINK
14 mL volume	SPECIFICATIONS

18 Random sub-samples of the powder, such as 4 replicates per species and distal/basal blade part, are weighed into aluminium cups (approximately 1 mg per sample),

encapsulated and added onto a 96 well plate (can also be stored in a desiccator filled with silica gel for shorter periods).

Equipment

Aluminium capsules/pans for solids	NAME
Aluminium capsules	TYPE
Elemental Microanalysis	BRAND
D3089	SKU
https://www.elementalmicroanalysis.com/index.php	LINK
Aluminium Capsules Pressed 8.75 × 3.5mm pack of 100	SPECIFICATIONS

Equipment

Corning™ Clear Polystyrene 96-Well Microplates	NAME
96-well plate	TYPE
Corning	BRAND
10377601	SKU
https://www.fishersci.co.uk/gb/en/home.html	LINK

19 Analysis of samples for ^{13}C and ^{15}N isotopes. For details of the results for this experiment see *PLOS ONE* article.

Note

UC Davis Stable Isotope Facility (SIF) performed ^{13}C and ^{15}N isotope analyses using an elemental analyser interfaced to a continuous flow isotope ratio mass spectrometer (IRMS). As per UC Davis SIF guidelines, small plant samples are analysed for ^{13}C and ^{15}N isotopes using a PDZ Europa ANCA-GSL elemental analyser interfaced to a PDZ Europa 20-20 isotope ratio mass spectrometer (Sercon Ltd., Cheshire, UK). Samples are combusted at 1000°C in a reactor packed with chromium oxide and silvered copper oxide. Following combustion, oxides are removed in a reduction reactor (reduced copper at 650°C). The helium carrier then flows through a water trap (magnesium perchlorate and phosphorous pentoxide). N_2 and CO_2 are separated from the samples via a Carbosieve GC column prior to entering the IRMS. Calibrated reference materials are added to the samples during analysis. During analysis, the samples are interspersed with multiple replicates of at least four distinct laboratory reference materials. These reference materials have undergone prior calibration against internationally recognized standards, including IAEA-600, USGS-40, USGS-41, USGS-42, USGS-43, USGS-61, USGS-64, and USGS-65. A sample's initial isotope ratio is assessed relative to a reference gas peak, which is analyzed alongside each sample. These initial values are then refined by adjusting them for the entire batch using the established values of the laboratory reference materials included in the analysis. Standard deviations are figured at 0.2 ‰ for ^{13}C and 0.3 ‰ for ^{15}N. The final delta values are expressed relative to international standards VPDB (Vienna Pee Dee Belemnite) and Air for carbon and nitrogen, respectively.

Citations

Step 1

M. D. Fortes & K. Lüning. Growth rates of North Sea macroalgae in relation to temperature, irradiance and photoperiod
https://doi.org/10.1007/BF01983538

Step 12

BC Parker. Translocation in the giant kelp Macrocystis. I. Rates, direction, quantity of C14-labeled products and fluorescein
https://doi.org/10.1111/j.1529-8817.1965.tb04554.x

Step 3

M. D. Guiry & E. M. Cunningham. Photoperiodic and temperature responses in the reproduction of north-eastern Atlantic Gigartina acicularis (Rhodophyta: Gigartinales)
https://doi.org/10.2216/i0031-8884-23-3-357.1

Step 7

Braeckman U, Pasotti F, Vázquez S, Zacher K, Hoffmann R, Elvert M, Marchant H, Buckner C, Quartino ML, Mác Cormack W, Soetaert K, Wenzhöfer F, Vanreusel A. Degradation of macroalgal detritus in shallow coastal Antarctic sediments.
https://doi.org/10.1002/lno.11125

Step 7

Rossi F, Gribsholt B, Gazeau F, Di Santo V, Middelburg JJ. Complex Effects of Ecosystem Engineer Loss on Benthic Ecosystem Response to Detrital Macroalgae.
https://doi.org/10.1371/journal.pone.0066650