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*Summary

The productivity and ecological distribution of freshwater plants can be controlled by the availability of inorganic carbon in water despite the existence of different mechanisms to ameliorate this, such as the ability to use bicarbonate. Here we took advantage of a short, natural gradient of CO_2 concentration, against a background of very high and relatively constant concentration of bicarbonate, in a spring-fed river, to study the effect of variable concentration of CO_2 on the ability of freshwater plants to use bicarbonate. Plants close to the source, where the concentration of CO_2 was up to 24-times air equilibrium, were dominated by *Berula erecta.* pH-drift results and discrimination against ^{13}C were consistent with this and the other species being restricted to CO_2 and unable to use the high concentration of bicarbonate. There was some indication from stable ^{13}C data that *B. erecta* may have had access to atmospheric CO_2 at low water levels. In contrast, species downstream, where concentrations of CO_2 were only about 5-times air-equilibrium were almost exclusively able to use bicarbonate, based on pH-drift results. Discrimination against ^{13}C was also consistent with bicarbonate being the main source of inorganic carbon for photosynthesis in these species. There was, therefore, a transect downstream from the source of increasing ability to use bicarbonate that closely matched the decreasing concentration of CO_2. This was produced largely by altered species composition, but partly by phenotypic changes in individual species.

1 Adaptation by macrophytes to inorganic carbon down a river with naturally variable

2 concentrations of CO_2

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23 Abstract

24 The productivity and ecological distribution of freshwater plants can be controlled by the

25 availability of inorganic carbon in water despite the existence of different mechanisms to

26 ameliorate this, such as the ability to use bicarbonate. Here we took advantage of a short,

27 natural gradient of CO_2 concentration, against a background of very high and relatively

28 constant concentration of bicarbonate, in a spring-fed river, to study the effect of variable

29 concentration of CO_2 on the ability of freshwater plants to use bicarbonate. Plants close to

30 the source, where the concentration of CO_2 was up to 24-times air equilibrium, were

31 dominated by *Berula erecta.* pH-drift results and discrimination against ^{13}C were consistent

32 with this and the other species being restricted to CO_2 and unable to use the high

33 concentration of bicarbonate. There was some indication from stable ^{13}C data that *B. erecta*

34 may have had access to atmospheric CO_2 at low water levels. In contrast, species

35 downstream, where concentrations of CO_2 were only about 5-times air-equilibrium were

36 almost exclusively able to use bicarbonate, based on pH-drift results. Discrimination against

37 ^{13}C was also consistent with bicarbonate being the main source of inorganic carbon for

38 photosynthesis in these species. There was, therefore, a transect downstream from the

39 source of increasing ability to use bicarbonate that closely matched the decreasing

40 concentration of CO_2. This was produced largely by altered species composition, but partly

41 by phenotypic changes in individual species.

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43 Keywords: bicarbonate, Fontaine de Vaucluse, photosynthesis, river Sorgue, stable carbon

44 isotope

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46 **Introduction**

47 The function and biodiversity of inland waters is controlled by interactions between the

48 physical and chemical environment and the physiological and biochemical acclimation and

49 adaptation of the organisms present as well as their short-term behavioural responses. High

50 biological demand for resources, created by high potential metabolic rates or high biomass

51 density, or both, can exceed the rate of re-supply and so alter the chemical and physical

52 conditions in inland waters. Conversely, uncoupling of biogeochemical cycles of production

53 and decomposition can generate a high supply of resource when the demand is low.

54 The primary producers at the base of the aquatic food web can be planktonic,

55 epiphytic or benthic. In rivers, benthic macrophytes, Plantae that are mainly derived from

56 land plants that have re-invaded the water, are often dominant because the rapid water

57 transit time can prevents the formation of large populations of phytoplankton, especially in

58 upstream regions where nutrient concentrations can be low (Reynolds and Descy, 1996).

59 Macrophytes can survive high flushing rates or low nutrient resources in the water, as in

60 many upland rivers, because most have roots, anchoring them to the substrate and allowing

61 them to access nutrients in the substrate.

62 However, freshwater macrophytes require high concentrations of CO_2 to saturate

63 photosynthesis because of a high transport resistance across relatively large external

64 boundary layers (Black et al., 1981, Maberly and Madsen, 1998). Photosynthesis can be

65 limited further by intermittent depletion of CO_2 produced when rates of photosynthetic

66 demand exceed rates of resupply and by the generation of high concentrations of oxygen

67 that can promote photorespiration (Maberly and Madsen, 2002, Pedersen et al., 2013).

68 However, aquatic photoautotrophs possess avoidance, exploitation and amelioration

69 strategies to minimize the effects of potential carbon limitation (Klavsen et al., 2011).

70 Avoidance strategies involve living in environments where the concentration of CO_2 is

71 naturally high, for example as a result of heterotrophic decomposition of organic matter

72 (Maberly, 1985). Exploitation strategies include gaining access to more reliable sources of

3

73   CO_2 such as the atmosphere (Maberly and Madsen, 2002). Just over half of the freshwater

74   macrophytes tested so far have amelioration strategies based on biophysical or biochemical

75   carbon concentrating mechanisms (CCMs) to minimise carbon limitation photorespiration

76   (Maberly and Madsen, 2002). The most widespread CCM is the use of bicarbonate as an

77   alternative source of carbon. For biochemical details see also (Kroth, 2015) and Sage ans

78   Stata (2015) . Because of physico-chemical equilibria, bicarbonate is less susceptible than

79   CO_2 to depletion during removal of inorganic carbon by photosynthesis and is present at

80   concentrations that exceed CO_2 at sites where the pH exceeds the pH representing the first

81   pK value of the carbonate system at approximately pH 6.3.

82   Although the use of bicarbonate is widespread, it is not universal, probably because

83   there are costs involved in its use. First, bicarbonate uptake involves moving bicarbonate

84   against its electrochemical potential gradient and across an inherently impermeable

85   plasmalemma and is therefore an active process requiring expenditure of ATP. In situations

86   where light energy is low, species therefore tend to lack the ability to use bicarbonate. One

87   example is the predominance of species at depth that lack an ability to use bicarbonate such

88   as sublittoral marine red macroalgae (Maberly, 1990) and in freshwater, aquatic bryophytes

89   (Krause-Jensen and Sand-Jensen, 1998). Secondly, there are further energetic costs

90   associated with the production and maintenance of the proteins involved in bicarbonate use

91   (Jones, 2005). Thirdly, there is also a 'permeability cost' involved in using bicarbonate:

92   species that use bicarbonate have a lower affinity for CO_2 than species that are restricted to

93   CO_2 (Maberly and Madsen, 1998).This is probably caused by a high internal resistance to

94   CO_2 uptake (Madsen and Maberly, 2003) that minimises the diffusive loss of carbon actively

95   pumped into the photosynthesising tissue, but consequently reduces rates of photosynthesis

96   at limiting concentrations of CO_2.

97   Comparisons of the ability of freshwater macrophytes to use bicarbonate or operate a

98   CCM are usually based on a comparison of plants collected from sites with very different

99   physical and chemical characteristics. An alternative comparative approach compares the

4

100   physiology of plants or algae at sites with locally high concentrations of CO_2 with those at

101   nearby sites with lower concentrations of CO_2. An example in the terrestrial environment

102   includes studies at natural CO_2 vents associated with volcanic activity (Korner and Miglietta,

103   1994). An example from the marine environment is the use of geothermal vents to study the

104   effects of high CO_2 and low pH as a surrogate for future ocean acidification (Hall-Spencer et

105   al., 2008). This approach does not appear to have been used for freshwater macrophytes at

106   a specific site despite the high variability in CO_2 concentrations in different surface waters

107   and the frequently high CO_2 concentrations found in groundwater and groundwater-fed

108   streams (Sand-Jensen and Staehr, 2012, Stets et al., 2009), although comparisons have

109   been made over wider spatial areas e.g. (Demars and Tremolieres, 2009). The aim of the

110   work reported here was to assess the ability of river macrophytes to use bicarbonate at

111   different sites down a natural gradient of high CO_2 concentration from the groundwater-fed

112   source of a river to lower concentrations downstream. The comparison is therefore made on

113   plants experiencing very similar background physico-chemical conditions apart from CO_2.

114   Furthermore, since this CO_2 gradient is likely to have been present for thousands of years, it

115   allows adaptive responses to varying CO_2 to be distinguished from acclimative responses to

116   short-term variability.

117

118 **Materials and methods**

119 *La Sorgue at Fontaine de Vaucluse*

120 Fontaine de Vaucluse, the source of the River Sorgue, is located in a Karst landscape in

121 Provence, in south-east France (Fig. 1). It is the largest spring in France, and one of the

122 largest in the world with a mean discharge of about 23 m^3 s^{-1} and minimum and maximum

123 discharges of 4 and over 100 m^3 s^{-1} respectively (Bonacci, 2007). The recharge area

124 comprises 1115 km^2 of Lower Carboniferous limestone, 1500 m thick, on largely uncultivated

125 land dominated by Mediterranean forest and 'garrigue'- dry limestone scrubland (Blavoux et

126 al., 1992) which on average receives an annual rainfall of 1096 mm (Bonacci, 2007). The

127 outflow has a very high concentration of bicarbonate of about 4.25 mmol L^{-1} (Emblanch et

128 al., 2003) with calcium as the dominant cation with a concentration of around 2 mmol L^{-1}

129 (Garry et al., 2008). The geometric mean pH of 7.25, calculated from the frequent outflow

130 data over a year presented in (Emblanch, Zuppi, 2003), was used to estimate an average

131 CO_2 concentration of about 0.6 mmol L^{-1} assuming a water temperature of 12°C and an ionic

132 strength of 0.0065 mol L^{-1} based on ionic composition from adjacent cave water given in

133 (Garry, Blondel, 2008) which had a similar bicarbonate concentration to the outflow.

134

135 *Field methods*

136 Samples were collected on 12/09/2013, between 11:00 and 16:00, down a transect from

137 close to the source of the spring at Fontaine de Vaucluse to about 20 km downstream on

138 one of the many distributaries in this region (Fig. 1). On this date, the discharge was

139 relatively low at about 9 m^3 s^{-1} (REALPACA, 2013) which is slightly less than half the annual

140 mean. At each site, water was collected in a large bucket and water temperature was

141 measured immediately and the concentration of CO_2 measured with a Vaisala non-

142 dispersive infrared gas analyser protected for use in water with a waterproof membrane as

143 described by (Johnson et al., 2010). Water was stored in two completely-filled 50 mL Falcon

144 polypropylene centrifuge tubes and kept in the dark in a cool box for analysis in the

6

145 laboratory on the next day. For stable carbon isotope measurement, 10 mL of lake or stream

146 water was injected via a syringe into evacuated (< 5 Pa) 12 mL exetainer (Labco) containing

147 0.15 mL degassed concentrated phosphoric acid (Maberly et al., 2013, Waldron et al.,

148 2007). Samples were collected in duplicate and kept inverted prior to analyses. At each site,

149 macrophytes were collected by hand or with a drag rake and placed, moist, in polythene

150 bags and stored in a cool box overnight.

151

152 *Laboratory analyses*

153 In the laboratory, macrophyte material for stable carbon isotope measurement was cleaned

154 carefully and dried at 80°C for 24 hours and stored in aluminium foil before analysis. The pH

155 of water from the river was measured with a combination pH-electrode (PHEL-GS2, Labbox)

156 and meter (pH201, Hanna Instruments). Gran titration was used to measure alkalinity by

157 duplicate titration of 15 mL samples with approximately 0.17 mol L^{-1} HCl that had been

158 standardised against 1 mol L^{-1} $NaHCO_3$ (Mackereth et al., 1978). Inorganic carbon

159 concentrations were calculated from measured temperature, pH and alkalinity following

160 (Maberly, 1996). Calcite saturation was calculated as in (Maberly, 1996) using the equations

161 of (Jacobson and Langmuir, 1974) to estimate the temperature-dependent calcite solubility

162 product.

163

164 *pH-drift experiments*

165 Macrophytes and benthic filamentous algae were identified, cleaned carefully in tap water

166 using a soft brush and rinsed in 1 mol L^{-1} $NaHCO_3$ for at least 20 minutes. This concentration

167 was chosen because it has been frequently used and while high enough to allow sufficient

168 bicarbonate, should it be present, it is not so high as to require extremely high pH values to

169 be generated to deplete inorganic carbon. Three species from each site were placed in 13

170 mL of fresh 1 mmol L^{-1} $NaHCO_3$ in 15 mL Falcon tubes that contained about 2 mL of air. The

7

171 tubes were capped and placed horizontally in an incubator (Innova 4230, New Brunswick

172 Scientific) at 17°C under continuous illumination from fluorescent tubes producing about 170

173 μmol m^{-2} s^{-1} photosynthetically active radiation (Macam Q201, Macam Photometrics,

174 Livingstone, Scotland). pH was measured with the combination pH-electrode after 24 hours

175 and roughly after every 12 hours until a maximum pH had been reached. Alkalinity was

176 measured at the end of the experiment on duplicate aliquots by Gran titration.

177

178 *Stable isotope analysis*

179 For the analysis of inorganic carbon δ^{13}C, four mL of helium (99.999%) was injected into the

180 headspace of each exetainer to overpressurise. After shaking and 30 minutes equilibration a

181 40 μL gas sample was removed for analysis. δ^{13}C of the CO_2 was measured on an Isoprime

182 Ltd Tracegas Preconcentrator coupled to an Isoprime Ltd Isoprime isotope ratio mass

183 spectrometer. Pulses of known reference CO_2 and blanks were run prior to each batch.

184 Small amounts of the plant were re-dried at 105°C and aliquots sealed into 6 x 5 mm tin

185 capsules and loaded into an autosampler (Eurovector Elemental Analyser, Eurovector,

186 Milano Italy) coupled in-line to a stable isotope ratio mass spectrometer (Isoprime,

187 Manchester, UK). Each sample was combusted at 1020°C with a pulse of oxygen and

188 products were carried by a flow of helium through a reduction reactor containing copper wire

189 at 650°C and dried with magnesium perchlorate. N_2 and CO_2 were separated by a packed

190 GC column and delivered, via an 'open-split' to the isotope ratio mass spectrometer. Values

191 were compared to pulses of CO_2 reference gas and to a solid working standard of known

192 isotopic composition. Stable isotope methods for inorganic and organic C were accredited to

193 UKAS ISO17025.

194 The δ^{13}C values of CO_2 and bicarbonate were calculated from the measured δ^{13}C of

195 inorganic carbon and carbonate speciation using the temperature-dependent equations in

196 (Mook et al., 1974) following (Maberly et al., 1992). Discrimination of plants against these

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197   potential carbon sources was calculated as in (Maberly, Raven, 1992) and against

198   atmospheric CO_2 with an assumed value of -8‰ (Verburg, 2007).

199   **Results**

200   The water temperature at the five sites varied by 2°C between 13.8°C at site A and 15.8°C

201   at site E (data not shown). Over the first 8 km downstream from the source, represented by

202   the first four sites, there was a large increase in pH from 7.35 to 8.05 (Fig. 2A). This was

203   linked to a decrease in concentration of CO_2 from about 0.46 to 0.09 mmol L^{-1} (24- to 5-times

204   air-equilibrium; Fig. 2B). The CO_2 concentration declined with distance downstream over the

205   first four sites, covering a distance of 6.6 km and a reduction in altitude of 15 m (i.e. a slope

206   of 2.27 m km^{-1}), at about $\log_e$ 0.188 mmol L^{-1} km^{-1}; R^2 = 0.96. Even at the fifth site, 20 km

207   downstream from the source, the concentration of CO_2 was about five times the air-

208   equilibrium concentration. These CO_2 concentrations were calculated from pH, alkalinity and

209   temperature, but direct measurements in the field with the non-dispersive infra-red sensor

210   gave very similar values and a comparison of the two methods across the five sites gave an

211   R^2 of 0.94, but these data from the sensor are not used further. The alkalinity was high at all

212   the sites and in contrast to the concentration of CO_2, its value only changed slightly from

213   4.84 to 4.73 mequiv L^{-1} down the transect. The concentration of bicarbonate ions was

214   correspondingly high and rather constant, varying between 4.83 and 4.68 mmol L^{-1}. The

215   concentration of carbonate was only 0.005 mmol L^{-1} at the source, but because of increasing

216   pH, the concentration had increased to 0.028 mmol L^{-1} (about 4.8-fold increase) at the fifth

217   site. Assuming a calcium concentration of 2 mmol L^{-1}, calcite was about 1.4-times over-

218   saturated at the source increasing to about 7-times over-saturated at the two lower sites

219   (saturation index for calcite 0.15 to 0.85) and this may have been one of the reasons for the

220   slightly lower alkalinity at the lower sites.

221   Nine species or genera of macrophytes and filamentous macroalgae were found at the

222   five sites (Table 1). At the top of the transect, the vegetation was dominated by large

223   underwater populations of *Berula erecta* (Fig. 3) along with the freshwater moss *Fontinalis*

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224 *antipyretica.* At sites D and E with the lowest concentrations of CO_2 these were largely

225 replaced by *Potamogeton nodosus, Stuckenia pectinata* (previously *Potamogeton*

226 *pectinatus*) *Ceratophyllum demersum* and *Ranunculus flutians* (Table 1).

227 All the species of macrophytes at the three sites nearest the source (sites A to C) had

228 final CO_2 concentrations at the end of the pH-drift experiment greater than 1 µmol L^{-1}, raised

229 the pH to less than 9.1 and had C_T/Alk quotients close to 1 (Table 2). The data are

230 consistent with these species being restricted to CO_2 as a source of inorganic carbon for

231 photosynthesis. The same was true for *B. erecta* at site D, but all the other species at this

232 site showed evidence of bicarbonate use with pH values over 10.0 and final CO_2

233 concentrations that were between 20- and 150-times below the CO_2 compensation

234 concentrations generated by the species restricted to CO_2. This was also the case for the

235 species from the fifth site (site E). One species, *Ceratophyllum demersum*, however, showed

236 signs of deterioration during the drift experiment and the final alkalinity at the end of the

237 experiment was over twice the starting alkalinity; pH-drift data from this species are not used

238 further.

239 The $\delta^{13}C$ value of the inorganic carbon was relatively constant: it was most depleted at

240 the source at -11.6‰ and varied between -10.5 and -10.9‰ at the four other sites- very

241 similar to values reported in (Emblanch, Zuppi, 2003). Plant $\delta^{13}C$ values were more negative

242 (depleted) at sites with high concentrations of CO_2 (Fig. 4A). Similarly plant $\delta^{13}C$ values were

243 more negative for plants that were unable to use bicarbonate and hence had higher final

244 CO_2 concentrations at the end of pH-drift experiments (Fig. 4B).The $\delta^{13}C$ values for *Berula*

245 *erecta* tended to be less negative than other plants that were restricted to CO_2.

246 Discrimination, Δ, was calculated for the different plant species for different putative

247 sources of inorganic carbon (Fig. 5). The wholly submerged species that were restricted to

248 CO_2 had an average discrimination value of 26.4 ‰ (SD = 1.4 ‰). If *B. erecta* was restricted

249 to CO_2 in water its average discrimination value would be 15.5 ‰ (SD = 3.0 ‰). If instead its

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250   organic carbon was produced solely from atmospheric CO_2, the average discrimination value

251   would be 28.6 ‰ (SD = 3.2 ‰). For species with the ability to use bicarbonate, two potential

252   forms of inorganic carbon are available. If these species were using CO_2, discrimination

253   against CO_2 would be only 11.2 ‰ (SD = 2.7 ‰) but their discrimination against bicarbonate

254   would be 21.6 ‰ (SD = 2.7 ‰).

255   There was a strong linear relationship between the final CO_2 in pH-drift experiments and

256   the CO_2 concentration at the collection site (Fig. 6). The correlation was even stronger if *B.*

257   *erecta* from site 4 was excluded from the data set (R^2 = 0.99; data not shown).

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259

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Discussion

261 Rivers generally have high concentrations of CO_2 because they receive large amounts of

262 terrestrial organic carbon that has been degraded to inorganic carbon in the soil and have

263 short residence times that limit evasion to the atmosphere (Cole et al., 2007, Raymond et al.,

264 2013, Sand-Jensen and Staehr, 2012) although some rivers can be undersaturated with CO_2

265 during periods of high productivity (Neal et al., 1998). Groundwater-fed rivers often have

266 particularly high concentrations of CO_2 at source because of sub-surface respiration and

267 restricted exchange with the atmosphere (Cole, Prairie, 2007). In the case of the Fontaine de

268 Vaucluse, data in (Emblanch, Zuppi, 2003) were used to calculate a mean concentration of

269 CO_2 of 0.61 mmol L^{-1} over a year at the source. On our sampling day, 0.3 km downstream

270 from the source, the concentration was about 0.46 mmol CO_2 L^{-1}, corresponding to the mean

271 in a compilation from groundwaters from New Zealand and Denmark (Sand-Jensen and

272 Staehr, 2012) and similar to the maximum values reported in high alkalinity rivers in eastern

273 France (Massabuau and Fritz, 1984). In limestone regions, these high concentrations of CO_2

274 are associated with high concentrations of bicarbonate, but high CO_2 associated with

275 groundwater inputs can also be found in non-limestone regions where alkalinity is relatively

276 low (Demars and Thiebaut, 2008).

277 Turbulent flow, especially in streams and rivers with steep slopes, leads to rapid

278 reduction in the concentration of CO_2 by evasion to the atmosphere and net photosynthetic

279 uptake causes the concentration to decline further (Butman and Raymond, 2011, Sand-

280 Jensen and Staehr, 2012). This reduction in concentration of CO_2 was also found here;

281 concentrations declined 2-fold over 3.7 km.

282 The species of macrophytes found in this study are typical of European rivers, especially

283 those with a groundwater influence. For example, all the macrophytes found here were

284 recorded in a survey of macrophytes from groundwater-fed regions of the rivers Rhine,

285 Rhône and Moosach, (Demars and Tremolieres, 2009) apart from *P. nodosus.* Furthermore,

12

286   (Demars and Tremolieres, 2009) showed that plant distribution at different sites was related

287   more to the concentration of CO_2 than to other chemical variables such as the concentration

288   of ammonium or phosphate. We show here that, as previously assumed based on literature

289   information, (Demars and Tremolieres, 2009), this pattern is caused by the differential ability

290   of the different species to utilize bicarbonate as a source of inorganic carbon in addition to

291   CO_2. At the first three sites below the spring down to about 3 km, the concentrations of CO_2

292   exceeded 10-times air-equilibrium (0.46 to 0.22 mmol L^{-1}) and all the species found were

293   unable to use bicarbonate. The amphibious macrophyte *Berula erecta* was dominant at

294   these sites and this species is known to be restricted to CO_2 (Sand-Jensen et al., 1992) and

295   this was confirmed here. Based on our measurements of CO_2 concentration and the kinetic

296   response of photosynthesis to CO_2 in (Sand-Jensen, Pedersen, 1992), we estimate that *B.*

297   *erecta* would be between 75% and 100% saturated with CO_2 at these sites. At site D, where

298   only small amounts of *B. erecta* were found, the CO_2 concentration of 0.09 mmol L^{-1} would

299   only support about 20% of the CO_2 saturated rates. The absence of species able to use

300   bicarbonate at the upper sites, despite substantial concentrations of bicarbonate, exceeding

301   4.8 mmol L^{-1}, is consistent with substantial costs linked to the reduced affinity for CO_2

302   (Maberly and Madsen, 1998), and increased costs of running the process and producing and

303   maintaining the extra machinery required (Jones, 2005). Conversely, at the lowermost sites,

304   all species found and tested, had the ability to use bicarbonate. Here, CO_2 concentrations

305   were only about 4.8-times greater than air-equilibrium which is likely to limit rates of

306   photosynthesis if CO_2 was the only source of inorganic carbon (Sand-Jensen and Frost-

307   Christensen, 1999).

308   The stable carbon isotope data can be used to give some indication of the source of

309   inorganic carbon for the different species of macrophytes at the different sites if putative

310   source values are known (Osmond et al., 1981). pH-drift experiments suggested that *F.*

311   *antipyretica* and *Vaucheria* sp. were restricted to CO_2. Discrimination against CO_2 was about

312   25 to 28 ‰ which is consistent with typical C3 photosynthesis with little diffusion limitation

13

313   (Lloyd and Farquhar, 1994). Although *B. erecta* was also limited to CO_2, discrimination

314   against this carbon source was only 12 to 19 ‰ which could indicate either diffusion

315   limitation or that *B. erecta* may have taken up CO_2 from the atmosphere at very low water

316   level even though the benefit is likely to be small at these very high CO_2 concentrations in

317   the river as suggested by work on another amphibious species (Madsen and Breinholt,

318   1995). The discrimination values for species shown to use bicarbonate in the drift

319   experiments were very low (8 to 16 ‰) against CO_2 but much more typical if expressed

320   against bicarbonate (18 to 26 ‰) which may indicate that this was the dominant source of

321   inorganic carbon even at moderate concentrations of CO_2.

322   *B. erecta* was found at four of the five sites, growing in CO_2 concentrations from 0.46 to

323   0.09 mmol L^{-1}. Although we do not have information on seasonal changes in CO_2 at the

324   sites, the downstream gradient is likely to be relatively stable because the annual variation of

325   discharge is relatively low and because the data in (Emblanch, Zuppi, 2003) show no

326   significant correlation between CO_2 concentration at the source and discharge (r = -0.039).

327   There was, however, some slight evidence for change in the CO_2 response of the different

328   populations. In pH-drift experiments, the *B. erecta* population at the site nearest the spring

329   had a final CO_2 concentration of about 19 μmol L^{-1} whereas the three downstream

330   populations sampled had final CO_2 concentrations of between 2 and 4 μmol L^{-1}. In contrast,

331   the final CO_2 concentration of the aquatic moss, *F. antipyretica,* that was found at the three

332   upper sites, was invariable at 2 to 3 μmol L^{-1}. More detailed work will be needed to

333   determine whether phenotypic or genotypic change is involved in the putative differences in

334   the *B. erecta* populations. Genotypic change is possible given the potentially large number of

335   generations that could have occurred at this site over thousands of years. However, a study

336   of soil algae at two high CO_2 (aerial) springs, found little evidence for genetic adaptation to

337   high CO_2 concentrations (Collins and Bell, 2006). The ability of plants such as *B. erecta* to

338   grow well in rivers is strongly linked to the high concentrations of CO_2 that can sometimes be

339   found there (Sand-Jensen, Pedersen, 1992) and also explains why these species are

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340   relatively rare in lakes which tend to have much lower CO_2 concentrations during most of the

341   year (Baattrup-Pedersen et al., 2013). Although bicarbonate users can down-regulate their

342   ability to use bicarbonate depending on inorganic carbon availability (Madsen et al., 1996)

343   this does not appear to have allowed them to compete with *B. erecta* when concentrations of

344   CO_2 were high at upstream sites.

345   Decomposition processes in the recharge zone can produce high concentrations of CO_2

346   in groundwater-fed rivers. This, coupled with a gradient of CO_2 loss downstream, produces a

347   natural experiment to test the effect of elevated CO_2 on the biodiversity and physiology of

348   aquatic plants in rivers which helps to explain the distribution of macrophytes in rivers.

349

357

358 **Figure legends**

359 Fig. 1. Location of the sampling site in France (A), showing the catchment area (dashed

360 line), the source (cross) and the downstream flow to the River Rhône (B) and the five sample

361 sites A to E (C).

362 Fig. 2. Changes in carbonate chemistry on the River Sorgue with distance downstream from

363 the source at Fontaine de Vaucluse. pH (A); alkalinity (open circles) and concentration of

364 bicarbonate (closed circles; B); concentration of carbon dioxide measured directly (open

365 circles) and calculated from pH, alkalinity and temperature (closed circles) with air-

366 equilibrium indicated by a dashed line (C) and concentration of carbonate (D).

367 Fig. 3. The River Sorgue downstream from site A. Showing general appearance of the river

368 (A) and the dominance of *Berula erecta* (B).

369 Fig. 4. Plant $\delta^{13}C$ values from the five sites for *Berula erecta* (open triangles), submerged

370 CO_2-users (open circles), and bicarbonate users (closed circles). A Plant $\delta^{13}C$ as a function

371 of site CO_2 concentration; B Plant $\delta^{13}C$ as function of final CO_2 concentration in pH-drift

372 experiments. The regression lines relate to all the combined data. Note the Log scale for

373 concentration of CO_2.

374 Fig. 5. Calculated discrimination, Δ, against air, (assumed -8‰; grey bars), carbon dioxide

375 (white bars) and bicarbonate (black bars) for the different species on the River Sorgue below

376 Fontaine de Vaucluse. Species are ordered by increasing ability to deplete inorganic carbon

377 in pH-drift experiments. The asterisk indicates the likely source of inorganic carbon based on

378 pH-drift experiments.

379 Fig. 6. Correlation between average final concentration of CO_2 at the end of a pH-drift

380 experiment and the concentration of CO_2 at the sites from which the samples were collected.

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16

383   References

384   Baattrup-Pedersen A, Madsen TV, Riis T, Cavalli G. Photosynthetic performance of submerged

385   macrophytes from lowland stream and lake habitats with contrasting CO_2 availability. New

386   Phytologist. 2013;198:1135-42.

387   Black MA, Maberly SC, Spence DHN. Resistances to carbon-dioxide fixation in four submerged

388   freshwater macrophytes. New Phytologist. 1981;89:557-68.

389   Blavoux B, Mudry J, Puig JM. The karst system of the Fontaine de Vaucluse (Southeastern France).

390   Environmental Geology and Water Sciences. 1992;19:215-25.

391   Bonacci O. Analysis of long-term (1878-2004) mean annual discharges of the karst spring Fontaine de

392   Vaucluse (France). Acta Carsologica. 2007;36:151-56.

393   Butman D, Raymond PA. Significant efflux of carbon dioxide from streams and rivers in the United

394   States. Nature Geoscience. 2011;4:839-42.

395   Cole JJ, Prairie YT, Caraco NF, McDowell WH, Tranvik LJ, Striegl RG, et al.Plumbing the global carbon

396   cycle: Integrating inland waters into the terrestrial carbon budget. Ecosystems. 2007;10:171-84.

397   Collins S, Bell G. Evolution of natural algal populations at elevated CO_2. Ecology Letters. 2006;9:129-

398   35.

399   Demars BOL, Thiebaut G. Distribution of aquatic plants in the Northern Vosges rivers: implications

400   for biomonitoring and conservation. Aquatic Conservation-Marine and Freshwater Ecosystems.

401   2008;18:619-32.

402   Demars BOL, Tremolieres M. Aquatic macrophytes as bioindicators of carbon dioxide in groundwater

403   fed rivers. Science of the Total Environment. 2009;407:4752-63.

404   Emblanch C, Zuppi GM, Mudry J, Blavoux B, Batiot C. Carbon 13 of TDIC to quantify the role of the

405   unsaturated zone: the example of the Vaucluse karst systems (Southeastern France). Journal of

406   Hydrology. 2003;279:262-74.

17

407 Garry B, Blondel T, Emblanch C, Sudre C, Bilgot S, Cavaillou A, et al.Contribution of artificial galleries

408   to the knowledge of karstic system behaviour in addition to natural cavern data. International

409   Journal of Speleology. 2008;37:75-82.

410 Hall-Spencer JM, Rodolfo-Metalpa R, Martin S, Ransome E, Fine M, Turner SM, et al.Volcanic carbon

411   dioxide vents show ecosystem effects of ocean acidification. Nature. 2008;454:96-99.

412 Jacobson RL, Langmuir D. Dissociation constants of calcite and $CaHCO_3^{+}$ from 0 to 50°C. Geochimica

413   et Cosmochimica Acta. 1974;38:301–18.

414 Johnson MS, Billett MF, Dinsmore KJ, Wallin M, Dyson KE, Jassal RS. Direct and continuous

415   measurement of dissolved carbon dioxide in freshwater aquatic systems-method and

416   applications. Ecohydrology. 2010;3:68-78.

417 Jones J. The metabolic cost of bicarbonate use in the submerged plant *Elodea nuttallii*. Aquatic

418   Botany. 2005;83:71-81.

419 Klavsen SK, Madsen TV, Maberly SC. Crassulacean acid metabolism in the context of other carbon-

420   concentrating mechanisms in freshwater plants: a review. Photosynthesis Research.

421   2011;109:269-79.

422 Korner C, Miglietta F. Long-term effects of naturally elevated CO_2 on mediterranean grassland and

423   forest trees. Oecologia. 1994;99:343-51.

424 Krause-Jensen D, Sand-Jensen K. Light attenuation and photosynthesis of aquatic plant communities.

425   Limnology and Oceanography. 1998;43:396-407.

426 Kroth P. The Biodiversity of Carbon Assimilation. J Plant Physiol 2015; 172: pp-pp

427 Lloyd J, Farquhar GD. C13 discriminationi during CO_2 assimilation by the terrestrial biosphere.

428   Oecologia. 1994;99:201-15.

429 Maberly SC. Photosynthesis by *Fontinalis antipyretica* .2. Assessment of environmental-factors

430   limiting photosynthesis and production. New Phytologist. 1985;100:141-55.

431 Maberly SC. Exogenous sources of inorganic carbon for photosynthesis by marine macroalgae.

432   Journal of Phycology. 1990;26:439-49.

18

433   Maberly SC. Diel, episodic and seasonal changes in pH and concentrations of inorganic carbon in a

434   productive lake. Freshwater Biology. 1996;35:579-98.

435   Maberly SC, Barker PA, Stott AW, De Ville MM. Catchment productivity controls CO_2 emissions from

436   lakes. Nature Climate Change. 2013;3:391-94.

437   Maberly SC, Madsen TV. Affinity for CO_2 in relation to the ability of freshwater macrophytes to use

438   HCO_3. Functional Ecology. 1998;12:99-106.

439   Maberly SC, Madsen TV. Freshwater angiosperm carbon concentrating mechanisms: processes and

440   patterns. Functional Plant Biology. 2002;29:393-405.

441   Maberly SC, Raven JA, Johnston AM. Discrimination between ^{12}C and ^{13}C by marine plants.

442   Oecologia. 1992;91:481-92.

443   Mackereth FJH, Heron J, Talling JF. Water analysis: some revised methods for limnologists.

444   Freshwater Biological Association Scientific Publication. 1978:1-120.

445   Madsen TV, Breinholt M. Effects of air contact on growth, inorganic carbon sources, and nitrogen

446   uptake by an amphibioius freshwater macrophyte. Plant Physiology. 1995;107:149-54.

447   Madsen TV, Maberly SC. High internal resistance to CO_2 uptake by submerged macrophytes that use

448   HCO_3-: measurements in air, nitrogen and helium. Photosynthesis Research. 2003;77:183-90.

449   Madsen TV, Maberly SC, Bowes G. Photosynthetic acclimation of submersed angiosperms to CO_2 and

450   HCO_3. Aquatic Botany. 1996;53:15-30.

451   Massabuau J-C, Fritz B. Respiratory gas content (O_2 and CO_2) and ionic composition of river water in

452   the plain of Alsace (eastern France). In: Eriksson E, editor. Hydrochemical balances of

453   freshwater systems. Uppsala: IAHS Press; 1984. p. 107-15.

454   Mook WG, Bommerso.Jc, Staverma.Wh. Carbon Isotope Fractionation between Dissolved

455   Bicarbonate and Gaseous Carbon-Dioxide. Earth and Planetary Science Letters. 1974;22:169-76.

456   Neal C, House WA, Jarvie HP, Eatherall A. The significance of dissolved carbon dioxide in major

457   lowland rivers entering the North Sea. Science of the Total Environment. 1998;210:187-203.

19

458   Osmond CB, Valaane N, Haslam SM, Uotila P, Roksandic Z. Comparisons of delta-C-13 values in

459   leaves of aquatic macrophytes from different habitats in Britain and Finland - some implications

460   for photosynthetic processes in aquatic plants. Oecologia. 1981;50:117-24.

461   Pedersen O, Colmer TD, Sand-Jensen K. Underwater photosynthesis of submerged plants - recent

462   advances and methods. Frontiers in Plant Science. 2013;4.

463   Raymond PA, Hartmann J, Lauerwald R, Sobek S, McDonald C, Hoover M, et al.Global carbon dioxide

464   emissions from inland waters. Nature. 2013;503:355-59.

465   REALPACA. Bulletin de situation hydrologique. REALPACA (http://wwwpacadeveloppement-

466   durablegouvfr/IMG/pdf/Bulletin_hydro_paca_Septembre_2013_cle53be71pdf). 2013;N180:11

467   Reynolds CS, Descy JP. The production, biomass and structure of phytoplankton in large rivers.

468   Archiv fuer Hydrobiologie Supplement. 1996;113:161-87.

469   Sage RF, Stata M. Photosynthetic Diversity meets Biodiversity: The C_4 plant Example. J Plant Physiol

470   2015: 172: pp-pp.

471   Sand-Jensen K, Frost-Christensen H. Plant growth and photosynthesis in the transition zone between

472   land and stream. Aquatic Botany. 1999;63:23-35.

473   Sand-Jensen K, Pedersen MF, Nielsen SL. Photosynthetic use of inorganic carbon among primary and

474   secondary water plants in streams. Freshwater Biology. 1992;27:283-93.

475   Sand-Jensen K, Staehr PA. CO_2 dynamics along Danish lowland streams: water-air gradients, piston

476   velocities and evasion rates. Biogeochemistry. 2012;111:615-28.

477   Stets EG, Striegl RG, Aiken GR, Rosenberry DO, Winter TC. Hydrologic support of carbon dioxide flux

478   revealed by whole-lake carbon budgets. Journal of Geophysical Research-Biogeosciences.

479   2009;114.

480   Verburg P. The need to correct for the Suess effect in the application of delta C-13 in sediment of

481   autotrophic Lake Tanganyika, as a productivity proxy in the Anthropocene. Journal of

482   Paleolimnology. 2007;37:591-602.

483   Waldron S, Scott EM, Soulsby C. Stable isotope analysis reveals lower-order river dissolved inorganic

484   carbon pools are highly dynamic. Environmental Science & Technology. 2007;41:6156-62.

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Table 1

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Table 1.

Species composition at the five sites on the River Sorgue (see Fig. 1 for location).

Species	A	B	C	D	E
Berula erecta (Huds.) Coville	■	■	■	■	
Fontinalis antipyretica Hedw.	■	■	■		
Vaucheria sp. DC.		■			
Lemna trisulca L.				■	
Stuckenia pectinata (L.) Böerner				■	
Cladophora sp. Kütz.				■	
Ceratophyllum demersum L.					■
Potamogeton nodosus Poir.					■
Ranunculus fluitans L.					■

Table 2

Table 2.

Mean carbonate equilibria from triplicate pH-drift experiments. Values are the maximal pH, the minimal concentration of total inorganic carbon (C_T) and CO_2 and the quotient of C_T/alkalinity.

Site	Species	pH	C_T (mmol L^{-1})	CO_2 (mmol L^{-1})	C_T/Alk
A	*Berula erecta*	7.61	0.348	1.98E-02	1.06
A	*Fontinalis antipyretica*	9.02	0.960	2.16E-03	0.95
B	*Berula erecta*	8.55	0.324	2.20E-03	0.99
B	*Fontinalis antipyretica*	8.91	0.971	2.81E-03	0.96
B	*Vaucheria* sp.	8.54	1.174	8.16E-03	0.99
C	*Berula erecta*	8.34	0.328	3.57E-03	1.00
C	*Fontinalis antipyretica*	9.05	0.957	1.99E-03	0.95
D	*Berula erecta*	8.29	0.328	4.03E-03	1.00
D	*Cladophora* sp.	10.73	0.458	6.33E-06	0.42
D	*Lemna trisulca*	10.31	0.734	4.61E-05	0.61
D	*Stuckenia pectinata*	10.43	0.339	1.62E-05	0.49
E	*Ceratophyllum demersum*	9.72	2.266	9.10E-04	0.81
E	*Potamogeton nodosus*	10.27	0.805	6.99E-05	0.60
E	*Ranunculus fluitans*	10.45	0.294	1.19E-05	0.48

Figure 1

Fig. 1.

Figure 2

Fig. 2

Figure 3

Fig. 3

Figure 4

Fig. 4

Figure 5

Fig. 5

Figure 6

$y = 0.028x - 0.002$
$R^2 = 0.968$

Fig. 6