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Acclimation of photosynthesis to supersaturated CO_2 in aquatic plant bicarbonate users

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SUMMARY

1. The photosynthesis of submerged aquatic plants can be limited by carbon dioxide (CO_2) because of the low diffusivity of dissolved gas in water. About half of the species are able to use bicarbonate (HCO_3^-) as an alternative source of dissolved inorganic carbon (DIC). HCO_3^- is usually available in several-fold higher concentrations than CO_2 at pH above seven, but its affinity is lower and the costs are higher than for CO_2 use. The effects of changes in CO_2 concentrations on the growth and physiology of bicarbonate users remain to be explored comparatively across a wide range of species.
2. We studied, in the laboratory, the plasticity in photosynthetic carbon kinetics and tissue composition of 10 submerged species, all able to use bicarbonate, acclimated to air saturated and 22 times supersaturated CO_2 concentrations with the same concentration of DIC (0.85 mM).
3. Growth rate of all was stimulated (1.1–2.5 fold) and plants allocated more biomass in roots under elevated CO_2 over the 35 days of the acclimation period. The photosynthetic affinity for HCO_3^- was higher in species acclimated to air saturated rather than supersaturated CO_2. Net photosynthesis was more stimulated (2–6 times) by CO_2 supersaturation concentrations after acclimation to supersaturated CO_2 concentration due to their reduced HCO_3^- use capacity.
4. Plants grown under elevated CO_2 showed reduced investment in tissue nitrogen, protein and free amino acids. Moreover, the high CO_2 treatments yielded overall lower gamma-aminobutyric acid (GABA) pools, an indicator of stress relief or changes in nitrogen metabolism.
5. The analysis of 10 freshwater plants confirms that availability of HCO_3^-, and CO_2 is a key regulator of plant physiology affecting growth. Inorganic carbon availability affects the energy budget of plants thereby influencing an array of ecological processes, and hence the distribution of submerged macrophytes in the wild.

Keywords: bicarbonate user, carbon dioxide, metabolite composition, nitrogen, submerged macrophytes

Introduction

Terrestrial plants have evolved different carbon concentration mechanisms (CCMs), such as C_4 and CAM photosynthesis to ensure efficient assimilation of CO_2 at the site of RubisCO, particularly in warm and dry climates where it is essential to reduce photorespiration and attain high CO_2 fixation relative to water loss (Sage, Sage & Kocacinar, 2012; Christin & Osborne, 2014; Sage & Stata, 2015). Even though many fresh waters are supersaturated with CO_2 (Titus, Feldman & Grisé, 1990; Butman & Raymond, 2011; Raymond *et al.*, 2013) and the partial pressure of CO_2 will increase due to the atmospheric CO_2 increase (Hasler *et al.*, 2016), aquatic plants face an even greater risk of CO_2 limitation of photosynthesis than land plants. This is due to the 99.99% lower diffusion coefficient of CO_2 in water and diurnal and spatial limitation of carbon availability caused by habitat conditions and the photosynthetic uptake of aquatic primary producers (Sand-Jensen, 1989; Santamaria, 2002; Pedersen, Colmer

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& Sand-Jensen, 2013; Maberly *et al.*, 2015). These differences in CO_2 availability affect macrophyte communities in freshwater ecosystems (Demars & Trémolières, 2009; Maberly *et al.*, 2015).

The most common adaptation to alleviate inorganic carbon limitation in submerged freshwater plants is to actively use dissolved HCO_3^- (bicarbonate), which is present in alkaline fresh waters at much higher concentrations than CO_2 at air saturation (Sand-Jensen, 1989; Santamaria, 2002). In addition, many freshwater plants have access to alternative carbon sources in the sediments (e.g., rosette species, Winkel & Borum, 2009) and the atmosphere (floating-leaved plants, Maberly & Madsen, 2002), or they are confined to streams enriched with seepage of CO_2-rich soil and ground water (Sand-Jensen & Staehr, 2012) or local lake habitats enriched with CO_2 from sediment respiration (Maberly, 1985). Despite these alternative carbon sources and physiological mechanisms to optimise the inorganic supply, photosynthesis of freshwater plants may still not be saturated by the ambient carbon supply (Maberly & Madsen, 2002).

Here, we tested the extent of carbon limitation of photosynthesis and growth of 10 submerged freshwater plants that can all utilise dissolved HCO_3^-. Photosynthesis and growth rate of these freshwater plants, despite their ability to use HCO_3^-, can be severely constrained by inorganic carbon supply in water at air saturation, and therefore, they will be stimulated by CO_2 supersaturation. While in the short term, submerged aquatic plants show a strong stimulation of photosynthesis under elevated CO_2 availabilities, and this stimulation is more pronounced in plants acclimated to CO_2 limitation and HCO_3^- use (Sand-Jensen & Gordon, 1986; Madsen, Maberly & Bowes, 1996; Hussner & Jahns, 2015), the long-term effects may be less as found in terrestrial plants. Terrestrial plants exhibit a relatively modest (<20%) stimulation of growth rates by elevated CO_2 (Long *et al.*, 2004; Leakey *et al.*, 2009). However, the few available growth studies suggest that the long-term growth rates of freshwater plants, which determine their ecological performance in the field, can be severely carbon limited (Madsen & Sand-Jensen, 1987; Titus *et al.*, 1990; Vadstrup & Madsen, 1995; Pagano & Titus, 2007).

According to cost–benefit principles, the investment in active uptake and internal CCMs as well as in carboxylating enzymes increases the costs of synthesis, maintenance and operation of the cells (von Caemmerer *et al.*, 2014; Raven, Beardall & Giordano, 2014). CO_2-rich conditions, in contrast, permit energy savings for carbon fixation, active transport and synthesis and maintenance of proteins (Madsen *et al.*, 1996) and, thus, can lead to decreased nitrogen content of leaves as shown for terrestrial plants (Leakey *et al.*, 2009).

Our overall objective was to examine the influence of supersaturated and air-saturated CO_2 concentrations on photosynthesis, respiration, growth, allocation and tissue concentrations of nitrogen, proteins and metabolites of freshwater plants. We kept the total concentration of dissolved inorganic carbon ($DIC = CO_2 + HCO_3^- + CO_3^{2-}$) constant and CO_3^{2-} extremely low in the experiments, but varied the proportion of CO_2 to HCO_3^- by manipulating pH (see Titus *et al.*, 1990) and, thereby, free CO_2 from air saturation (LC) to 22-fold supersaturation (HC). Because freshwater plants have a much greater affinity for CO_2 than HCO_3^- (Maberly & Spence, 1983; Pedersen *et al.*, 2013), CO_2 kept at the same limiting concentration as HCO_3^- can sustain a much higher photosynthesis without involving active transmembrane processes (Raven & Beardall, 2014). We predict that elevated CO_2 concentrations, under moderate bicarbonate concentrations, should: (i) stimulate growth; (ii) reduce investment in active HCO_3^- uptake and, thus, reduce HCO_3^- affinity in photosynthesis; (iii) reduce nitrogen and protein content and (iv) alter the composition of metabolites (including organic acids of the tricarboxylic acid cycle and photorespiratory intermediates).

Methods

Laboratory culture

Ten species were selected among the Hydrocharitaceae (*Elodea canadensis* and *Elodea nuttallii*, *Egeria densa*, *Egeria najas*, *Hydrilla verticillata* and *Vallisneria spiralis*), Haloragaceae (*Myriophyllum heterophyllum*, *Myriophyllum spicatum*), Ceratophyllaceae (*Ceratophyllum demersum*) and Potamogetonaceae (*Stuckenia pectinata*). *Hydrilla verticillata* came from a New Zealand strain (National Institute of Water and Atmospheric Research). *Egeria najas* (Hydrocharitaceae) was provided by an aquatic plant trader. The remaining eight species were collected nearby Heinrich Heine University, Düsseldorf, Germany. Prior to the experiments, the plants were cultivated for at least 4 weeks under standard conditions in 150-L aquaria. The plants were rooted in nutrient-rich natural sediment and compost mixture (Düsseldorf Botanic Garden). The aquaria were filled with the general purpose culture solution of Smart & Barko (1985), with pH adjusted to 7.2–7.5 units by continuously adding low amounts of CO_2, adjusted with a regulator. The water was filtered by aquarium filters (type 2213; Eheim, Deizisau). Irradiance was set at about 100 μmol photons m^{-2} s^{-1} in a 16:8 light dark cycle at 23 °C.

Low and high CO_2 experimental acclimations

Rootless apices of the same length (10 ± 1 cm, except for small intact plants of *V. spiralis*) were used with initial dry masses of 0.029–0.178 g. Initial dry mass was determined using the proportion of fresh mass to dry mass (drying at 105 °C to constant mass) for a subsample of plants of similar size to those used in the growth experiments. Plants were potted in 200-mL glass containers filled with the same nutrient-rich sediment used for the laboratory culture (see above). A half centimetre thick layer of washed sand was placed on top of the sediment to reduce leaching of solutes to the water. Each glass container with a single plant was placed in a 5-L pot filled with the same culture solution as for the laboratory culture. Water was filtered by a small water pump (Trixie 200, Tarp) in each pot to prevent phytoplankton growth. The initial DIC was set at 0.85 mM in the LC (according to the protocol of Smart & Barko, 1985) and at 0.40 mM in the HC treatment, in order to provide similar final concentrations of DIC in the air-saturated CO_2 (LC) and supersaturated CO_2 (HC) treatments during growth. CO_2 was added continuously to the pots equipped with a needle regulator from a commercially available 10-L CO_2 tank to the HC treatments. CO_2 addition was adjusted for each pot with the needle regulator. The pH in the LC treatment was held constant at 8.2 ± 0.2, while pH in the HC treatment was fixed at 6.7 ± 0.2, resulting in CO_2 concentrations of 0.012 mM in the LC and 0.263 mM in the HC treatment (Hussner & Jahns, 2015). Due to the continuous addition of CO_2 in the HC treatment, DIC reached 0.8–0.95 mM, close to the 0.85 mM of the ambient air bubbled LC treatments.

One pot was used as one experimental unit with four replicates for each of the two different treatments. The plants grew for 35 days under the different CO_2 conditions.

Plant growth rate

Plants were harvested at the end of the experimental acclimation period (35 days), and shoots (including leaves) and roots were separated. Plant material was dried at 90 °C until constant dry mass (DM). Relative growth rate (RGR, g g^{-1} DM d^{-1}) was calculated according to the following:

$$\mathrm{RGR} = \left(\ln(W_f) - \ln(W_i)\right) \times T^{-1}$$

where W_i is the initial DM and W_f is the final DM after T days.

Plant leaf analyses

Leaf dry matter content (LDMC) was measured on 10–20 leaves of each species at the end of the experimental acclimation period. Fresh mass (FM) of the leaves was measured, leaves were frozen in liquid nitrogen, lyophilised using an Alpha 1–2 LD Freeze Dryer (Christ, Osterode am Harz, Germany) and DM determined. The LDMC was calculated as LDMC = DM/FM. The dried leaf material was subsequently ground to a powder using a Mixer mill MM301 (Retsch, Haan, Germany), and the powder was used for pigment, C, N, metabolite and protein analyses.

Chlorophyll content was determined on the freeze-dried leaf material following the method of Arnon (1949). Chlorophyll *a* and *b* from about 2 mg leaf material was extracted in 80% acetone for 12 h in the dark at 4 °C; the sample was centrifuged at 13 000 rpm and the chlorophyll content of the supernatant was measured spectrophotometrically at 645 and 663 nm. The pellet was used for analysis of total protein. The pellet was extracted in a SDS buffer containing 1.6% SDS, 1 M urea and 50 mM Tris/HCl at pH 7.6 by incubation for 20 min at 70 °C and 10 min at 95 °C. Total protein content was determined with the BioRad DC assay of Lowry *et al.* (1951) using a bovine serum albumin (BSA) standard as control.

Leaf nitrogen and carbon content were determined from a fine powder of lyophilised leaf material with an elemental analyser (Elementar Analysensysteme, Hanau).

Aliquots of lyophilised leaf material used for the elemental analysis were extracted with a mixture of chloroform–methanol–water for analysis of sugars, organic acids and amino acids by gas chromatography-mass spectrometry (GC-MS) according to Fiehn *et al.* (2000) using an Agilent (Santa Clara, California) 7890A GC coupled to a Waters (Milford, Massachusetts) GCT Premier TOF-MS as described in Gu *et al.* (2012). Analysis of chromatograms was done using the Quant Lynx Software (Waters, Milford, Massachusetts). For relative quantification, all metabolite peak areas were normalised to the amount of extracted plant material and the peak area of the internal standard ribitol added prior to extraction.

Net photosynthesis and dark respiration

Net photosynthesis and dark respiration of the plants acclimated to the different carbon conditions were measured 1 day prior to plant harvest. Net photosynthesis was measured at pH 6 and pH 9, where CO_2 and HCO_3^-, respectively, were the main carbon species. A

6-cm-long shoot apex from each plant was inserted into a 100-mL glass-stoppered flask filled using the same laboratory culture medium with a DIC of 0.85 mM and adjusted to pH 9.0 (with 0.1 N NaOH) or pH 6 (with 0.1 N HCl). Plants were then exposed to 100 μmol photons m^{-2} s^{-1} for 60 min and changes in the oxygen concentration were measured with photo-optical oxygen sensors (Fibox, Presens, Regensburg). Dark respiration was measured on the same plants after 120 min in the dark. Blanks without plant material were used as controls. Rates of net photosynthesis and dark respiration were expressed as mg O_2 mg^{-1} Chl h^{-1} and mg O_2 g^{-1} DM h^{-1}.

Statistical analyses

We used two-way ANOVA to test the effects of treatments and species (and their interactions) on response variables. Because of the high number of response variables, an alpha of 0.01 was chosen to avoid type II error. When significant, the tests were followed by a Tukey HSD post hoc test for the CO_2 treatments ($\alpha = 0.01$). We used R statistical software version 3.2.3. (R Core Team 2015). Pearson correlation was used to test for the significance of the correlation between the RGR ratio at pH 6.7 and 8.2 and the photosynthetic rates at pH 6 and 9.

Results

Growth response during the acclimation period

Overall, all species had significantly higher relative growth rates (RGR) under HC than LC conditions, and significant differences between the species were found (Fig. 1). The ratio of RGR at LC to HC ranged from 0.4 for *M. heterophyllum* to more than 0.9 for *H. verticillata* and *E. densa*, documenting the high efficiency of HCO_3^- use for the latter species. Leaf dry matter contents (LDMC) and root: shoot ratios were also significantly higher at HC compared with LC and differed between the species (Fig. 1).

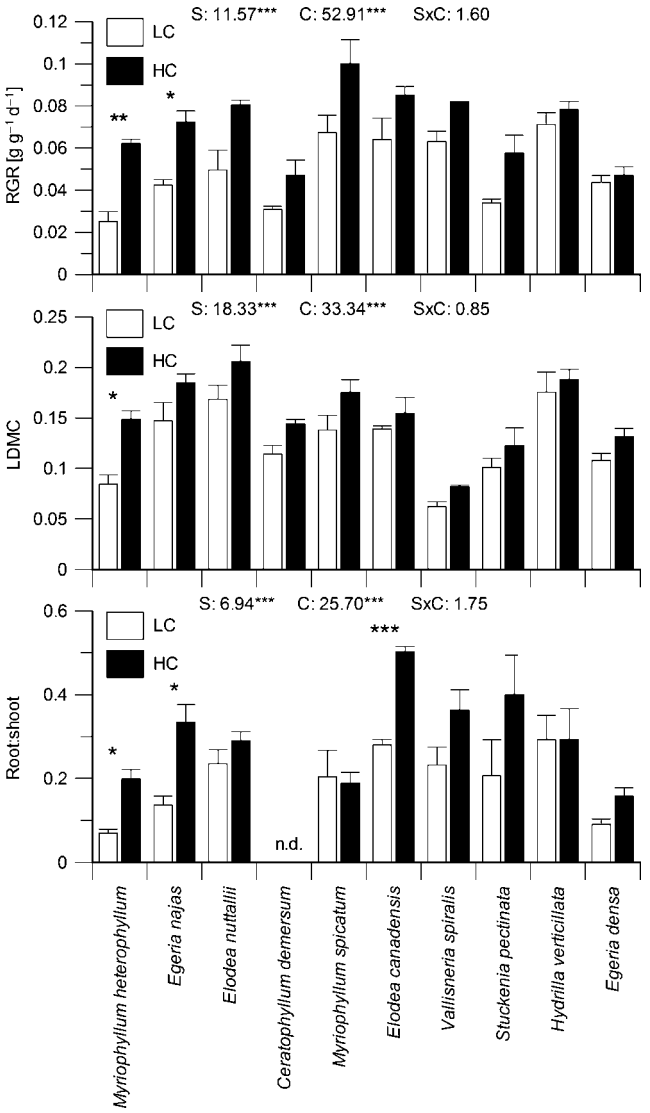

Fig. 1 Relative growth rates (RGR), leaf dry matter content (LDMC) and root:shoot ratio of 10 submerged plant species growing at two different pH values (and CO_2 and HCO_3^- concentrations) for 35 days (mean ± SE are shown). Asterisks indicate significant differences between the LC and HC treatment of each species (ANOVA followed by the Tukey HSD post hoc test). The *F*-values and level of significances for differences between the different carbon treatments (C), between the species (S) and their interaction (S × C) are given from two-way ANOVA (significance levels: $*P < 0.01$; $**P < 0.001$; $***P < 0.0001$).

Physiological response after acclimation period

Net photosynthesis at pH 9 relative to chlorophyll and DM was overall significantly higher for plants acclimated to LC condition than to HC condition (Fig. 2). Species differed significantly in their ability to photosynthesise at pH 9 with *M. heterophyllum* and *C. demersum* showing low rates and *E. nuttallii*, *E. densa* and *H. verticillata* showing high rates. Only *H. verticillata* and *E. densa* maintained high photosynthetic rates at pH 9.0 when acclimated to HC conditions. In contrast, net photosynthesis at pH 6 relative to chlorophyll exhibited no overall differences between plants acclimated to LC or HC conditions, but significant differences between the species were found (Fig. 2). However, net photosynthesis at pH 6 relative to DM of LC plants was significantly higher than for HC plants and differed significantly between the species (Fig. 2). Dark respiration on

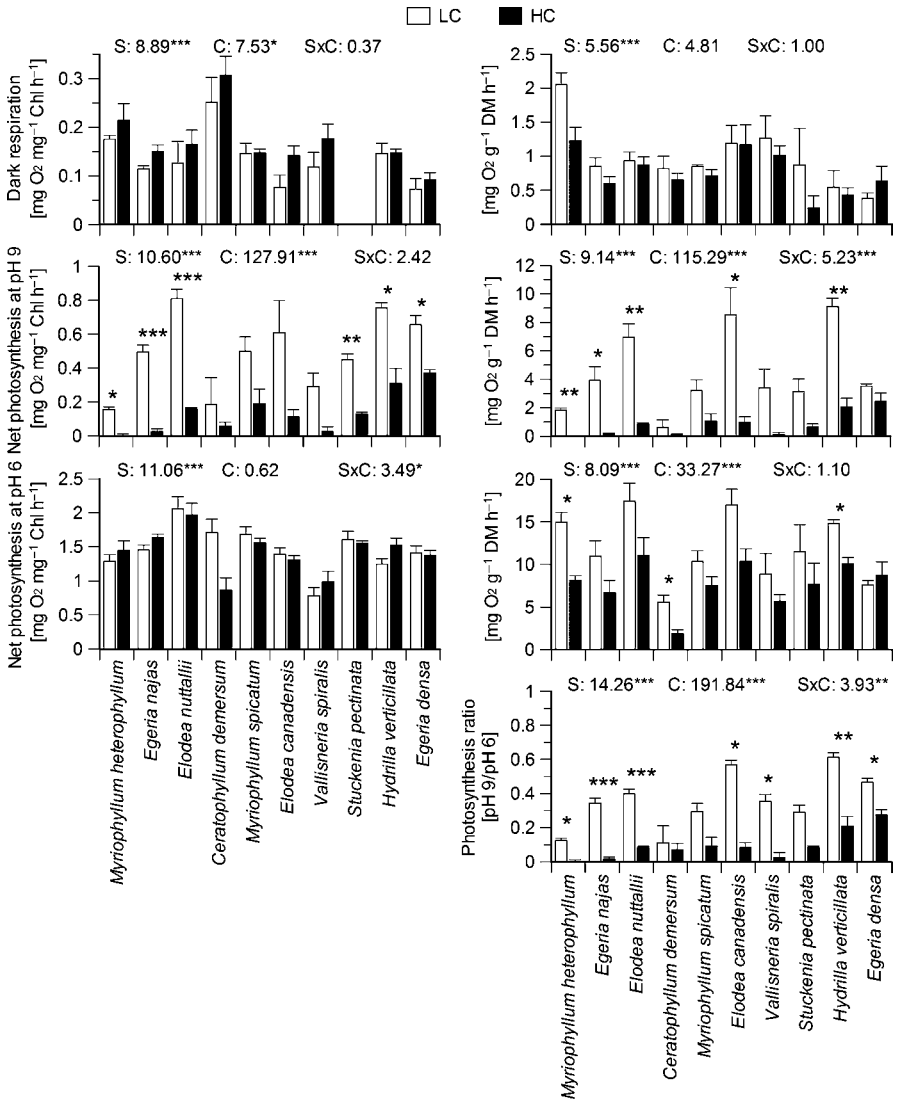

Fig. 2 Dark respiration and net photosynthesis at pH 6 and pH 9 and 0.85 mM DIC relative to chlorophyll (left column) and DM (right column) and the ratio of net photosynthesis at pH 9/pH 6 of 10 submerged plant species that had been acclimated for 35 days to low CO_2 (LC, 0.012 mM, open bars) and high CO_2 (HC, 0.28 mM, closed bars). Mean ± SE are shown. Probabilities and *F*-values as in Fig. 1.

chlorophyll basis was significantly different between the species and the LC and HC treatments, while on DM basis, only significant differences between the species were present.

Net photosynthesis at pH 9 relative to pH 6 is a measure of HCO_3^--affinity normalised to photosynthetic capacity (Fig. 2). Bicarbonate affinity was significantly different between the species and significantly higher for LC-treated plants than for HC-treated plants. The bicarbonate affinity ranged from 0.11 to 0.61 among species grown at LC conditions and from 0.02 to 0.28 grown at HC conditions, with significant differences between the species. These results demonstrate the substantial plasticity of HCO_3^- affinity and its suppression by high CO_2 concentrations. Accordingly, net photosynthesis was stimulated (1.6–8.9 times) by high relative to low CO_2 concentrations (pH 6/pH 9) at constant DIC for the 10 species acclimated to air-saturated CO_2 concentrations (LC). However, the stimulation was even greater (3.6–48.7 times) when species had been acclimated to 22-times CO_2 supersaturation (HC) due to their reduced HCO_3^- use capacity.

Bicarbonate affinity expressed in photosynthesis measurements (LC plants at pH 9/HC plants at pH 6) was significantly positively related to HCO_3^- affinity expressed in growth experiments as the ratio of RGR under LC to RGR under HC (Fig. 3). The different pH values and thus CO_2 availabilities during photosynthesis and growth measurements might explain the generally lower net photosynthesis ratios compared to the RGR ratios and the relatively low net photosynthetic rates of *C. demersum* compared to its growth rates (Figs 1 & 2). *Myriophyllum heterophyllum* showed the lowest, while *H. verticillata* and *E. densa* showed the highest affinity

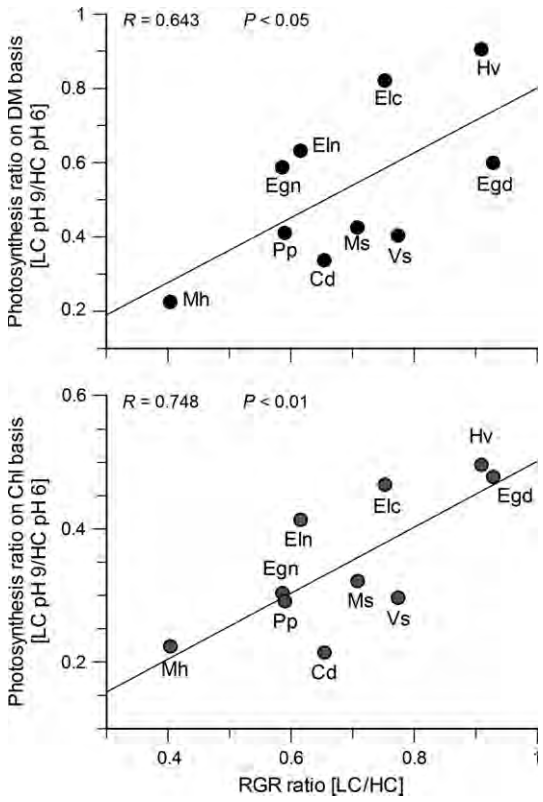

Fig. 3 The correlation of the RGR at pH 8.2/RGR at pH 6.7 and the net photosynthesis ratio (LC plants at pH 9/HC plants at pH 6) expressed on a DM basis (above) and chlorophyll basis (below) for 10 submerged aquatic plants. The p value and the Pearson correlation coefficient (*r*) are given.

for HCO_3^- both in photosynthesis and growth measurements. The latter two species also showed the lowest plasticity of HCO_3^- affinity in response to changes in CO_2 concentrations during growth.

Tissue composition

Plants grown at LC conditions showed significantly higher leaf nitrogen concentrations than plants acclimated to HC conditions, and significant differences between the species were also found. In contrast, leaf carbon content only differed significantly between the species. Between LC and HC treatments, no overall significant differences were found with carbon values of 37.5–44.5% of DM (Fig. 4).

Along with the higher leaf nitrogen content, the protein content also differed significantly between the LC and HC treatments, and significant differences existed between the species (Fig. 4). Similarly, the chlorophyll content was significantly higher in LC than HC treatments and differed significantly between the species.

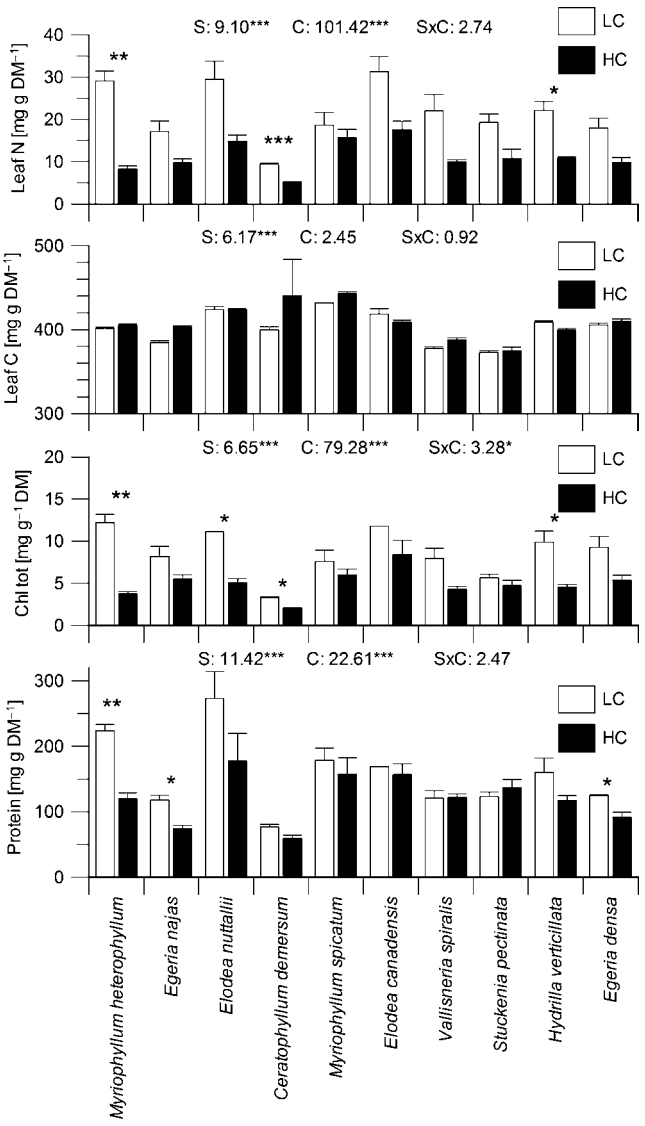

Fig. 4 Nitrogen, carbon, chlorophyll and protein content of leaves of 10 submerged plant species growing at two different pH values (resp. CO_2 and HCO_3^- concentrations) for 35 days (mean ± SE are shown). Probabilities and *F*-values as in Fig. 1.

Metabolite concentrations

Strikingly, all measured metabolite pools were significantly different among species. This metabolic diversity is likely due to the wide morphological diversity among species and their different photosynthetic types.

Among the measured photorespiratory intermediates, significant differences due to the different CO_2 treatments were found in glycerate and serine pool sizes, whereas glycine and glycolic acid pool sizes remained unchanged (Fig. 5). Glycerate levels of *M. heterophyllum* and *E. najas* at LC were 3- to 10-fold higher than those of the other species at LC. *M. heterophyllum* and *E. najas*

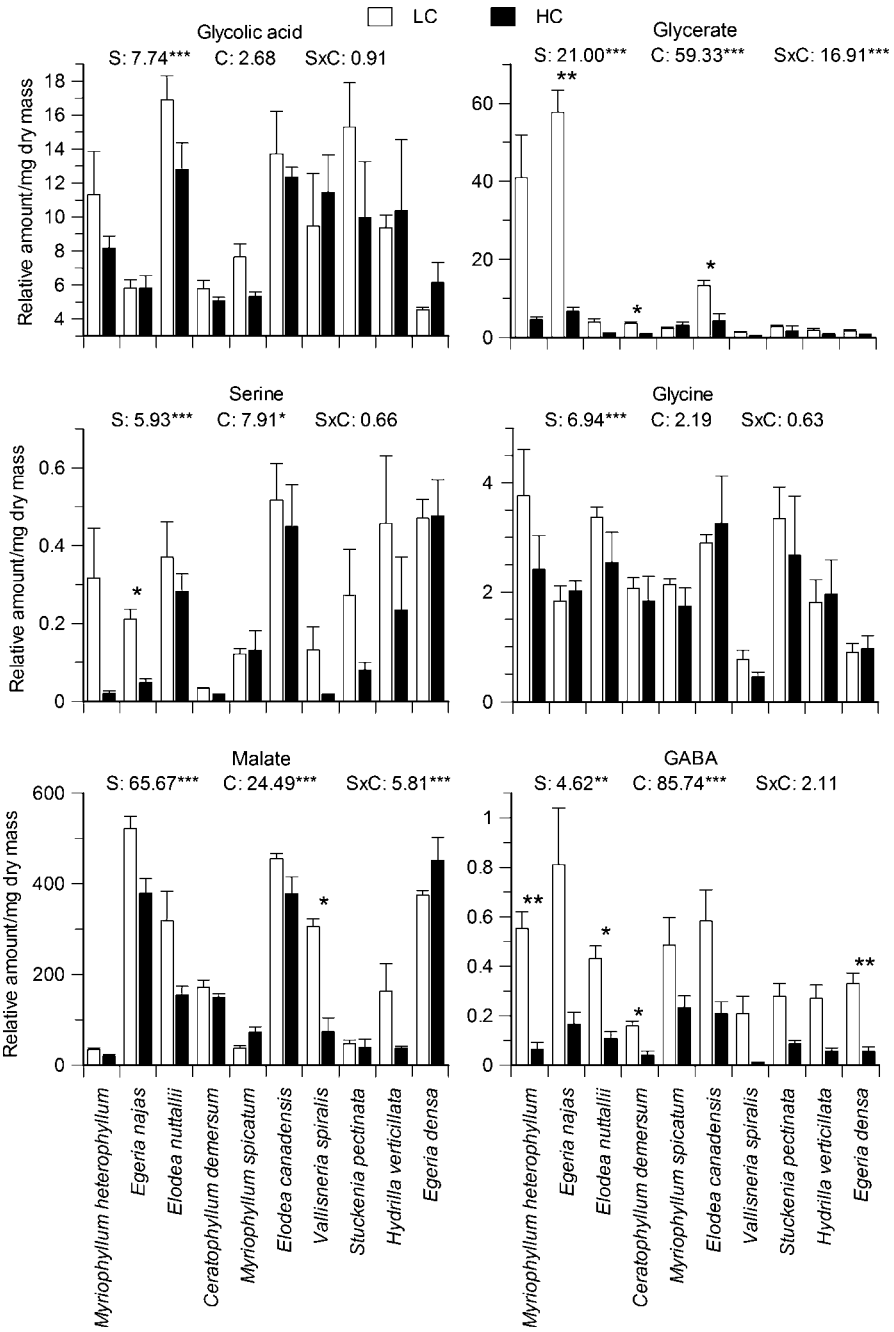

Fig. 5 Relative content of GABA and the photorespiratory metabolites glycolic acid, glycerate, glycine, serine and malate in leaves of 10 submerged plant species growing at two different pH values (resp. CO_2 and HCO_3^- concentrations) for 35 days (mean ± SE are shown). Probabilities and *F*-values as in Fig. 1.

also showed more than eight times higher glycerate levels under LC than HC conditions. These patterns could reflect the high photorespiratory rate of these two species under LC. There were large differences in malate level between species (Fig. 5). A higher malate level at LC than at HC was found in four species (*E. najas*, *E. nuttallii*, *V. spiralis* and *H. verticillata*) although it was only significant for *V. spiralis*.

Overall, 10 amino acids (alpha-alanine, aspartic acid, GABA, glutamate, isoleucine, leucine, phenylalanine, serine, threonine and valine) of the 18 amino acids measured were significantly higher under LC compared with HC conditions (Fig. 5, Figure S1). The largest changes occurred in the pool size of GABA (Fig. 5).

One of the main direct products of photosynthesis, sucrose, was also significantly increased under LC condition compared with HC condition (Fig. 6). Interestingly, *C. demersum* and *M. spicatum* had consistently low sucrose levels, but high raffinose levels under both growth conditions.

The two-way ANOVA tested the interaction between species and CO_2 treatment to be significant for glycerate

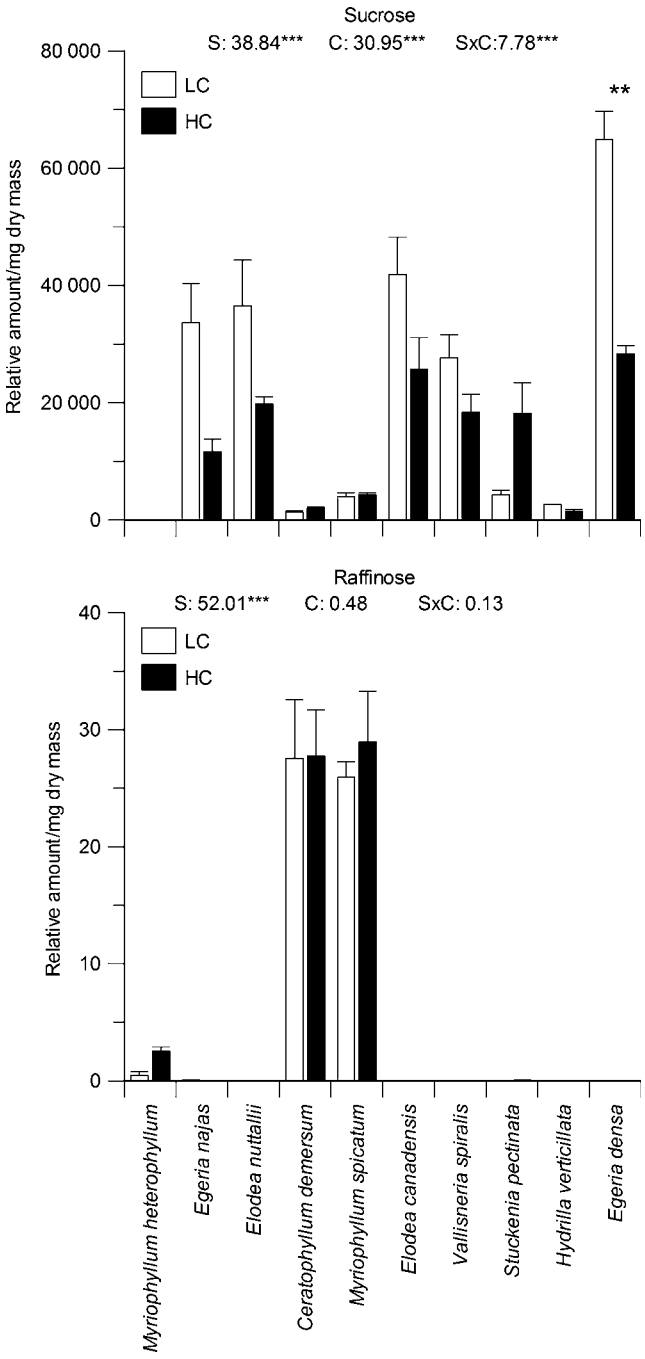

Fig. 6 Relative amounts of sucrose and raffinose in leaves of 10 submerged plant species grown under two different pH conditions (resp. different CO_2 and HCO_3^- concentrations) for 35 days (mean ± SE are shown). Probabilities and *F*-values as in Fig. 1.

and malate, suggesting that these two pool sizes may reflect different strategies to deal with different CO_2 supply among the 10 species. Additionally, the interaction between species and CO_2 treatment was also significant for alpha-alanine and sucrose.

Discussion

Photosynthesis and inorganic carbon

Even though this study only tested the influence of two CO_2 concentrations on photosynthesis and growth, our data confirm that the acquisition of inorganic carbon and the availability of CO_2 are major factors for regulating submerged plant photosynthesis, growth, allocation and tissue contents even among species capable of efficient HCO_3^- use.

It is common among freshwater plants that net photosynthesis is severely limited by field concentrations of inorganic carbon availability for amphibious plants, obligate CO_2 users and HCO_3^- users (Sand-Jensen, Pedersen & Nielsen, 1992; Pedersen *et al.*, 2013). This situation implies that access to additional CO_2 sources in air or sediments and CO_2 supersaturation in the water by input of CO_2-rich soil water or CO_2 from sediment respiration will greatly stimulate submerged plant photosynthesis (Maberly, 1985; Sand-Jensen & Frost-Christensen, 1998). Many amphibious plants have CO_2 compensation points for submerged photosynthesis close to air-saturated concentrations (Sand-Jensen *et al.*, 1992), and they are unable to grow and survive under water unless it is supersaturated with CO_2 (Pedersen *et al.*, 2013). Obligate CO_2 users usually have CO_2 compensation points 2- to 5-fold lower than CO_2 concentrations at air saturation, but photosynthesis is, nonetheless, very low at air saturation, while CO_2 saturation of photosynthesis often requires 10–50 times CO_2 supersaturation (Madsen, 1991). Accordingly, also obligate CO_2 users prefer CO_2 supersaturated conditions in streams and above lake sediments (Maberly, 1985; Sand-Jensen & Frost-Christensen, 1998).

Surprisingly, the stimulation by CO_2 enhancement was less pronounced for net photosynthesis measurements than relative growth rates. This is most likely the result of downregulation of the photosynthetic apparatus due to the enrichment of non-structural carbohydrates under long-term CO_2 enrichment. This response has been documented for terrestrial plants, especially under nitrogen limitation (den Hertog *et al.*, 1998; Stitt & Krapp, 1999; Long *et al.*, 2004). It seems likely that CO_2 enrichment leads to starch accumulation in the leaves, which is suggested by the increased leaf dry matter content and a concomitant decrease of the chlorophyll content (den Hertog *et al.*, 1998).

Freshwater plants capable of using HCO_3^-, such as the 10 species examined here, constitute a high percentage of submerged plant species in the NW European

flora (about 50%, Maberly & Madsen, 2002; Madsen & Sand-Jensen, 1991), and they can grow well in HCO_3^--rich waters even though CO_2 is depleted below air saturation (Madsen & Sand-Jensen, 1987). Nonetheless, the higher affinity for CO_2 than HCO_3^- implies that even HCO_3^- users are substantially stimulated in their photosynthesis by elevated CO_2 concentrations (Madsen & Sand-Jensen, 1987) as shown here by the 10 examined species.

The affinity ratio for HCO_3^- relative to CO_2 during photosynthesis was highly variable among the 10 tested species (0.11–0.61), and it was suppressed for all species (to 0–0.28) by acclimation to elevated CO_2. This phenomenon has been shown before for a few freshwater plants and marine macroalgae (Sand-Jensen & Gordon, 1984, 1986; Hussner & Jahns, 2015), but it is confirmed here for the first time as a general phenomenon for many species acclimated to specific air-saturated and supersaturated CO_2 concentrations. It can be regarded as a suitable cost–benefit response because investment in costly HCO_3^-use mechanisms is futile, when free CO_2 is readily available. Moreover, high HCO_3^- affinity can be re-established if CO_2 once again is reduced to limiting low concentrations. The decrease in HCO_3^- affinity under high CO_2 availability for the plant species will depend on their existing CCM mechanism. In freshwater vascular plants, the most common CCM is the use of external HCO_3^- by direct uptake or extracellular leaf acidification and conversion of HCO_3^- to CO_2 (Raven *et al.*, 2014). Only the known single-cell C_4 species (with a C_4 pathway between cytoplasmic space and chloroplasts in a single cell, Bowes, 2011), *H. verticillata* and *E. densa* maintained high photosynthetic rates at pH 9.0 when acclimated to HC conditions. In *E. densa* and *H. verticillata*, the single-cell C_4 metabolism runs concomitantly with HCO_3^- use (Raven *et al.*, 2014). This might explain why the latter species are less affected by changes in CO_2 availability and exhibited the highest HCO_3^- use efficiency both during photosynthesis and growth.

The variability of HCO_3^- affinity among species and in response to acclimation to either air-saturated or supersaturated CO_2 concentrations was not just a short-term physiological response but was accompanied by sustained long-term changes of the relative growth rate. Under steady-state growth conditions, it is expected that the short-term metabolic response of photosynthesis and respiration is reflected by the long-term growth response, which is ecologically a more relevant feature for species performance under field conditions. Thus, variable HCO_3^- use among species and between LC and HC conditions will influence the colonisation, dispersal and competitive strength among the species between low and high DIC waters. In habitats where pH regularly exceeds 9, efficient HCO_3^- use is required to sustain photosynthesis and growth and ensure survival (Santamaria, 2002).

Growth and inorganic carbon

The 10 freshwater species grew 1.1–2.5 times faster under supersaturated than air-saturated CO_2 conditions for the same DIC concentration confirming the importance of CO_2 as an important growth stimulating factor for HCO_3^--using freshwater plants. Earlier studies suggest that the strength of this CO_2 stimulation of growth would increase with higher light (Eusebio Malheiro, Jahns & Hussner, 2013; Hussner & Jahns, 2015) and nutrient supplies (but see Titus, 1992), while it would decrease at higher HCO_3^- concentrations in more alkaline waters. There are contrasting results as to whether growth stimulation by CO_2 elevation should be even stronger for sole CO_2 or HCO_3^- users (Pagano & Titus, 2007; Hussner *et al.*, 2015). Recent findings indicate that sole CO_2 users are more stimulated by increased CO_2 concentrations than HCO_3^- users, because Demars & Trémolières (2009) documented the dominance of amphibious plants without HCO_3^- use capacity in CO_2 rich groundwater fed streams.

The rising CO_2 concentrations in the atmosphere will increase equilibrium concentrations of CO_2 in fresh waters proportionally and through chemical equilibrium reactions will also increase HCO_3^- and DIC concentrations (Bowes, 1991). Invasion of CO_2 from the atmosphere to CO_2 depleted surface waters is common during intense photosynthesis in eutrophic lakes and dense plant beds (Christensen, Sand-Jensen & Staehr, 2013), while CO_2 evasion usually prevails during darker and colder autumn to spring seasons (Staehr & Sand-Jensen, 2007). Our present findings and cited literature suggest that all freshwater plants can experience stimulation of summer growth by higher atmospheric CO_2 concentrations and that the strongest stimulation is expected for HCO_3^- users in low-alkaline waters and for CO_2 users in all waterbodies where limitation by inorganic carbon is likely to prevail.

While there are obviously severe inorganic carbon constraints on growth rates of sole CO_2 users among freshwater plants, only few studies examine the consequences of carbon constraints and rising atmospheric CO_2 concentrations for HCO_3^- users (Titus, 1992; Olesen & Madsen, 2000; Pagano & Titus, 2004; Pagano & Titus, 2007). First, the growth stimulation by higher CO_2

concentrations has not been widely documented before and the higher HCO_3^- concentrations accompanying higher CO_2 concentrations have often been overlooked. It should be stressed that growth limitation by inorganic carbon availability is important for the performance of freshwater plants and much more important for their distribution and performance (Sand-Jensen, 1989; Santamaria, 2002) than for terrestrial plants, for which only a weak influence of elevated CO_2 has been reported (Long *et al*., 2004; Leakey *et al*., 2009).

We found increasing root: shoot ratios from low to supersaturated CO_2 concentrations in the waters. A similar root:shoot response with increasing DIC (Pagano & Titus, 2007; Hussner & Jahns, 2015; Hussner *et al*., 2015) and increasing light availability (Barko, Hardin & Matthews, 1982) has been observed before. A proportionally larger root system could have several advantages. It ensures better root anchorage and, thereby, reduces the risk of dislodgment of the plants exposed to strong waves and currents (Schutten, Dainty & Davy, 2005). Higher biomass allocation to roots has also been observed in turbulent than stagnant conditions (Strand & Weisner, 2001). Large root systems reduce the risk of nutrient shortage by increasing the sediment volume from which nutrients can be derived (Mantai & Newton, 1982). Moreover, roots can act as storage organs during winter (Wersal *et al*., 2011) and benefit plant regrowth in spring. These considerations stress that inorganic carbon availability affects the energy budget of the plants thereby influencing an array of ecological processes.

Elemental composition and metabolites

The internal nitrogen content in submerged aquatic plants is positively related to nitrogen availability in the habitat, although the correlation is often weak (Demars & Edwards, 2007). Our results confirm for many species, the interaction of inorganic carbon supply with leaf tissue nitrogen as elevated CO_2 apparently allows savings in tissue nitrogen (Titus & Andorfer, 1996; Madsen, Hahn & Johansen, 1998). Under field conditions, high nutrient availabilities often result in more luxuriant plant growth and stronger CO_2 depletion, which activates the CCM mechanisms and, thus, increases nitrogen demands. A low pH in the water and sediment can reduce the nitrogen uptake capacity of reed plants (Dyhr-Jensen & Brix, 1996), and this effect cannot be ruled out in our study. However, the ammonia uptake of *Typha latifolia* was constant across the pH range of 5–8 (Dyhr-Jensen & Brix, 1996), which is wider than the pH range from 6.7 to 8.2 in the waters of our study. Aquatic plants can influence sediment pH (Blossfeld & Gansert, 2007) and thus limit the pH effects on nutrient uptake.

Terrestrial plants experiencing CO_2 enrichment reduce C_4 cycle amino acids (e.g. aspartate) and photorespiratory intermediates (e.g., serine and glycine; Timm *et al*., 2012). Indicators of different CCMs operating in the studied species include the marked differences in malate concentrations. Malate is transported from the cytosol to the chloroplast in C_4 species and has been found at relatively high concentrations in LC acclimated plants of all tested hydrocharitaceans (see also Salvucci & Bowes, 1983). Outside this group, we found malate accumulation to a lower extent in *C. demersum*, while malate concentrations remained low in *M. spicatum*, *M. heterophyllum* and *S. pectinata* irrespective of inorganic carbon supply. Malate is the C_4-compound thought to be transported from the cytosol to the chloroplast in water plants with single-cell C_4 photosynthesis such as *E. densa* (Bowes, 2011). Indeed, malate concentrations in *E. densa* were 10- and 22-fold higher than in *M. heterophyllum*, a known C_3-photosynthesis species (Beer & Wetzel, 1982), under LC and HC treatments, respectively. However, malate was slightly higher at LC in the second single-cell C_4 photosynthetic species *H. verticillata*, suggesting that malate pool size is not a sufficient parameter to identify single-cell C_4 photosynthesis types and that metabolism of *H. verticillata* and *E. densa* is not identical. There might be underlying differences between the single-cell C_4 photosynthesis strategies of these two species. This idea is also suggested by the much lower levels of sucrose in *H. verticillata* compared with *E. densa* (Fig. 6).

Gamma-aminobutyric acid is a non-protein amino acid formed by decarboxylation of glutamate in higher plants (Shelp, Bown & Mc Lean, 1999). The observed increase of the stress-related amino acid GABA under CO_2 depletion indicates that CO_2 depletion generally causes some type of stress in submerged plants even among HCO_3^- users. Alternatively, the increased GABA levels could be related to its involvement in nitrogen metabolism (Shelp *et al*., 1999; Bouché & Fromm, 2004). The glutamine synthetase/glutamate synthase (GS/GOGAT) pathway assimilates most inorganic nitrogen. Its product glutamate is then the starting point for amino acid biosynthesis, including GABA (Bouché & Fromm, 2004). In our study, the overall higher leaf nitrogen content under CO_2 depletion, implying higher nitrogen assimilation relative to growth rate, is accompanied by increased pool sizes of most amino acids with GABA increasing most. These findings agree with the proposed role of

GABA as a transient nitrogen storage compound (Masclaux-Daubresse *et al*., 2002). The striking species differences between high concentrations of sucrose in some species (all studied Hydrocharitaceans and *S. pectinata*) and high raffinose levels in other species (*M. spicatum*, *M. heterophyllum* and *C. demersum*) could be related to differences in phloem loading strategies (Rennie & Turgeon, 2009), but the suggestion remains speculative as no thorough studies are available.

The observed metabolic responses could reflect the chain of reactions where CO_2 enrichment initially increases photosynthetic rates (Pedersen *et al*., 2013) and higher photosynthesis increases starch accumulation in the leaves (Bowes, 1991), as indicated by higher leaf dry matter content (LDMC). The increased starch allocation in turn may induce lower affinity for both CO_2 and HCO_3^- and lower chlorophyll content (Bowes, 1991).

In summary, supersaturated CO_2 availabilities cause higher photosynthetic rates and growth rates, but decrease the HCO_3^- affinity. In natural waters, high photosynthetic rates of submerged plants lead to self-induced CO_2 depletion and activation of CCMs and increase in their HCO_3^- affinity. The observed increase in leaf nitrogen and protein content at air-saturated CO_2 concentrations seems to be a key factor for the functioning of the CCMs. When nitrogen limitation reduces the ability to activate CCM, it could reduce the competitive strength of potential HCO_3^- users relative to obligate CO_2 users under nitrogen-limiting conditions. Thus, availabilities of CO_2, HCO_3^- and nutrients together should influence growth rates and competition hierarchies among freshwater plant species with different ecophysiological traits.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. Relative amounts of selected amino acids in leaves of 10 submerged plant species grown under two different pH conditions (resp. different CO_2 and HCO_3^- concentrations) for 35 days (mean ± SE are shown). Probabilities and *F*-values as in Fig. 1.

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