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# Experimental assessment of the macroalgae *Ascophyllum nodosum* and *Fucus vesiculosus* for monitoring N sources at different time-scales using stable isotope composition

Inés G. Viana [a,b,*], Antonio Bode [a], Megan Bartholomew [b], Ivan Valiela [b]

[a] Instituto Español de Oceanografía, Centro Oceanográfico de A Coruña, Apdo. 130, E15080 A Coruña, Spain
[b] The Ecosystems Center, Marine Biological Laboratory, Woods Hole, MA 02534, USA

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ABSTRACT

Stable isotope composition of brown macroalgae has been widely used to monitor N loading during the last decades but some of the required assumptions when using them to detect anthropogenic inputs remain untested. In this study several experiments were run with two key species, *A. nodosum* and *F. vesiculosus*, to determine internal nitrogen isotope dynamics. First, the equilibration of the isotopic values of the different parts of the thallus of these species was tested by growing them under different water sources. Then, nitrate uptake capacity and N transport along the frond were tested by $^{15}N$ enrichment experiments. The results indicate that although the growing tips had the highest uptake rates, older parts of the frond of both species have the capacity to incorporate N at low rates. No evidence of N transport along the thallus, from the tip to the basal segment of the frond or the converse was found. These results show that the growing tips of these macroalgae can be used to monitor N loadings at time scales from weeks (*F. vesiculosus*) to months (*A. nodosum*). The use of non-growing parts of the thallus to do retrospective studies cannot be recommended because of their measurable exchange of N with the surrounding water.



## 1. Introduction

Concern with coastal eutrophication has increased in the last decades due to higher N loading associated with the growing human population in these areas. The ratio of the stable isotopes of N ($^{15}N$:$^{14}N$) in macroalgal tissues allows detecting the presence of anthropogenic N that is available for macroalgae in coastal waters, but also allows estimating the intensity of the effluents and detect disturbances before alteration in structure and function occur in the ecosystem (Carballeira et al., 2013; Costanzo et al., 2001; García-Sanz et al., 2010, 2011; Gartner et al., 2002; McClelland and Valiela, 1998a,b; McClelland et al., 1997). The basis for the use of macroalgae and other biota for monitoring anthropogenic water sources is that different water sources may show characteristic isotopic signatures (Xue et al., 2009) due to different fractionation processes occurring through the N cycle (Montoya, 2007). All the different sources of N may also alter the baseline $\delta^{15}N$ of the macroalgae, as they use N as part of their metabolism, to synthetize structural components or to gain energy for growth (Gruber, 2008).

Among macroalgae, Fucaceae, as *Fucus vesiculosus* and *Ascophyllum nodosum,* have been widely used for monitoring loads of N and other substances (e.g. heavy metals) (Viana et al., 2010, 2011). As these species show apical growth, the tips have been traditionally used in monitoring studies. The growing tips can be feasibly related with previous weeks of growth (Viana et al., 2014, 2015) and hence with the environmental status at a particular time. First studies with Fucaceae were focused on detecting wastewater effluents (Hobbie et al., 1990; Savage and Elmgren, 2004) but later, they were reliable used to discern anthropogenic from natural sources (Carballeira et al., 2013; García-Sanz et al., 2010, 2011; Viana and Bode, 2013). Their high tolerance to broad salinity ranges have also enable to study the status of estuaries and rias in both native populations (Bode et al., 2011, 2014; Raimonet et al., 2013; Viana et al., 2011) and transplant studies in the field (Deutsch and Voss, 2006).

In any case, long-term monitoring is needed to track the ecological status of the ecosystem or to feasibly interpret data obtained at a particular moment. Obtaining a reliable and long-time monitoring series would require of a careful sampling plan implemented during decades. Consequently there are only few examples of time series using stable isotopes (Viana et al., 2011). That is the reason why some authors have taken advantage of the long lifespan of the species considered, up to 15 years in the case of *A. nodosum* (Niell, 1979), and their apical growth to do retrospective studies. If growth rates are known (Viana et al., 2014, 2015), different segments along the frond can be related with past environmental or water conditions (Carballeira et al., 2014;

* Corresponding author at: Instituto Español de Oceanografía, Centro Oceanográfico de A Coruña, Apdo. 130, E15080 A Coruña, Spain. Tel.: +34 981218584; fax: +34 981229077.
*E-mail address:* inesgviana@gmail.com (I.G. Viana).

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Raimonet et al., 2013; Savage and Elmgren, 2004). Moreover, *A. nodosum* fronds develop a gas bladder in the tip that generally occurs once a year (David, 1943; Viana et al., 2014). This annual bladder enables estimation of the minimum age of an individual and definition of its annual growth (Niell, 1979; Viana et al., 2014). Retrospective analysis would allow reducing the sampling effort in monitoring programs (Carballeira et al., 2014).

The use of stable isotopes in the growing tips of these species for monitoring N loadings requires some assumptions related to their physiology. For instance, net fractionation processes (i.e. the preferential use of light against heavy isotopes) in macroalgae are poorly understood. Fractionation during uptake in macroalgae is the best studied. Experimental studies on different macroalgal species demonstrated that, at least those macroalgae, did not exhibit concentration dependent N isotope fractionation (Cohen and Fong, 2005; Dudley et al., 2010; García-Sanz, 2009). But there is no information about fractionation processes during the subsequent processes within the tissues, as absorption, accumulation or release of nitrogen. This is important as if fractionation factor is not known; the isotopic values in macroalgae can lead to misinterpretation of the contribution of anthropogenic sources (Bode et al., 2014).

The main assumption of retrospective studies is that only the growing tips of the thallus take up nitrogen and, therefore, the isotopic composition of a given section of the thallus would reflect the isotopic composition of the dissolved nitrogen in the surrounding water at the time of growth. To fully interpret the data obtained in these studies, some questions need to be answered. First, Fucaceae do not have specific transport tissues, but the pores of the sieve plates should enable a continuous system of cytoplasm for longitudinal translocation of materials (Moss, 1983). There is experimental evidence of such transport of organic $^{14}C$, $^{86}Rb$ or $^{32}P$ (Diouris and Floc'h, 1984; Penot and Penot, 1979; Raven, 2003). If transport of nitrogen along the thallus also takes place, it would directly affect the retrospective identification of past nitrogen sources. Second, most studies assume that isotopic composition of tissues does not change for at least several months, given that these species generally show low variability in $\delta^{15}N$ values at monthly time scales (Gartner et al., 2002; Raimonet et al., 2013), but no data of N-specific uptake and turnover rate were available for this species.

To assess the feasibility of using *A. nodosum* and *F. vesiculosus* for isotopic differentiation of local N sources, two sets of experiments were made under laboratory conditions. The first experiment aimed to determine the equilibration of N isotopes in the growing tips and older parts of the fronds by growing them under water with different N origins. The second experiment aimed to detect nitrogen transport along their thalli and to test if all the parts of the frond have the capacity of taking up $NO_3^-$ by using artificially $^{15}N$-enriched water. The latter approach also allowed the estimation of N turnover rates in different sections of the thallus.

## 2. Material and methods

### 2.1. Experiment 1: N isotope equilibration

#### 2.1.1. Water samples

The first laboratory experiment was conducted with water from 3 different sites: water from an urbanized watershed, from a forested watershed, and from an oceanic influenced site which was considered the control. The first two sites are Childs River (CR) and Sage Lot Pond (SLP), which are part of the Waquoit Bay National Estuarine Research Reserve, Massachusetts (Fig. 1b). The Waquoit Bay estuarine system is a complex of sub-estuaries with different N inputs from their watersheds, and thus, with differing ambient N concentration and origin (Valiela et al., 1992, 1997). The CR estuary (41°34′ N, 70°32′W) is surrounded by the most urbanized watershed in the Waquoit Bay system. Nutrients (primarily nitrate) are delivered to the CR estuary from the watershed via groundwater flow (Valiela et al., 1992). In contrast, SLP (41°55′′N, 70°50′W) has a forested watershed receiving a low N load, with $NH_4^+$ as the dominant dissolved inorganic nitrogen (DIN) form (Valiela et al., 1997), and the estuary is surrounded by salt marshes. The control site was at Nobska Beach (41°51′N, 70°65′W), which water is marine with no terrestrial or anthropogenic inputs draining in the area (Fig. 1a).

#### 2.1.2. Experimental design

Individual fronds of *A. nodosum* and *F. vesiculosus* were collected at Quissett Harbor and Nobska Beach respectively, in Woods Hole, Massachusetts (Fig. 1a); and were transported in coolers to the laboratory. Macroalgae were kept in tanks with continuous seawater flow (15.7 ± 1.6 °C) and low light intensities during the night (less than 12 h) until the start of the experiment. *A. nodosum* fronds of 14.6 ± 2.6 cm long and with 2 or 3 gas bladders, and *F. vesiculosus* fronds of 10.7 ± 2 cm long were selected to run the experiment. Individuals with visible damage or epiphytes were avoided.

For each set of water treatments, macroalgae ($n = 4$ for *A. nodosum*, $n = 3$ for *F. vesiculosus*) were placed in three different 1 L Erlenmeyer flasks containing CR, SLP or Nobska unfiltered water. The study was run in triplicate with each replicate in a separate flask for each of the three treatments over a period of 22 days for *A. nodosum* and 12 days for *F. vesiculosus*. Samples were taken at the start of the experiment ($t = 0$) and at subsequently exponential times, 4 times for *A. nodosum* and 3 times for *F. vesiculosus*. At each time, a macroalgal frond of each flask was sampled and frozen (−20 °C) before processing. The different time scales for each species were chosen based on the previous knowledge of growth rates. A control flask with no macroalgae was established for each water treatment and maintained under the same conditions as the experimental flasks.

For comparison with experimental individuals, native individuals of *F. vesiculosus* were collected along with water samples where present (i.e. CR and SLP) and analyzed for stable isotope composition. Local populations of *A. nodosum* were not found at the sites selected for water collection.

Experiments were carried out in a culture chamber with 18:6 light: dark cycle at light intensities varying between 390 and 450 μE $m^{-2}$ $s^{-1}$ under 18–20 °C air temperature oscillation between night and day respectively. Water aeration was maintained with air pumps and diffusers and water temperature set at 24.08 ± 0.06 °C.

Water was replaced every 2 days to avoid nutrient depletion. Samples of water were collected before and after replacement to quantify the variation in DIN concentrations among times and sites and to check macroalgal consumption. Salinity and temperature were measured with a portable conductivity meter (YSI Model 30) every time the water was changed.

The macroalgal samples used for $\delta^{15}N$ and N and C contents were separated with a glass spatula. The growing tip (1 cm) was sampled at all sampling dates during the experiment for both species. Additionally to the tip, at the start of the experiment ($t = 0$) and at the endpoint, all intervesicular segments were sampled in *A. nodosum* individuals, while for *F. vesiculosus* individuals only the basal segment of the frond was additionally sampled. All macroalgal samples were rinsed with Milli Q water and frozen (−20 °C) before processing. Later, samples were defrosted and dried (50 °C) until constant weight before grinding into a homogeneous powder prior to isotopic and elemental analysis.

#### 2.1.3. Macroalgal growth

To measure macroalgal growth response to the different water samples, the wet biomass of each frond was recorded at the beginning of the experiment and at the time the frond was sampled. Individual growth rates ($\mu$) were calculated as a percent increase in biomass per day (% $d^{-1}$):

$$\mu = \frac{100\left[\mathrm{Ln}\left(\frac{N_t}{N_0}\right)\right]}{t}$$