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RESEARCH ARTICLE

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Isotopically labelled macroalgae: A new method for determining sources of excess nitrogen pollution

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Rationale: Stable nitrogen isotope ratios ($\delta^{15}N$) can be used to discern sources of excess nitrogen pollution in water. The $\delta^{15}N$ values of nitrate in water often do not reflect the true $\delta^{15}N$ source value owing to high temporal variation, and there are high analytical costs associated with obtaining $\delta^{15}N$ values from water nitrate. To find alternative solutions, we isotopically labelled macroalgae (i.e. seaweed) beyond natural variation as a new method for determining sources of excess nitrogen pollution in seawater.

Methods: *Fucus vesiculosus* (bladder wrack) non-fertile tips were collected from Easington Colliery, County Durham, UK, and cultured in two isotopically enriched solutions containing ammonium sulphate with $\delta^{15}N$ values of 170 ± 5‰ and −60 ± 3‰ for a period of 19 days. The macroalgae were cultured in separate opened glass jars in an incubator with set temperature (11°C) and light (125 μmol photons m^{-2} s^{-2} on a light/dark rhythm of 16 h/8 h). The oven-dried tips were analysed for $\delta^{15}N$ over the 19-day experiment.

Results: The macroalgal tips incorporated the isotopically enriched solutions rapidly, reaching 50% of the isotopically enriched seawater after *ca* 11 days for the ^{15}N-enriched solution and *ca* 15 days for the ^{14}N-enriched solution. $\delta^{15}N$ values were incorporated more into the torn base of the macroalgal tips than into the middle and apex regions.

Conclusions: *F. vesiculosus* rapidly incorporates the isotopic ratio of the artificial seawater solution to which it is translocated. The laboratory-developed isotopically labelled macroalgae can be manufactured to generate 'unnatural' $\delta^{15}N$ values for translocation into coastal environments. This approach can provide an efficient, low-cost alternative to current analytical methods for determining and monitoring nitrogen pollution.

1 | INTRODUCTION

The intensification of urbanization and a growing human population around coasts have increased the amount of dissolved inorganic nitrogen (DIN) delivered to estuaries and coastal waters.[1] High concentrations of DIN can cause eutrophication in estuaries, creating environmental disturbances such as blooms of opportunistic macroalgae (i.e. seaweed) and anoxic conditions.[2,3] Traditional physicochemical analysis of water and sediments fails to offer any information regarding the origin of pollutants.[4]

$\delta^{15}N$ values of DIN sampled directly from seawater have been used to identify sources of nitrogen;[5] however, because natural

environments such as estuaries have complex flow patterns with high temporal variability, the results do not always represent the true $\delta^{15}N$ value of the source.[6,7] Increasingly, $\delta^{15}N$ values in macroalgal tissues are used as a low-cost alternative to $\delta^{15}N$-DIN sampling to monitor nitrogen pollution.[4,8,9] This approach is based on the assumption that the $\delta^{15}N$ values measured in macroalgal tissues are representative of an integrated $\delta^{15}N$ value from sources in the water column over time.[7,10,11] Fractionation processes in the water column, or during nitrogen uptake, could mean that this assumption is invalid.[12,13] Nitrogen uptake is often determined by many different environmental factors such as light, temperature, nitrogen species and morphological factors.[14–16] Despite these issues, $\delta^{15}N$ values in macroalgae are generally believed to be representative of biologically available nitrogen in estuaries.[15] Native macroalgae can experience different tidal regimes that could influence the uptake of nitrogen;[17] therefore, translocating macroalgae is considered the optimal method to determine the average $\delta^{15}N$ values of sources in an area.[7]

The use of experimental macroalgae to monitor nitrogen pollution in another area should be referred to as 'translocation'. We advocate translocation over transplantation on the basis that translocation experiments are used to determine the chemical exchange and alteration of the macroalgae (e.g. 'the movement of chemicals'). Transplantation, on the other hand, relates to moving an organism from one region to another region where it remains.

Anthropogenically derived nitrogen sources, such as sewage, fish farm effluent, livestock manure and terrestrial runoff, tend to be enriched in ^{15}N relative to seawater (Figure 1).[6,18–21] Nitrogen sources from agricultural activities (e.g. chemical fertilizers) tend, however, to be depleted in ^{15}N relative to seawater as they are synthesized from atmospheric N_2.[19] Synthetic inorganic fertilizers tend to have $\delta^{15}N$ values close to that of atmospheric N_2 (0‰), whereas organic fertilizers have higher and more variable $\delta^{15}N$ values.[18,22,23] However, $\delta^{15}N$ values can vary as a result of freshwater inputs and biogeochemical processes.[24] Therefore, because different combinations of sources could produce similar $\delta^{15}N$ values, additional information about the nitrogen dynamics of an area is required to accurately identify potential anthropogenic nitrogen pollutant sources.[24]

The need to monitor nitrogen pollution arises from legislative measures in place to ensure that water bodies are being managed correctly and pollution levels are managed and/or reduced. In particular, the European Water Framework Directive (WFD, 2000/60/EC)[25] was developed to assess the ecological status of water bodies. Hence, monitoring and identifying nitrogen pollution in coastal environments and estuaries are essential to conform to these legislative measures.

2 | MACROALGAL PHYSIOLOGY

2.1 | Nitrogen assimilation in macroalgae

Nutrient uptake (i.e. nitrogen) in macroalgae is linearly controlled by concentration until a plateau is reached, following a Michaelis–Menten curve.[16] In the natural environment, ammonium and nitrate ions are considered the most common nitrogen sources, and as such are frequently used in laboratory experiments.[26] Macroalgae have been shown to preferentially uptake ammonium over nitrate.[14,16] Alwyn and Rees[14] found that K_m (the concentration of a nutrient that gives half of the maximum rate of uptake) and V_{max} (the maximum rate of uptake achieved at saturating conditions of the nutrient) are greater for ammonium uptake than for nitrate uptake for a range of macroalgal species. However, Gröcke et al[7] concluded that nitrate was taken up at a higher rate than ammonium by *Fucus vesiculosus*. This finding may be explained by the fact that the initial $\delta^{15}N$ value of the macroalgae was closer to that of the nitrate solution than that of the ammonium solution. Therefore, nitrate uptake appeared to happen at a faster rate than ammonium uptake because isotopic equilibrium was achieved faster using the nitrate solution.

Ammonium (NH_4^+) can be assimilated directly into macromolecules and later be used for growth, whereas nitrate (NO_3^-) must be reduced first to nitrite (NO_2^-) and then subsequently to NH_4^+ before it can be assimilated into macromolecules.[16] Reduction occurs through reducing enzymes present in macroalgae, such as nitrate and nitrite reductase.[16,26] These nitrate-reducing enzymes can be temporarily switched off as a result of ammonium assimilation; therefore, ammonium assimilation can inhibit nitrate being simultaneously assimilated in macroalgae.[16,26] This is particularly relevant to this study, whereby transplanted macroalgae could preferentially assimilate the $\delta^{15}N$ signal of ammonium present in an estuary rather than that of the nitrate present, leading to inaccurate interpretations of the nitrogen sources present.[11]

2.2 | Growth of *F. vesiculosus*

Macroalgae in temperate habitats experience large variations in temperature, irradiance and nutrient concentration that can affect

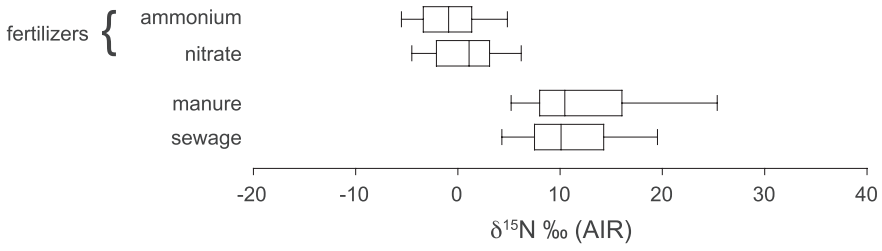

FIGURE 1 Box-and-whisker plots of $\delta^{15}N$ values from various nitrogen sources clearly showing the distinct difference between effluent sources and industrial fertilizers (adapted from Xue et al[18])

their physiology.[17,27] Particularly in intertidal zones, macroalgae are periodically exposed to tidal cycles, resulting in a disjunction between optimal light, nutrient availability and temperature for growth.[17] Perennial species, such as *F. vesiculosus*, can store nutrients internally, so that growth can still occur during the winter, when conditions are not favourable for growth, i.e. light levels and temperature are low.[27] Several species of brown macroalgae (e.g. *F. vesiculosus*, *F. serratus*, *F. spiralis*, *Laminaria digitate*) have been documented as accumulating nitrogen during the autumn and winter, when external nitrogen concentrations are high, and storing it in the vacuoles of the thallus.[17] The stored nitrogen is then utilized in the spring and summer when conditions are more favourable for growth and external nitrogen concentrations are typically lower.[28,29] The ability of macroalgae to store nutrients uncouples growth from nutrient availability.[30]

2.3 | Factors affecting nitrogen isotope fractionation in macroalgae

Fractionation of nitrogen isotopes can occur in the water column or through uptake processes in macroalgae. Concentration-dependent fractionation in macroalgae has been debated; Cohen and Fong[11] and Naldi and Wheeler[31] found that it does not occur, whereas other studies have suggested that it does occur at high concentrations of nitrogen.[7,13,32] Light intensity can also produce a small amount of fractionation, with higher light intensity producing more elevated $\delta^{15}N$ values.[15] This is important to consider when designing translocation experiments, as the depth of the submerged macroalgae will affect the amount of light that can reach them.[7] Gröcke et al[7] found that *F. vesiculosus* tips submerged at a depth of 1 m below the surface did not isotopically shift as much as tips submerged at 0.2 m from the surface. This could be caused by isotopic fractionation resulting from different environmental conditions between the two depths, or depth stratification of nitrogen sources leading to varying $\delta^{15}N$ values.[7] It is unknown whether temperature-dependent fractionation affects $\delta^{15}N$ values in macroalgae, and thus further investigation is needed into this and other environmental factors.[33]

There are a number of studies that have investigated the uptake of nitrogen isotopes by culturing several different species of macroalgae in solutions with varying forms of nitrogen. Several studies have cultured macroalgae in a ^{15}N-enriched solution *in vitro*.[11,31,34] There are, however, many other studies that have used both ^{15}N-enriched and ^{14}N-enriched solutions to culture macrophytes.[35,36] Some studies have introduced solutions with known $\delta^{15}N$ values *in situ*, demonstrating that the macroalgal $\delta^{15}N$ values are representative of the source of nitrogen pollution.[37–39] Others have cultured macroalgae in solutions containing nitrogen compounds with known $\delta^{15}N$ values *in vitro*.[7,13,40,41] The experiments that have involved isotopic labelling of macroalgae are those which aim to model the nutrient transfer between macroalgae and intertidal sediments.[42–45] This study is the first to culture macroalgal tips *in vitro* in both ^{15}N-enriched and ^{14}N-enriched solutions that have unnatural $\delta^{15}N$ values.

In this study, we cultured *F. vesiculosus* (bladder wrack) in ^{15}N-enriched and ^{14}N-enrichedartificial seawater solutions, with the hypothesis that macroalgal $\delta^{15}N$ values will converge on the $\delta^{15}N$ value of the pollutant source (e.g. industrial fertilizer *versus* effluent). Creating artificial macroalgal $\delta^{15}N$ signatures in the laboratory will assess their suitability to be translocated into the marine environment to determine and monitor nitrogen sources.

3 | MATERIALS AND METHODOLOGY

3.1 | Macroalgal selection

Brown macroalgae are photoautotrophic, and are composed mainly of sulphated fucans and alginates, with cellulose accounting for only a small fraction of the dry algal weight (1–8%).[46] *F. vesiculosus* is a perennial, intertidal brown macroalga with growth rates of approximately 4.5 mm per week.[47] Furthermore, its widespread around Europe and is believed to integrate DIN variations over approximately 16 days *in vitro*.[6] Thus, due to its robustness, commonality and use in research, *F. vesiculosus* was chosen for this isotopic experiment. Non-fertile tips of *F. vesiculosus* were collected from Easington Colliery beach (54°47′18.2″ N, 1°18′37.9″ W), County Durham, UK, in September 2019. The non-fertile tips were taken back to the laboratory in a container filled with local seawater, and then cleaned using distilled water; any visible epiphytes were physically removed.

3.2 | Artificial seawater

For the experiment it was decided to use artificial seawater over natural seawater so that the nitrogen content was controlled. Two isotopically labelled stock solutions were prepared by dissolving ammonium sulphate, $(NH_4)_2SO_4$, in distilled water to create artificial solutions. One ammonium sulphate solution contained 99.99 at% ^{14}N (Sigma-Aldrich, Gillingham, UK; CAS: 1196157-83-1; PubChem Substance ID: 24872071) and the other contained 60 at% ^{15}N (Sigma-Aldrich; CAS: 43086-58-4; PubChem Substance ID: 24872330) (Table 1). The nitrogen-enriched solutions were then diluted in artificial seawater made using PRO-REEF salt (Tropic Marin, Chorleywood, UK) (Table 1). The artificial seawater solutions were made so that the nitrogen concentration would be just above 50 μM, which is consistent with typical nitrogen concentrations found in coastal areas.[26]

TABLE 1 Nitrogen concentrations and $\delta^{15}N$ values of the artificial seawater solutions used in this study

	Nitrogen concentration (μM)	$\delta^{15}N$‰ (AIR) of solution
^{15}N-enriched	57.6	170 ± 5
^{14}N-enriched	54.0	−60 ± 3

3.3 | Growth incubator

Non-fertile tips of *F. vesiculosus* were added to specimen jars containing isotopically labelled artificial seawater. In order to prevent the non-fertile tips from congregating, plastic mesh was placed in the jars to create four sections (Figure 2). Each section contained three non-fertile tips. The specimen jars were covered with stocking material to ensure exchange with the atmosphere in the growth incubator (Figure 2). Two control specimen jars were set up with only artificial seawater. The growth incubator used was a MaxQ™ 6000 (Thermo Fisher Scientific, Bremen, Germany) housed in the Department of Biosciences, Durham University. The incubator was set at a temperature of 11°C with a light/dark rhythm of 16 h/8 h, and a light intensity of 125 μmol photons m^{-2} s^{-2}; these were the same settings as used by Gröcke et al.[7] The specimen jars were arranged as shown in Figure 3. The non-fertile tips were sampled at approximately the same time each day over the course of 19 days. The artificial seawater was changed every three days (days 3, 6, 9, 12, 15, 18). Each day, five macroalgal tips were sampled (removed): two tips from the ^{14}N-enriched jars, two tips from the ^{15}N-enriched jars and one from the control jar.

3.4 | Sample preparation and isotopic analysis

Macroalgal tips were removed from the jars and dried using paper towels to remove any salt; following this the tips were placed into labelled, small brown envelopes. The envelopes were then placed into a drying oven set at 60°C for at least 24 h until completely dry. The tips were then removed and subsampled for stable isotope analysis by cutting two small fragments from the tip apex to generate replicate samples. We chose this method over grinding the entire tip as it is not currently known how much of the tip region exchanges nitrogen with the seawater solution. Macroalgal samples were weighed into 6 mm × 4 mm tin capsules with a weight range between 1.5 and 2.5 mg. Replicate analyses were performed for the majority of the samples. In addition, it was decided that the bottom and middle sections of the non-fertile tips should be analysed to determine whether there was exchange in other parts of the experimental sample. Only tips from days 1, 5, 10 and 15 were analysed in this respect in order to gain a representative sample set of changes in nitrogen uptake of the artificial seawater solutions.

Isotopic analysis was performed using a ECS 4010 elemental analyser (Costech, Valencia, CA, USA) connected to a Thermo

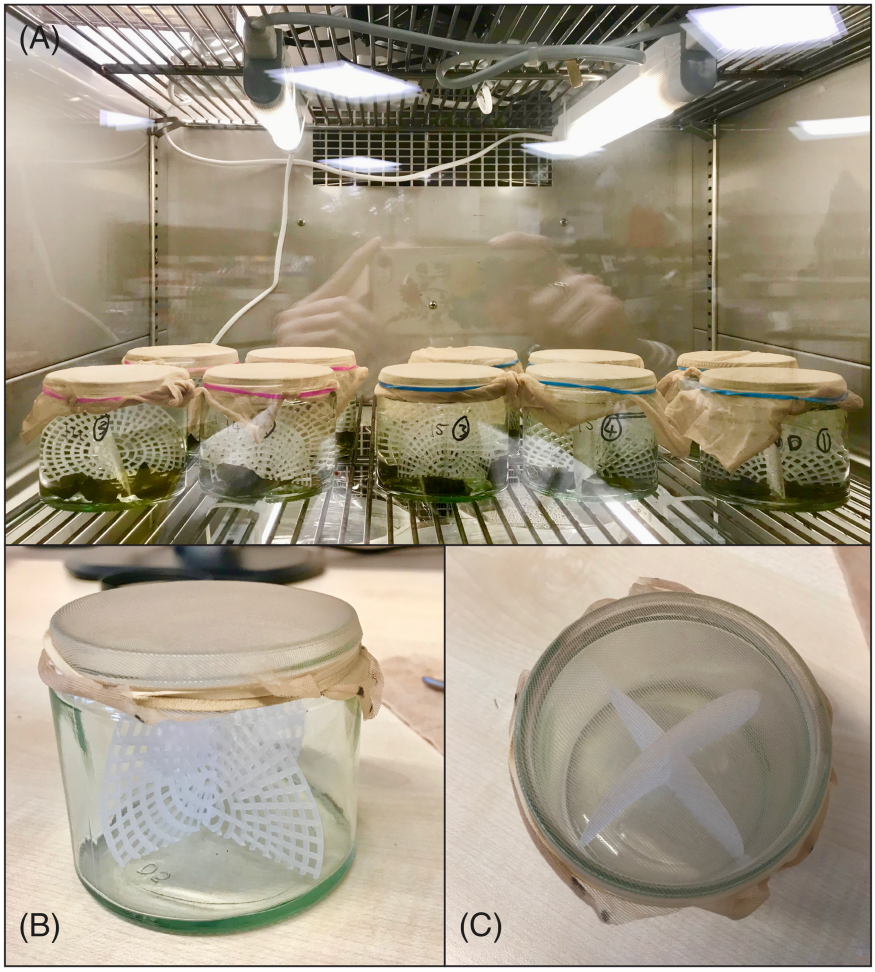

FIGURE 2 Photographs of the experimental jars used to culture the macroalgae tips in this study. A, The position of the jars in the incubator during the experiment. B, Side view of a jar with plastic mesh to separate macroalgal samples. C, Aerial view of a jar with plastic mesh and stocking material to allow atmospheric exchange and light penetration; in Gröcke et al[7] the solid plastic lids were placed loosely on top which would have caused reduced light intensity [Color figure can be viewed at wileyonlinelibrary.com]

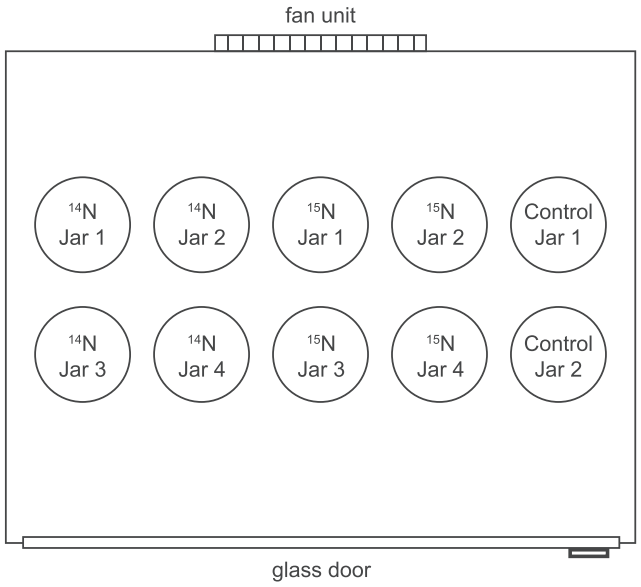

FIGURE 3 Aerial view of the incubator unit showing the position of the experimental jars with respect to the glass door and fan unit

Scientific Delta V Advantage isotope ratio mass spectrometer. The mass spectrometer was calibrated using internal reference samples (e.g. glutamic acid, α-cellulose, IVA urea) and international reference standards (e.g. IAEA-600, IAEA-CH-3, IAEA-CH-6, IAEA-N-1, IAEA-N-2, NBS 19, USGS40, USGS24). All these samples were analysed in duplicate with every run. The standard deviation of the standards was better than ±0.1‰ (1σ) for carbon and nitrogen isotope ratios.

4 | RESULTS

F. vesiculosus collected from Easington Colliery had an average δ^{15}N value of 10.79‰ ± 0.81‰ (n = 40). This δ^{15}N value is elevated in comparison with that of *F. vesiculosus* from unpolluted, natural regions (i.e. 4‰ to 6‰).[9,48,49] Using the dominant nitrogen inputs that can affect macroalgae (see Figure 1) this would suggest that Easington Colliery beach is affected by inputs of livestock manure and/or effluent. All isotopic and elemental data are provided in the supporting information.

4.1 | Isotope analysis of experimental macroalgal tips

4.1.1 | ^{15}N-enriched seawater

Over the course of the experiment, the δ^{15}N values of the *F. vesiculosus* tips cultured in the ^{15}N-enriched solution show a gradual increase towards the value of the enriched artificial seawater solution (Figure 4). The regression lines for the macroalgal tips cultured in ^{15}N-enriched solutions are high. The standard deviations on some data points, particularly towards the end of the experiment, are large owing to the elevated δ^{15}N values or intra-variation in uptake and deposition in the macroalgal tip. The non-fertile tips harvested between days 8 and 10 deviate away from the trendline; this is probably the result of an unobserved power failure to the incubator resetting the temperature. As shown in Figure 4, the δ^{15}N values of the non-fertile tips have shifted between days 9 and 11 by over 50% from the initial macroalgal δ^{15}N value to that of the solution. They reach isotopic equilibrium with the ^{15}N-enriched seawater between days 16 and 19. The trendline for each jar is similar in all cases except for ^{15}N-Jar 3, which was positioned in the middle front of the incubator (see Figure 3).

4.1.2 | ^{14}N-enriched seawater

The δ^{15}N values of *F. vesiculosus* tips cultured in the ^{14}N-enriched artificial seawater solution show a decrease towards the value of the enriched solution at a much slower rate than the ^{15}N-enriched solution jars (Figure 4). This is probably caused by the excess of ^{14}N enrichment in this artificial solution compared with the ^{15}N-enriched solution. As a result, even after 19 days the macroalgal tips have not reached isotopic equilibrium with the artificial seawater solution (see Figure 4), but between days 12 and 15 the δ^{15}N values have shifted by over 50% from the initial macroalgal value to that of the solution. The trendline for each jar is very similar in all cases except for ^{14}N-Jar 3, which deviates from day 10; this jar is positioned in the front left of the incubator (see Figure 3).

4.1.3 | Control seawater solution

The *F. vesiculosus* tips cultured in the control artificial seawater show very little deviation away from the initial average δ^{15}N value of the Easington Colliery macroalgal tips at the date of collection (δ^{15}N ∼ 10.8‰) (Figure 4). The artificial seawater solution contains no traceable nitrogen (PRO-REEF salt, Tropic Marin), and thus we would expect to see no change through the experiment. However, it is noted that there is a cyclic change in δ^{15}N values in the control experiment (see Figure 4). The seawater solutions were changed on days 3, 6, 9, 12, 15, 18. During each 3-day period the δ^{15}N values of the tips become more elevated (Figure 4); this is most extreme in the first three days of the experiment.

4.2 | Isotope analysis of experimental macroalgal sections

Due to the higher standard deviation of the δ^{15}N values of the samples towards the end of the experimental period, it was questioned whether the isotopic signature was spatially inhomogeneous. In order to ascertain this, we performed nitrogen isotope analysis on different parts of the non-fertile tip (e.g. apex, middle and torn base). Figure 5 shows that for both the ^{15}N-enriched

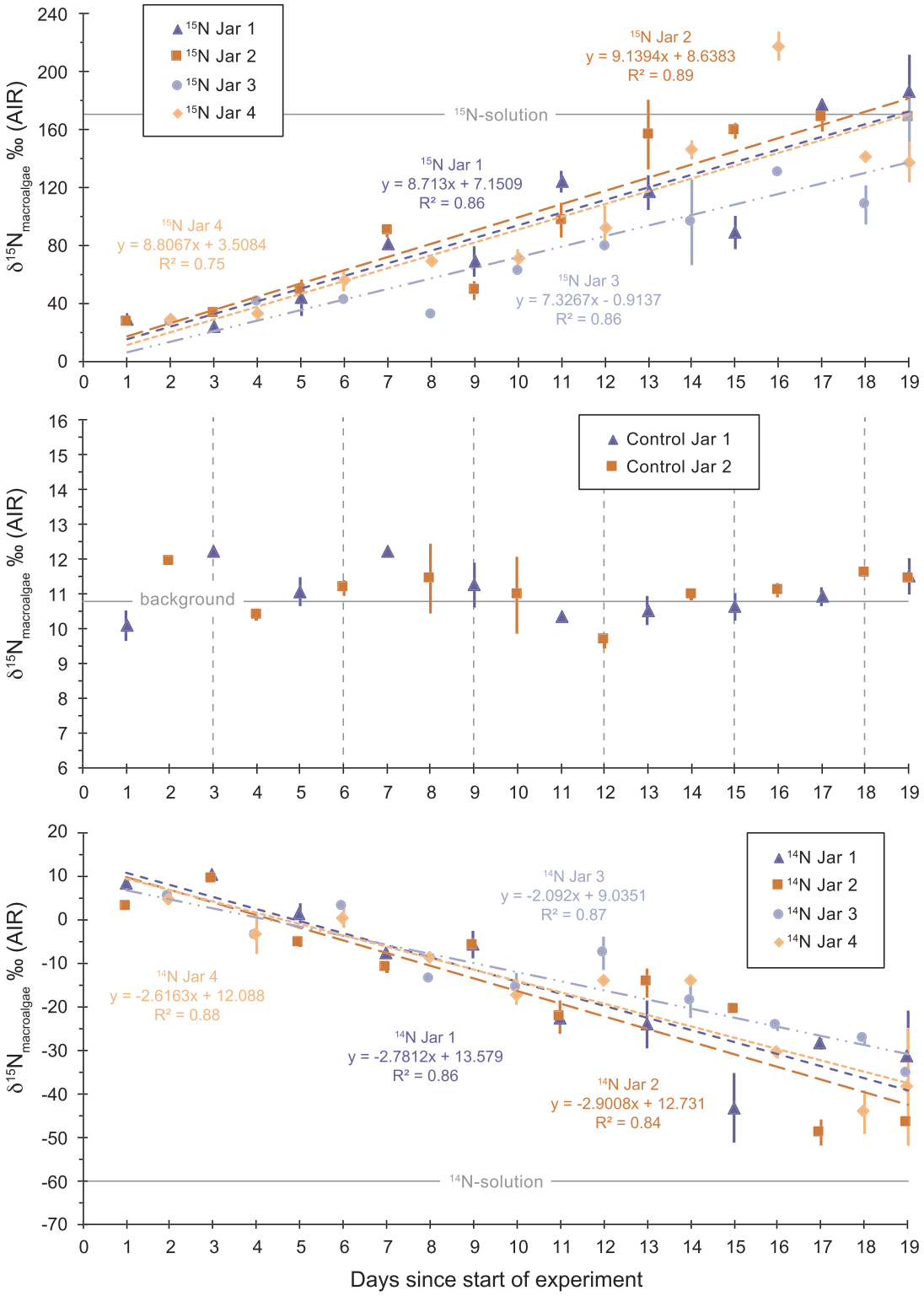

FIGURE 4 $\delta^{15}N$ values of *F. vesiculosus* cultured in ^{15}N-enriched (top) and ^{14}N-enriched (bottom) artificial seawater solutions over an experimental period of 19 days. The central graph shows the results of the control experiment. Grey solid lines represent the $\delta^{15}N$ value of the ^{15}N-enriched and ^{14}N-enriched solutions, whereas in the control graph the solid grey line represents the background $\delta^{15}N$ value of the macroalgae collected at Easington Colliery. The dashed grey lines in the control experiment graph represent the days when the artificial seawater solutions were replaced. Error bars represent standard deviation on multiple fragments of the sampled growing tip [Color figure can be viewed at wileyonlinelibrary.com]

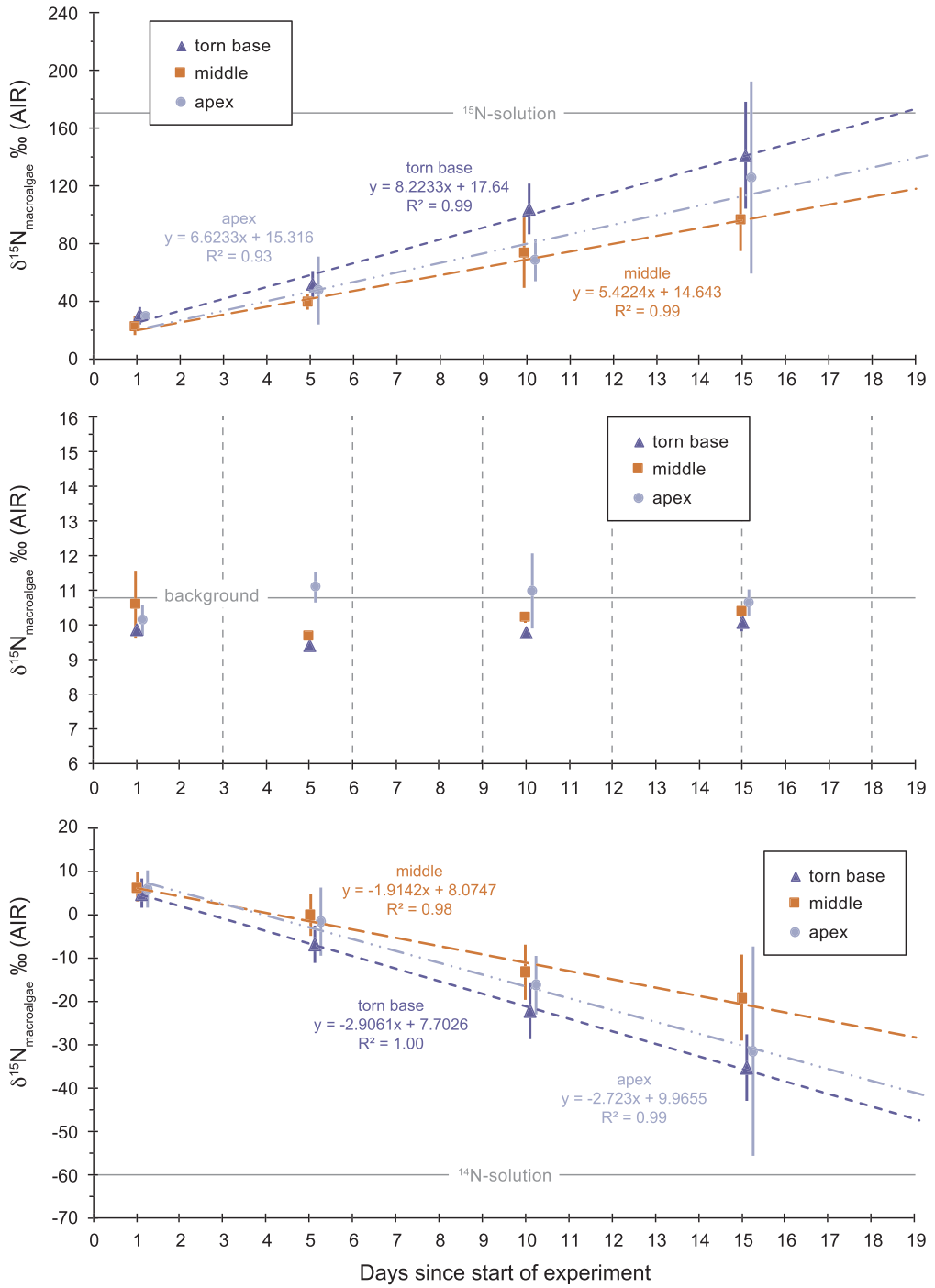

FIGURE 5 $\delta^{15}N$ values of the torn base, middle and apex areas of *F. vesiculosus* tips cultured in ^{15}N-enriched (top) and ^{14}N-enriched (bottom) artificial seawater solutions over an experimental period of 19 days. The central graph shows the results of the control experiment. Only days 1, 5, 10 and 15 were sampled for these data. The data points for torn base and apex have been slightly moved to the right in order to see the standard deviation of each group. [See Figure 4 for description of graphs.] Error bars represent standard deviation on multiple fragments of the sampled area. Large standard deviations suggest that there is microscale variation. Note that for each experimental solution the torn base was closest to the solution $\delta^{15}N$ value whereas the middle was the least close [Color figure can be viewed at wileyonlinelibrary.com]

and the ^{14}N-enriched seawater solutions the torn base of the tips incorporated the isotopic ratio of the artificial seawater more efficiently than the apex, followed by the middle of the tips. We predict that this occurred due to the breakage and opening up of the tissue meristoderm, thus allowing easier exchange with the artificial seawater solution. Even within these analyses there is significant scatter in $\delta^{15}N$, indicating that isotopic incorporation is not homogeneous. Although grinding the entire non-fertile tip may produce smaller standard deviations it may not accurately reflect the isotopic uptake amount. On the other hand, the control tips do not

show any systematic difference in $\delta^{15}N$ values for each sample area during the experiment.

5 | DISCUSSION

5.1 | Assimilation of isotopically enriched nitrogen seawater solutions

Non-fertile tips of *F. vesiculosus* cultured in ^{14}N-enriched or ^{15}N-enriched artificial seawater systematically incorporated these isotopic solutions linearly over a 19-day experimental period (see Figures 4 and 5). The ^{15}N-enriched seawater solution appears to have been taken up at a faster rate than the ^{14}N-enriched seawater. This equilibrium effect is caused by a greater uptake rate in the ^{15}N-enriched experiment as the solution is further away from the starting point, and thus assimilation happens faster. The ^{14}N-enriched solution is closer to the macroalgal starting point ($\delta^{15}N = 10.8‰$), and hence the assimilation happens slower. The non-fertile tips cultivated in the ^{15}N-enriched solution reached isotopic equilibrium within the 19-day experiment, and in some cases actually became more enriched than the solution (day 16, ^{15}N-Jar 3; days 17 and 19, ^{15}N-Jar 1; see Figure 4). Overshooting of the solution $\delta^{15}N$ value may be generated by a release of ^{14}N in the macroalgae in order to enable uptake of ^{15}N to form stronger chemical bonds with macromolecules within the macroalgae. It is unclear at present why the macroalgae would choose to release ^{14}N in favour of ^{15}N, since the consequence of this would require significant amounts of energy. This experimental study suggests that macroalgae can be artificially cultured to develop 'unnatural' $\delta^{15}N$ values for translocation experiments.

5.2 | Other factors affecting nitrogen assimilation

An oscillation pattern is recorded in $\delta^{15}N$ values in the control jars every 3 days (Figures 4 and 5), which coincides with replacement of the artificial seawater solution. The increase in ^{15}N in macroalgal tips over the 3-day period in the control experiment may relate to a build-up of metabolites (e.g. ammonium) and reprocessing (breaking down) of ^{14}N-containing bonds within organic molecules in the macroalgae that are subsequently released, hence leaving the tissues more ^{15}N-enriched. The seawater solutions were not analysed at the end of each 3-day period, although future research should incorporate this. During the experimental period only one 3-day interval shows an inverse relationship (decreasing $\delta^{15}N$ values over the 3-day period; Figure 4). This time interval coincides with the power failure on day 8 that reset the temperature of the incubator to factory settings (e.g. from 11 to 19°C). This was not realised until day 11 when the temperature was reset to 11°C. So, does temperature alter the uptake rate and breakdown of nitrates and ammonium in macroalgae? Further research investigating the effects of temperature on the uptake rates of ^{15}N-enriched and ^{14}N-enriched solutions is required.

Although the $\delta^{15}N$ values vary highly between non-fertile tips of the same sample and different jars we suspect that this is through a combination of factors, such as spatial variation in macroalgae uptake, micro-climate in the incubator and isotopic analysis of enriched samples. However, there are other environmental factors that could cause variation in $\delta^{15}N$ values. In particular, the surface area to volume ratio of the macroalgae is important in determining nutrient uptake.[14] Although this was not measured directly in this study, all macroalgal non-fertile tips were visually similar in length (*ca* 3 cm) and thickness. Isotopic analysis of the in-house and international standards was performed regularly during the generation of this dataset; hence, we do not believe this to be an issue. Despite this, the $\delta^{15}N$ values and slopes are relatively consistent.

The variation between the experimental jars may be explained by their position in the incubator (see Figure 3) and potential differences in the micro-climate of the incubator: for example, ^{15}N-Jar 3 had a slope that was most different from those of the other jars (see Figure 4). Light and temperature are known to be very important parameters determining nutrient uptake in macroalgae.[50,51] However, light would not have been an issue since the jars had no lids and the lighting is consistent throughout the incubator (see Figure 2). Although the incubator is relatively small, there may have been subtle variations in temperature between the jars. During the experiment it was assumed that the temperature of the seawater solutions was equal to the set temperature of the incubator; in future studies, the temperature of the solutions should also be continuously recorded. We recommend that temperature should be considered as an important factor in nitrogen-enrichment experiments with respect to nutrient uptake.[33] Therefore, spatial variation in the macroalgae may be the cause of the higher standard deviation.

5.3 | Intra-variation in uptake of ^{14}N and ^{15}N by *F. vesiculosus* tips

F. vesiculosus exhibits apical growth[47] and, therefore, nutrients are concentrated in metabolically active areas of the thallus.[6,52] Previous studies have recorded varying trends in $\delta^{15}N$ values along the length of a non-fertile tip; some reporting an increase in $\delta^{15}N$ values towards the apex,[6] whereas others report a decrease.[1,9] These differing trends in $\delta^{15}N$ values could simply be explained by different harvesting times of the macroalgae, alterations in environmental conditions and/or adjustments in the uptake and release of nitrogen throughout the growing thallus.

In this study, the base of the non-fertile tips (i.e. torn base) records $\delta^{15}N$ values that are closest to those of the artificial seawater solutions during the experiment (Figure 5). This is possibly due to breaking the cell membrane in this region during collection, hence allowing an easier pathway for the artificial seawater to pass directly into the macroalgal tissues. In addition, the diffusion boundary layer that exists around macroalgal cells may have also been damaged, thus allowing more uptake.[14] The $\delta^{15}N$ value of the non-fertile apex is also very close to that of the solutions. The middle of the non-fertile tips

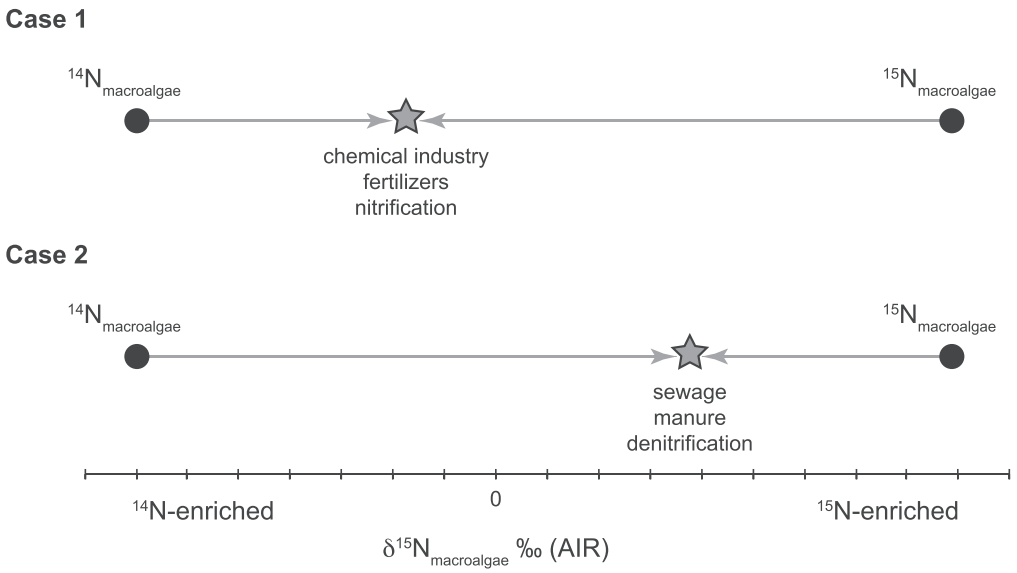

FIGURE 6 Schematic illustration of how ^{15}N-enriched and ^{14}N-enriched *F. vesiculosus* tips translocated into an environmental setting could assimilate the dominant nitrogen source of the region and move towards the δ^{15}N value associated with that source. For example, in an area affected by effluent from chemical industries (case 1), the δ^{15}N values in the translocated macroalgae are more likely to move towards 0‰ whereas in areas affected by sewage effluent (case 2) the δ^{15}N values of the macroalgae are more likely to be more positive. However, each of these scenarios may also record the background oceanographic environment of the area, for example, one dominated by nitrification (case 1) *versus* denitrification (case 2)

was the least enriched in either ^{14}N or ^{15}N (Figure 5). Although the apex and torn base have the highest exchange rates with the artificial seawater solutions, there is still significant variation in δ^{15}N values. This points towards sub-millimetre variation in uptake and distribution of nitrogen isotopes in the macroalgal tissue (a typical sample size for isotopic analysis was *ca* 3 mm). Future experiments in the laboratory and in field translocation should consider this variation between different parts of the macroalgae, and primarily focus on sampling the torn base of the tip for isotopic analysis.

5.4 | Use of isotopically labelled macroalgal tips for translocation experiments

Studies that pre-culture macroalgae before translocation do so in a low-nutrient solution with a δ^{15}N value of approximately 0‰.[4] This is performed on the basis that depleting the internal nitrogen reserves will make the macroalgae more sensitive to δ^{15}N values upon translocation. In this study, the internal nitrogen concentration of the macroalgae has not increased using >50 μM nitrate (e.g. measured by wt% nitrogen), but has incorporated an 'unnatural' δ^{15}N value (see Figure 4). Macroalgal tips grown in very high-nutrient solutions could potentially cause them to accumulate and store nitrogen reserves, as they do in the winter.[17,27–29] Hence, when the macroalgae are translocated they will not exchange with the local environment, but instead rely on their reserves. On the other hand, macroalgae release both organic nitrogen[53] and inorganic nitrogen reserves[31] during active growth and so have the potential to assimilate nutrients in the environment upon translocation. Experimental macroalgae cultured with ammonium may use this internal reserve for growth instead of assimilating nitrate in the water column,[16] and may therefore not integrate the local δ^{15}N signature completely.

Due to SARS-CoV-2 (Covid-19) restrictions we, unfortunately, were unable to conduct the entire field translocation experiment of this study. A suite of ^{15}N- and ^{14}N-enriched macroalgalsampleswastranslocated to the River Tees (same study site as reported in Gröcke et al[7]), but we were only able to sample macroalgae after 1 day prior to the national shutdown. Although the generated isotopic data from these samples indicated change, the data are not adequate to determine if these isotopically enriched samples cultured in 50 μM ammonium would be successful. Figure 6 illustrates our working hypothesis of how ^{15}N- and ^{14}N-enriched macroalgal samples would work in a field translocation experiment. In this scenario, the ^{15}N- and ^{14}N-enriched macroalgal samples (translocated together) will incorporate the local δ^{15}N signature and move towards an end point. That end point would inform the investigator whether the location was affected by industrial (e.g. farming fertilizers) or effluent (e.g. manure or sewage) processes. Both ^{14}N- and ^{15}N-enriched macroalgal samples need to be translocated in the same location to ensure the δ^{15}N values can be analysed such that our working hypothesis would be satisfied (Figure 6). In addition, there needs to be enough samples cultured initially so that translocated samples are collected over a period of time greater than 19 days (i.e. based on this study using extreme δ^{15}N values). Furthermore, the translocation experiment must generate δ^{15}N values geospatially in order to discern local variations and/or source points.

6 | CONCLUSIONS

In this experimental study, *F. vesiculosus* was cultured in ^{15}N-enriched and ^{14}N-enriched ammonium solutions (δ^{15}N = 160‰ and −60‰, respectively) for a period of 19 days. The macroalgae incorporated the enriched solutions linearly over the 19-day period with the ^{15}N-enriched experiment reaching equilibrium with the solution within *ca* 16 days. The δ^{15}N signal of the isotopically labelled solutions is incorporated most effectively at the torn base of non-fertile tips, followed by the apex and then the middle. The torn base allows the solution to enter the cells much more easily. However, even within these regions there is isotopic variation due to differential uptake and incorporation in the tissues. This study highlights the potential value of isotopically labelled macroalgal tips using enriched ammonium solutions. Despite this, additional research is required to identify optimal conditions for growing isotopically enriched macroalgae for translocation experiments.

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