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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1. Introduction

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

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

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

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Given the potential importance of DON as source of nitrogen in macrophyte-dominated systems, it is imperative that we gain more insight in the role of this highly complex and little understood ecosystem component. Hence, we conducted an uptake experiment in the laboratory with three co-occurring temperate macrophyte species from Cadiz Bay (Spain): *Zostera noltii*, *Cymodocea nodosa* (both seagrasses), *Caulerpa prolifera* (a rhizoid forming 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.

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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 ($[NH_4^+] = 0.3 \pm 0.06$ µmol l^{-1}; $[NO_3^-] = 0.7 \pm 0.38$ µmol l^{-1}; [urea] = 2.2 ± 0.24 µmol

118　　l^{-1}; [DON] = 16.4 ± 0.77 µmol l^{-1}; [Gly] 23.7 ± 3.6 nmol l^{-1}; [Leu] 0.6 ± 0.4 nmol l^{-1}; [Phe] 4.8

119　　± 0.7 nmol l^{-1}; mean ± 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}NH$_4$Cl, and one from an axenic culture of *Skeletonema costatum* grown on NaH^{13}CO$_3$

166 and Na^{15}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 (DIN = NH_4^+ + NO_2^- + 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 (DCAA = THAA - 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                         $E_{sample} = F_{sample} - F_{nat}$

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

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

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) mg DW^{-1} 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   (mg DW)$^{-1}$ h^{-1} (Fig. 2):

213                 $\%V_{sample} = 100 \times V_{sample} / C_{added}$ (or N_{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 $$I_{sample} = E_{sample} \times POM,$$

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_{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 15N 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 × incubation ×

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

17

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. $NH_4^+:NO_3^-$, 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.
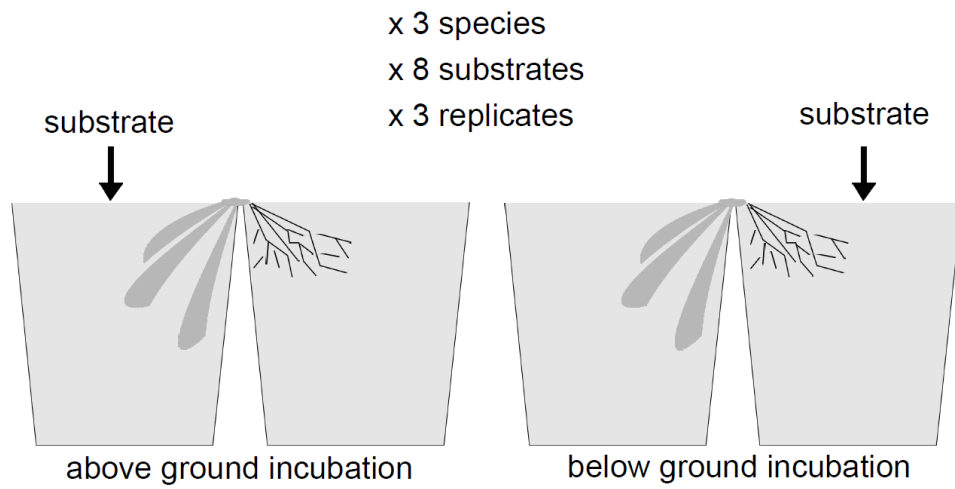
427 2008).

428

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.

445

454   Figures and tables

x 3 species
x 8 substrates
x 3 replicates

substrate

substrate

above ground incubation

below ground incubation

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
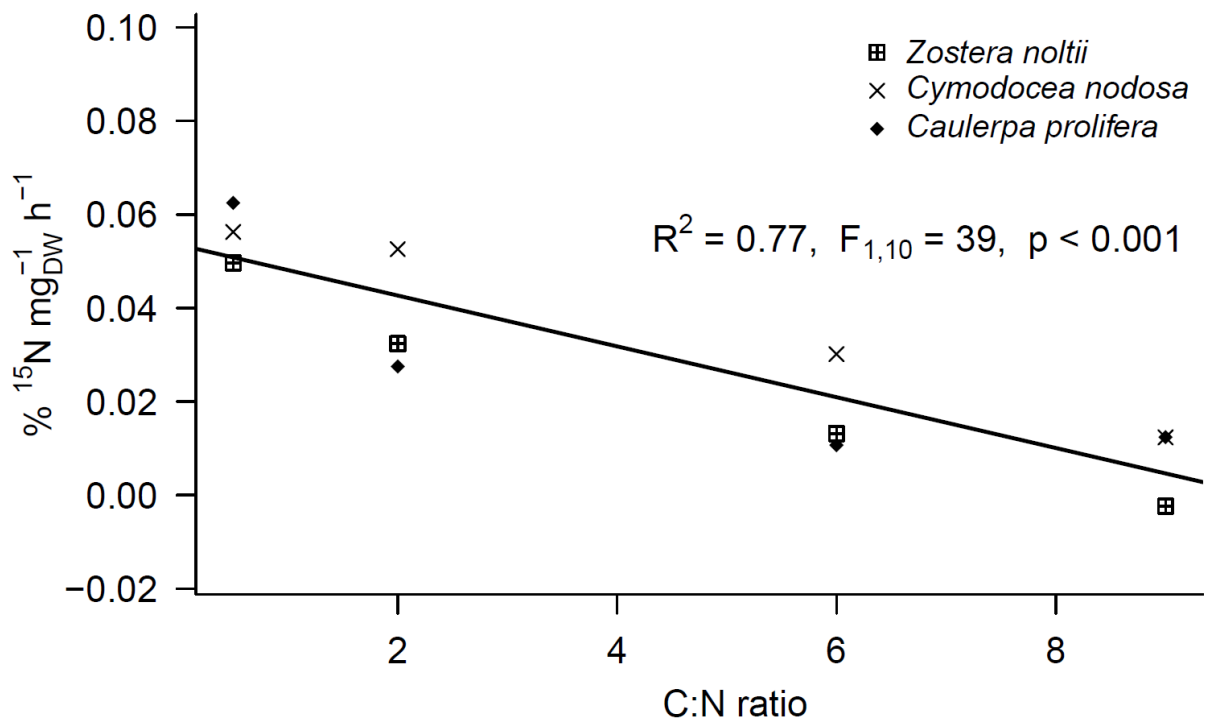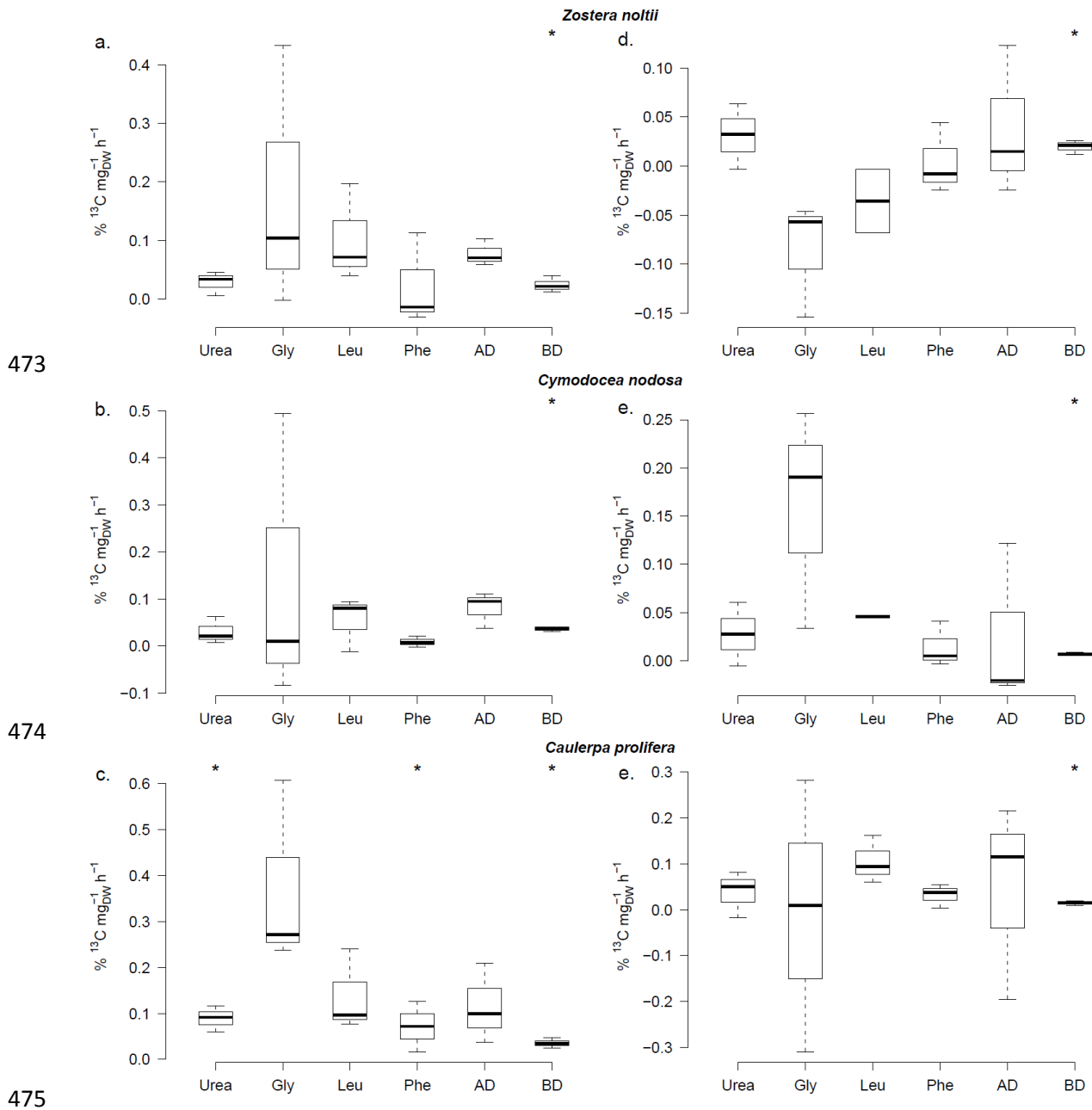
458   substrates.

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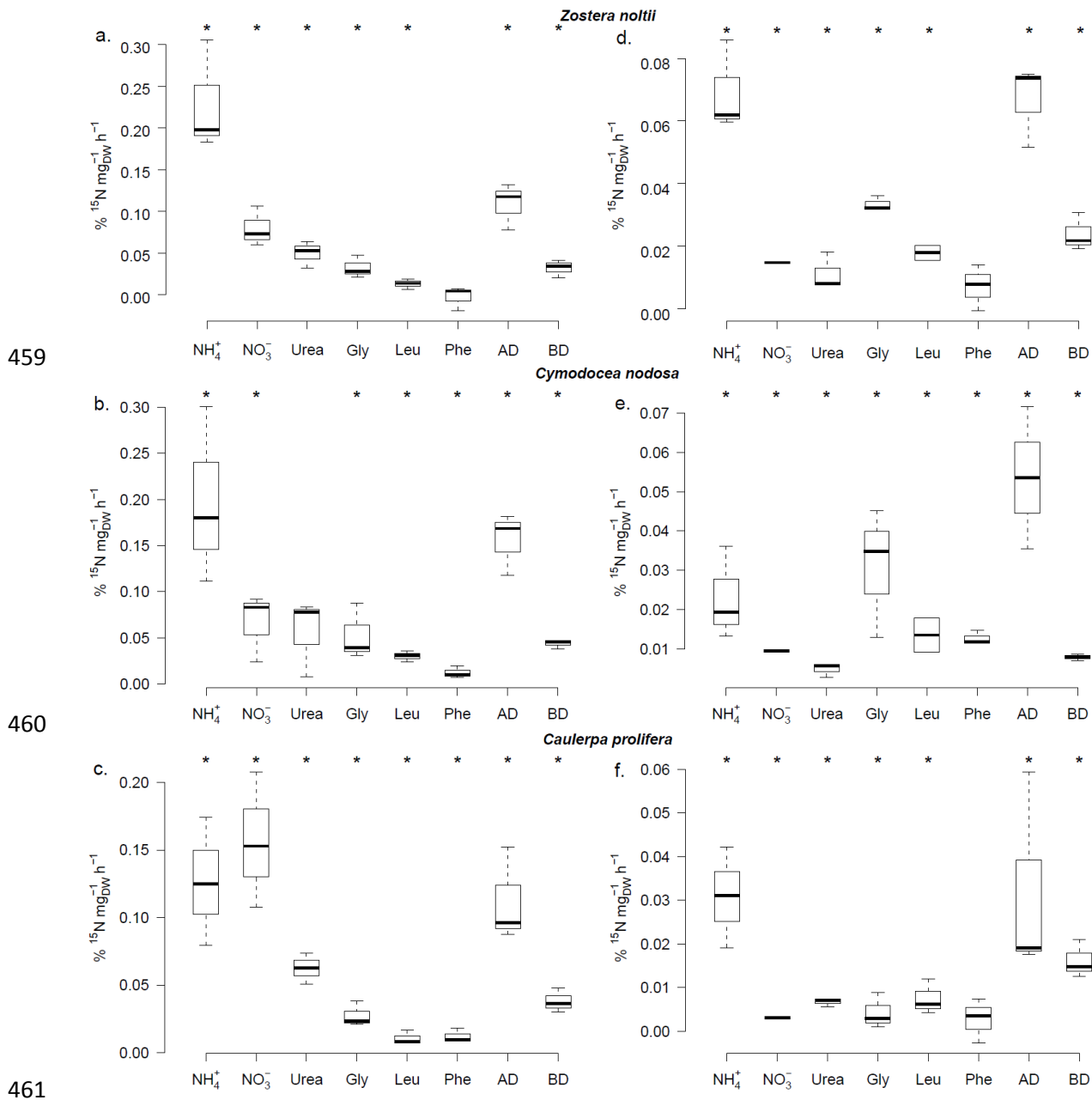
Fig. 2: ^{15}N substrate preferences (cf. table 1 for abbreviations on x-axis) by the aboveground (left) and belowground tissue (right) of *Zostera noltii* (a, d), *Cymodocea nodosa* (b, e), and *Caulerpa prolifera* (c, f), expressed as a percentage of the added substrate per milligram of tissue dry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

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

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

23

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 (μmol-N l^{-1})	Final C concentration (μmol-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			½	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				

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

	aboveground incubations			belowground incubations		
	Treatment	t	p	Treatment	t	p
Zostera noltii	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
	Treatment	t	p	Treatment	t	p
Cymodocea nodosa	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
	Treatment	t	p	Treatment	t	p
Caulerpa prolifera	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.

$\mathbf{Df_{denom}}$: 102	$\mathbf{df_{num}}$	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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