

1 Potential uptake of dissolved organic matter by seagrasses and macroalgae

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11 Running head: Dissolved organic matter utilization by macrophytes

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29 Abstract

30 Dissolved organic nitrogen (DON) acts as a large reservoir of fixed nitrogen. Whereas DON
31 utilization is common in the microbial community, little is known about DON utilization by
32 macrophytes. We investigated the ability of coexisting temperate marine macrophytes
33 (*Zostera noltii*, *Cymodocea nodosa*, and *Caulerpa prolifera*) to take up nitrogen and carbon
34 from small organic substrates of different molecular complexities (urea, glycine, L-leucine,
35 and L-phenylalanine) and from DON derived from algal and bacterial cultures (substrates
36 with a complex composition). In addition to inorganic nitrogen, nitrogen from small organic
37 substrates could be taken up in significant amounts by all macrophytes. Substrate uptake by
38 the aboveground tissue differed from that of the belowground tissue. No relationships
39 between carbon and nitrogen uptake from small organics were found. Preference for
40 individual organic substrates was related to their structural complexity and C:N ratio. Uptake
41 of algae-derived organic nitrogen was of similar magnitude as inorganic nitrogen, and was
42 preferred over bacteria-derived nitrogen. These results add to the growing evidence that direct
43 or quick indirect DON utilization may be more widespread among aquatic macrophytes than
44 traditionally thought.

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49 Keywords: uptake / dissolved organic nitrogen / stable isotopes / *Zostera noltii* / *Cymodocea*
50 *nodosa* / *Caulerpa prolifera* / seagrasses / macroalgae

51

52 1. Introduction

53 Seagrass ecosystems are highly productive and exhibit a strong nutrient retention capacity
54 (Stapel et al. 2001). The affinities and uptake rates of seagrasses for dissolved inorganic
55 nitrogen (DIN) are high (Stapel et al. 1996), keeping ambient DIN concentrations low and
56 potentially limiting for growth (Bulthuis et al. 1992). Under such conditions, efficient nutrient
57 recycling represents a vital ecosystem function. Regenerated nitrogen can be supplied to
58 primary producers in two ways: (1) DIN uptake after remineralization of dissolved organic
59 matter (Zehr and Ward 2002), and (2) direct uptake of dissolved organic nitrogen (DON;
60 Bronk et al. 2007). Therefore, DON may occupy a central position in nitrogen cycling in
61 systems characterized by low inorganic nitrogen concentrations.

62

63 Despite its long-standing recognition as potential nutrient, DON utilization by primary
64 producers is not well understood (Bronk et al. 2007). Many phytoplankton taxa can directly
65 use DON through processing mechanisms, such as urease activity and amino acid oxidation
66 (Palenik and Morel 1991, Mulholland et al. 2002, Stoecker and Gustafson 2003, Solomon and
67 Glibert 2008). But it is not clear yet to what extent macrophytes share these capabilities.
68 Although small organic compounds were until recently not considered a significant direct
69 nutrient source for seagrasses (Romero et al. 2006), uptake of organic matter derived nitrogen
70 has been demonstrated in macroalgae and seagrasses (Bird et al. 1998, Tyler et al. 2003).
71 Availability of detritus-derived compounds to seagrasses was demonstrated by Evrard et al.
72 (2005), and Barrón et al. (2006), but these studies did not indisputably show DON uptake
73 without prior remineralization by the present bacterial community. However, macrophytic
74 DON uptake in axenic cultures has been observed (Bird et al. 1998, Tarutani et al. 2004).
75 Hence, DON uptake may be more common than is generally perceived.

76

77 Studying uptake of natural DON (a complex mixture of organic nitrogen compounds) is
78 inherently complicated. Not all compounds are treated the same way by a primary producer.
79 Tyler et al. (2005) demonstrated clear differences in the processing of two amino acids
80 (alanine versus glycine) by a macroalgae. Harrison et al. (2007) found relationships between
81 amino acid complexity and preference by terrestrial plants. Not all primary producers are
82 equally capable of taking up organic nitrogen. Uptake capabilities even vary seasonally in
83 certain microphytobenthic taxa (Nilsson and Sundbäck 1996). The fact that different primary
84 producers distinguish between different types of organic molecules presents a complicating
85 factor in studies on DON variability and uptake. This can be extrapolated to entire DON
86 pools. DON pools of different composition and origin (e.g. algal versus bacterial, benthic
87 versus pelagic, or spring floods versus baseflow conditions in rivers) may exhibit different
88 bioavailabilities, and may be subject to different processing mechanisms (Ziegler and Benner
89 1999, Stepanauskas et al. 2000, McCarthy et al. 2004). Understanding the DON processing
90 mechanisms may help in explaining the large seasonal variability that is observed in DON
91 concentrations and differences in reactivity between studies and ecosystems (Bronk et al.
92 2002, Van Engeland et al. 2010).

93

94 Given the potential importance of DON as source of nitrogen in macrophyte-dominated
95 systems, it is imperative that we gain more insight in the role of this highly complex and little
96 understood ecosystem component. Hence, we conducted an uptake experiment in the
97 laboratory with three co-occurring temperate macrophyte species from Cadiz Bay (Spain):
98 *Zostera noltii*, *Cymodocea nodosa* (both seagrasses), *Caulerpa prolifera* (a rhizoid forming
99 macroalga). The main objectives of this study are: (1.) to assess whether uptake of organic

100 matter derived nitrogen and/or carbon by the macrophytes occurs, (2.) to compare uptake of
101 different inorganic (NH_4^+ and NO_3^-) and organic (urea, glycine, leucine, phenylalanine, algae-
102 derived DOM, and bacteria-derived DOM) nitrogen sources of contrasting complexity and
103 C:N ratio, and (3.) to compare uptake by aboveground and belowground plant parts of the
104 three temperate macrophyte species under investigation, because macrophytes have shown
105 difference in their ability to acquire nitrogen with above and belowground organs (Thursby
106 and Harlin 1984, Vonk et al. 2008). Isotope dual-labeled substrates (^{13}C and ^{15}N) allowed for
107 the separation of uptake from other fluxes out of the substrate pool, and to track carbon uptake
108 and nitrogen uptake separately.

109

110 2. Materials and Methods

111 2.1. Experimental setup

112 Specimens of *Zostera noltii* Hornem, *Cymodocea nodosa* Ucria (Ascherson), and *Caulerpa*
113 *prolifera* (Forsskål) J. V. Lamouroux were collected in the field near Santibañez (36° 28'
114 12.79" N, 6° 15' 7.07" W; Cádiz, Spain) and immediately brought to the laboratory.
115 Epiphytes were removed from the aboveground plant parts by gently scraping with a razor
116 blade. Filtered water (GF/F filter, Whatman) from the same location was used as incubation
117 medium ($[\text{NH}_4^+] = 0.3 \pm 0.06 \mu\text{mol l}^{-1}$; $[\text{NO}_3^-] = 0.7 \pm 0.38 \mu\text{mol l}^{-1}$; [urea] = $2.2 \pm 0.24 \mu\text{mol}$
118 l^{-1} ; [DON] = $16.4 \pm 0.77 \mu\text{mol l}^{-1}$; [Gly] $23.7 \pm 3.6 \text{ nmol l}^{-1}$; [Leu] $0.6 \pm 0.4 \text{ nmol l}^{-1}$; [Phe] 4.8
119 $\pm 0.7 \text{ nmol l}^{-1}$; mean \pm s.e.). Note that a GF/F filter retains a part of the bacterial community
120 but not all bacteria. The incubations were performed in a climate-controlled room.
121 Macrophytes were left intact with their aboveground and belowground parts in separate
122 plastic cups (123 ml). Cups were filled almost to the top so as to minimize local desiccation of
123 the plants where they protruded out of the water, while care was taken to prevent mixing of

124 water between cups via capillary effects or spilling. Inorganic and organic compounds, further
125 referred to as substrates (substances that are acted upon by an enzyme) of various molecular
126 complexities were added to the 'aboveground cup' in the aboveground incubation and to the
127 'belowground cup' in the belowground incubation (Fig. 1) to final concentrations as indicated
128 in table 1. Plants were incubated for approximately 3 hours in a full factorial design with 3
129 species, 8 substrates, 2 incubations (aboveground vs. belowground), and 3 replicates. Extra
130 plants were collected to measure the natural abundance (isotope fractions) of ^{13}C and ^{15}N in
131 the relevant plant parts. The water in the cups was constantly stirred to prevent local depletion
132 of substrate. To avoid experimental artifacts, substrate concentrations were kept close to those
133 found in the water column of the bay (see above and Tab. 1). Although this means that the
134 substrate could get depleted, this was not a problem since our first objective was to determine
135 if macrophytes could take up DON of different complexities rather than accurately
136 quantifying the uptake kinetics. After incubation, the plants were rinsed with clean filtered
137 seawater, dabbed with tissues, dissected, frozen at -20°C , freeze dried, weighed and ground to
138 a fine powder. *C. nodosa* leaves, sheaths, rhizome, and roots were processed separately.
139 Because of the low plant biomass, *Z. noltii* was dissected into an aboveground and a
140 belowground (i.e., rhizome + root) part. *C. prolifera* individuals were dissected into the
141 assimilators, the stolons, and the rhizoids (analogs of leaves, stems, and roots respectively).

142

143 2.2. Inorganic and organic substrates

144 This study was based on stable isotope labeled substrates, which has the advantage over a
145 mass balance approach that uptake can be distinguished from other effluxes out of the source
146 pool. NH_4^+ and NO_3^- served as reference for comparison with organic nitrogen uptake. Urea
147 and the amino acids glycine, L-leucine and L-phenylalanine (all ^{13}C and ^{15}N double labeled)

148 were used as small individual organic N sources with contrasting complexity, C:N ratio and
149 molar mass (Tab. 1). Glycine is a fairly simple achiral amino acid with hydrogen as R-group.
150 Leucine is more complex and chiral with an iso-butyl group on the α -carbon, and often used
151 as a tool to measure bacterial activity (Kirchman et al. 1985). Phenylalanine has a benzene-
152 like ring (phenyl) as R-group, which is considered chemically stable (resistant to breakdown).
153 We cautiously assume that this chemical stability affects the overall biological reactivity, at
154 least to some extent. The abundance of these amino acids in organisms and bacteria may also
155 hint at their overall bioavailability (Tab. 1), assuming a certain correspondence between
156 abundance in particulate organic matter (POM) and exchange (uptake/release) with the
157 dissolved organic matter (DOM) pool. Finally, two composed DOM pools were used to
158 mimic naturally complex DOM from the environment (also double labeled; see section
159 2.32.3). Both the nitrogen and carbon were present as heavy isotopes in the organic substrates
160 (Tab. 1), thus enabling us to study the potential coupling of carbon and nitrogen uptake.

161

162 2.3. Preparation of algae and bacteria-derived DOM

163 Besides the individual organic compounds mentioned above, two DOM substrates with a
164 complex composition were created: one from a culture of soil bacteria grown on ^{13}C -glucose
165 and $^{15}\text{NH}_4\text{Cl}$, and one from an axenic culture of *Skeletonema costatum* grown on $\text{NaH}^{13}\text{CO}_3$
166 and $\text{Na}^{15}\text{NO}_3$. After incubation, the cells were freeze dried, added to 10 ml of milli-Q water
167 and shaken for 48 hours at room temperature after addition of Devarda's alloy and MgO to
168 remove inorganic nitrogen. Particles were removed by centrifugation. The supernatants were
169 diluted to 20 ml and kept frozen at -20°C until further use (i.e. addition to the incubations).

170

171 The DON concentrations in the concentrated algae-derived and bacteria-derived substrates
172 were 14.6 mmol-N l⁻¹ and 19.3 mmol-N l⁻¹ respectively. The DOC concentrations were 124
173 mmol-C l⁻¹ and 690 mmol-C l⁻¹. Final concentrations of added DON and DOC in the
174 incubations are listed in table 1. Dissolved combined amino acids (DCAA) comprised 84 ± 24
175 % (algae-derived) and 47 ± 11 % (bacteria-derived) of the DON, and 34 ± 11 % (algae-
176 derived) and 46 ± 12 % (bacteria-derived) of the DOC. The dissolved free amino acid
177 (DFAA) fraction was for algae-derived DON dominated by L-arginine and L-glutamine, and
178 for bacteria-derived DON by L-arginine and L-glutamate (data not shown). L-alanine and L-
179 leucine were dominant in the DCAA fraction of both bacteria and algae-derived DON (data
180 not shown). These analyses confirmed the difference in chemical composition of the algae-
181 derived and bacteria-derived DOM substrates.

182

183 2.4. Stable isotope and nutrient measurements

184 Dissolved inorganic nitrogen concentrations ($\text{DIN} = \text{NH}_4^+ + \text{NO}_2^- + \text{NO}_3^-$) and urea were
185 determined colorimetrically in filtered incubation medium (GF/F filter; Whatman). DON was
186 calculated as the difference between total dissolved nitrogen (TDN), determined as NO_3^- after
187 an alkaline persulphate destruction (Hansen and Koroleff 1999), and DIN. Dissolved organic
188 carbon (DOC) concentrations were measured with an auto-analyzer (Skalar SK12 organic
189 carbon analyzer) after filtering the water over a GF/6 filter (Whatman). Dissolved free amino
190 acids (DFAA) were determined prior to hydrolysis by HPLC on a Waters HPLC system with
191 a 996 photodiode array detector, total hydrolysable amino acids (THAA) post hydrolysis, and
192 dissolved combined amino acids by difference ($\text{DCAA} = \text{THAA} - \text{DFAA}$).

193

194 Tissue carbon and nitrogen content and relative abundances of ^{13}C and ^{15}N and the
195 concentrated DON substrates were measured using a Thermo EA 1112 elemental analyzer
196 coupled to a Thermo Delta V Advantage isotope ratio mass spectrometer with a ConFlo II
197 interface (EA-IRMS).

198

199 2.5. Data treatment

200 Due to the wide range of labeling intensities our calculations were based on isotope fractions
201 (F) instead of δ -values (Fry 2006). Isotope excesses (E_{sample}) were calculated as the difference
202 between the isotope fraction in the sample (F_{sample}) and the natural abundance (i.e. initial
203 isotope fractions; F_{nat}):

$$204 \quad E_{\text{sample}} = F_{\text{sample}} - F_{\text{nat}}$$

205 Specific uptake rates of heavy isotope, V_{sample} , were calculated as the quotient:

$$206 \quad V_{\text{sample}} = \text{POM} \times E_{\text{sample}} / (\text{time} \times \text{dry weight}),$$

207 where POM is organic matter content of the plant in terms of carbon or nitrogen. V_{sample} is
208 expressed in μmol (^{13}C or ^{15}N) $\text{mg DW}^{-1} \text{h}^{-1}$. These specific uptake rates allow for a
209 comparison between different species and individuals with varying biomass. Next, corrections
210 for varying substrate concentrations were accomplished by dividing V by the substrate
211 concentration in terms of nitrogen or carbon added, and multiplying by 100 to convert to %
212 $(\text{mg DW})^{-1} \text{h}^{-1}$ (Fig. 2):

$$213 \quad \%V_{\text{sample}} = 100 \times V_{\text{sample}} / C_{\text{added}} \text{ (or } N_{\text{added}})$$

214 This specific uptake rate normalized to the amount of substrate initially available gives a
215 rough indication of the preference for a particular nutrient source, and can be interpreted as an

216 uptake rate per amount available. Note that preferences in treatments, with an equal amount of
217 substrate added, can be compared as if they were specific uptake rates. The total amount of
218 heavy isotope taken up (^{15}N incorporation; I_{sample}) during the incubation (~ 3 hours) was
219 calculated as:

$$220 \quad I_{\text{sample}} = E_{\text{sample}} \times \text{POM},$$

221 expressed in μmol , where POM is the amount of nitrogen or carbon in the macrophytes (in
222 μmol). This incorporation was statistically checked against the amounts that were added to the
223 incubation to assess the potential for depletion, and therefore underestimation of specific
224 uptake rates and preferences. Incubations that became depleted in substrates cannot be used
225 for comparison of uptake rates.

226

227 2.6. Statistical analyses

228 The occurrence of ^{15}N (or ^{13}C) uptake (research question 1) and translocation was tested with
229 t-tests by comparing initial isotope fractions (natural abundance) to the isotope fractions after
230 incubation. Corrections for differences in variance per treatment were successfully performed
231 by means of a variance function ($\sim 1/s^2$, with s the within-group standard deviation). After this
232 correction, no overall deviations from normality were detected in the residuals (Kolmogorov-
233 Smirnov tests). Since we found indications of translocation, ^{15}N incorporation from all plant
234 parts were added together to account for the total amount of label taken up. The remainder of
235 the statistics applies to the total amounts of label incorporated.

236

237 Total ^{15}N incorporation (I) after the incubation was compared to the amount added at the
238 beginning by means of 1-sample tests. Absence of significant differences between those two

239 might hint at substrate depletion. This is important because substrate depletion would lead to
240 underestimation of the true specific uptake rates.

241

242 Effects of substrate, macrophyte species and incubation (aboveground vs. belowground) on
243 the preference ($\%V_{\text{sample}}$), were investigated by means of variance analyses (research
244 questions 2 and 3). This substrate preference was chosen as response variable, because it
245 includes corrections for biomass and differences in initial substrate concentrations.
246 Corrections for differences in variance were performed by logarithmic transformation of the
247 response variable (preference). All tests were done at the 5 %-significance level. Differences
248 between species \times incubation \times substrate combinations were tested by Tukey's HSD tests
249 (honest significant difference).

250

251 The relationship of substrate preference with C:N ratio as a measure of chemical complexity
252 and molecule size was tested for the individual organic compounds (urea, glycine, leucine,
253 phenylalanine) by means of linear regression on the average values per species and
254 incubation. The coefficient of determination of this model is reported.

255

256 3. Results

257 3.1. Nitrogen enrichment and incorporation

258 ^{15}N enrichments (E) in the aboveground parts in the aboveground incubations of the three
259 macrophyte species were significant, except for the phenylalanine addition to *Zostera noltii*
260 and the urea additions to *Cymodocea nodosa* (Fig. 2 a - c, Tab. 2 left column). After substrate

261 addition to the belowground organs ^{15}N tissue enrichments were significant in the
262 belowground organs, except for the phenylalanine addition to *Z. noltii* and to *Caulerpa*
263 *prolifera* (Fig. 2 d - f, Tab. 2 right column). There was thus significant uptake in the plant
264 parts.

265

266 Occurrence of **translocation** was tested as enrichments in the plant parts that did not directly
267 receive label in their medium. In *Caulerpa prolifera* downward translocation was only
268 encountered in the NH_4^+ and algae-derived DON additions (t-tests, $p < 0.05$). Inorganic
269 nitrogen, algae-derived, and bacteria-derived DON were also transported downward in the
270 seagrasses (t-tests, $p < 0.05$). Upward translocation was found in all *Cymodocea nodosa*
271 substrate treatments (t-tests, $p < 0.05$), except phenylalanine. In *Zostera noltii* upward
272 translocation was significant (t-tests, $p < 0.05$), except for the individual organic compounds
273 (urea, glycine, leucine, and phenylalanine). Because of this translocation, the ^{15}N and ^{13}C
274 uptake in the remainder of the results section is considered for the entire plants.

275

276 The potential for substrate depletion was investigated by comparing total ^{15}N incorporations
277 (I) to the amounts of ^{15}N added. In the aboveground incubations of *Caulerpa prolifera* the
278 amount of ^{15}N in the tissue after 3 hours of incubation was close to the amount of ^{15}N added
279 as NH_4^+ , NO_3^- and algae-derived DON (i.e. the ^{15}N incorporation did not significantly differ
280 from the amounts added, 1-sample t-tests, $p > 0.05$). This means that substrate depletion might
281 have occurred in these treatments, and that comparison of uptake rates or preferences should
282 be done with caution. The same holds for the algae-derived DON addition to *Cymodocea*
283 *nodosa* aboveground parts (1-sample t-test, $p > 0.05$). ^{15}N incorporation in the rest of the
284 plants remained well below the amount added (1-sample t-tests, always $p < 0.05$), indicating

285 that no significant depletion occurred there. The tissue in the belowground incubations did not
286 exhibit any signs of depletion in any of the species either (1-sample t-tests, always $p < 0.05$).

287

288 3.2. Nitrogen preference

289 A 3-way analysis of variance (macrophyte species \times incubation \times substrate) illustrated
290 significant factor effects and first-order interactions (Tab. 3), indicating ^{15}N substrate
291 preferences with macrophyte species and macrophyte tissue.

292

293 In the aboveground incubations the preferences for NH_4^+ were significantly higher than for all
294 other substrates (Fig. 2 a - c; Tukey HSD, always $p < 0.05$), except for the algae-derived DON
295 additions to *Cymodocea nodosa* and *Caulerpa prolifera*, and the NO_3^- addition to *C. prolifera*
296 (but remember the potential for depletion in *C. prolifera*). Uptake of algae-derived DON
297 exhibited a preference similar to NO_3^- in *Zostera noltii* and *C. prolifera*, and similar to NH_4^+
298 in *C. nodosa* (Tukey HSD, always $p > 0.05$). In the belowground incubations *Z. noltii*
299 exhibited a significantly higher preference for NH_4^+ than for urea, leucine, phenylalanine, and
300 bacteria-derived DON (Tukey HSD, always $p < 0.05$; a tendency was found for the difference
301 with NO_3^- preference). Algae-derived DON preference by the belowground tissue was
302 significantly higher than the urea and phenylalanine preference in both seagrasses.

303

304 Algae-derived DON was in the aboveground incubations always preferred over bacteria-
305 derived DON (Tukey HSD, always $p < 0.01$; Fig. 2 a - c). In the belowground incubations a
306 significant difference between algae-derived DON and bacteria-derived DON preferences was
307 only detected for *Cymodocea nodosa*. (Tukey HSD, $p < 0.05$; a tendency existed for *Zostera*

308 *noltii*: $p = 0.07$). These results indicate that a distinction was made by the macrophytes
309 between algae-derived DON and bacteria-derived DON as N source.

310 The preferences for individual organic compounds (urea, glycine, leucine, and phenylalanine)
311 were in the aboveground incubations significantly lower than for algae-derived DON and
312 NH_4^+ (Fig. 2 a - c; Tukey HSD, always $p < 0.05$), except for the urea addition to *Caulerpa*
313 *prolifera*. Differences in the preferences among these individual compounds were not always
314 significant, but urea preference differed significantly from the phenylalanine preference in *C.*
315 *prolifera* and *Zostera noltii* (Tukey HSD, $p < 0.01$), while in *Cymodocea nodosa* a strong
316 tendency was found (Tukey HSD, $p = 0.055$). In the aboveground incubations, a significant
317 negative linear relationship existed between the C:N ratio of the individual organic
318 compounds and the preference (Fig. 3). No such relationship existed for the belowground
319 incubations.

320

321 3.3. Carbon enrichments

322 For the double-labeled (^{15}N and ^{13}C) treatments, we also assessed the potential coupling
323 between ^{13}C and ^{15}N uptake. Generally, the carbon signals were weaker than the
324 corresponding nitrogen signals, and more variable per treatment (Fig. 4). Significant excesses
325 in heavy carbon relative to natural isotope fractions were found in all bacteria-derived DON
326 additions (t-tests, $p < 0.001$), which also received the highest ^{13}C amounts. Additions of urea,
327 and phenylalanine to the aboveground *Caulerpa prolifera* tissue also resulted in significant
328 ^{13}C enrichments (t-tests, $p < 0.05$). No relationships between carbon and nitrogen isotope
329 enrichments, preferences, or incorporation were found for any species \times incubation \times
330 substrate combination, except for the bacterial DON additions (data not shown).

331

332 4. Discussion

333 4.1. DON as nutrient source

334 This experiment has illustrated the ability of three temperate marine macrophytes to take up
335 nitrogen from both inorganic and organic sources within hours. Above ground as well as
336 belowground organs took up organic nitrogen. The specific uptake rates per amount of
337 substrate (here called 'preference') for DON derived from an axenic diatom culture were
338 comparable to those for NH_4^+ , which is assumed to be the preferred nitrogen source for
339 seagrasses (Romero et al. 2006). This highlights the potential importance of DON as an
340 immediate source of bioavailable nitrogen. The potential of detritus-derived nitrogen
341 utilization by seagrasses was already illustrated by Evrard et al. (2005) and by Barrón et al.
342 (2006) for oligotrophic systems. However, their incubations lasted one to several days and
343 focused on sediment associated processes, thus including direct uptake of dissolved organic
344 nitrogen and uptake of dissolved inorganic nitrogen following remineralization. By restricting
345 the incubations to 3 hours, remineralization was minimized or is at least lower in our
346 experiment. Recently, short-term (~ hours) organic nitrogen uptake through aboveground and
347 belowground organs was also demonstrated for seagrass species in a tropical oligotrophic
348 system (Vonk et al. 2008). To our knowledge, our study is the first to systematically address
349 short-term organic nitrogen utilization from specific compounds in temperate seagrasses.

350

351 Our study shows that, although N uptake occurred from both inorganic and organic substrates,
352 not all substrates were treated similarly by the macrophytes. Inorganic nitrogen was preferred
353 over individual organic compounds, mainly due to the high ammonium preference (nitrate
354 preference was sometimes not higher than urea or glycine preference). Similar patterns were
355 found for tropical seagrasses (Vonk et al. 2008). Ammonium is readily available for

356 incorporation in amino acids which gives an advantage over other substrates that have to be
357 reduced or split. Interactions between ammonium and nitrate uptake were for *Zostera noltii*
358 demonstrated, with a preference for ammonium when both substrates were present (Alexandre
359 et al. 2010). Assimilatory nitrate reduction will in many cases still be cheaper than
360 extracellular ammonification, because in the former case enzymes are re-used, and the
361 resulting ammonium (after reduction) is not lost to the same extent as when it would be
362 produced in the environment by exo-enzymes.

363

364 Different small organic substrates were treated differently by the macrophytes. Based on this
365 small set of compounds, there appeared to be a relationship with C:N ratio in the uptake by
366 aboveground organs. Given the absence of significant carbon uptake in many cases, one
367 would not expect that the C:N ratio plays a direct role. However, chemical structure (Tab. 1),
368 relative abundance in organisms and seagrass-dominated systems (Cowie and Hedges 1992,
369 Hansen et al. 2000), and molar mass were for our substrates related to this C:N ratio and
370 might provide explanations for this consistent variation in preference. The chemical structure
371 may be important in enzymatic breakdown because of the associated resistance to breakdown
372 (e.g. the increased chemical stability of an aromatic ring), but also because of substrate-
373 specificity in enzymes. Based on the decreasing relative abundance of the amino acids from
374 glycine to phenylalanine (Tab. 1, Cowie and Hedges 1992, Hansen et al. 2000), one might
375 hypothesize that expression of enzymes may have been fine tuned to make optimal use of
376 what is present in the environment. DON concentration and/or composition are indeed known
377 to influence activity of specific enzymes (Stepanauskas et al. 1999).

378

379 Although we found statistically significant differences between the spectrum of nitrogen
380 sources preferred by the belowground organs, relative to aboveground organs, we were not
381 able to detect significant differences between most of the individual treatments due to limited
382 statistical power. Nevertheless, our results give some indications that preferences for
383 ammonium, glycine and algae-derived DON were roughly similar and tended to be higher
384 than for the other substrates in the seagrasses. *Caulerpa prolifera* seemed to prefer ammonium
385 and DON (algae-derived and bacteria-derived) over other substrates. Although more
386 replication will be needed to validate these patterns, they are in line with the results from
387 Vonk et al. (2008). They also found higher uptake rates by seagrasses for ammonium and an
388 amino acid mixture than for urea and nitrate. They suggested that root-mediated amino acid
389 uptake might give them a competitive advantage over some macroalgae. Our results indeed
390 seem to indicate that seagrasses roots tend to take up amino acids more easily than *C.*
391 *prolifera* rhizoids. Altogether the present study supports the idea that, besides
392 microphytobenthos, marine macrophytes are able to directly influence organic nitrogen fluxes
393 from the sediment to the water column by uptake (Nilsson and Sundbäck 1996, Tyler et al.
394 2003, Linares and Sundbäck 2006)

395

396 Algae-derived DON was most of the time preferred over bacteria-derived DON, irrespective
397 of macrophyte species. The lower fraction of amino acids in bacteria-derived DON relative to
398 algae-derived DON (section 2.3) may explain why algae-derived DON was the preferred
399 DON pool, since it implies that other compounds, which may be more resistant to breakdown,
400 are more abundantly present in the bacteria-derived DON. These findings are in line with the
401 observation that bacterial DON comprises a substantial fraction of the refractory part of
402 oceanic DOM (McCarthy et al. 1998, 2004), although their results concerned mainly
403 autotrophic cyanobacteria and much larger time scales. Our findings are thus in good

404 agreement with the dependence of DON reactivity on DON composition and origin, found for
405 other ecosystems (Ziegler and Benner 1999, Seitzinger et al. 2002, McCallister et al. 2006),
406 and in DOM derived from different phytoplankton cultures (Pete et al. 2010). DON may thus
407 play a role in community composition (e.g. seasonal succession patterns), just as other
408 nutrients and their relative abundances do (e.g. $\text{NH}_4^+:\text{NO}_3^-$, N:P ratios, DIN/DON; Berg et al.
409 2003).

410

411 4.2. Do macrophytes take up DON directly or only after remineralization to DIN?

412 This study has revealed an uptake of nitrogen, but not carbon, originating from various
413 organic substrates in a matter of hours. These results contrast those found for other
414 macrophytes by Harrison et al. (2007) and Mozdzer et al. (2010), who found relationships
415 between carbon and nitrogen uptake from amino acids related to their C:N ratios. Past
416 research on organic carbon uptake in phytoplankton showed that this process and its coupling
417 with organic nitrogen uptake strongly varies with species and substrates, and throughout the
418 season (Mulholland et al. 2004, Andersson et al. 2006). In principle the absence of clear ^{13}C
419 enrichment means that in our experiment either the nitrogen alone entered the plants, or the
420 carbon was lost after breakdown of the substrates in the plant, with subsequent carbon
421 exudation. The uptake of nitrogen only requires degradation of the DOM by exo-enzymes or
422 cell-surface associated enzymes, and a subsequent transport of the smaller degradation
423 products. The remaining carbon in the medium would be lost in a large background pool of
424 DIC, or as carbon-rich organic matter. However, the fact that no relationship between carbon
425 and nitrogen uptake was detected in our study, may also be due to the high carbon background
426 in the macrophytes, which basically results in a low signal-to-noise ratio (von Felten et al.
427 2008).

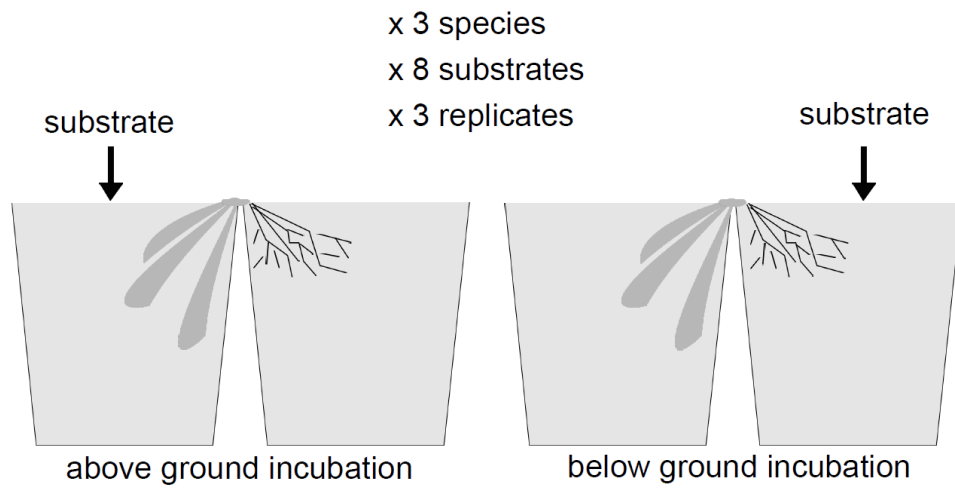
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429 The differences in the substrate preference between species and between plant parts implies
430 that consistent distinctions were made by either the plants themselves or by associated
431 bacteria. Seagrasses may indeed have specific bacterial communities attached to the root
432 surface (Jensen et al. 2007). Although *Caulerpa prolifera* effectively repels epiphytes by the
433 production of caulerpenine (Paul and Fenical 1987), endosymbiotic prokaryotes have been
434 shown to provide nitrogen to *Caulerpa taxifolia* (Chisholm et al. 1996). However, this
435 concerned fixation of dinitrogen, which could enter the cells by diffusion. Provided that
436 endosymbionts can only process organic compounds within the tissue/cells, these compounds
437 obviously have to be taken up or produced by the plant itself. Note that macroalgae-bacteria
438 interactions are highly species-specific and not well understood (Goecke et al. 2010). It is not
439 clear to what extent bacteria influence our results. But even with the removal of epiphytes,
440 phytoplankton and a part of the bacterial community, macrophytes are practically able to take
441 up nitrogen from an organic origin immediately. A complex pool of DON can, therefore,
442 immediately supply nitrogen in similar or larger quantities than inorganic pools at close-to-
443 ambient concentrations found in temperate seagrass-dominated systems. Therefore, DON
444 should be included in future N studies on seagrass systems.

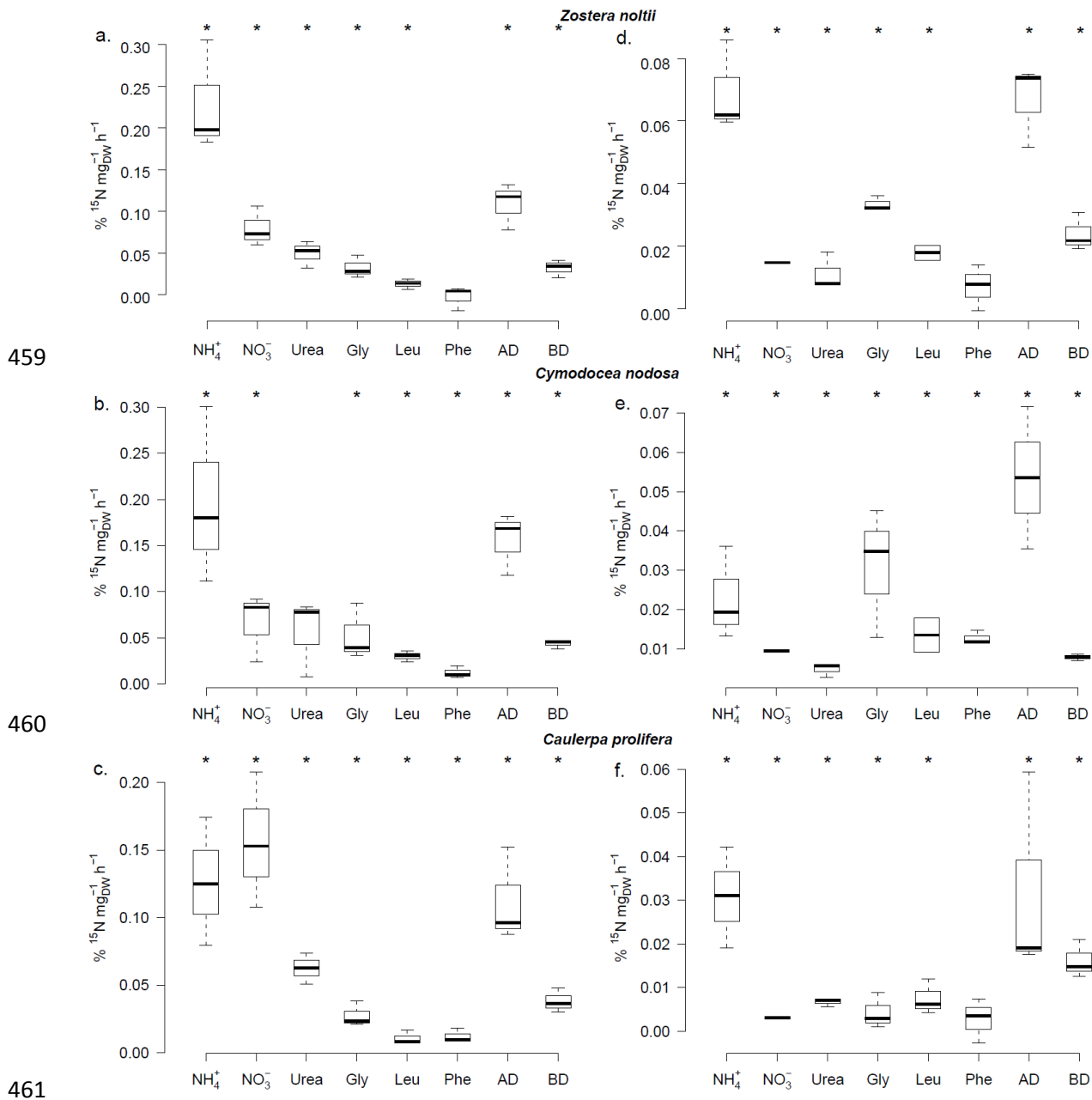
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446 Acknowledgements

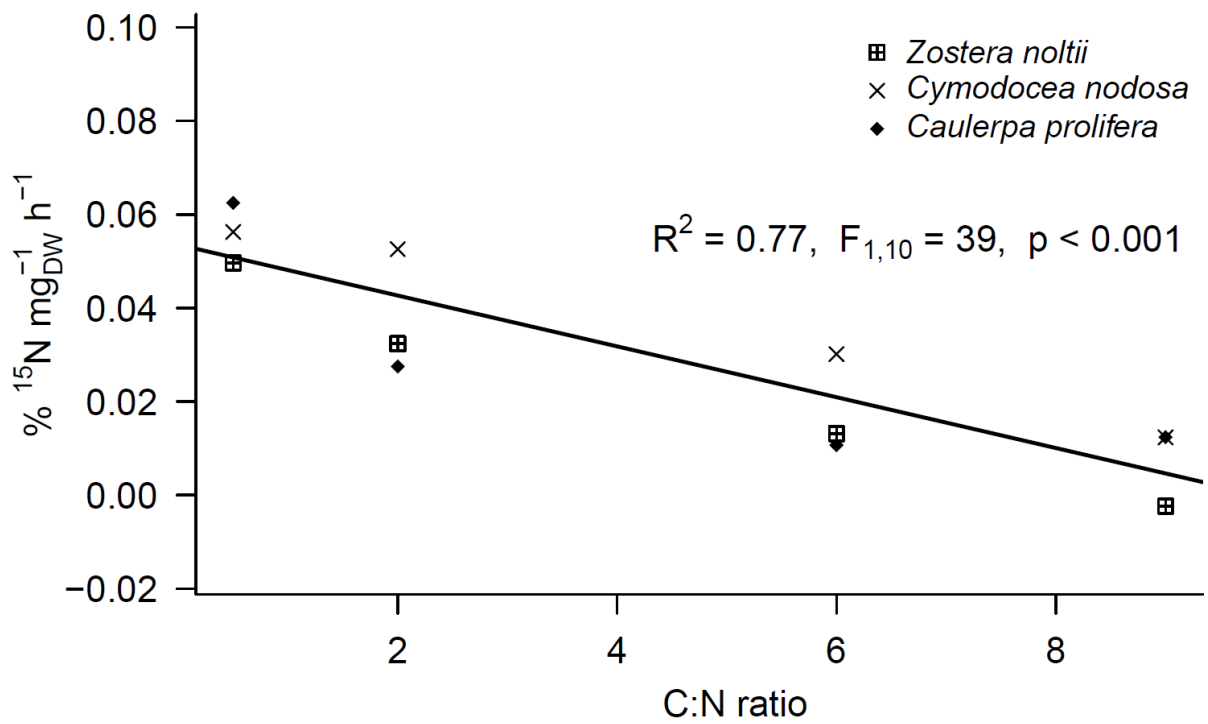
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455
456 Fig. 1: Experimental setup. Substrate was added to the relevant cups in the aboveground and
457 belowground incubation. This was performed in triplicate for the three macrophytes and eight
458 substrates.

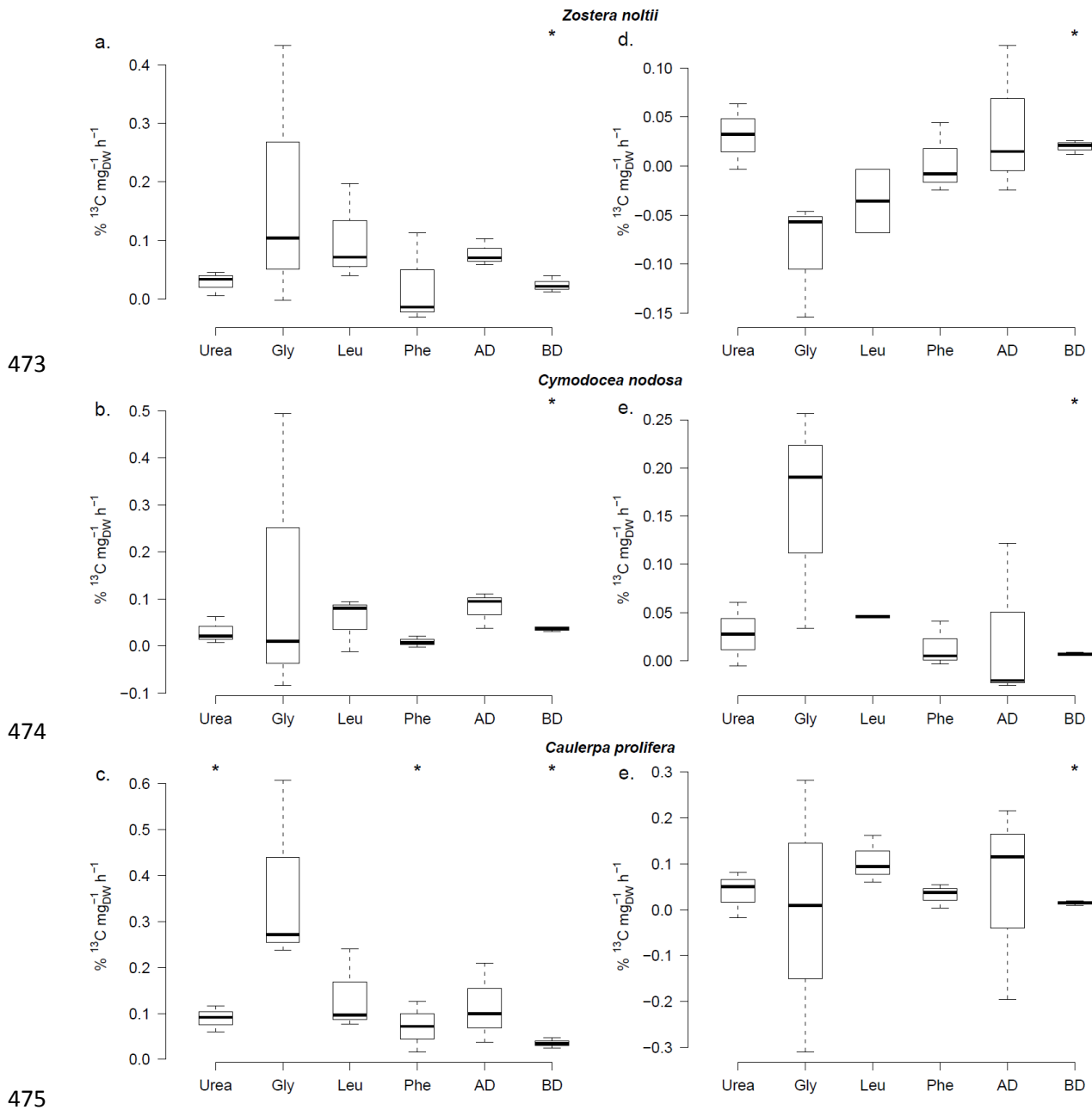


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 461
 462 Fig. 2: ^{15}N substrate preferences (cf. table 1 for abbreviations on x-axis) by the aboveground
 463 (left) and belowground tissue (right) of *Zostera noltii* (a, d), *Cymodocea nodosa* (b, e), and
 464 *Caulerpa prolifera* (c, f), expressed as a percentage of the added substrate per milligram of
 465 tissue dry weight and per hour of incubation time (translocation was taken into account).
 466 Significant excesses (95%-confidence level) in heavy isotope fractions per treatment relative
 467 to the natural heavy isotope fractions in the macrophyte tissue are indicated with an asterisk
 468 (on top of the graph). Note the difference in scales between the different graphs.







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470 Fig. 3: Linear regression of the ¹⁵N substrate preference versus the C:N ratio of the organic
 471 substrates urea (1/2), glycine (2), leucine (6), and phenylalanine (9), and the coefficient of
 472 determination of the regression model.



476 Fig. 4: ^{13}C substrate preferences (cf. table 1 for substrate abbreviations) by the aboveground
 477 (left) and belowground tissue (right) of *Zostera noltii* (a, d), *Cymodocea nodosa* (b, e), and
 478 *Caulerpa prolifera* (c, f) expressed as a percentage of the substrate added per milligram of
 479 tissue dry weight and per hour of incubation time (translocation was considered. Significant
 480 excesses (95%-confidence level) in heavy isotope fractions per treatment relative to the
 481 natural heavy isotope abundance in the macrophyte tissue are indicated with an asterisk (on
 482 top of the graphs). Note the difference in scales between the different graphs.

483 Tab. 1: The substrates (Cambridge Isotope Laboratories) used for this experiment, their labeling and abbreviations used in this study, their final
 484 concentrations in the incubations, the chemical structure of the small organic compounds, and the approximate molar mass of the small organic
 485 molecules. The substrates marked by a * are only used in the DOM preparation. For the amino acids their range in relative abundance in total
 486 hydrolysable amino acids in organisms is also given, based on data from Cowie and Hedges (1992).

Substrates	Isotopes	Abbrev.	Final N concentration ($\mu\text{mol-N l}^{-1}$)	Final C concentration ($\mu\text{mol-C l}^{-1}$)	Structure	C:N ratio	Molar mass (g mol^{-1})	Abundance in tissue (mole % N)
NaHCO ₃	(¹³ C, 99 %)		*					
NH ₄ Cl	(¹⁵ N, 99 %)	NH ₄ ⁺	1					
NaNO ₃	(¹⁵ N, 98 %)	NO ₃ ⁻	1					
Urea	(¹³ C, 99%; ¹⁵ N ₂ , 98%)	Urea	2			1/2	63	
Glycine	(U ¹³ C ₂ , 98%; ¹⁵ N, 98%)	Gly	0.1			2	78	7.9 – 14.5
L-Leucine (SILAC)	(U ¹³ C ₆ , 98%; ¹⁵ N, 98%)	Leu	0.1			6	138	6 – 9.3
L-Phenylalanine	(U ¹³ C ₉ , 98%; ¹⁵ N, 98%)	Phe	0.1			9	175	2.4 – 4.5
D-glucose	(U- ¹³ C ₆ , 99%)		*					
Algae-DOM	(¹³ C, 8%; ¹⁵ N, 66%)	AD	0.5	4.3				
Bacteria-DOM	(¹³ C, 46%; ¹⁵ N, 46%)	BD	6.7	24				

487 Tab. 2: Results from the t-tests per macrophyte species and per incubation (aboveground or
 488 belowground) for the difference in ^{15}N atomic fractions (with consideration of translocation)
 489 after incubation relative to the natural abundance in the plant parts that directly received
 490 labeled substrate. Significant (95% confidence level) results indicate ^{15}N uptake from the
 491 substrates, ammonium, nitrate, urea (UR), glycine (Gly), leucine (Leu), phenylalanine (Phe),
 492 algae-derived DON (AD), and bacteria-derived DON (BD) by the respective species and plant
 493 parts.

	aboveground incubations			belowground incubations		
	Treatment	t	p	Treatment	t	p
<i>Zostera noltii</i>	NH_4^+	9.2	< 0.001	NH_4^+	7.2	< 0.001
	NO_3^-	9.8	< 0.001	NO_3^-	15.4	< 0.001
	UR	2.9	0.009	UR	5.1	< 0.001
	Gly	2.7	0.015	Gly	7.1	< 0.001
	Leu	3.3	0.004	Leu	5.9	< 0.001
	Phe	0.0	0.975	Phe	1.5	0.143
	AD	4.3	< 0.001	AD	6.9	< 0.001
	BD	2.8	0.012	BD	6.5	< 0.001
<i>Cymodocea nodosa</i>	Treatment	t	p	Treatment	t	p
	NH_4^+	13	< 0.001	NH_4^+	14.3	< 0.001
	NO_3^-	2.2	0.042	NO_3^-	553	< 0.001
	UR	1.9	0.076	UR	6.6	< 0.001
	Gly	3	0.007	Gly	3.4	0.004
	Leu	3.4	0.003	Leu	12.1	< 0.001
	Phe	4	0.001	Phe	11	< 0.001
	AD	4.9	< 0.001	AD	8.9	< 0.001
BD	5.5	< 0.001	BD	52.8	< 0.001	
<i>Caulerpa prolifera</i>	Treatment	t	p	Treatment	t	p
	NH_4^+	10.7	< 0.001	NH_4^+	4.7	< 0.001
	NO_3^-	4.1	0.001	NO_3^-	4.3	< 0.001
	UR	5.4	< 0.001	UR	8.3	< 0.001
	Gly	4.4	< 0.001	Gly	2.7	0.015
	Leu	4.3	< 0.001	Leu	5.3	< 0.001
	Phe	4.9	< 0.001	Phe	2	0.058
	AD	22	< 0.001	AD	3.8	0.001
BD	35	< 0.001	BD	3.5	0.002	

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497 Tab.3: Analysis of variance table from the 3-factor model with substrate preference as
498 response variable and species, incubation (aboveground vs. belowground) and substrate main
499 effects and all possible interactions.

Df_{denom}: 102	df_{num}	F	p
Species	2	3.4	0.036
Incubation	1	176	< 0.001
Substrate	7	56	< 0.001
Species × Incubation	2	8.4	< 0.001
Species × Substrate	14	3.3	< 0.001
Incubation × Substrate	7	17	< 0.001
Species × Incubation × Substrate	14	1.0	0.49

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