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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 CO₂ 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 CO₂ on the ability of freshwater plants to use bicarbonate. Plants close to the source, where the concentration of CO₂ was up to 24-times air equilibrium, were dominated by *Berula erecta*. pH-drift results and discrimination against ¹³C were consistent with this and the other species being restricted to CO₂ and unable to use the high concentration of bicarbonate. There was some indication from stable ¹³C data that *B. erecta* may have had access to atmospheric CO₂ at low water levels. In contrast, species downstream, where concentrations of CO₂ were only about 5-times air-equilibrium were almost exclusively able to use bicarbonate, based on pH-drift results. Discrimination against ¹³C 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 CO₂. 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 CO₂

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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 CO₂ 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 CO₂ on the ability of freshwater plants to use bicarbonate. Plants close to
30 the source, where the concentration of CO₂ was up to 24-times air equilibrium, were
31 dominated by *Berula erecta*. pH-drift results and discrimination against ¹³C were consistent
32 with this and the other species being restricted to CO₂ and unable to use the high
33 concentration of bicarbonate. There was some indication from stable ¹³C data that *B. erecta*
34 may have had access to atmospheric CO₂ at low water levels. In contrast, species
35 downstream, where concentrations of CO₂ were only about 5-times air-equilibrium were
36 almost exclusively able to use bicarbonate, based on pH-drift results. Discrimination against
37 ¹³C 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 CO₂. This was produced largely by altered species composition, but partly
41 by phenotypic changes in individual species.

42

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

45

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 prevent 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 CO₂ 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 CO₂ 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 (Klavnsen et al., 2011).
70 Avoidance strategies involve living in environments where the concentration of CO₂ 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

73 CO₂ 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 and
78 Stata (2015). Because of physico-chemical equilibria, bicarbonate is less susceptible than
79 CO₂ to depletion during removal of inorganic carbon by photosynthesis and is present at
80 concentrations that exceed CO₂ 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 CO₂ than species that are restricted to
93 CO₂ (Maberly and Madsen, 1998). This is probably caused by a high internal resistance to
94 CO₂ 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 CO₂.

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

100 physiology of plants or algae at sites with locally high concentrations of CO₂ with those at
101 nearby sites with lower concentrations of CO₂. An example in the terrestrial environment
102 includes studies at natural CO₂ 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 CO₂ 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 CO₂ concentrations in different surface waters
107 and the frequently high CO₂ 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 CO₂ 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 CO₂.
114 Furthermore, since this CO₂ gradient is likely to have been present for thousands of years, it
115 allows adaptive responses to varying CO₂ 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 $23 \text{ m}^3 \text{ s}^{-1}$ and minimum and maximum
123 discharges of 4 and over $100 \text{ m}^3 \text{ s}^{-1}$ 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 $0.0065 \text{ mol L}^{-1}$ 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 $9 \text{ m}^3 \text{ s}^{-1}$ (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

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

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 $\mu\text{mol m}^{-2} \text{s}^{-1}$ 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 $\delta^{13}\text{C}$, 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. $\delta^{13}\text{C}$ 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 $\delta^{13}\text{C}$ values of CO_2 and bicarbonate were calculated from the measured $\delta^{13}\text{C}$ 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

197 potential carbon sources was calculated as in (Maberly, Raven, 1992) and against
198 atmospheric CO₂ 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 CO₂ from about 0.46 to 0.09 mmol L⁻¹ (24- to 5-times
204 air-equilibrium; Fig. 2B). The CO₂ 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; R² = 0.96. Even at the fifth site, 20 km
207 downstream from the source, the concentration of CO₂ was about five times the air-
208 equilibrium concentration. These CO₂ 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 R² 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 CO₂, 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*

224 *antipyretica*. At sites D and E with the lowest concentrations of CO₂ these were largely
225 replaced by *Potamogeton nodosus*, *Stuckenia pectinata* (previously *Potamogeton*
226 *pectinatus*) *Ceratophyllum demersum* and *Ranunculus fluitans* (Table 1).

227 All the species of macrophytes at the three sites nearest the source (sites A to C) had
228 final CO₂ 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 C₇/Alk quotients close to 1 (Table 2). The data are
230 consistent with these species being restricted to CO₂ 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 CO₂
233 concentrations that were between 20- and 150-times below the CO₂ compensation
234 concentrations generated by the species restricted to CO₂. 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 δ¹³C 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 δ¹³C values were more negative
242 (depleted) at sites with high concentrations of CO₂ (Fig. 4A). Similarly plant δ¹³C values were
243 more negative for plants that were unable to use bicarbonate and hence had higher final
244 CO₂ concentrations at the end of pH-drift experiments (Fig. 4B). The δ¹³C values for *Berula*
245 *erecta* tended to be less negative than other plants that were restricted to CO₂.

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 CO₂ had an average discrimination value of 26.4 ‰ (SD = 1.4 ‰). If *B. erecta* was restricted
249 to CO₂ in water its average discrimination value would be 15.5 ‰ (SD = 3.0 ‰). If instead its

250 organic carbon was produced solely from atmospheric CO₂, 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 CO₂, discrimination
253 against CO₂ 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 CO₂ in pH-drift experiments and
256 the CO₂ 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).

258

259

260 **Discussion**

261 Rivers generally have high concentrations of CO₂ 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 CO₂
265 during periods of high productivity (Neal et al., 1998). Groundwater-fed rivers often have
266 particularly high concentrations of CO₂ 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 CO₂ 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 CO₂ 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 CO₂
274 are associated with high concentrations of bicarbonate, but high CO₂ 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 CO₂ 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 CO₂ 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,

286 (Demars and Tremolieres, 2009) showed that plant distribution at different sites was related
287 more to the concentration of CO₂ 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 CO₂. At the first three sites below the spring down to about 3 km, the concentrations of CO₂
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 CO₂ (Sand-Jensen et al., 1992) and
295 this was confirmed here. Based on our measurements of CO₂ concentration and the kinetic
296 response of photosynthesis to CO₂ in (Sand-Jensen, Pedersen, 1992), we estimate that *B.*
297 *erecta* would be between 75% and 100% saturated with CO₂ at these sites. At site D, where
298 only small amounts of *B. erecta* were found, the CO₂ concentration of 0.09 mmol L⁻¹ would
299 only support about 20% of the CO₂ 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 CO₂
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, CO₂ concentrations
305 were only about 4.8-times greater than air-equilibrium which is likely to limit rates of
306 photosynthesis if CO₂ 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 CO₂. Discrimination against CO₂ was about
312 25 to 28 ‰ which is consistent with typical C3 photosynthesis with little diffusion limitation

313 (Lloyd and Farquhar, 1994). Although *B. erecta* was also limited to CO₂, 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 CO₂ from the atmosphere at very low water
316 level even though the benefit is likely to be small at these very high CO₂ 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 CO₂ 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 CO₂.

322 *B. erecta* was found at four of the five sites, growing in CO₂ concentrations from 0.46 to
323 0.09 mmol L⁻¹. Although we do not have information on seasonal changes in CO₂ 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 CO₂ concentration at the source and discharge ($r = -0.039$).
327 There was, however, some slight evidence for change in the CO₂ response of the different
328 populations. In pH-drift experiments, the *B. erecta* population at the site nearest the spring
329 had a final CO₂ concentration of about 19 μmol L⁻¹ whereas the three downstream
330 populations sampled had final CO₂ concentrations of between 2 and 4 μmol L⁻¹. In contrast,
331 the final CO₂ 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 CO₂ (aerial) springs, found little evidence for genetic adaptation to
337 high CO₂ 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 CO₂ that can sometimes be
339 found there (Sand-Jensen, Pedersen, 1992) and also explains why these species are

340 relatively rare in lakes which tend to have much lower CO₂ 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 CO₂ were high at upstream sites.

345 Decomposition processes in the recharge zone can produce high concentrations of CO₂
346 in groundwater-fed rivers. This, coupled with a gradient of CO₂ loss downstream, produces a
347 natural experiment to test the effect of elevated CO₂ on the biodiversity and physiology of
348 aquatic plants in rivers which helps to explain the distribution of macrophytes in rivers.

349

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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 $\delta^{13}\text{C}$ values from the five sites for *Berula erecta* (open triangles), submerged
 370 CO_2 -users (open circles), and bicarbonate users (closed circles). A Plant $\delta^{13}\text{C}$ as a function
 371 of site CO_2 concentration; B Plant $\delta^{13}\text{C}$ 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.

381

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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
<i>Berula erecta</i> (Huds.) Coville	■	■	■	■	
<i>Fontinalis antipyretica</i> Hedw.	■	■	■		
<i>Vaucheria</i> sp. DC.		■			
<i>Lemna trisulca</i> L.				■	
<i>Stuckenia pectinata</i> (L.) Böerner				■	
<i>Cladophora</i> sp. Kütz.				■	
<i>Ceratophyllum demersum</i> L.					■
<i>Potamogeton nodosus</i> Poir.					■
<i>Ranunculus fluitans</i> L.					■

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

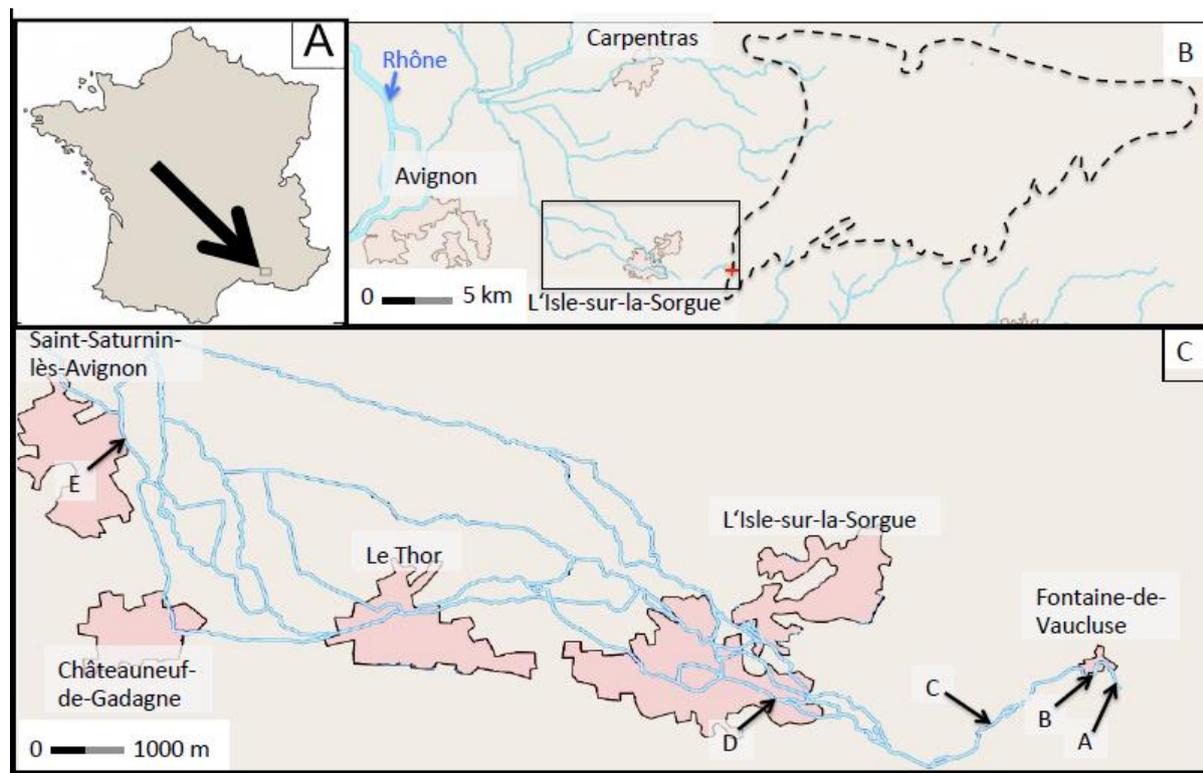


Fig. 1.

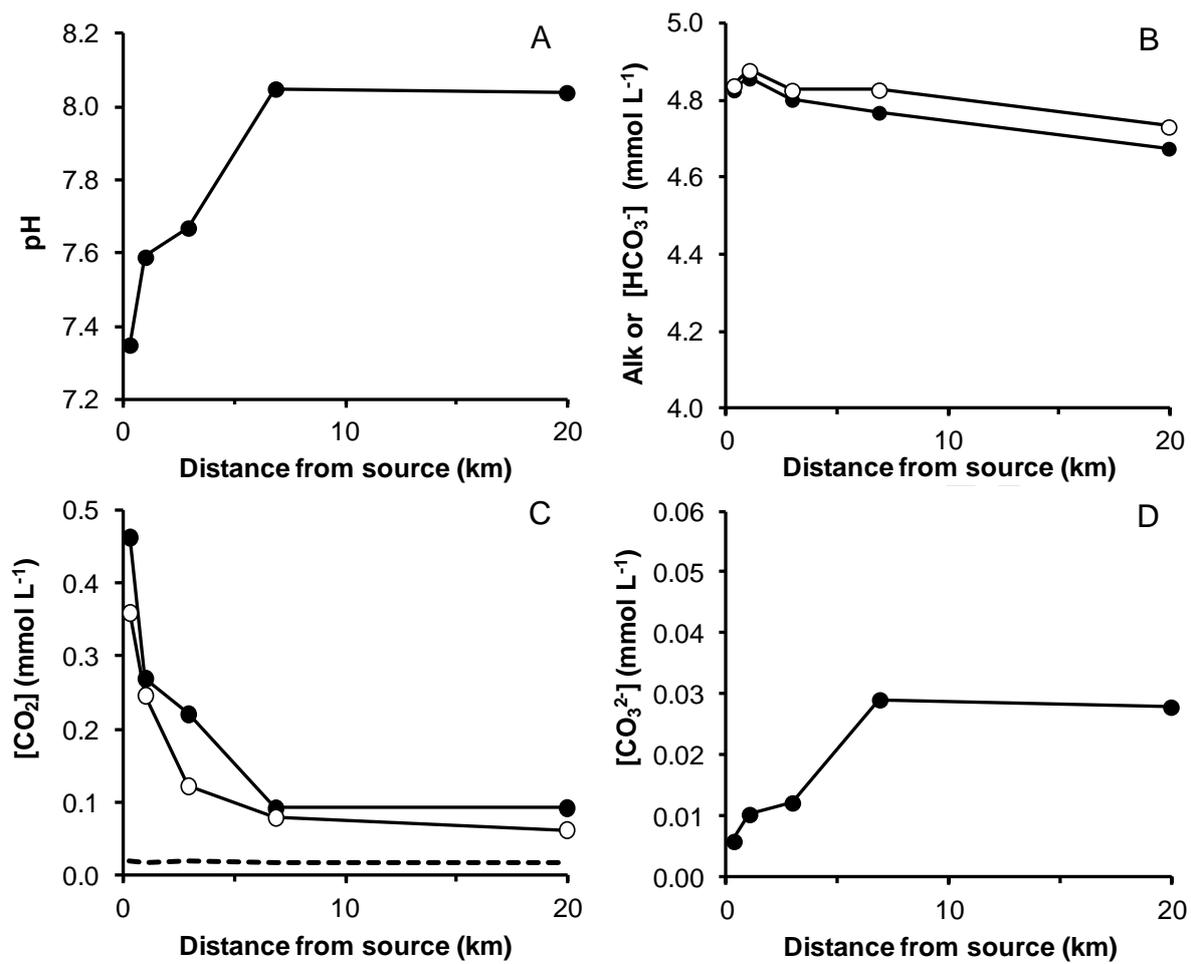


Fig. 2



Fig. 3

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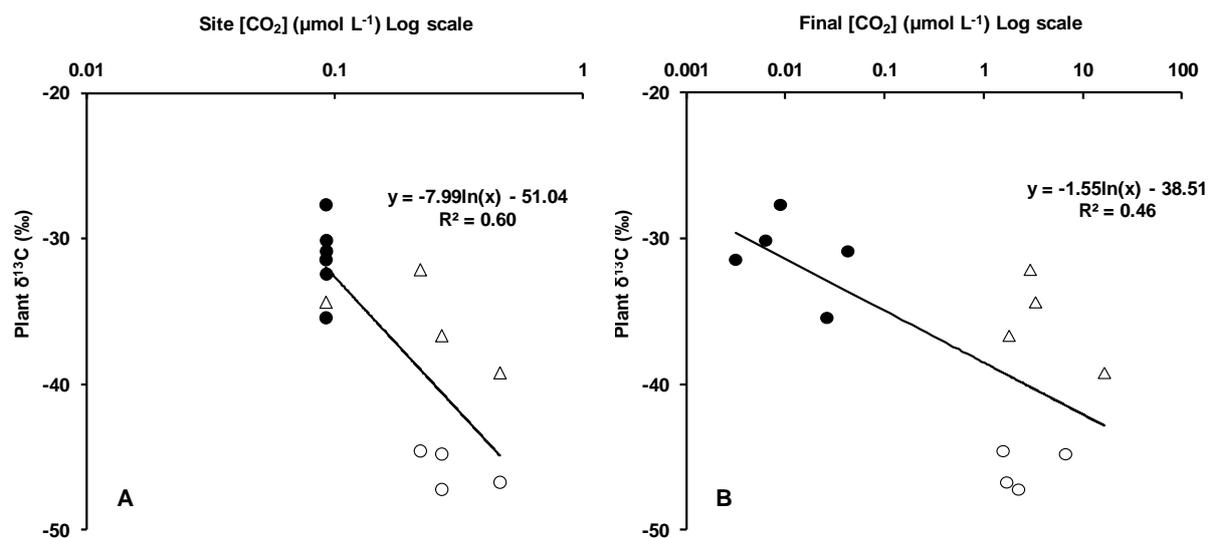


Fig. 4

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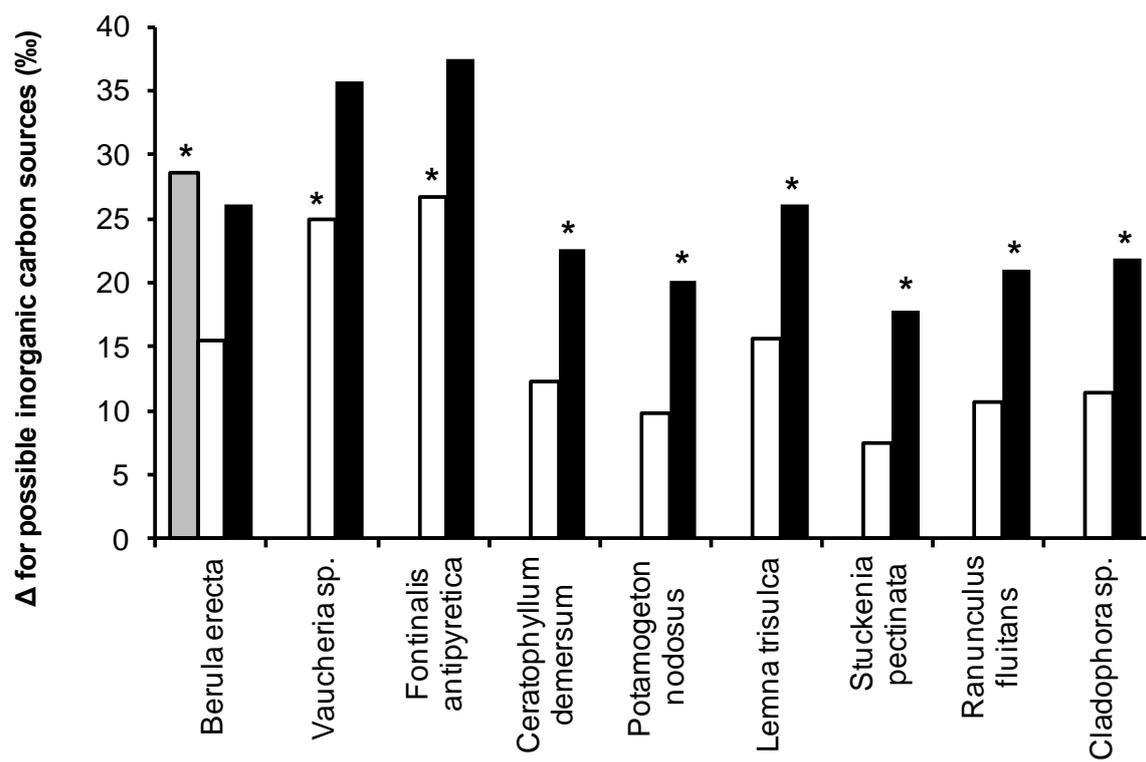


Fig. 5

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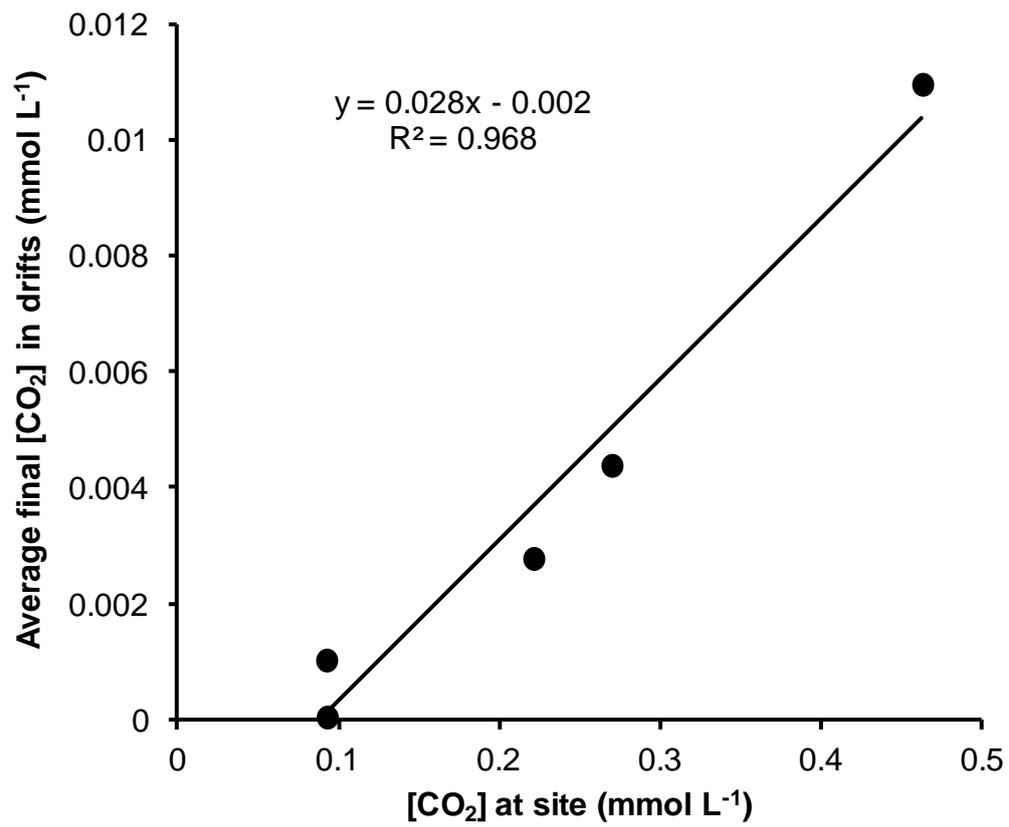


Fig. 6