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9   **Experimental assessment of the macroalgae *Ascophyllum nodosum* and *Fucus***

10   ***vesiculosus* for monitoring N sources at different time-scales using stable isotope**

11   **composition**

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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 stipe 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 (McClelland et al., 1997; McClelland and Valiela, 1998a, 1998b; Costanzo et al., 2001; Gartner et al., 2002; García-Sanz et al., 2010, 2011; Carballeira et al., 2013). 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 δ^{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 for monitoring studies. The growing tips can be feasibly related with previous weeks of growth (Viana et al., 2014, in review) and hence with the environmental status at a particular time. First studies 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 (García-Sanz et al., 2010, 2011; Carballeira et al., 2013; Viana and Bode, 2013). Their high tolerance to broad salinity ranges have also enable to study the status of estuaries and rias in both

68   native populations (Bode et al., 2011, 2014; Viana et al., 2011; Raimonet et al., 2013)

69   and transplant studies (Deutsch and Voss, 2006).

70   In any case, to feasibly interpret the data obtained at a particular moment, long-term

71   monitoring is needed to track the ecological status of the ecosystem or to contextualize

72   current observations. Obtaining a reliable and long-time monitoring series would

73   require of a careful sampling plan implemented during decades. Consequently there are

74   only few examples of time series using stable isotopes (Viana et al., 2011). That is the

75   reason why some authors have taken advantage of the long lifespan of the species

76   considered, up to 15 yr in the case of *A. nodosum* (Niell, 1979), and their apical growth

77   to do retrospective studies. If growth rates are known (Viana et al., 2014, in review),

78   different segments along the frond can be related with past environmental or water

79   conditions (Savage and Elmgren, 2004; Raimonet et al., 2013; Carballeira et al., 2014).

80   Moreover, *A. nodosum* fronds develop a gas bladder in the tip that generally occurs

81   once a year (David, 1943; Viana et al., 2014). This annual bladder enables estimation of

82   the minimum age of an individual and definition of its annual growth (Niell, 1979;

83   Viana et al., 2014). This approach would allow reducing the sampling effort in

84   monitoring programs (Carballeira et al., 2014).

85   The use of stable isotopes in the growing tips of these species for monitoring N

86   loadings requires some assumptions related to their physiology. For instance, net

87   fractionation processes (i.e. the preferential use of light against heavy isotopes) in

88   macroalgae are poorly understood. Fractionation processes during uptake in macroalgae

89   are the best studied. Experimental studies on different macroalgal species demonstrated

90   that, at least those macroalgae, did not exhibit concentration dependent N isotope

91   fractionation (Cohen and Fong, 2005; García-Sanz, 2009; Dudley et al., 2010). But

92   there is no information about fractionation processes during the subsequent processes

93   within the tissues, as absorption, accumulation or release of nitrogen. This is important

94   as if fractionation factor is not known; the isotopic values in macroalgae can lead to

95   misinterpretation of the contribution of anthropogenic sources (Bode et al., 2014). .

96   The main assumption of retrospective studies is that only the growing tips of the

97   thallus take up nitrogen and, therefore, the isotopic composition of a given section of

98   the thallus would reflect the isotopic composition of the dissolved nitrogen in the

99   surrounding water at the time of growth. To fully interpret the data obtained in these

100   studies, some questions need to be answered. First, Fucaceae do not have specific

101   transport tissues, but the pores of the sieve plates should enable a continuous system of

102   cytoplasm for longitudinal translocation of materials (Moss, 1983). There is

103   experimental evidence of such transport of organic ^{14}C, ^{86}Rb or ^{32}P (Penot and Penot,

104   1979; Diouris and Floc´h, 1984; Raven, 2003). If transport of nitrogen along the thallus

105   also takes place, it would directly affect the retrospective identification of past nitrogen

106   sources. Second, most studies assume that isotopic composition of tissues does not

107   change for at least several months, given that these species generally show low

108   variability in δ^{15}N values at monthly time scales (Gartner et al., 2002; Raimonet et al.,

109   2013), but no data of N-specific uptake and turnover rate were available for this species.

110   To assess the feasibility of using *A. nodosum* and *F. vesiculosus* for isotopic

111   differentiation of local N sources, two sets of experiments were made under laboratory

112   conditions. The first experiment aimed to determine the equilibration of N isotopes in

113   the growing tips and older parts of the fronds by growing them under water with

114   different N origins. The second experiment aimed to detect nitrogen transport along

115   their thalli and to test if all the parts of the frond have the capacity of taking up NO_3^- by

116   using artificially ^{15}N-enriched water. The latter approach also allowed the estimation of

117   N turnover rates in different sections of the thallus.

118   **2. Material and Methods**

119   *2.1. Experiment 1: N isotope equilibration*

120   **Water samples –**The first laboratory experiment was conducted with water from 3

121   different sites: water from an urbanized watershed, from a forested watershed, and from

122   an oceanic influenced site which was considered the control. The first two sites are

123   Childs River (CR) and Sage Lot Pond (SLP), which are part of the Waquoit Bay

124   National Estuarine Research Reserve, Massachusetts (Fig. 1). The Waquoit Bay

125   estuarine system is a complex of sub-estuaries with different N inputs from their

126   watersheds, and thus, with differing ambient N concentration and origin (Valiela et al.,

127   1992; Valiela et al., 1997). The CR estuary (41º34' N, 70º32'W) is surrounded by the

128   most urbanized watershed in the Waquoit Bay system. Nutrients (primarily nitrate) are

129   delivered to the CR estuary from the watershed via groundwater flow (Valiela et al.,

130   1992). In contrast, SLP (41º55'N, 70º50'W) has a forested watershed receiving a low N

131   load, with NH_4^+ as the dominant dissolved inorganic nitrogen (DIN) form (Valiela et al.,

132   1997), and the estuary is surrounded by salt marshes. The control site was at Nobska

133   Beach (41º51'N, 70º65'W), which water is marine with no terrestrial or anthropogenic

134   inputs draining in the area (Fig. 1a).

135   **Experimental design–** Individual fronds of *A. nodosum* and *F. vesiculosus* were

136   collected at Quissett Harbor and Nobska Beach respectively, in Woods Hole,

137   Massachusetts (Fig. 1a); and were transported in coolers to the laboratory. Macroalgae

138   were kept in tanks with continuous seawater flow (15.7±1.6ºC) and low light intensities

139   during the night (less than 12 hours) until the start of the experiment. *A. nodosum* fronds

140   of 14.6±2.6cm long and with 2 or 3 gas bladders, and *F. vesiculosus* fronds of 10.7±2

141   cm long were selected to run the experiment. Individuals with visible damage or

142   epiphytes were avoided.

143   For each set of water treatments, macroalgae (n=4 for *A. nodosum*, n=3 for

144   *F. vesiculosus*) were placed in three different 1 L Erlenmeyer flasks containing CR, SLP

145   or Nobska unfiltered water. The study was run in triplicate with each replicate in a

146   separate flask for each of the three treatments over a period of 22 days for *A. nodosum*

147   and 12 days for *F. vesiculosus*. Samples were taken at the start of the experiment (t=0)

148   and at subsequently exponential times, 4 times for *A. nodosum* and 3 times for

149   *F. vesiculosus*. At each time, a macroalgal frond of each flask was sampled and frozen

150   (-20 °C) before processing. The different time scales for each species were chosen

151   based on the previous knowledge of growth rates of the species. A control flask with no

152   macroalgae was established for each water treatment and maintained under the same

153   conditions as the experimental flasks.

154   For comparison with experimental individuals, native individuals of *F. vesiculosus*

155   were collected along with water samples where present (i.e. CR and SLP) and analyzed

156   for stable isotope composition. Local populations of *A. nodosum* were not found at the

157   sites selected for water collection.

158   Experiments were carried out in a culture chamber with 18:6 light:dark cycle at light

159   intensities varying between 390-450 μE m^{-2} s^{-1} under 18-20 °C air temperature

160   oscillation between night and day respectively. Water aeration was maintained with air

161   pumps and diffusers and water temperature set at 24.08±0.06 °C.

162   Water was replaced every 2 days to avoid nutrient depletion. Samples of water were

163   collected before and after replacement to quantify the variation in DIN concentrations

164   among times and sites and to check macroalgal consumption. Salinity and temperature

165   were measured with a portable conductivity meter (YSI Model 30) every time the water

166   was changed.

167   The macroalgal samples used for $\delta^{15}N$ and N and C content were separated with a

168   glass spatula. The growing tip (1 cm) was sampled at all sampling dates during the

169   experiment for both species. Additionally, at the start of the experiment (t=0) and at the

170   endpoint, the growing tip (1 cm) and all intervesicular segments were sampled in

171   *A. nodosum* individuals, while for *F. vesiculosus* individuals only the growing tip (1

172   cm) and the basal segment of the frond were sampled. All macroalgal samples were

173   rinsed with Milli Q water and frozen (-20 °C) before processing. Later, samples were

174   defrosted and dried (50 °C) until constant weight before grinding into a homogeneous

175   powder prior to isotopic and elemental analysis.

176   **Macroalgal growth–**To measure macroalgal growth response to the different water

177   samples, the wet biomass of each frond was recorded at the beginning of the experiment

178   and at the time the frond was sampled. Individual growth rates (μ) were calculated as a

179   percent increase in biomass per day (% d^{-1}):

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

180   where N_t is the biomass on day t, N_0 is the initial biomass, and t is time in days of

181   incubation (Lobban and Harrison, 1994).

182   **Nutrient sampling and analysis–**Changes in concentration of $NO_3^- + NO_2^-$, NH_4^+, and

183   PO_4^{3-} were determined during the experiment to quantify differences in ambient nutrient

184   concentrations among water samples. Water samples were frozen until analysis of

185   nutrient concentrations. Nitrate and phosphate were determined using standard

186   colorimetric assays in a Lachat Auto Analyzer (Cd reduction). Ammonium

187   concentrations were determined by spectrophotometry following the indophenol

188   method. Detection limit was 0.25 µM for any of the three nitrogen species.

189   *2.2. Experiment 2: ^{15}N enrichment experiment*

190   An enrichment experiment was done to determine N-turnover rates in different

191   sections of the thallus and to test: i) the occurrence of transport of N along the thallus,

192   from the tip to the basal segment of the stipe, ii) the occurrence of transport of N from

193   the basal segment of the stipe to the tip, and iii) to quantify the uptake rates of the

194   growing tips and mature parts of the thallus.

195   As in the previous experiment, *A. nodosum* and *F. vesiculosus* were collected at

196   Quissett Harbor and Nobska Beach respectively (Fig. 1a). Macroalgae were transported

197   in coolers to the laboratory and maintained under the same pre-incubation conditions as

198   previously described. For these experiments *A. nodosum* individuals were 23.2±0.9 cm

199   long and had 4 gas bladders, and *F. vesiculosus* individuals were 12.7±1.1 cm. The

200   selected individuals did not show apparent damage or epiphytes. Treatment water was

201   created by adding a stock solution of 10 mM $K^{15}NO_3$ (99 atom % ^{15}N) to 2 L of a final

202   volume of seawater (from Nobska). The final concentration was ~120 µM, with 98.8%

203   atom % ^{15}N enrichment. Nitrate was selected as the tested nutrient as it is a dominant

204   inorganic nitrogen compound entering these estuaries.

205   To test i) and ii), experiments were divided in two periods: a first 4-h period under

206   the stock solution, followed by a 24 h period under control seawater. During the first

207   period, only the tips (i) or the basal segment of the frond (ii) of three different fronds of

208   each species were submerged, while the non-submerged parts of the thallus were

209   manually vaporized with control seawater at regular intervals (~20 min) to avoid

210   desiccation. Macroalgae were maintained inside the culture chamber under the same

211   light and temperature conditions as in the previous experiment. After this first 4-hour

212   period, individuals were gently washed with seawater and transferred individually to an

213   Erlenmeyer flask with 1 L of control seawater. They were kept during 24 hours under

214   the same conditions of temperature, light and aeration as in the previous experiment.

215   After both incubation periods, all individuals were immediately subsampled for

216   stable isotope determinations. Each *A. nodosum* individual was divided into tip (1-1.5

217   cm fragment measured from the distal part) and intervesicular segments, and those of *F.*

218   *vesiculosus* were divided into tip (1 cm fragment from the distal part) and regular length

219   segments (~3 cm) from the tip to the base. The lateral vegetative or reproductive

220   branches of *A. nodosum* or reproductive tips of *F. vesiculosus* were discarded.

221   To test iii) the uptake capacity of the tip and non-growing parts of the thallus, three

222   fronds of each species were completely submerged in the treatment solution for 2 h.

223   Macroalgae were maintained inside the culture chamber under the light and temperature

224   conditions as in the previous experiment. To exclude the possible transport of inorganic

225   N along the thallus, macroalgae were subsampled immediately after the incubation

226   period. Macroalgae were subsampled following the same procedure as previously

227   described for i) and ii).

228   During each of the three treatments, control individuals of *A. nodosum* (n=3) and *F.*

229   *vesiculosus* (n=3) were maintained in the same conditions as the experimental

230   individuals but in 1L Erlenmeyer flasks with control seawater.

231   *2.3. Internal nutrient content and $\delta^{15}N$ analysis*

232   N stable isotope and elemental analyses for N and C content to estimate the tissue

233   C:N were performed for all samples. Aliquots of ca. 2.5 mg of macroalgae samples

234   were used. Samples were placed in tin capsules and introduced into an isotope-ratio

235   mass spectrometer (Thermo Finnigan Mat Delta Plus) via an element analyzer (Carlo

236   Erba CHNSO 1108). Isotopic results are expressed in delta notation:

237     $\delta^{15}N = [(^{15}N_{sample}:^{14}N_{sample}/^{15}N_{std}:^{14}N_{std})-1] \times 1000$

238   where the standard (std) is atmospheric N_2. Precision (se of 5 replicates) was better than

239   0.05‰ for either IAEA-N-2, IAEA-N-1 or IAEA-NO-3 standards. The coefficient of

240   variation of triplicate sample aliquots was always $<2\%$.

241   *2.4. Statistical analyses and calculations*

242   Comparison of nutrient concentrations among water samples was done by analysis of

243   variance (one-way ANOVA). Differences in the growth, $\delta^{15}N$ and C:N of the growing

244   tips of macroalgae over the experiments were also tested using one-way ANOVA at

245   each time separately using the site as fixed factor.

246   This test was also used to analyze differences among sites and macroalgal segments

247   along the thallus at the end of the isotope equilibration experiment, and to study

248   differences between macroalgal segments within individuals from the same site. When

249   significant differences were detected, *a posteriori* Student-Neuman-Keuls (SNK) tests

250   for multiple comparisons were used to detect differences among groups.

251   Experimental samples of the ^{15}N enrichment experiments were compared with the

252   control samples to test the atom % ^{15}N enrichment using a paired-samples t-test, which

253   compares two measurements of the same sample before and after the treatment. All tests

254   were carried out with SPSS Statistical Software.

255   To estimate N uptake in the enrichment experiment we used the N specific uptake

256   rate, which was calculated from appearance of the ^{15}N in the macroalgal tissue:

$$\text{N specific uptake} = \frac{\text{atom\% }^{15}N_f - \text{atom\% }^{15}N_i}{R \cdot t}$$

257   where atom % $^{15}N_f$ and atom % $^{15}N_i$ are the final and initial atom % ^{15}N enrichment of

258   macroalgal thallus, R (%) is the calculated exponential average of the initial and final

259   atom % enrichment of dissolved NO_3^-, and t is the time in hours.

260   The inverse of the N specific uptake-rate was used to estimate the turnover time (tr)

261   in days that would take to renovate the total N of a particular macroalgal fragment.

262   **3. Results**

263   *3.1.   Experiment 1: N isotope equilibration rates*

264   Concentrations of all inorganic nitrogen compounds during the experiment with *A.*

265   *nodosum* in September were higher than those found during the *F. vesiculosus*

266   experiment in August (Table 1). In the former case, water from CR had more nitrate and

267   ammonium than water from the other sites but showed similar phosphate

268   concentrations. In contrast, during the *F. vesiculosus* experiment, the oceanic-influenced

269   site (Nobska) held larger nitrate and lower ammonium and phosphate concentrations

270   than those at the other experimental sites, which had similar concentrations of all

271   nutrients. In all cases, $DIN:PO_4^{3-}$ values were low, indicating potential nitrogen

272   limitation of algal growth.

273   The macroalgal growth response to nutrient changes differed between species,

274   although the pattern was very similar among sites within the same species (Fig. 2).

275   Overall growth of *A. nodosum* was higher than growth of *F. vesiculosus*. In all cases

276   there was positive growth, but maximum growth was recorded after 6 d for *A. nodosum*

277   and after 12 d for *F. vesiculosus* (Table 2). During the experiment with *F. vesiculosus*,

278   no significant differences between sites were observed (Table 2). While during *A.*

279   *nodosum* experiment, significant differences were detected after 6 days of incubation,

280   when maxium growth was observed (Table 2).

281   The response of N isotope composition was different for each species (Table 2) but

282   similar for all water types assayed (Fig. 2). $\delta^{15}N$ values in the growing tips of both

283   species significantly differed during the experiment from initial values, especially in *F.*

284   *vesiculosus* (Table 2). Nevertheless, differences among fronds cultivated in different

285   water treatments were slight and remained close to the range of variation of the initial

286   values (6.7±0.1‰ in *A. nodosum* and 8.5±0.2‰ in *F. vesiculosus*, Fig. 2). These

287   changes were not large enough to reach the N isotopic values observed in native

288   individuals of *F. vesiculosus* in CR (6.9±0.1‰) or SLP (5.0±0.3‰).

289   As observed in the case of growth rates, tissue C:N of both species increased during

290   the experiment but there was no significant effect of culture water and only *F.*

291   *vesiculosus* maintained in SLP water had lower C:N values than those individuals

292   maintained in other water types (Fig. 2, Table 2). For all treatments, however, final C:N

293   values measured exceeded the range of values observed in the site of collection.

294   At the end of the experiment, differences between initial (t=0) and final values along

295   the thallus were especially noticeable in the tips, both for $\delta^{15}N$ and tissue C:N values

296   (Fig. 3, Table 3). In all parts of the frond, and for both species, the lowest isotopic

297   values were observed generally in individuals cultured in SLP water and the highest

298   values in those cultured in CR water (Fig. 3) thus approaching the isotopic values of

299   native macroalgae. The $\delta^{15}N$ values for growing tips of *A. nodosum* individuals

300   maintained in Nobska and SLP water were significantly different from other segments,

301   while no significant differences between segments from the same individual exposed to

302   CR water appeared (ANOVA, post hoc SNK test, p≤0.01). *F. vesiculosus* showed

303 significant differences between tip and the basal segment of the frond in individuals

304 under all culture regimes (ANOVA, post hoc SNK test, p≤0.01).

305   As *F. vesiculosus* was cultivated in its original water (Nobska), this can be used as

306 a control to find differences when macroalgae was moved from its original water to two

307 other water treatments (Sage Lot Pond and Childs River). N isotopic values of the

308 growing tips of macroalgae cultivated under water from Childs River were not

309 significantly different from the control at the endpoint of the experiment, while there

310 were statistical differences between the control and Sage Lot Pond. No significant

311 differences were found in C:N of the growing tips of macroalgae under the control and

312 the two other water treatments.

313 *3.2. Experiment 2: ^{15}N enrichment experiment*

314   The growing tip and the basal segment of the frond of both species when submerged

315 in ^{15}N enriched seawater significantly increased their ^{15}N content relative to non-

316 submerged parts of the frond and to control segments (Fig. 4a, b). Tips increased from

317 natural levels to average enrichments of 1.1% and 1.7% in *A. nodosum* and *F.*

318 *vesiculosus* respectively, while enrichment of the basal segment were only 0.4 and

319 0.8%, respectively. No evidence of enrichment was found in the emerged sections of the

320 thallus during this experiment.

321   The ^{15}N content in wholly-submerged fronds of both species significantly changed

322 after the treatment (Fig. 4c). As in the previous experiment, higher enrichment was

323 observed for *F. vesiculosus* than for *A. nodosum* individuals, and consequently N-

324 specific uptake rates were lowest in the latter (Table 4). Among *A. nodosum* individuals,

325 the basal segment showed the lowest enrichment, while in *F. vesiculosus* the segment

326 immediately under the growing tip showed the lowest enrichment together with the

327 basal segment. The tips of both species were more enriched relative to other segments.

328 N uptake proceeded at low rates and N turnover times estimated from these rates

329 were in general higher than the duration of the isotope equilibration experiments (Fig.

330 2).The average N turnover time of tip-submerged individuals was about 30 and 16 d for

331 *A. nodosum* and *F. vesiculosus* respectively (Table 4). In contrast, when the basal

332 segment was submerged, N turnover times averaged 7 months and 19 days for *A.*

333 *nodosum* and *F. vesiculosus* respectively. Finally, when all frond segments were

334 submerged, turnover time of the tip for *A. nodosum* was longer (up to 6 months) than in

335 the other treatments, although turnover at the basal segment of the fronds was

336 maintained (Table 4). Turnover for intermediate segments was slightly faster (4-5

337 months) than at the tip or at the basal segment. In the case of *F. vesiculosus*, N turnover

338 at the tip would need on average 11 d and only 21 d at the basal segment of the frond,

339 while other algal segments showed intermediate turnover values.

340 **4. Discussion**

341 *4.1.   Variation of $\delta^{15}N$ in macroalgal growing tips*

342 As both macroalgae show apical growth, isotope composition of the tips was

343 expected to change according to the isotope composition of the surrounding water at

344 faster rates than other parts of the thallus. These changes would ideally lead to a

345 complete isotope equilibration between the algal tissue and the water in absence of

346 isotope fractionation. The results of the experiments in this study revealed that the tips

347 of both *A. nodosum* and *F. vesiculosus* required a long time to converge with the $\delta^{15}N$

348 values typical of native plants when exposed to water with different isotopic

349 composition. The time required largely exceeded the duration of the experiments (up to

350   22 d), as N turnover rates varied between 11 d (*F. vesiculosus*) and 6 months (*A.*

351   *nodosum*). Similar delays in the equilibration of δ^{15}N values in apical tissues of *F.*

352   *vesiculosus* when changing the surrounding water were reported in other *in situ*

353   transplant studies with *F. vesiculosus* (Deutsch and Voss, 2006) while much faster

354   equilibration was observed for other brown (García-Sanz, 2009), red or green

355   macroalgal species (Naldi and Wheeler, 2002; Teichberg et al., 2008). Such delays can

356   be due to low growth and N uptake rates, strong isotope fractionation, low ambient N or

357   to the initial nitrogen content, and isotope composition of the individuals assayed.

358   Both macroalgae evidence logistic growth, with highest rates during their first year

359   of life. *F. vesiculosus* can grow in length up to 2 cm month^{-1} at the season of maximum

360   growth but more often rates are as low as 0.6 cm month^{-1} (Viana et al., in review a). The

361   growth for *A. nodosum* is much slower, but individuals of this species can live for more

362   than 10 yr (Viana et al., 2014). Low growth rates also imply lower N requirements and

363   uptake than fast growing species (Pedersen and Borum, 1997). Such low requirements

364   would explain N-specific uptake rates <0.1 d^{-1} even at high ambient N concentrations as

365   those employed in the enrichment experiment in this study (Table 4), and consequently

366   long N turnover times in these macroalgae.

367   Strong isotope fractionation is not likely to occur. Previous studies with Fucaceae

368   (García-Sanz, 2009) and other macroalgae (Cohen and Fong, 2005) did not find

369   significant N isotope fractionation related to nutrient concentrations, in contrast with

370   diatoms (Wada and Hattori, 1978; Pennock et al., 1996). The rates of change in δ^{15}N in

371   our experiments would have been faster than observed if fractionation were a significant

372   factor, as the light isotopes would have been preferred. For instance, the assayed *F.*

373   *vesiculosus* with mean initial δ^{15}N = 8.5‰ would have converged to values typical of

374 individuals native of the water origin locations (5.0 to 6.9‰) but they did not show

375 significant changes in their isotopic composition after 12 d.

376 The concentration of ambient N may have also affected changes in macroalgal $\delta^{15}N$.

377 The water employed in the experiments had nutrient concentrations typical of summer

378 in the study area, when uptake by primary producers depletes nutrients (Tomasky et al.,

379 1999). N sources, rather than total N concentration determines $\delta^{15}N$ in the water and

380 ultimately in primary and secondary producers (McClelland and Valiela, 1998b; Viana

381 and Bode, 2013). Experiments with other species showed that macroalgal $\delta^{15}N$ did not

382 change with water N concentrations as long as the $\delta^{15}N$ of dissolved N was constant

383 (Cohen and Fong, 2005; García-Sanz, 2009). Furthermore, nutrient uptake in *F.*

384 *vesiculosus* is less dependent on substrate concentration than in green or red algae

385 (Pedersen and Borum, 1997). In our experiment with water of different origins, the low

386 concentrations of dissolved N did not prevent the individuals of both species from

387 growing in weight and maintaining C:N values characteristic of non N-limited algae

388 (Niell, 1976), thus suggesting that the slight changes in $\delta^{15}N$ were not a direct

389 consequence of water N concentration.

390 The relatively high nitrogen content (1.2±0.3% for *A. nodosum*, 1.4±0.1% for *F.*

391 *vesiculosus*) and the enriched $\delta^{15}N$ values of macroalgae at the starting point could have

392 also influenced isotopic equilibration. Slow-growing brown macroalgae usually rely on

393 their internal N pools during periods of low nutrient supply, as in summer seasons in

394 temperate areas (Lehvo et al., 2001; Villares et al., 2013). During these periods growth

395 rates and external nutrient demand are lowered while the macroalgae, eventually

396 profiting from high light levels, develop carbon reserves, thus increasing tissue C:N, as

397 observed in our experiments (Fig. 2). Naldi and Wheeler (2002) also observed that high

398 total N content of thalli influenced nitrate uptake rates in green and red macroalgal

399   species. Low external N demand along with large difference in $\delta^{15}N$ values between the

400   macroalgal tissue and the surrounding water (as suggested by the $\delta^{15}N$ values of native

401   macroalgae), may be the main determinants of the rate of isotopic equilibration in our

402   incubations with *F. vesiculosus*. Other experiments with transplanted individuals of this

403   species in the field also found small or no changes in their tissue $\delta^{15}N$ after days of

404   incubation (Deutsch and Voss, 2006). In contrast, and despite the longer turnover time,

405   *A. nodosum* started to show differences in $\delta^{15}N$ after 12 days of incubation, likely

406   because the initial values for this species were much lower than those for *F. vesiculosus*.

407   *4.2.   N uptake and turnover along the thallus*

408   The results of the enrichment experiments showed that both species do not transport

409   recently absorbed N along their thallus, at least during 24 h after uptake (Fig. 4).

410   Despite their internal structure (i.e. symplastic pathway) suited for transport (Raven,

411   2003), only carbon photosynthetic assimilates were reported to translocate along the

412   thallus of some Fucaceae (Diouris and Floc´h, 1984). Inorganic nitrogen transport,

413   however, was reported for other brown macroalgae, such as Laminariales (Mizuta et al.,

414   1996; Hepburn et al., 2012). These algae have nutrient requirements different from

415   those of Fucales as they show basal meristematic growth, which means that they grow

416   where the blade and the stipe meet (Lobban and Harrison, 1994). In contrast, Fucales

417   show mostly apical growth and therefore concentrate N demands in the tips of the

418   thallus (Topinka, 1978), although as demonstrated by our enrichment experiment (Fig.

419   4c), all sections of the thallus are able to take up inorganic N from the water. As N

420   transport have relatively high energy and oxygen requirements (Raven, 2003), this

421   process can be avoided if both assimilation and uptake occur in the same part of the

422   thallus. In Laminariales, N uptake and assimilation occur at different rates in the

423   different parts of the thallus, resulting in gradients along the frond (Mizuta et al., 1996).

424   Despite their apical growth, variation in $\delta^{15}N$ values along the thallus has been

425   reported for *Fucus* species (Savage and Elmgren, 2004; Raimonet et al., 2013) and in

426   the present study (Fig. 3). If transport is excluded, such intra-individual variation might

427   be due to differential uptake and growth, or to isotope fractionation in the different

428   sections of the thallus.

429   In the enrichment experiment we showed that both species were able to incorporate

430   dissolved nitrogen when submerged (Fig. 4). The process of nitrogen uptake and

431   assimilation in macroalgae involves transport from the water column and then

432   assimilation into organic compounds, followed by incorporation into proteins and

433   macromolecules for growth (McGlathery et al., 1996). Growth is the most important N

434   sink in macroalgae. In mature segments, N demand for structural pools is not as

435   important as in growing tips, this would explain why N uptake at the non-growing

436   segments was only half the uptake rate measured at the tips of *F. vesiculosus*, when all

437   the frond was submerged (Table 4). For *A. nodosum* there was also a marked difference

438   in the uptake rates of the tip and those of the mature segments, at least when only one of

439   the sections was submerged. These results agree with studies reporting higher N uptake

440   in apical fronds and whole young plants or germlings and lowest in slower-growing

441   older fronds and stipes of *F. spiralis* (Topinka, 1978; Rosenberg et al., 1984) and

442   differential ^{15}N enrichment along thalli regions of *F. vesiculosus* (Döhler et al., 1995).

443   Non-apical segments of *A. nodosum* and *F. vesiculosus* individuals can store N to

444   use in metabolic processes other than growth. For instance, N can be accumulated as

445   inorganic (NO_3^- and NH_4^+) and organic compounds (as phycobiliproteins) and can be

446   found in algal pigments (Hanisak, 1983) although NH_4^+ storage capacity is limited due

447   to toxicity (Haines and Wheeler, 1978; Lotze and Schramm, 2000).

448   The net short-term N uptake recorded along the thallus implies that δ^{15}N values of

449   different sections would change with the isotopic composition of the surrounding water

450   at rates depending on their initial δ^{15}N value, and of the processes affecting isotope

451   fractionation within each section. Nitrogen release, both in organic and inorganic forms,

452   has been observed for some green and red macroalgae (Naldi and Wheeler, 2002; Tyler

453   and McGlathery, 2006) and was interpreted as the result of isotopic equilibration of

454   internal and external pools (Fujita et al., 1988) or to stress due to sudden changes in the

455   proportion of different N sources (Naldi and Wheeler, 2002). Fractionation during

456   uptake in brown macroalgae is not likely to occur (García-Sanz, 2009), although in

457   other primary producers it was observed to result in lower nitrogen isotopic values in

458   the tissues than in the water (Pennock et al., 1996). On the other hand, the release of

459   preferentially light N isotopes may explain the higher enrichment of the tip sections

460   compared to other parts of the thallus, as found in our experiments (Figs. 3 and 4) and in

461   other studies (Raimonet et al., 2013). As far as we know, there are no reports of N

462   release in the species considered in our study, but it can be expected that this process is

463   restricted to the most metabolically active tissues.

464   *4.3.   Implications for the use of* A. nodosum *and* F. vesiculosus *to monitor land-*

465   *derived nitrogen sources*

466   The results of the present study are of application when using *A. nodosum* and *F.*

467   *vesiculosus* to study the impact of anthropogenic N sources on littoral ecosystems both

468   analyzing native populations and in incubation experiments, the latter applicable when

469   these species are not naturally present in the impacted area. Taking advantage of the

470   apical growth and long life span of both species, Savage and Elmgren (2004) interpreted

471   δ^{15}N values in different sections of the thallus of *F. vesiculosus* in a retrospective study

472   to monitor changing N loadings. The underlying assumptions were that annual growth

473   occurred only at the tips and, by knowing the rate of growth, each section of the thallus

474   could be dated and associated to a particular period of exposure to the ambient N. Thus,

475    $\delta^{15}N$ of the sections would reflect past N sources if mature segments do not equilibrate

476   N contents with the surrounding water and if there is no transport of N along the thallus.

477   Other studies, however, questioned this application for retrospective studies as they

478   found contrasting patterns of change along the thallus that could not be related to

479   ambient N (Raimonet et al., 2013).

480   The enrichment experiment in this study demonstrated that all sections of the thallus

481   of both species take up N from the ambient water when submerged. Even when there

482   was no transport of the N along the thallus and the rates of uptake at the mature parts of

483   the frond were lower that at sections located at or near the tip this uptake would affect

484   the $\delta^{15}N$ of the sections. These results explain why previous studies found contrasting

485   patterns of change of $\delta^{15}N$ along the thallus of *F. vesiculosus* as the $\delta^{15}N$ of each section

486   changes with the isotopic composition of the water at different rates. Therefore, it is not

487   possible to obtain unbiased estimates of past N sources from the $\delta^{15}N$ of different

488   sections of the thallus of these macroalgae. Furthermore, determinations of $\delta^{15}N$ from

489   pooled samples of different sections would produce $\delta^{15}N$ values resulting from a

490   mixture of past and present N sources, depending on the amount of matter from sections

491   with different turnover rates. Pooled samples of the whole individual can be also be

492   misinterpreted if individuals of different lengths (i.e. ages) are used. $\delta^{15}N$ of the tips

493   can, however, be used as monitors of N sources in ambient water averaged over scales

494   of 15 days (*F. vesiculosus*) and up to 6 months (*A. nodosum*). This range of integration

495   times is particularly appropriate to differentiate chronic pollution from point discharges

496   that may have little impact on the macroalgae.

497   Besides the use of natural populations, these macroalgae can be used in

498   transplantation or laboratory experimental incubations with different water types to

499   determine potential impacts of different N sources (Deutsch and Voss, 2006). In this

500   case, the turnover and equilibration times of the tips, as determined in the present study,

501   need to be taken into account when determining the duration of the incubations.

502   Otherwise the results will not reflect the actual impact of the ambient N sources.

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667

Table 1. Sampling dates, and mean (±se) values of salinity, nutrient concentrations (µM) and $DIN:PO_4^{3-}$ during the N isotope equilibration experiments with *A. nodosum* and *F. vesiculosus* exposed to water from Childs River, Sage Lot Pond and Nobska (Fig. 1). Significant differences among nutrient concentrations in the different sites are shown (***: $p \leq 0.001$, **: $p \leq 0.01$, *: $p \leq 0.05$).

	A. nodosum			*F. vesiculosus*		
	Childs River	Sage Lot Pond	Nobska	Childs River	Sage Lot Pond	Nobska
Dates	29 August- 20 September 2013			2 August- 14 August 2013		
Salinity	24.57±0.89	27.04±0.45	31.04±0.05	25.85±0.40	26.33±1.28	31.10±0.32
Nutrient concentrations (µM)						
$NO_3^- + NO_2^-$	5.98±2.58	2.08±0.29	1.85±0.14*	1.07±0.13	1.28±0.15	2.03±0.18**
NH_4^+	5.12±1.46	3.12±0.65	1.15±0.09**	2.19±0.01	0.85±0.13	0.57±0.04***
PO_4^{3-}	1.70±0.51	1.06±0.12	1.25±0.12	1.55±0.24	0.75±0.15	1.23±0.09**
$DIN:PO_4^{3-}$	7.02±2.38	4.99±0.79	2.20±0.3	1.11±0.33	2.29±0.78	2.39±0.55*

Table 2. Results of one-way ANOVA analysis at each sampling time (2, 6, 12 and 22 days from the start of the experiment) to analyze the variation in growth (% d^{-1}), δ^{15}N (‰) or C:N in the tips of *A. nodosum* and *F. vesiculosus*. The variability in the tips of both species is compared when grouped by sites as fixed factors (Childs River, Sage Lot Pond or Nobska) and the initial values (t0). Significant differences at different times are shown (***: $p \leq 0.001$, **: $p \leq 0.01$ *: $p \leq 0.05$).

	2 days				6 days				12 days				22 days			
	SS	df	MS	F	SS	df	MS	F	SS	df	MS	F	SS	df	MS	F
A. nodosum																
Growth	8.27	8	0.00	-	55.04	8	0.15	178.72***	5.21	8	0.58	1.49	29.23	8	3.66	0.99
δ^{15}N	3.2	11	0.18	3.4	1.42	11	0.11	1.7	2.19	11	0.13	3.15	3.57	11	0.11	8.31**
C:N	744.59	11	26.86	6.58**	1323.73	11	37.81	9.00**	1307.43	11	106.14	1.44	673.71	11	49.79	1.84
F. vesiculosus																
Growth	0.02	8	0.00	-	0.00	8	0.00	-	1.26	8	0.12	2.45	-	-	-	-
δ^{15}N	1.57	12	0.047	8.01**	1.71	12	0.07	5.62*	2.17	12	0.08	6.02**				
C:N	164.87	12	10.31	2.33	430.98	12	18.56	4.74*	1146.96	12	39.64	6.65**				

Table 3. Results of analysis of variance (one-way ANOVA) and SNK post-hoc comparison tests of $\delta^{15}N$ (‰) and C:N in different segments of *A. nodosum* and *F. vesiculosus* fronds (n=3) at the endpoint of the study, compared with the initial values (t0) (Fig. 3). Site set as fixed factor: CR, Childs River; SLP, Sage Lot Pond and N, Nobska. P values are significant when ≤0.05. n.s.: non significant. The tip and BS segments correspond to the growing apical segment and the basal segment respectively. S1 and S2 segments for *A. nodosum* correspond to the intervesicular segments numbered from the tip to the base.

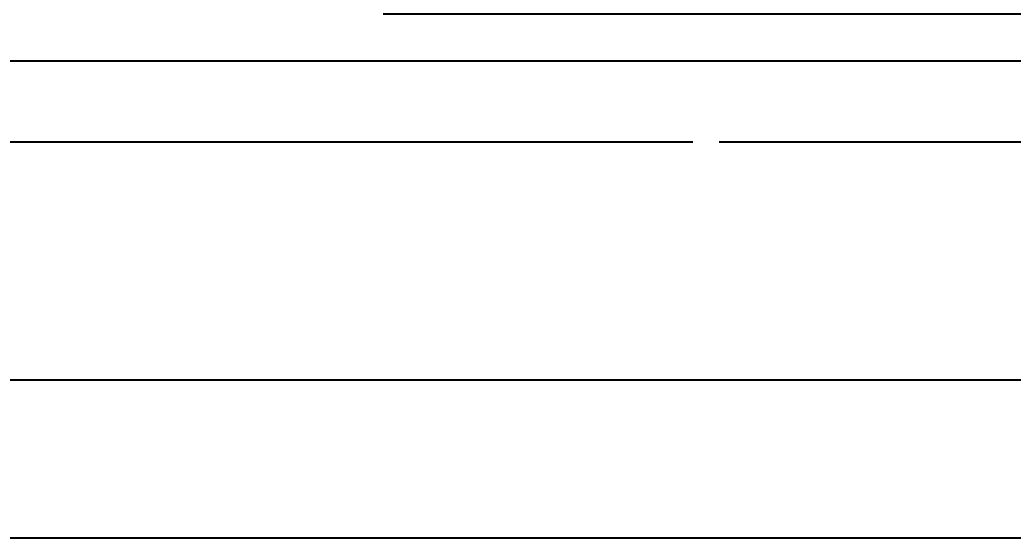| Species	Macroalgal segment	$\delta^{15}N$				C:N			
		df	F	p value	post-hoc	df	F	p value	post-hoc
A. nodosum									
	Tip	11	8.308	0.008	t0<SLP<CR=N	11	1.8	0.218	n.s.
	S1	11	13.7	0.002	t0<SLP<CR=N	11	1.0	0.428	n.s.
	S2	11	8.7	0.007	CR>t0=SLP=N	11	1.3	0.34	n.s.
	BS	11	15.6	0.001	CR>SLP=N>t0	11	8.7	0.007	t0<CR=SLP=N
F. vesiculosus									
	Tip	12	6.0	0.016	t0=SLP<CR=N	12	6.6	0.012	t0=SLP<CR=N
	BS	11	0.6	0.625	n.s.	11	1.7	0.238	n.s.

Table 4. Variation of mean±se N specific uptake (days^{-1}) and turnover time (days) in the different macroalgal segments of *A. nodosum* and *F. vesiculosus* when, i) the tip was submerged in an enriched seawater solution (Fig. 4a), ii) the basal segment of the frond (BS) was submerged in an enriched seawater solution (Fig. 4b), and iii) the entire frond was submerged in an enriched seawater solution (Fig. 4c). The tip in both species corresponds to the growing apical segment. S1, S2 and S3 segments correspond to intervesicular segments and 3-cm segments in order from the tip to lower down the frond in *A. nodosum* and *F. vesiculosus* respectively.

Species	Experiment	Macroalgal segment	N specific uptake (days^{-1})	Turnover time (days)
A. nodosum				
	i	Tip	0.0409±0.0104	29.27±9.62
	ii	BS	0.0048±0.0004	209.17±14.66
	iii	Tip	0.0053±0.0001	188.15±5.33
		S1	0.0085±0.0002	118.04±2.44
		S2	0.0087±0.0007	115.97±10.27
		S3	0.0062±0.0004	162.43±10.8
		BS	0.0044±0.0001	227.67±5.57
F. vesiculosus				
	i	Tip	0.0665±0.0100	15.74±2.35
	ii	BS	0.0525±0.0100	19.06±2.35
	iii	Tip	0.0949±0.0047	10.59±0.5
		S1	0.0522±0.0017	19.2±0.62
		S2	0.0722±0.0050	13.98±0.9
		S3	0.0721±0.0036	13.95±0.73
		BS	0.0476±0.0021	21.1±0.96

Figure legends

Fig. 1. Location of the study sites at Cape Cod, Massachusetts, USA (Basemap: USGS). Open symbols indicate the sites where the water samples were taken while the black symbol indicates where *A. nodosum* were sampled.

Fig. 2. Changes in mean±se (n=3) growth in wet biomass (% d^{-1}), $\delta^{15}N$ (‰) and tissue C:N in *A. nodosum* (a, c, e) and *F. vesiculosus* (b, d, f) during 22 and 12 d incubations respectively using water of three different locations. Square symbols are the mean values at time 0 and the dashed lines the range of variation. Analysis of variance results shown in Table 2.

Fig. 3. Variation between initial (Time 0) and endpoint $\delta^{15}N$ values ($\Delta\delta^{15}N$, mean±se, ‰) and tissue C:N (ΔC:N, mean±se) for different sections of the thallus of *A. nodosum* (a, c) and *F. vesiculosus* (b, d) individuals (n=3) growing under water of three different locations (Childs River, Sage Lot Pond and Nobska). Tip, intervesicular segments numbered from the tip to the base and basal (BS) segments are shown for *A. nodosum* and tip and basal segment (BS) for *F. vesiculosus*.

Fig. 4. Mean (±se) variation of atom % ^{15}N enrichment (mean±se) along the fronds of *A. nodosum* (a, c, e) and *F. vesiculosus* (b, d, f) individuals (n=3) when either: the tip was submerged in an enriched seawater solution (a, b), the basal segment of the frond (BS) was submerged in an enriched seawater solution (c, d), or the entire frond was submerged in an enriched seawater solution (e, f). The tip in both species corresponds to the growing apical segment. S1, S2 and S3 segments correspond to the intervesicular segments and to 3-cm segments in order to the closeness to the tip in *A. nodosum* and *F. vesiculosus* respectively. Significant differences between the experimental and the

control frond values are indicated by asterisks (*: p≤0.05, **: p≤0.01, ***: p≤0.001,
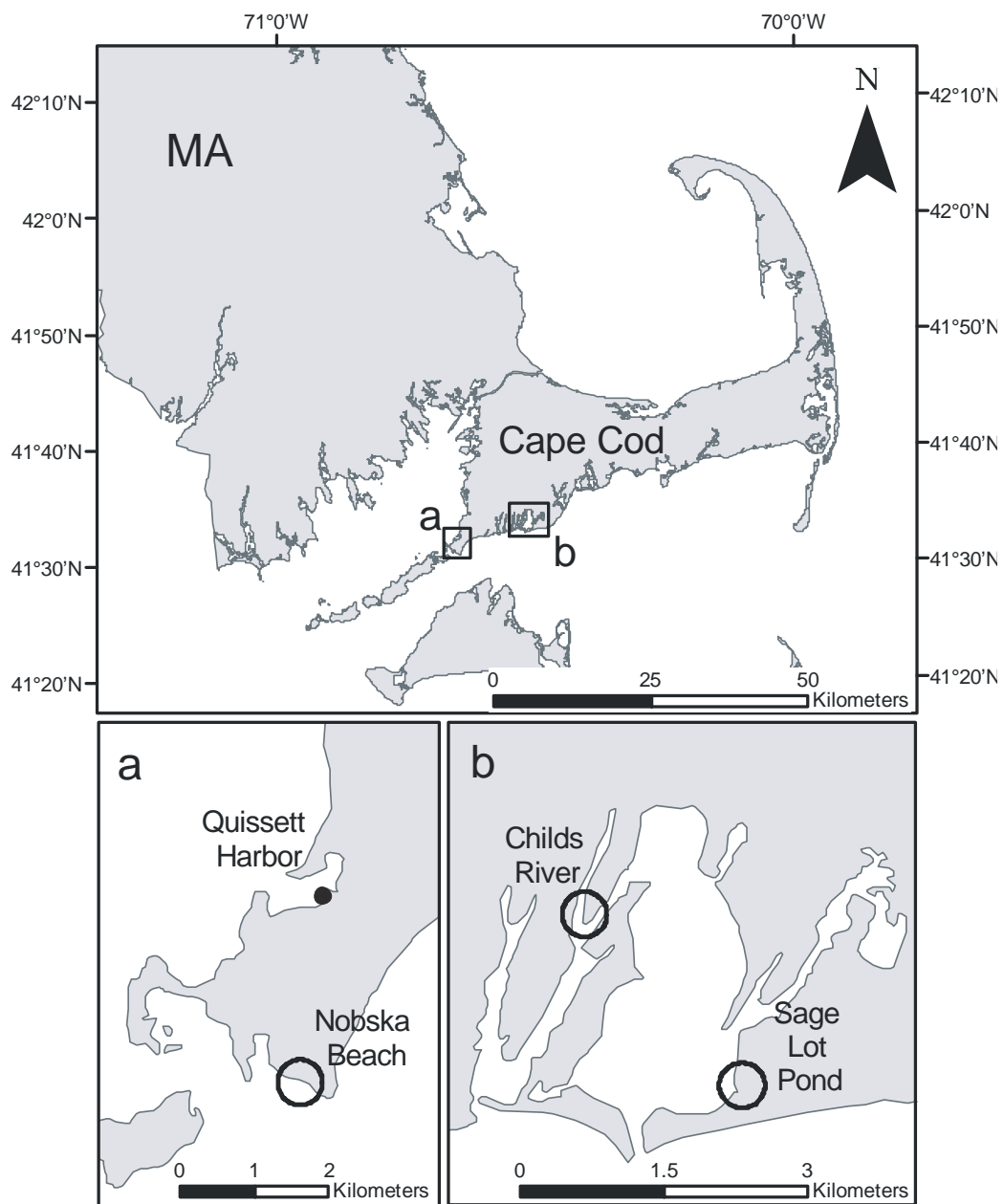
paired-samples t-test).

Fig. 1.

Fig. 2.

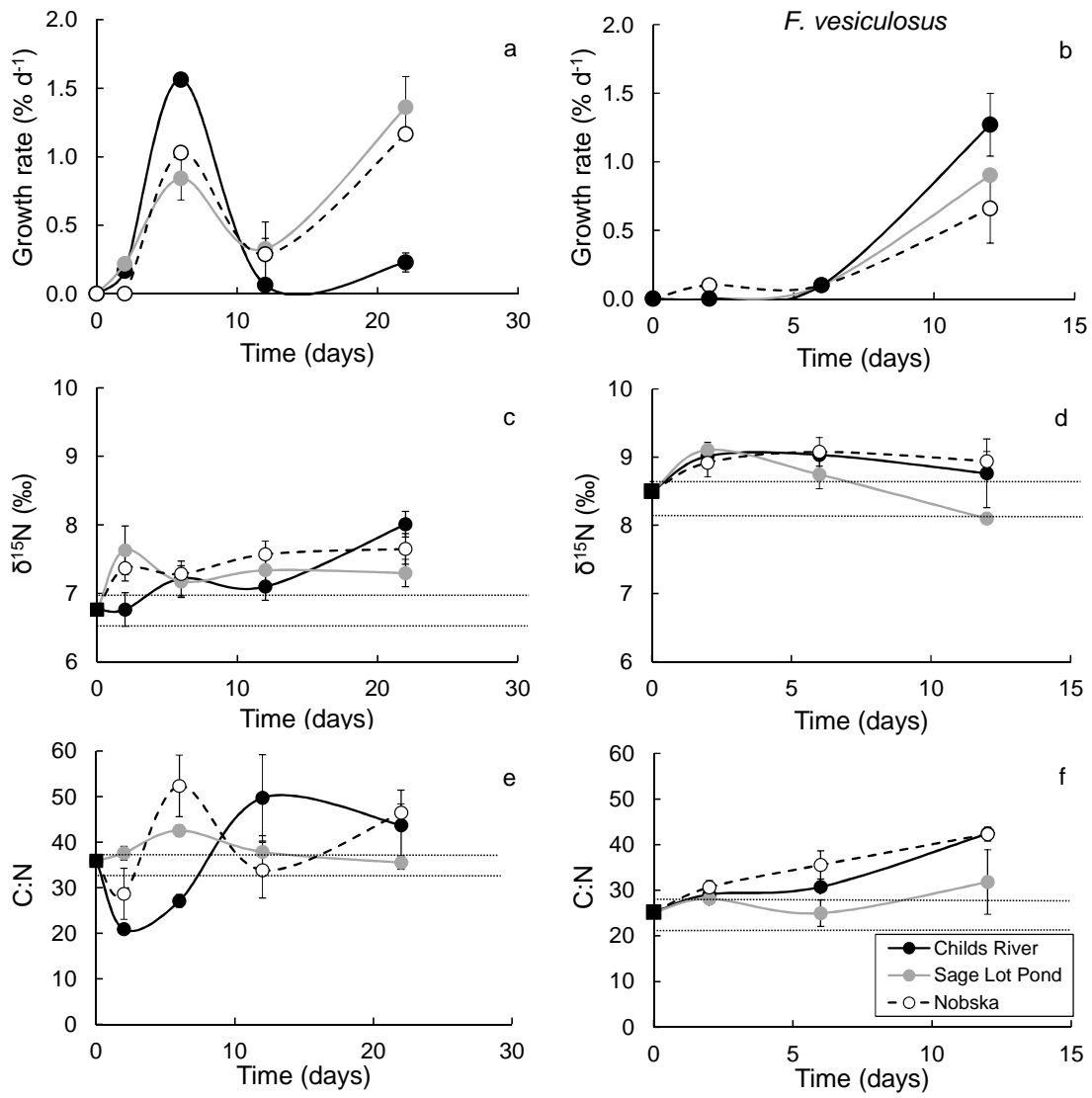**Fig. 3**
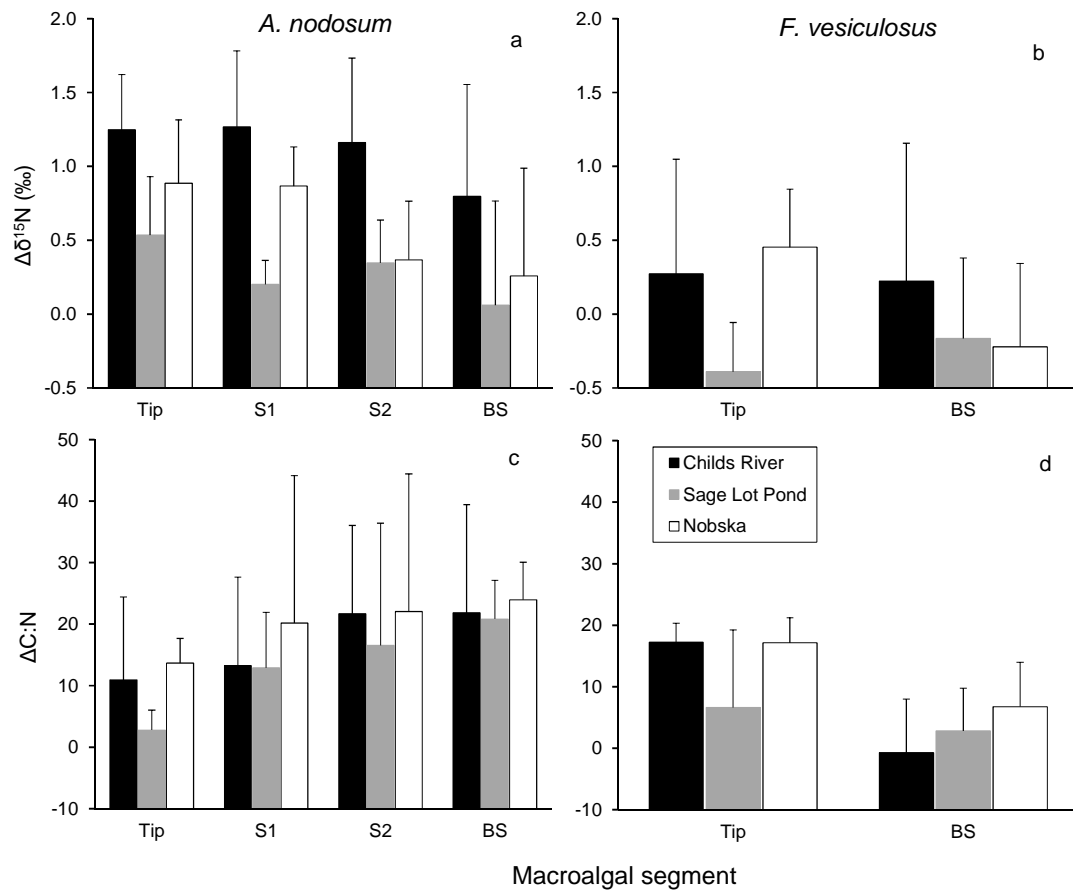
Fig. 4.