



# Response of Submerged Macrophyte Growth, Morphology, Chlorophyll Content and Nutrient Stoichiometry to Increased Flow Velocity and Elevated CO<sub>2</sub> and Dissolved Organic Carbon Concentrations

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It is expected that climate change will cause more frequent extreme events of heavy precipitation and drought, changing hydrological conditions in riverine ecosystems, such as flow velocity, evapotranspiration (drought) or runoff (heavy precipitation). This can lead to an increased input of terrestrial organic matter and elevated levels of dissolved organic carbon (DOC) and CO<sub>2</sub> due to degradational processes in water. Consequences for submerged macrophytes, as essential organism group, are still poorly understood. The combined effects of changing flow velocity, DOC and CO<sub>2</sub> have not been studied before, so this was tested in a racetrack flume experiment on the macrophyte *Berula erecta* using a trait-based approach. The plants were exposed to two different flow velocities, two DOC concentrations and two CO<sub>2</sub> concentrations in a full factorial design. Apart from individual dose-response tests, two climate change scenarios were tested: a wet scenario simulating heavy precipitation and runoff with high flow velocity, high DOC and CO<sub>2</sub> concentrations and a dry scenario simulating evapotranspiration with low flow velocity, high DOC and high CO<sub>2</sub> concentrations. Growth rate, biomass, morphology, chlorophyll and nutrient content (C, N, and P) were measured. *B. erecta* responded strongly to both scenarios. Biomass and the relative growth rate increased and stems were shorter, especially in the wet scenario, and vegetative reproduction (the number of stolons) decreased. In both scenarios, the N content was lower and P content higher than in conditions without climate change. It can be concluded that climate change effects, especially shading by DOC, strongly influence macrophytes: macrophyte abundance will probably be negatively affected by climate change, depending on the macrophyte species and abundance of epiphytic algae. This may have consequences for other components of the aquatic ecosystem.

**Keywords:** aquatic plants, *Berula erecta*, climate change, carbon dioxide, brownification, humic substances, flow velocity, multiple stressors

## INTRODUCTION

As a result of human-induced climate change, worldwide precipitation patterns are altering. In Europe, for example, the frequency of heavy precipitation events is increasing in winter, whereas there is an increased risk of drought in summer at the same time in some regions (Hoegh-Guldberg et al., 2018). If temperatures increase by 1.5°C, it has been predicted that heavy precipitation intensity (annual maximum 5-day precipitation) increases by at least 5–10% in many parts of Europe, whereas precipitation may decrease by 5–15% in some periods, especially in the Mediterranean area (Jacob et al., 2018). Because precipitation is an important driver of changes in river discharge (Dai et al., 2009), more extremes in discharge can be expected in the future, which can profoundly affect water quality and riverine ecosystems (van Vliet et al., 2013). Aquatic macrophytes play a key role in those ecosystems as they affect nutrient cycling and sedimentation (Clarke, 2002), oxygen dynamics (Uehlinger et al., 2000) and organise stream structure and functioning (Schoelynck et al., 2012b).

Macrophytes can be affected in several ways by changing river discharge. Firstly, aquatic macrophytes are directly affected by changes in river discharge. Dry periods with slow flowing or standing water can lead to warmer water and a lower water level with more eutrophic conditions including high algae growth and relatively more fish, and in some cases more saline conditions; leading to a decline of submerged macrophytes (Short et al., 2016). When discharge and flow velocity are high, macrophytes can break or uproot due to increased pulling forces acting on the plants (Schutten et al., 2005). Hydrodynamic stress caused by increased flow velocity can also affect plant physiology: photosynthesis can decrease by 30–60% (Madsen et al., 1993). Macrophytes can adapt to hydrodynamic stress by changing their morphology. There are two strategies: the first strategy is stress avoidance, which involves becoming more streamlined or smaller, so this affects plant biomass. The second strategy is stress tolerance, which involves increasing resistance to breakage by increasing their cross-sectional area and forming stronger tissue (Puijalón et al., 2011), for example by increasing their silica content (Schoelynck et al., 2012a). Altered plant biomass and nutrient stoichiometry can indirectly affect other organisms that depend on macrophytes.

Secondly, changing precipitation patterns can affect the amount of organic and inorganic carbon in water. From 1990 an increase in dissolved organic carbon (DOC) concentrations in surface waters has been observed, especially in Europe and North America (Monteith et al., 2007), which often leads to an increase in water colour called “brownification” (Kritzbeg and Ekström, 2012). This is probably caused by a complex interaction of different factors, but two main mechanisms have been proposed: due to better regulation of sulphate pollution in the atmosphere, atmospheric acid deposition decreased which caused higher soil organic matter solubility (Pagano et al., 2014). The second mechanism is the effects of climate change: with increasing temperature and increased atmospheric carbon dioxide (CO<sub>2</sub>) concentrations, more terrestrial organic matter is produced and with increased precipitation intensity this material can be flushed

into rivers (Pagano et al., 2014). The flux of terrestrial carbon to inland waters is 5.1 Pg C year<sup>-1</sup>, and this is increasing with 0.3 Pg year<sup>-1</sup> (Drake et al., 2018). On the other hand, drought can be a driver of DOC as well: when the water level is lowered, in some cases more aerobic conditions are created which can stimulate the production of DOC (Porcal et al., 2009). Increased DOC concentrations in the water can have several effects on macrophytes. DOC from terrestrial sources like tree leaves often mainly consists of humic substances that give the water a brown colour (Sachse et al., 2005) and may thus be a main driver for brownification. Moreover, it is expected that as a result of climate change more DOC will consist of humic substances in the future (Creed et al., 2018). Humic substances can directly negatively affect macrophytes as they diminish light availability to primary producers (Karlsson et al., 2009; Choudhury et al., 2019) and reduce macrophyte colonisation depth (Chambers and Prepas, 1988). Moreover, some humic substances may directly affect macrophytes by entering the plant's cells and causing damage by production of reactive oxygen species (Grigutyte et al., 2009) or by interfering with photosynthesis (Pflugmacher et al., 2006). Even though DOC may cause a major threat to macrophytes, research about the magnitude of the problem and the exact effects on macrophytes is still limited (Reitsemá et al., 2018).

Upon degradation, DOC can also be a source of CO<sub>2</sub> (Sobek et al., 2005). Mainly due to the high quantity of carbon entering from terrestrial soil or wetlands the world average CO<sub>2</sub> concentration in rivers and streams is 3100 ppm (Raymond et al., 2013), which is substantially higher than the concentration of 400 ppm in the atmosphere. Despite the fact that riverine CO<sub>2</sub> concentrations are relatively high, a further rise is expected in the future (Sobek et al., 2005; Phillips et al., 2015). DOC degradation is one of the mechanisms behind this, together with a reduced CO<sub>2</sub> efflux from the water as a result of higher atmospheric CO<sub>2</sub> concentrations, caused by a rise in CO<sub>2</sub> emissions (Phillips et al., 2015). It is difficult to predict future CO<sub>2</sub> levels in freshwater ecosystems because the exact factors controlling aquatic CO<sub>2</sub> concentrations and their response to climate change are not yet well understood. Moreover, current CO<sub>2</sub> and total inorganic carbon levels in rivers are highly variable and can depend on the catchment (Iversen et al., 2019), and location within the river (Maberly et al., 2015). As a consequence, it is hard to predict future CO<sub>2</sub> levels and how freshwater organisms will respond (Hasler et al., 2016). Research on the effects of CO<sub>2</sub> mainly focusses on marine ecosystems, where the resulting ocean acidification is relatively well studied (Boyd et al., 2016). Studies looking at the effects of elevated CO<sub>2</sub> concentrations on freshwater macrophytes observed increased plant growth rates under high CO<sub>2</sub> concentrations (Eusebio Malheiro et al., 2013; Dülger et al., 2017; Lv et al., 2019), increased biomass production (Hussner et al., 2016), and an increase in root:shoot ratio (Madsen et al., 1996; Hussner et al., 2016; Dülger et al., 2017). Moreover, the nitrogen (N) content of macrophyte tissue was found to be lower (Dülger et al., 2017; Hussner et al., 2019), the phosphorus (P) content was higher (Yan et al., 2006), chlorophyll content was lower (Madsen et al., 1996; Dülger et al., 2017), their dry matter content higher (Eusebio Malheiro et al., 2013) and specific leaf area (SLA) lower (Madsen et al., 1996).

Although the separate effects of varying flow velocity, increased DOC and increased CO<sub>2</sub> concentration have been studied before, their combined effects have not. However, macrophytes will probably be affected by a combination of different climate change effects. Often, complex ecological drivers like climate change are simplified in experiments (Knapp et al., 2018), so by testing the interactions between three factors a more realistic situation can be approached. This is important because contrasting results may be expected for the different factors that are tested. Macrophytes exposed to high DOC concentrations may remain smaller (Szmaja and Bociąg, 2004), whereas macrophytes exposed to high CO<sub>2</sub> concentrations may produce more biomass (Hussner et al., 2016) and show more clonal growth (Yan et al., 2006). Larger plants may be more vulnerable when flow velocity increases (Puijalón et al., 2011). Studying multiple aspects of climate change may result in more accurate predictions about how macrophytes may respond to climate change.

This study aims to test how macrophytes respond to flow velocity, DOC, CO<sub>2</sub> and their interactions. Besides individual dose-response tests, the effects of two climate change scenarios were tested: a wet scenario with high flow velocity, high DOC and high CO<sub>2</sub> concentrations, and a dry scenario with low flow velocity, high DOC and high CO<sub>2</sub> concentrations. A trait-based approach was used with analysis of growth rate, morphology, biomass allocation, chlorophyll production and C, N and P content of the plant. We hypothesised that in both scenarios plants would produce more biomass, especially more reproductive biomass like stolons, and that they would have a lower N and chlorophyll content and higher P content due to the increased CO<sub>2</sub> concentration. In contrast, we hypothesised that DOC would partially counteract the effect of elevated CO<sub>2</sub> due to shading, resulting in decreased plant growth. We also hypothesised that the stems would be shorter and thicker in the wet scenario as an adaptation to hydrodynamic stress. Lastly, we hypothesised that there would be interaction effects between flow velocity, DOC and CO<sub>2</sub> concentrations, due to the contrasting effects they can have as described in earlier paragraphs.

## MATERIALS AND METHODS

### Plant Material

In this experiment *Berula erecta* (Hudson) Coville (Apiaceae) was chosen as model species, since it is a sub-cosmopolitan species that can grow in many different lotic and lentic freshwater habitats (de Belair and Lansdown, 2013), and it is not a floating species, which makes it relatively vulnerable to the effects of climate change (Short et al., 2016). *B. erecta* is a homophyllous amphibious species, but at the sampling location it grew only submerged. Although many macrophyte species can take up two forms of inorganic carbon [bicarbonate (HCO<sub>3</sub><sup>-</sup>) and CO<sub>2</sub>], *B. erecta* can only take up CO<sub>2</sub> (Sand-Jensen et al., 1992), so we expected that this species would respond strongly to changes in CO<sub>2</sub> availability. Young plants were collected in the Fischa River in Austria close to the village of Pottendorf (47.91° N, 16.39° E). Plants of similar size were selected with initial dry

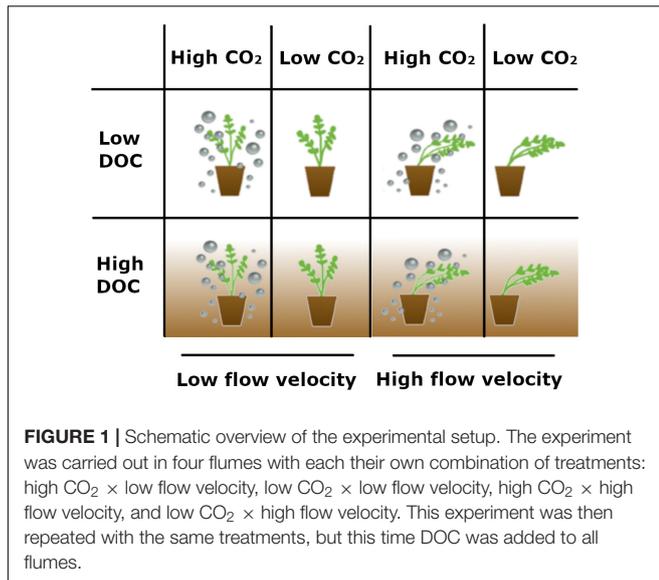
mass of 0.11 ± 0.06 g. This was determined on 12 representative individuals that were not used in the experiment: from those 12 plants fresh and dry weight was measured and the conversion factor between fresh and dry weight was used to estimate dry weight of the experimental plants, based on their fresh weight. 384 plants (48 pseudo replicates per treatment) were each placed in 9 × 9 × 10 cm square pots filled with 0–2 mm grain size cleaned river sand (commercially bought: Cobo gardens, Niel, Belgium) and with a layer of gravel on top to prevent erosion of the sand.

### Experimental Design

The experiment was carried out in a greenhouse at the University of Antwerp (Belgium), where the plants were exposed to the natural day/night cycle. Plants were divided over four 400 × 120 cm racetrack flumes, in a 155 × 36 cm test section with a water height of 44 cm. Tap water was used [initial nutrient concentrations: 0.002 mg L<sup>-1</sup> phosphorus (PO<sub>4</sub><sup>3-</sup>-P), 0.03 mg L<sup>-1</sup> ammonium (NH<sub>4</sub><sup>+</sup>-N), 0.002 mg L<sup>-1</sup> nitrite (NO<sub>2</sub><sup>-</sup>-N), and 2.308 mg L<sup>-1</sup> nitrate (NO<sub>3</sub><sup>-</sup>-N)] and temperature was kept constant at 18°C. After 19 days of acclimatisation the plants in two flumes were exposed to higher flow velocity (0.4 m s<sup>-1</sup>) and the other two flumes to low flow velocity (0.04 m s<sup>-1</sup>), measured with a Valeport 801 ElectroMagnetic Flowmeter at 5, 10, 15, and 20 cm above the sediments in the middle of the flume, at 10 cm left from the middle and at 10 cm right from the middle, afterward the average was calculated. Moreover, CO<sub>2</sub> gas from a commercial bottle was added to two flumes with an airstone at approximately 2 L h<sup>-1</sup> (gas pressure 2 bar). Gas flux was regulated with a Skalar GT1355 Sho-Rate G flowmeter. This resulted in four different treatments: high CO<sub>2</sub> (1000 ppm) with high flow velocity (HC-HF), high CO<sub>2</sub> with low flow velocity (HC-LF), low CO<sub>2</sub> (400 ppm) with high flow velocity (LC-HF), and low CO<sub>2</sub> with low flow velocity (LC-LF). The experiment was done between May 19, 2017 and July 24, 2017 without any added DOC (low DOC or LD treatment) and the experiment was repeated the year after between May 24, 2018 and July 31, 2018, this time DOC was added to all treatments (high DOC or HD treatment), see **Figure 1** for experimental setup. Solar radiation on the roof of the greenhouse was measured and in 2017 the total amount of radiation received during the experiment was 4.32 MW m<sup>-2</sup> (224 W m<sup>-2</sup> d<sup>-1</sup>) and in 2018 this was 4.65 MW m<sup>-2</sup> (237 W m<sup>-2</sup> d<sup>-1</sup>).

### DOC

In this study it was decided to use leaf and peat leachate as DOC source. In some other studies artificial humic acid is used, but when we tested this material it did not dissolve well and only resulted in low DOC values that did not correlate with the amount of artificial humic acid added to the water. DOC was created in two tanks of approximately 2000 litres of water. To each tank, four 100 L bags of leaf litter (a mix of *Fagus sylvatica* L. and *Quercus robur* L.) and 30 L of peat (commercially bought: Aveve) was added. This was done on the 25th of May (the second day of the experiment). The tanks were covered with cloth to prevent photodegradation of the DOC. On day 21, 30, and 54 of the experiment, approximately 200 L of DOC-water was



added to each flume after being filtered through muslin cloth, in order to establish a DOC concentration of 5 mg C L<sup>-1</sup>. In total, eight different treatments were tested (one flume per treatment), with the LD-LC-LF treatment as “no climate change scenario,” and the HD-HC-HF and HD-HC-LF treatments as two climate change scenarios; in both scenarios increased CO<sub>2</sub> and increased DOC were tested, with heavy precipitation and drought being simulated in the HF and LF scenario, respectively. The other five treatments help in understanding the relative contribution of the three tested aspects of climate change to the response of the macrophytes.

### Water Quality Measurements

The concentration of CO<sub>2</sub> in the water was measured continuously with a Pro-Oceanus Digital Mini CO<sub>2</sub> probe which alternated between the flumes. In addition, pH was measured weekly on approximately the same time of the day (early afternoon) (multiline F/set-3 multimeter) Alkalinity was measured four times during the experiment (SAN++, Skalar, Breda, Netherlands). Nutrient concentrations in the water were measured on day 6, 20, 50, and 67 of experiment 1 and on day 12, 26, 40, 54, and 68 of experiment 2; water samples were filtered with 0.45 μm filters (Chromafil® Xtra MV-45/25, Macherey-Nagel, Düren, Germany) and the concentration of PO<sub>4</sub><sup>3-</sup>-P, NH<sub>4</sub><sup>+</sup>-N, NO<sub>2</sub><sup>-</sup>-N, NO<sub>3</sub><sup>-</sup>-N was measured (SAN++, Skalar, Breda, Netherlands). The concentration of DOC was measured on day 6 (first experiment) and day 6, 12, 22, 26, 33, 40, 47, 54, 61, and 68 (second experiment). In order to measure DOC quality a sample from the DOC stock (see earlier paragraph) was filtered with a 0.45 μm filter and subsequently the sample was characterised by LC-OCD (liquid chromatography – organic carbon detection) (Huber et al., 2011). With this technique different size class fractions can be determined: biopolymers (large molecules like polysaccharides and proteins), humic substances (humic and fulvic acids), building blocks (oxidation products of humics) and low molecular weight neutrals and acids.

The effect of DOC on photosynthetically active radiation (PAR) availability was measured as well. On two cloudy days, plastic transparent 5 L buckets (diameter 19 cm, height 20 cm) were filled with water from each flume and another bucket was filled with tap water in order to be able to compare to a control. A light sensor (MQ-210 Apogee underwater quantum PAR meter) was mounted to a frame to keep the sensor in the same position in all buckets. The frame was put in the middle of each bucket and the amount of PAR was measured. PAR availability was measured in buckets to avoid effects of shading from the macrophytes, the lid of the flume and the roof of the greenhouse. Additionally, a light profile was made in each flume in the middle of the test section by measuring PAR at every 5 cm, starting at the bottom.

### Plant Growth and Morphology Measurements

Before planting on day 1, the total fresh mass (roots and shoots together) was determined for each individual. On day 1, 28, 46, and 67 (experiment 1) and day 1, 30, 47, and 68 (experiment 2) all plants were measured: number of stems and leaves, length and stem diameter of the longest stem, number of stolons (if visible) and the number of stems and leaves on the new ramets were counted (all are non-disturbing measurements). After harvesting the plants, stems, leaves and roots were separated and weighed fresh, and after drying the plant material for 48 h at 70°C the dry mass was determined. Before drying the samples, a subsample of 10 randomly chosen plants from each treatment was selected. The leaves of those plants were separated from the stems and photographed on a white background, after which the surface area of the leaves was calculated using the image processing programme ImageJ.

During the experiment periphytic algae started growing and covered the inner walls of the flumes and parts of the macrophytes. The algae were removed from the macrophytes twice by carefully taking them off the leaves by hand (see **Supplementary Figure S1**), but often started growing again within a few days. The amount of algae growing in the flumes was not quantified, but on pictures that have been taken it can be seen that in the treatment with high DOC there appear to be more algae growing in the flumes than in the treatment with low DOC (**Supplementary Figure S1**). The dissolved CO<sub>2</sub> pattern in the water also suggests that there were more algae in the high DOC treatment, as the day-night fluctuations were more pronounced than in the low DOC treatment (**Supplementary Figure S2**), despite a lower plant biomass.

### Chlorophyll Analysis

From the subset of 10 plants per treatment used for the leaf surface area calculations, approximately 150 mg of fresh leaf material was ground with 80% acetone and quartz sand. The sample was centrifuged once at 4000 rpm and twice at 3000 rpm, after which the chlorophyll content (a, b, total and carotenoids) was determined spectrophotometrically. The samples were kept in the dark on ice during the extraction. The absorbance of the samples was measured at four different wavelengths (710, 663.2,

646.8, and 470 nm) which were used to calculate chlorophyll according to the following formulas ( $A_x$  = absorbance at specific wavelength):

$$Chl_a = 12.25 * (A_{663.2} - A_{710}) - 2.79 * (A_{646.8} - A_{710})$$

$$Chl_b = 21.5 * (A_{646.8} - A_{710}) - 5.1 * (A_{663.2} - A_{710})$$

$$Chl_{a+b} = 7.15 * (A_{663.2} - A_{710}) - 18.71 * (A_{646.8} - A_{710})$$

Total carotenoids =

$$\frac{1000 * (A_{470} - A_{710}) - 1.82 * (Chl_a - 85.02 * Chl_b)}{198}$$

The rest of the subsample plant material was dried in the same way as the other material and the dry weight was

**TABLE 1** | F-values of the three-way ANOVA tests and z values of the generalised linear models of growth and morphological parameters (n = 48).

	CO <sub>2</sub>	Flow	DOC	CO <sub>2</sub> *Flow	CO <sub>2</sub> *DOC	Flow*DOC	CO <sub>2</sub> *Flow*DOC
Number of stems	-12.96***	-3.44***	9.89***	ns	ns	ns	ns
Number of leaves	178.69***	0.19	61.51***	0.11	17.41***	0.11	9.49**
Length longest stem	163.81***	51.14***	12.03***	19.01***	95.30***	23.43***	ns
Diameter longest stem	133.80***	20.91***	131.70***	5.72*	7.33**	5.88*	9.86**
Dry mass total	379.93***	4.08*	234.89***	0.019	21.69***	2.73	5.32*
Leaf stem ratio	11.19***	5.88*	57.26***	ns	37.66***	ns	ns
Root shoot ratio	0.17	9.96**	60.97***	0.11	70.79***	1.76	8.01**
Number of stolons	-8.40***	-4.00***	8.49***	ns	ns	ns	ns
Average stolon length	-2.97**	-3.27**	9.22***	2.57*	-4.15***	2.33*	-3.07**
Total stolon length	-2.97**	-3.27**	12.31***	2.57*	-6.33***	1.35	-2.37*
Relative growth rate	640.62***	25.60***	117.12***	7.81**	48.70***	4.71*	21.83***
Dry matter content leaves	1.83	9.45**	43.52***	4.83*	17.82***	11.88***	9.93**
Dry matter content stems	1.16	12.40***	48.53***	3.95*	18.15***	12.93***	7.55**
Dry matter content roots	-0.10	0.45	-2.03*	ns	ns	ns	ns

Interaction effects that were not significant have been removed from the model (ns). Number of stems, number of stolons and average and total stolon length and DMCR have been tested with a GLM. Some variables have been transformed: number of leaves:  $x^{1/4}$ , length of the longest stem:  $x^{1/2}$ , stem diameter:  $x^{1/2}$ , dry mass total:  $x^{1/15}$ , leaf:stem ratio:  $x^{1/2}$ , root:shoot ratio:  $x^{1/5}$ , relative growth rate:  $100 + x^{1.1}$ , dry matter content leaves and dry matter content stems:  $1/x$ . Signif. codes: \* < 0.05, \*\* < 0.01 \*\*\* < 0.001.

**TABLE 2** | F-values of the three-way ANOVA tests of morphological parameters, chlorophyll and nutrient stoichiometry parameters (n = 5–10).

	CO <sub>2</sub>	Flow	DOC	CO <sub>2</sub> *Flow	CO <sub>2</sub> *DOC	Flow*DOC	CO <sub>2</sub> *Flow*DOC
% N Leaves	107.68***	0.29	144.92***	ns	33.79***	ns	ns
% N Stems	539.56***	0.67	56.31***	ns	ns	ns	ns
% C Leaves	76.25***	0.96	5.05*	ns	ns	7.03*	ns
% C Stems	1.85	0.02	6.43*	8.31**	58.07***	8.05*	ns
C:N leaves	132.20***	4.57*	89.07***	ns	17.91***	ns	ns
C:N stems	221.05***	1.70	36.95***	ns	5.73*	ns	ns
% P leaves	0.14	3.24	38.14***	ns	ns	ns	ns
% P stems	3.68	2.61	235.58***	ns	ns	ns	ns
C:P leaves	0.28	3.75	24.35***	ns	ns	ns	ns
C:P stems	0.55	3.58	120.90***	ns	ns	ns	ns
N:P leaves	18.55***	2.68	9.49**	ns	ns	ns	ns
N:P stems	111.42***	0.93	56.32***	ns	ns	ns	ns
Total leaf area	87.07***	1.12	56.85***	5.51*	17.39***	ns	ns
Average leaf area	155.39***	0.84	9.55**	ns	41.59***	6.77*	ns
Specific leaf area	Na	2.47	5.28*	na	na	ns	na
Chlorophyll a	10.77**	0.91	0.92	7.40**	6.38*	4.37*	ns
Chlorophyll b	30.33***	0.15	4.50*	6.14*	ns	ns	ns
Chlorophyll a:b	19.45***	0.04	19.94***	ns	45.10***	ns	ns
Chlorophyll a + b	30.21***	0.07	ns	6.77*	ns	ns	ns
Carotenoids	32.68***	0.41	ns	7.30**	ns	ns	ns
Total plant chlorophyll	63.55***	0.027	28.22***	11.65**	28.48***	ns	ns

Interaction effects that were not significant have been removed from the model (ns). Some variables have been transformed: % N leaves:  $x^{-1}$ , % N stems:  $\log(x/0.8)$ , % C leaves:  $\log$ , % C stems:  $x^6$ , C:N leaves:  $1/x^{1/2}$ , C:N stems:  $x^{1/2}$ , % P leaves:  $x^2$ , C/P leaves:  $1/x$ , C/P stems:  $\log$ , N/P leaves:  $\log(x^{1/3})$ , N/P stems:  $\log$ , total leaf area:  $x^{1/4}$ , mean leaf area:  $\log$ , SLA:  $x^2$ , chlorophyll B:  $\log$ , chlorophyll A/B:  $x^2$ , total carotenoids and total plant chlorophyll:  $\log$ . Signif. codes: \* < 0.05, \*\* < 0.01 \*\*\* < 0.001.

**TABLE 3** | Omega squared values for the growth and morphological parameters ( $n = 48$ ).

	CO <sub>2</sub>	Flow	DOC	CO <sub>2</sub> *Flow	CO <sub>2</sub> *DOC	Flow*DOC	CO <sub>2</sub> *Flow*DOC
Number of leaves	0.43	0.012	0.257	0.009	0.016	0.004	0.006
Length longest stem	0.205	0.113	0.308	0.014	0.069	0.016	0
Diameter longest stem	0.241	0.021	0.482	0	0	0	0.006
Dry mass total	0.4	0.006	0.458	0.001	0.006	0	0.002
Leaf stem ratio	0.002	0.012	0.049	0	0.087	0	0
Root shoot ratio	0.216	0.023	0.031	0.025	0.125	0.002	0.005
Relative growth rate	0.567	0.021	0.276	0	0.009	0	0.007
Dry matter content leaves	0.02	0.001	0.069	0	0.017	0.005	0.022
Dry matter content stems	0.032	0.005	0.069	0	0.023	0.011	0.016

**TABLE 4** | Omega squared values for the morphological parameters, chlorophyll and nutrient stoichiometry parameters ( $n = 5-10$ ).

	CO <sub>2</sub>	Flow	DOC	CO <sub>2</sub> *Flow	CO <sub>2</sub> *DOC	Flow*DOC	CO <sub>2</sub> *Flow*DOC
% N Leaves	0.759	0	0.139	0	0.037	0	0
% N Stems	0.824	0	0.085	0	0	0	0
% C Leaves	0.436	0.016	0.183	0	0	0.034	0
% C Stems	0.134	0	0.186	0.038	0.296	0.037	0
C:N leaves	0.804	0.005	0.099	0	0.021	0	0
C:N stems	0.892	0	0.039	0	0.005	0	0
% P leaves	0	0	0.411	0	0	0	0
% P stems	0.009	0.006	0.806	0	0	0	0
C:P leaves	0	0.04	0.305	0	0	0	0
C:P stems	0	0	0.689	0	0	0	0
N:P leaves	0.221	0.023	0.108	0	0	0	0
N:P stems	0.506	0	0.256	0	0	0	0
Total Area	0.401	0	0.413	0.009	0.03	0	0
Mean Area	0.271	0.002	0.451	0	0.089	0.013	0
SLA Total	na	0.085	0.19	na	na	0	Na
Chlorophyll a	0.207	0.062	0.006	0.052	0.04	0.029	0
Chlorophyll b	0.231	0.065	0.033	0.045	0	0	0
Chlorophyll a:b	0	0	0	0	0.393	0	0
Chlorophyll a + b	0.22	0.067	0	0.054	0	0	0
Carotenoids	0.251	0.019	0	0.06	0	0	0
Total plant chlorophyll	0.374	0.02	0.352	0.026	0.064	0	0

determined. Beside chlorophyll concentration, total chlorophyll content per plant was calculated by multiplying the total chlorophyll concentration with the total fresh weight of the leaves of each plant (as chlorophyll was measured in fresh biomass).

## Plant Carbon, Nitrogen and Phosphorus Analysis

The dried plant material (leaves and stems separately) from each flume was combined into five samples (nine plants per sample), in order to have enough material for the analyses. Those combined samples were ground with an Ultra Centrifugal Mill ZM 200 (Retsch, Germany). The ground material was analysed for C and N content on a FLASH 2000 Organic Elemental Analyser, based on Flash Dynamic Combustion (Thermo Fisher Scientific, Waltham, MA, United States). P content was determined by acid digestion and subsequently

measured on ICP-OES (iCAP 6300 Duo view, Thermo Fisher, Waltham, MA, United States).

## Statistical Analyses

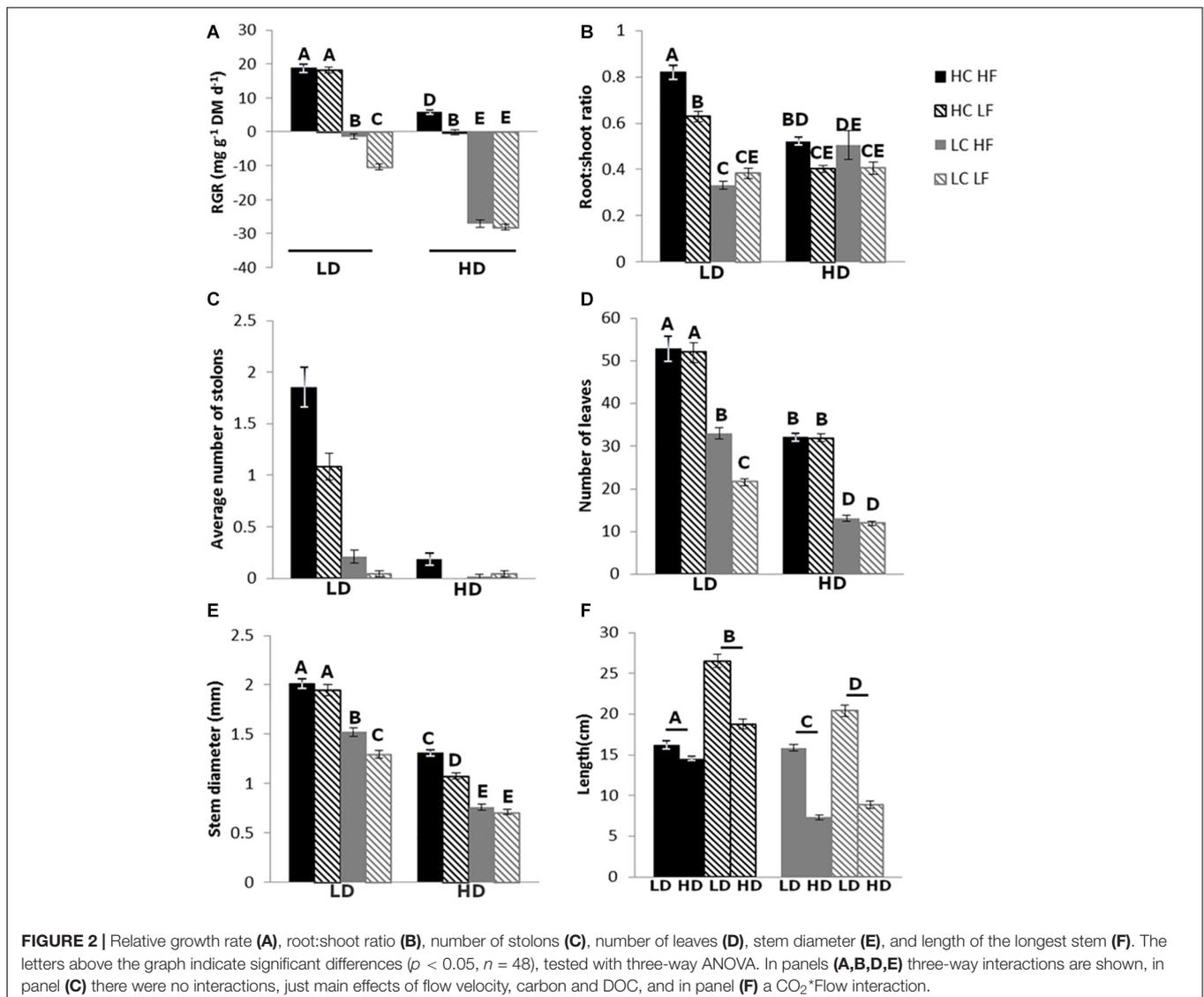
All statistical analyses were carried out in R statistics version 3.4.3. The effects of elevated CO<sub>2</sub>, DOC and flow velocity on growth and morphology parameters, chlorophyll, C, N and P content (35 traits in total, **Tables 1, 2**) were tested with a three-way ANOVA with type III sums of squares. Normal distribution of the residuals was tested with Shapiro–Wilk tests and checked visually with Q-Q plots, homogeneity was tested with Levene's tests, and if necessary, data were transformed to meet the assumptions. When significant, a Tukey HSD *post hoc* test was performed. Variables with count data (number of stems and number of stolons) were analysed with poisson regression and variables with a severe positive skew (average and total stolon length and leaf, stem and root dry matter content) were analysed with gamma regression. In order to test the relative importance

of the treatments and their interactions omega squared ( $\omega^2$ ) was calculated, which shows the proportion of the variance that is explained by every treatment and interaction. Negative values were set to zero as it can be assumed that those values signify that the effect was negligible (Graham and Edwards, 2001). R package “sjstats” (Lüdecke, 2019) was used to calculate  $\omega^2$  values and the values were visualised with Venn diagrams. To test how the plants responded to the treatment over time a Principal Response Curve (PRC) was used, which is a special case of the Redundancy Analysis (RDA) and was developed by Van den Brink and Ter Braak (1999). This was done using the “vegan” (Oksanen et al., 2019) package in R. In a PRC plot the effect of the different treatments is shown over time, relative to a control treatment that has been assigned before the analyses. The control treatment that was chosen is the “no climate change” scenario, with low CO<sub>2</sub>, low DOC and low flow velocity.

## RESULTS

### CO<sub>2</sub> and DOC Concentrations

The average CO<sub>2</sub> concentration in the HC-LD and HC-HD treatments was 1494 ppm ± 299 (62 ± 12 μM) and 1086 ppm ± 948 (45 ± 39 μM), respectively. In the LC-LD treatment it was 449 ppm ± 51 (19 ± 2 μM) and in the LC-HD treatment 183 ± 153 (8 ± 6 μM). The concentrations followed a day-night rhythm with the most pronounced fluctuations in the high DOC treatment (**Supplementary Figure S2**). The DOC added to the flumes had the following consistence: 72.6% humic substances, 11.64% neutrals with small molecular weight, 7.1% building blocks, 7.0% biopolymers and <5.5% acids with small molecular weight. The DOC concentration in the first experiment was very low (1.4 ± 0.3 mg L<sup>-1</sup>), whereas in the second experiment, where DOC was added regularly, it reached a reasonably constant value of 5.9 ± 0.8 mg L<sup>-1</sup>



(**Supplementary Figure S3A**). In the stock solution of DOC the amount of nutrients was relatively high, especially phosphate and ammonium. In a DOC solution of  $5 \text{ mg C L}^{-1}$  there was  $0.29 \text{ mg L}^{-1}$  phosphate ( $\text{PO}_4^{3-}\text{-P}$ ) and  $0.87 \text{ mg L}^{-1}$  ammonium ( $\text{NH}_4^+\text{-N}$ ). However, in the flumes the measured concentrations were far lower (**Supplementary Figure S4**), suggesting that nutrients were consumed rapidly.

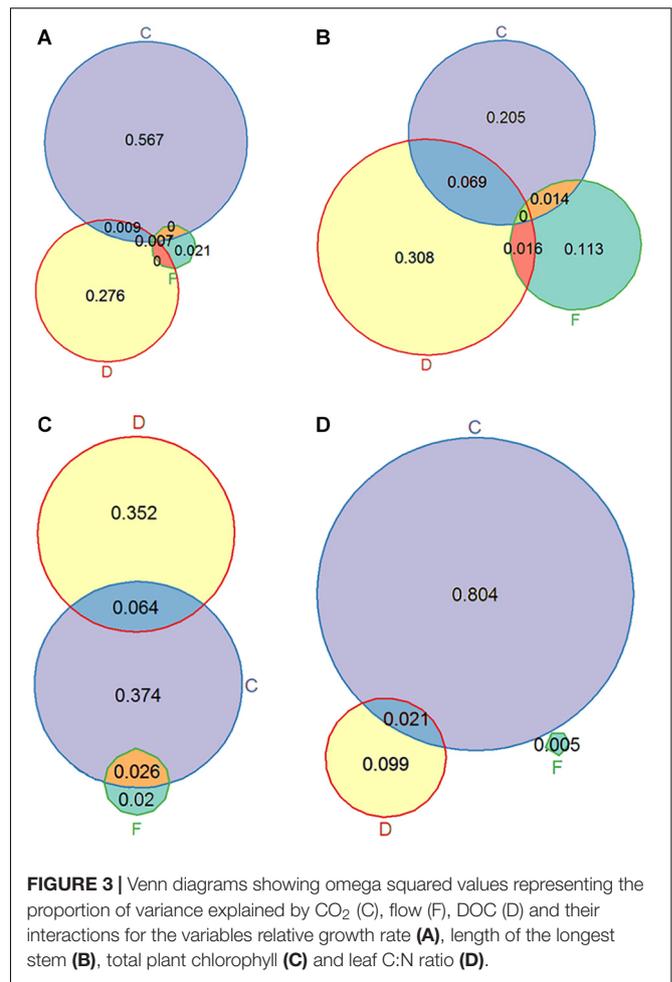
Light availability was lower in the second experiment compared to the first; this ranged (average for all flumes) from  $437.5 \pm 28.5 \mu\text{mol m}^{-2} \text{ s}^{-1}$  just below the water surface to  $163 \pm 22.7 \mu\text{mol m}^{-2} \text{ s}^{-1}$  at the bottom of the flumes (**Supplementary Figure S3B**). PAR availability decreased with 23.8% in water with increased DOC concentrations (measured in a 20 cm deep bucket, see section “Materials and Methods”). For more details on water quality (pH, alkalinity and nutrients) see **Supplementary Information** “detailed results and discussion” and **Supplementary Figure S4**.

## Effects of the Treatments and Interactions

Flow velocity,  $\text{CO}_2$  and DOC all affected *B. erecta*. Out of the 35 traits measured, in 32 of them there was a significant effect of DOC, in 25 of them there was a significant effect of  $\text{CO}_2$ , in 13 traits a significant effect of flow, in 20 a significant effect of the  $\text{CO}_2$ \*DOC interaction, in 14 a significant effect of the  $\text{CO}_2$ \*flow interaction, in nine a significant effect of the flow\*DOC interaction and in nine traits a three-way interaction (**Tables 1, 2**). When looking at the relative importance of the treatments (omega squared values), in most cases DOC and  $\text{CO}_2$  had the greatest effect, relative to the other treatments, followed by the  $\text{CO}_2$ \*DOC treatment. Flow velocity and the other interactions had lower omega squared values in most traits (**Tables 3, 4**).

## Macrophyte Growth and Morphology

In the following paragraphs the main results will be highlighted, for a more detailed overview of the results, see **Supplementary Information** “detailed results and discussion”.  $\text{CO}_2$  and DOC had a pronounced effect on the relative growth rate (RGR) (**Table 3**) which was significantly higher in plants exposed to HC compared to LC and higher in LD compared to HD (**Figures 2A, 3A**). Flow velocity had a smaller effect: plants growing under low flow velocity (LF) had a lower RGR than plants growing under high flow velocity (HF), but this was only significant in the LC-LD and HC-HD treatment (**Tables 1, 2**). In nearly all LC and HD treatments the average RGR was negative. Biomass allocation was also affected by the treatments: the root:shoot ratio was mainly affected by  $\text{CO}_2$ , and to a smaller extent by the  $\text{CO}_2$ \*DOC interaction (**Table 3** and **Figure 2B**). This can be seen in the LD treatment, where there is a positive effect of  $\text{CO}_2$  on root:shoot ratio, whereas there is no  $\text{CO}_2$  effect in the HD treatment. Moreover, the high flow, high  $\text{CO}_2$  and low DOC treatments resulted in more and longer stolons (**Figure 2C**). In most morphological traits a positive effect of  $\text{CO}_2$  and a negative effect of DOC was observed. This was most pronounced in the number of leaves (**Figure 2D** and **Table 3**). The number of stems, stem length, stem diameter (**Figure 2E**), total and average leaf

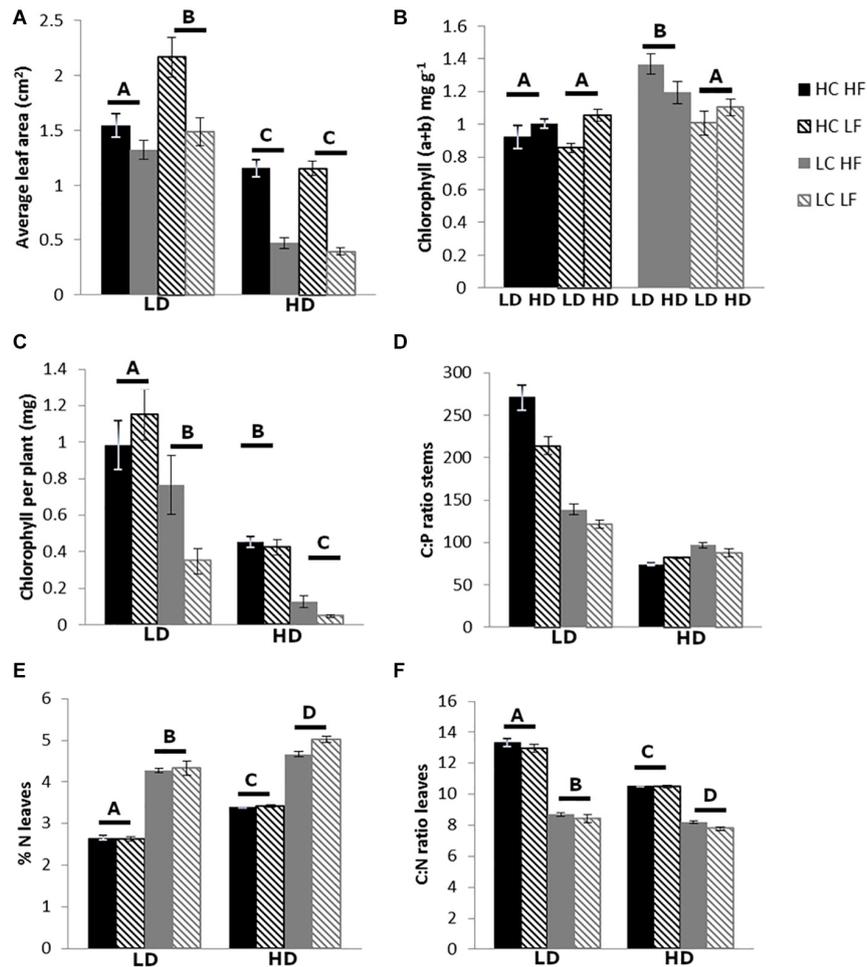


area and total dry mass were also significantly more numerous or larger in HC and LD than in LC and HD conditions, SLA was also higher in the HD than the LD treatment.

Flow velocity had a smaller effect on plant morphology: plants exposed to HF had more leaves and more and thicker stems than plants growing under LF, but this was only significant in the LC-LD treatment (**Figures 2D,E**). In the LD treatment, leaves exposed to HF were smaller than leaves exposed to LF. However, in the HD treatment there was no effect of flow velocity (**Figure 4A**). The clearest effect of flow velocity was observed in the stem length, with the longest stems in the LF treatment (**Figures 2F, 3B**).

## Chlorophyll and Nutrient Stoichiometry

For chlorophyll a, chlorophyll b and total chlorophyll ( $\text{a} + \text{b}$ ) concentration ( $\text{mg g}^{-1} \text{ FM}$ ) similar results were observed. Plants growing in the LC-HF treatment had a higher chlorophyll concentration than plants growing in other treatments (**Figure 4B**). When looking at the total chlorophyll content per plant, a different pattern was observed: plants exposed to HC appeared to have more chlorophyll than plants exposed to LC, and in the LD treatment they had more chlorophyll than in the HD treatment (**Figures 3C, 4C**), as the plants in the HC-LD



**FIGURE 4 |** Average leaf area (A), total chlorophyll concentration (B), total amount of chlorophyll per plant (C), C:P ratio of the stems (D), % N in the leaves (E), and C:N ratio of the leaves (F). The letters above the graph indicate significant differences ( $p < 0.05$ ,  $n = 5-10$ ), tested with three-way ANOVA. In panels (A,C,E,F),  $\text{CO}_2$ \*DOC interactions are shown, in panel (B) a  $\text{CO}_2$ \*flow interaction and in panel (D) there were no interactions.

treatment had more biomass. The chlorophyll a: chlorophyll b ratio was higher in the LC than the HC treatment when plants were exposed to LD, but this was the other way around when plants were exposed to HD.

The DOC treatment affected every component of nutrient stoichiometry: in the high DOC treatment plants had higher N, C, and P concentrations than in the low DOC treatment; for P the differences were most pronounced, leading to lower C:P and N:P ratios in the HD treatment (Figure 4D and Table 2). The  $\text{CO}_2$  treatment mainly affected plant N concentrations, which were lower in the HC than the LC treatment, resulting in higher C:N ratios in both leaves and stems (Table 4 and Figures 3D, 4E,F), especially in the LD treatment.

## Differences Over Time

For eight plant traits that have been measured four times during the experiment a PRC diagram was made (Figure 5) to show how the traits developed over time. This was done for: number of leaves and stems (total, on the main plants and on the newly

formed ramets at the end of the stolons), the number of stolons and the length of the longest stem. 30% of the treatment variance could be explained by the model ( $F = 82.264$ ,  $p = 0.001$ ). All plant traits had positive weights, indicating a positive relationship with the treatments in the diagram. This means that, especially toward the end of the experiment, all plant traits (especially the number of leaves) were favoured by most treatments except for the LC-LF-HD and LC-HF-HD treatments. The differences between the treatments become more pronounced toward the end of the experiment. While  $\text{CO}_2$  and DOC had a relatively large effect, the effect of flow velocity was limited for the traits measured in this analysis.

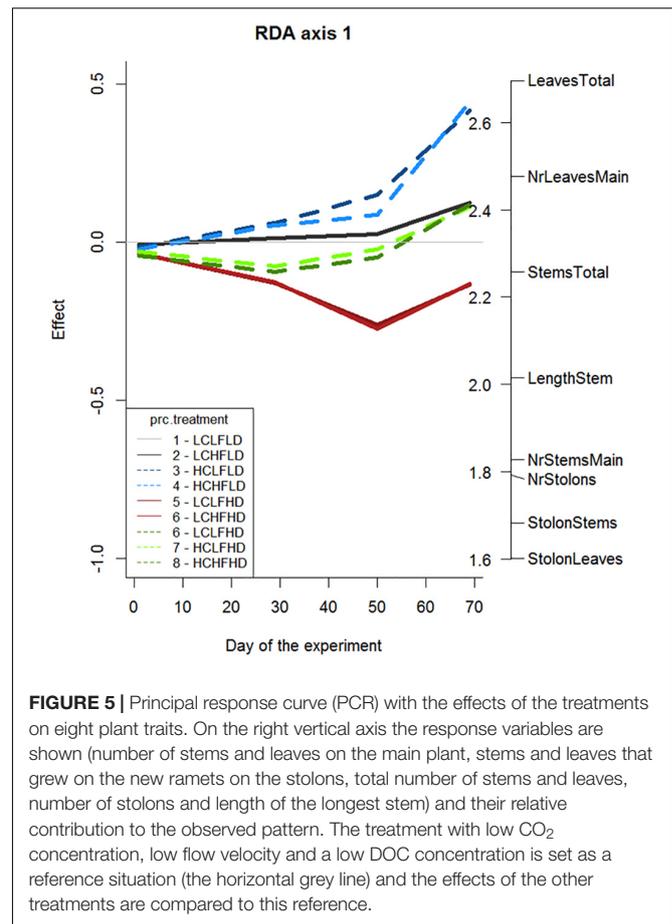
## DISCUSSION

In this study  $\text{CO}_2$ , DOC and, to a smaller extent, flow velocity (all potential effects of climate change) had strong effects on the growth and development of *B. erecta*, which is consistent

with what was found in literature (Steinberg et al., 2006; McElarney et al., 2010; Cao and Ruan, 2015; Reitsemá et al., 2020). Macrophytes that grew in the wet climate change scenario with increased heavy precipitation intensity (HD-HC-HF) had a higher RGR, more biomass, shorter stems, a higher root:shoot ratio, lower N content and higher P content than the plants growing in the no climate change scenario. The higher RGR and biomass production, especially belowground, seemed to be mainly caused by the increased CO<sub>2</sub> availability and this effect is also found in other studies (Dülger et al., 2017; Gufu et al., 2019). This effect is partly compensated by the negative effect of DOC on RGR and biomass production, which is probably caused by light limitation (Szmeja and Bociąg, 2004; Karlsson et al., 2009; Thrane et al., 2014), although DOC can also interfere with oxygen production (Pflugmacher et al., 2006) and cause oxidative stress (Steinberg et al., 2006). Additionally, the low, even negative, RGR in the HD treatment may have been caused indirectly by carbon limitation, besides the shading effect of elevated DOC. Measured CO<sub>2</sub> concentrations in the HD-HC treatment were lower than in the LD-HC, possibly caused by growth of epiphytic algae on the macrophytes (Supplementary Figure S1). *B. erecta* is an homophyllous amphibious plant that is unable to take up other forms of inorganic carbon than CO<sub>2</sub> and therefore it needs a high concentration of CO<sub>2</sub> to sustain photosynthesis (Nielsen, 1993).

The higher root:shoot ratio under HC has also been observed in other studies (Madsen et al., 1996; Yan et al., 2006; Hussner et al., 2016) and can be explained by root carbohydrate storage for overwintering (Dülger et al., 2017) and investment in clonal reproduction, which is regarded as a strategy to increase the plants' potential nutrient uptake (Yan et al., 2006). This last hypothesis seems to be most consistent with the results of the current study, as the stolons, which are used for clonal reproduction, were more numerous and longer in the LD-HC treatment.

In the HD-HC-HF scenario stems were shorter, and this may be explained by the plants' adaptation strategy to develop a more compact growth form in order to avoid hydrodynamic stress (breakage or uprooting), which has been observed in other research studying *B. erecta* (Puijalón et al., 2005). This idea is supported by the high root:shoot ratio in the HF treatment, which can be explained by the fact that roots enable plant anchoring (Schutten et al., 2005). The lower plant N content in the HD-HC-HF scenario seemed to be caused by the increased CO<sub>2</sub> treatment, which has been found in other studies as well (Titus and Pagano, 2002; Hussner et al., 2016). This may be explained by accumulation of carbohydrates under high CO<sub>2</sub> concentrations, leading to nitrogen savings (Dülger et al., 2017), although no evidence was found in the current study as the dry matter content in the leaves was similar under HC and LC and leaf C content was even smaller under HC compared to LC. The higher stem P content, which seemed to be caused by the high DOC treatment, is more difficult to explain. In literature the opposite is found: due to light limitation plants elongate their stems and in this structural tissue the relative amount of C is high and P is low (Su et al., 2016). In the current study P originating from DOC may explain the high P content. After adding DOC, a high P



peak was observed in the water, whereas this was less pronounced for N (Supplementary Figure S4D).

In the second climate change scenario with increased drought (HD-HC-LF) most of the results were comparable to the first climate change scenario with heavy precipitation (HD-HC-HF). The RGR was lower in the HD-HC-LF treatment, suggesting that there was a negative effect of increased boundary layers due to low flow velocity on biomass production (Westlake, 1967). However, the RGR was higher than in the no climate change scenario, suggesting that this negative effect of increased boundary layers was partially compensated by the increased CO<sub>2</sub> availability. The root:shoot ratio was smaller and stems were longer in the HD-HC-LF scenario compared to the HD-HC-HF scenario and were more similar to the no climate change scenario (LD-LC-LF), suggesting that flow velocity had a major impact on those morphological traits due to a stress avoidance response (see previous paragraph). With regard to nutrient stoichiometry, plants responded similarly to both climate change scenarios; flow velocity had a negligible effect on nutrient stoichiometry.

Most of the plant traits were strongly affected by CO<sub>2</sub>. However, it should be taken into account that most rivers and streams are supersaturated with CO<sub>2</sub> (Raymond et al., 2013), so *in situ* concentrations are likely always higher than the ones used in this experiment. Aquatic CO<sub>2</sub> enhancement due to

climate change may be relatively limited, and the effects on macrophytes less pronounced than in this experiment (Andersen and Pedersen, 2002). This means that the relative effects of flow velocity and DOC may be higher in natural situations. In this study, DOC had a negative effect on plant growth, but this is not observed for all macrophyte species: fast-growing potentially invasive species like *Hydrilla verticillata* (L. f.) Royle or *Elodea nuttallii* (Planch.) H. St. John show a positive growth response to DOC, due to accelerated growth rates under light limitation (Xu et al., 2018).

Although DOC is usually degraded in water by microorganisms, which results in CO<sub>2</sub> production (Sobek et al., 2005), this was not observed in this experiment: CO<sub>2</sub> concentrations in the LC treatment were lower in HD than LD conditions. Although additional tests confirmed that respiration increases when DOC is added to the water, in the flumes the extra amount of CO<sub>2</sub> was consumed fast. The macrophytes may have taken up this CO<sub>2</sub>, but it is more likely that algae used the main part, as in general DOC had a negative effect on macrophyte growth and algae growth was more pronounced in the HD than the LC treatment. Moreover, the higher biomass of periphytic algae may also have caused additional shading (**Supplementary Figure S1**). A second factor to take into account concerning the HD treatment, besides periphytic algae growth, is that this treatment was done a year later than the LD treatment. Still, as the experiments were done in the same time of the year for an equal number of days, with equal constant water temperature, and a comparable amount of solar radiation we think that this difference was very small and did not significantly affect the results of this study.

Dissolved organic carbon also had a negative effect on vegetative reproduction: the number of stolons in the HD treatment is very low compared to the LD treatment. This may be explained by light limitation caused by brownification: although the effect of DOC on stolon formation has not been studied before, it has been found that there is a negative effect of water depth on stolon formation in *Vallisneria spiralis* (L.) H. Hara (Xiao et al., 2007), which suggests that in low light conditions, in this case caused by DOC, macrophytes produce fewer stolons. These results show why it is important to study multiple aspects of climate change in experiments, as different climate change aspects can have contrasting results, which makes it difficult to predict the response of macrophytes and the rest of the aquatic ecosystem.

To conclude, in this study it was found that *B. erecta* strongly responds to climate change. High flow velocity mainly affected plant morphology; stems were shorter and belowground biomass relatively larger. Biomass production was stimulated by CO<sub>2</sub> and limited by DOC, and there were strong interaction effects between those two stressors. As CO<sub>2</sub> has a large positive effect and DOC has a small negative effect on biomass production, compared to the control situation, one would expect a positive effect of the combination of CO<sub>2</sub> and DOC. However, in this study the combined effects of CO<sub>2</sub> and DOC are less positive than the sum of the two effects separately (a positive antagonistic effect) (Piggott et al., 2015). This means that elevated DOC concentrations can form a major

reduction of performance in *B. erecta*, and this cannot be completely compensated by increased CO<sub>2</sub>. Therefore, if DOC levels rise in the future, it can be expected that, depending on the macrophyte species and abundance of epiphytic algae, macrophyte biomass production and reproduction is negatively affected, and it can also indirectly influence ecological functions of the ecosystem, because macrophytes play an important role in riverine ecosystems. For example, a reduction in macrophyte biomass may imply reduced nutrient cycling between sediments and water column (Clarke, 2002), a reduction in dissolved oxygen (Carpenter and Lodge, 1986) and reduced diversity of macroinvertebrates and small fish (Camp et al., 2014). It is important that more studies investigate changes in the DOC and CO<sub>2</sub> concentrations, flow velocity and other parameters that will change due to climate change in rivers and how these changes correlate with macrophyte growth and the health of the ecosystem.

## DATA AVAILABILITY STATEMENT

The datasets generated for this study are available on request to the corresponding author.

## AUTHOR CONTRIBUTIONS

All authors contributed to the study design, commented on previous versions of the manuscript, and read and approved the final manuscript. RR, JS, J-WW, and SP performed the material preparation, data collection, and analysis. RR drafted the first manuscript.

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## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fenvs.2020.527801/full#supplementary-material>

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The handling editor is currently organising a Research Topic with the authors JS and TH, and confirms the absence of any other collaboration.

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