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Effects of climate change on growth and development of *Berula erecta* as model species for freshwater macrophytes

Effecten van klimaatverandering op de groei en ontwikkeling van *Berula erecta* als modelsoort voor zoetwaterplanten

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Promotoren: Prof. dr. Jonas Schoelynck

Prof. dr. Patrick Meire

Cover design: Ann Roelant
Drawing on cover: Rosanne Reitsema
Lay-out: Jan-Willem Wolters & Rosanne Reitsema

Tales from the seas

Cathedral of green

The very core of life

The soaring high of truth and light

The music of this awe

Deep silence between the notes

Deafens me with endless love

Nightwish – Shudder before the beautiful

Tuomas Holopainen (2015)

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Abstract

Freshwater ecosystems are one of the most diverse, but also one of the most threatened ecosystems in the world. Aquatic macrophytes are highly affected by consequences of climate change like increased concentrations of dissolved organic carbon (DOC) and carbon dioxide (CO_2), but also changes in flow dynamics and eutrophication. Knowledge on the effects of DOC and CO_2 on macrophytes, and especially their interaction effects with other effects of climate change, is relatively limited. Therefore, the aim of this thesis was to study effects of climate change, like increases in carbon concentrations, using a holistic approach that also focused on their interaction effects with other environmental variables, rather than only studying effects separately.

The main macrophyte species studied in this thesis is *Berula erecta* (lesser water parsnip). Under natural conditions in a temperate lowland stream, this species was found to be highly variable in its biomass, morphology and nutrient content throughout the growing season, and there were interactions between plant growth (biomass and morphology) and environmental parameters like flow velocity and fine sediment depth. This implies that if flow velocity increases due to climate change, it can be expected that macrophyte morphology changes.

The effects of climate change were tested in a greenhouse experiment by exposing two macrophytes species, *B. erecta* and *Myriophyllum spicatum* to different concentrations of CO_2 and DOC in a wide range. The macrophytes responded to both treatments, with the strongest effects in the highest doses. There were large differences between the two species, with regard to growth and morphological responses.

In order to study interaction effects among CO_2, DOC, flow velocity and nutrients, a series of experiments was done in racetrack flumes. First, *B. erecta* was exposed a combination of CO_2, eutrophication and increased flow velocity. Those stressors sometimes had opposing effects: CO_2 stimulated growth, eutrophication indirectly limited growth due to shading by epiphytic algae and increased flow velocity led to a more compact growth form. Due to the combination of CO_2 and flow velocity macrophytes developed in a more horizontal way. Plants exposed to CO_2 also had a higher C:N ratio, which decreases the quality of the biomass, which can cause a problem when it serves as a food source to other organisms. Combined effects of the three stressors

may lead to a decrease in macrophyte abundance. Additionally, *B. erecta* was exposed to a combination of CO_2, DOC and increased flow velocity. Again, stressors had opposing effects where CO_2 stimulated growth, DOC limited growth due to shading and increased flow velocity led to a more compact growth form. DOC also had a negative effect on vegetative reproduction (the number of stolons). From studying the interactions between CO_2 and DOC effects, it can be concluded that especially elevated DOC concentrations can form a major threat to macrophyte growth.

From this thesis it can be concluded that climate change can have a large effect on macrophytes. Different aspects of climate change often have opposing effects, with many interaction effects occurring among them. Taking all aspects of climate change together, the results from this thesis indicate that submerged macrophytes in temperate lowland streams and rivers will decrease in biomass quantity and quality under continuing climate change. This will in turn have negative consequences for ecosystem processes and organisms that depend on the macrophytes.

Samenvatting

Zoetwaterecosystemen zijn een van de meest diverse maar ook een van de meest bedreigde ecosystemen op aarde. Aquatische macrofyten worden sterk beïnvloed door de gevolgen van klimaatverandering, verhoogde concentraties van opgeloste organische koolstof (DOC) en koolstofdioxide (CO_2), maar ook veranderingen in stroomsnelheidsdynamiek, en eutrofiëring. Kennis over effecten van DOC en CO_2 op waterplanten, en vooral over interacties met andere effecten van klimaatverandering is relatief beperkt. Daarom was het doel van deze thesis om effecten van klimaatverandering, zoals stijgende koolstofconcentraties, met een holistische benadering te onderzoeken waarbij ook naar interacties met andere milieufactoren is gekeken, in plaats van alleen afzonderlijke effecten te onderzoeken.

In deze thesis is vooral macrofytensoort *Berula erecta* (kleine watereppe) onderzocht. Onder natuurlijke omstandigheden in een laaglandbeek bleek dat biomassa, morfologie en het nutriëntengehalte sterk varieerden doorheen het groeiseizoen, en waren er interacties tussen plantengroei (biomassa en morfologie) en omgevingsfactoren zoals stroomsnelheid en de diepte van de fijne sedimentlaag. Dit betekent dat als stroomsnelheid stijgt door klimaatverandering, het verwacht kan worden dat de morfologie van macrofyten verandert.

Effecten van klimaatverandering zijn getest in een experiment waarbij twee macrofytensoorten (*B. erecta* en *Myriophyllum spicatum*) zijn blootgesteld aan een wijde CO_2 en DOC gradiënt. De macrofyten reageerden op beide behandelingen, met de sterkste effecten bij de hoogste doses. Er waren grote verschillen tussen de twee soorten wat betreft hun groei- en morfologierespons.

Interacties tussen CO_2, DOC, stroomsnelheid en nutriënten zijn onderzocht in een aansluitend experiment in stroomgoten. Eerst is *B. erecta* blootgesteld aan een combinatie van verhoogde CO_2, nutriënten en stroomsnelheid. Deze drie factoren hadden soms tegengestelde effecten: CO_2 stimuleerde de groei, nutriënten limiteerden indirect de groei door beschaduwing van epifytische algen en verhoogde stroomsnelheid leidde tot een compactere groeivorm. Door de combinatie van CO_2 en stroomsnelheid gingen de planten zich meer in de breedte ontwikkelen. Planten die warden blootgesteld aan CO_2 hadden ook een hogere C:N ratio, wat betekent dat de kwaliteit van de biomassa lager wordt,

wat een probleem kan vormen als het als voedselbron dient voor andere organismen. Wanneer de drie bestudeerde effecten tegelijkertijd plaatsvinden zou dit kunnen leiden tot een lagere abundantie van macrofyten. In een volgend experiment werd *B. erecta* blootgesteld aan een combinatie van verhoogde CO_2, DOC en stroomsnelheid. Ook hier hadden de factoren tegengestelde effecten waarbij CO_2 groei stimuleerde, DOC groei limiteerde door beschaduwing en stroomsnelheid leidde tot een compactere groeivorm. DOC had ook een negatief effect op vegetatieve reproductie (het aantal stolonen van de plant). Wanneer gekeken wordt naar interacties tussen CO_2 en DOC kan er geconcludeerd worden dat met name verhoogde DOC concentraties een aanzienlijke bedreiging voor de groei van macrofyten kan vormen.

Aan de hand van deze thesis kan er geconcludeerd worden dat klimaatverandering grote effecten op macrofyten kan hebben. Verschillende aspecten van klimaatverandering hebben vaak tegengestelde effecten en er treden interacties op. Wanneer alle aspecten van klimaatverandering tegelijkertijd optreden kan er, op basis van de resultaten in die in deze thesis werden gevonden, verwacht worden dat ondergedoken macrofytenbiomassa in rivieren zal afnemen in kwantiteit en kwaliteit. Dit kan vervolgens negatieve gevolgen hebben voor aquatische processen en voor organismen die afhankelijk zijn van macrofyten.

Chapter 1.

General introduction

Freshwater ecosystems

Freshwater ecosystems are one of the most diverse ecosystems in the world, with different types of biomes and habitats, like surface water, groundwater or riparian systems and they can be lentic or lotic. Abiotic factors like temperature, light availability, nutrients and dissolved gases are also highly variable (Geist 2011). As a consequence, biodiversity in freshwater ecosystems is relatively high compared to other ecosystems. A third of all vertebrate species live in freshwater (Dudgeon et al. 2006). Whereas (surface) freshwater habitats cover only 0.8% of the earth's surface (Gleick 1996), it has been estimated that almost 6 % of all species known to science live in freshwater systems (Hawksworth and Kalin-Arroyo 1995). It is likely that this percentage is even higher, as many freshwater species, especially invertebrates and microbes, have not been described yet (Dudgeon et al. 2006). Freshwater ecosystems are also particularly valuable systems to humans as they provide a wide range of ecosystems services: they provide clean water for humans, animals and surrounding ecosystems, they protect us from hazards like flooding, they highly contribute to biogeochemical cycles, support our economies (e.g. by enabling transportation by ships and hydropower), and deliver cultural services through recreation, tourism and education (Durance et al. 2016).

As freshwater systems are economically important, they are heavily used, posing a threat to its biodiversity but also to ecosystem services. Especially species living in lotic systems are under threat (Collen et al. 2014). Many groups of species show relatively fast declines, e.g. the decline of freshwater megafauna is twice as high as the decline of terrestrial and marine macrofauna (He et al. 2019). There are five major categories of threats to biodiversity: overexploitation (mainly of vertebrates), water pollution (nutrients, chemicals, plastic, noise and light), flow modification (e.g. dam building), habitat degradation (e.g. sand excavation or removal of riparian vegetation) and invasion by exotic species and pathogens (Dudgeon et al. 2006, Reid et al. 2019). Those stressors also interact with each other, which often implies that together they are more dangerous than when acting separately (Jackson et al. 2016). Currently, 82 % of the human population is served by freshwater provisions of which the upstream area is exposed to threats, which highly affects water availability and safety and food security (Green et al. 2015). Freshwater vertebrate populations have declined by 83 % between 1970 and 2014, and as

a consequence, many species are at risk of extinction. Although trends in biodiversity of other organisms is less well studied, freshwater taxa like amphibians, fishes, invertebrates, microbes, plants, turtles and waterbirds are under threat too. In terrestrial and marine systems population declines are less severe (Reid et al. 2019). 2018, 2019 and 2020 have been relatively dry years in Europe with regional low groundwater tables and water shortages. In order to make freshwater availability more sustainable and climate proof, there are initiatives to combat drought at the local scale, like the Blue Deal in Flanders (De Witte and Torfs 2020) and the Delta Programme in The Netherlands (2020).

Macrophytes

Macrophytes are important organisms in many freshwater ecosystems. The term 'macrophytes' literally means 'large plants' and can be defined as aquatic photosynthetic organisms that can be seen with the naked eye, which vegetative parts grow permanently or periodically in water (Chambers et al. 2007). Traditionally, they are classified based on growth form, and according to this classification there are four different types of macrophytes: emergent, floating-leaved, submerged, and free-floating plants. However, in streams they are often classified based on their habitat: obligate submerged, amphibious and terrestrial plants (Bowden et al. 2017). This thesis is mainly about macrophyte species *Berula erecta* (Huds.) Coville, which is an amphibious species: it can live emergent on land, but also fully submerged (Bowden et al. 2017), although this thesis mainly deals with submerged individuals. *B. erecta* is used as model species for submerged macrophytes in this thesis. It is a sub-cosmopolitan species that can grow in both lentic and lotic systems (de Belair and Lansdown 2013). *B. erecta* can only use CO_2 as inorganic carbon source (Sand-Jensen et al. 1992). About 50% of all macrophyte species can also take up HCO_3^-, when (local) CO_2 concentrations are low (Madsen and Sand-Jensen 1991). Therefore, *B. erecta* may not be representative for all species, so in one of the experiments of this thesis another species was used: *Myriophyllum spicatum* L., an obligate submerged species. This macrophyte species can also take up HCO_3^- (Maberly and Madsen 1998). This thesis focusses on submerged macrophytes, because of their important role in ecosystems (see below) and because they are relatively vulnerable to climate change (Short et al. 2016). As emergent and floating

macrophytes are directly exposed to the atmosphere, they can be expected to be less affected by changing conditions in the water due to climate change.

Macrophytes are important species in many aquatic ecosystems, though in this thesis there will be a focus on macrophytes in rivers and streams. In those systems there are many interactions between macrophytes and their environment (figure 1.1). In macrophyte-dominated streams, there is a natural fluctuation in oxygen levels, depending on light availability (Uehlinger et al. 2000, Desmet et al. 2011). Macrophytes can drive stream metabolism; plant cover is often strongly correlated to primary production in the system (O'Brien et al. 2014). During a dry summer in eutrophic rivers, algae- and phytoplankton blooms occur, and they can

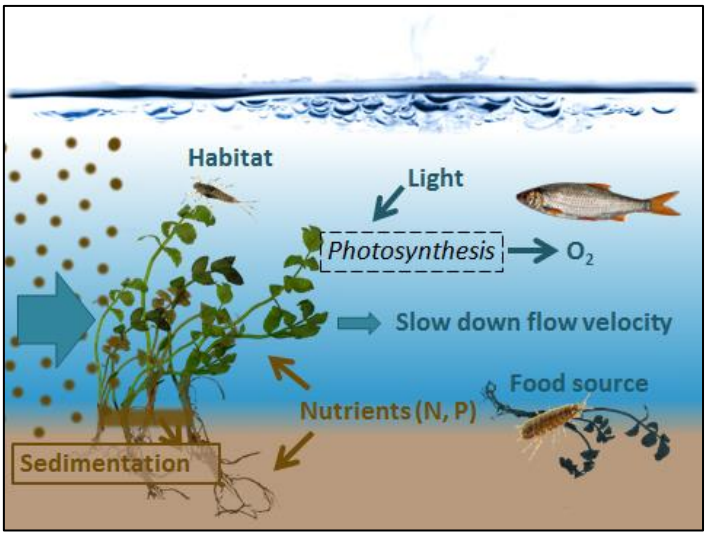

Figure 1.1 Interactions between macrophytes and their environment in rivers and streams. Macrophytes provide oxygen through photosynthesis using light, they take up nutrients from water and sediments, they slow down flow velocity and stimulate sedimentation of suspended matter. Within macrophyte patches, fish and macroinvertebrates find a habitat and dead plants form a food source.

change oxygen dynamics, leading to oversaturated or hypoxic peaks (Desmet et al. 2011). This can cause a decrease in diversity and abundance of other aquatic species, like macroinvertebrates or fish, that depend on oxygen availability (Kalogianni et al. 2017). After plant senescence, macrophytes enter the foodweb as detritus and form a food source for many organisms (Vannote et al. 1980). Macrophytes also affect nutrient cycling and sedimentation: macrophytes take up nutrients, if they have roots they form a link between the water column and the sediments and as they slow down flow velocity in streams, they stimulate sedimentation of suspended matter (Clarke 2002). Due

to the interaction with flow velocity, macrophytes organise in patches, with low flow velocity and high sedimentation within a patch and high flow velocity and low sedimentation outside of the patch (Schoelynck et al. 2012b). Macrophyte patches can consist of one or multiple species, they can be clearly or poorly delineated, and they can act as one hydrodynamic patch in their interaction with flow velocity, which increases their influence in river processes (Schoelynck et al. 2018). Macrophyte patches are habitats for macroinvertebrates and fish, and the roots improve sediment stability (Sand-Jensen 1998). All in all, by modifying the aquatic ecosystem, macrophytes can be seen as physical engineers in riverine systems (Gurnell 2014).

As stated above, freshwater ecosystems are currently under threat and this also includes macrophytes. Many macrophyte species only have a narrow distributional range and a high degree of endemism (Murphy et al. 2019), which makes them particularly vulnerable to extinction. Macrophytes are highly threatened by changes in hydromorphology caused by restructuring of water courses, water pollution (Steffen et al. 2013), changes in land use and invasive species (Hofstra et al. 2020). Currently, 19% of the macrophytes species assessed by the International Union for Conservation of Nature (IUCN) are at risk (Murphy et al. 2019).

Climate change

Besides the stressors described above, there is another main stressor acting on freshwater ecosystems and macrophytes within them: climate change. According to the Intergovernmental Panel on Climate Change (IPCC), the temperature on our planet has increased by 1°C, relative to the period 1850-1900 due to anthropogenic emissions of greenhouse gases like carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O), and it is expected that this will further increase (Allen et al. 2018). This has various direct consequences and a cascade of indirect consequences for freshwater ecosystems.

Temperature

Water temperatures are sensitive to changes in atmospheric temperature, so water temperatures rise as well (van Vliet et al. 2013). In general, increased temperatures lead to increased biomass production when tested in experiments, but only if sufficient amounts of light, nutrient and inorganic

carbon are available (Barko et al. 1982, Zhang et al. 2020). In many cases climate change is not only about general warming, but also about fluctuations in temperatures and seasons. Fluctuating temperatures can reduce the number of flowers and decrease sexual reproduction in macrophytes (Li et al. 2017, Xu et al. 2020). When winters are less severe due to climate change, macrophytes can develop sooner in spring (Barko et al. 1982), which gives macrophytes and advantage over phytoplankton, but when warming is combined with high nutrient concentrations, phytoplankton can outcompete macrophytes (Moss et al. 2011). Increasing temperatures can also impact nutrient dynamics, for example by stimulating internal eutrophication, which enhances phosphorus release from the sediments (Short et al. 2016). As a result of climate change, a higher frequency of heatwaves is expected, which can change macrophytes species composition and decrease sexual reproduction (Li et al. 2017).

Discharge

Indirectly, there is a second important effect: changes in river discharge (Hoegh-Guldberg 2018). This is caused by changes in precipitation and increased frequency of extreme events like heat waves or extreme precipitation (Jacob et al. 2018), which lead to periods of lower or higher flow velocity. As stated above, many river courses have been restructured, which limits the amount of habitat with slower flowing water (Steffen et al. 2013). If pulses of high flow velocity occur more frequently in those rivers, many species that are intolerant to hydrodynamic stress may disappear. Events of extreme precipitation can also affect water quality by increasing runoff, which causes higher input of nutrients (Jeppesen et al. 2011) and organic matter (Pagano et al. 2014).

Nutrient concentrations

With increased surface runoff nutrient loading in aquatic ecosystems can increase and this can lead, especially in cultivated catchments, to eutrophic conditions (Jeppesen et al. 2011, Coffey et al. 2019). Secondly, nutrient concentrations can also increase during droughts due to evapoconcentration (Jeppesen et al. 2011). Although nutrients are required for macrophyte growth, too many nutrients can give a competitive advantage to other primary producers. Periphyton (Sand-Jensen and Borum 1991), and in stagnant conditions, non-rooted macrophytes (Hough et al. 1989) can limit submerged

macrophyte growth due to shading (Hilton et al. 2006). Eventually this can lead to a shift from a system dominated by macrophytes to a system dominated by algae (Scheffer et al. 1993). Although the critical nutrient value triggering this shift depends on the characteristics of the system, there are definitions of trophic states. In rivers and streams, the system is called eutrophic if the N concentration is higher than 1.5 mg L^{-1}, and if the P concentration is higher than 0.075 mg L^{-1} (Smith et al. 1999). Climate change can aggravate nutrient problems, as increased temperatures affect nutrient flows and thereby it can increase eutrophication (Beklioglu et al. 2010).

Organic matter input: effects on aquatic carbon

Input of organic matter has two major consequences for the aquatic ecosystem and macrophytes. Firstly, dissolved organic carbon (DOC) leaches from organic matter. DOC consists of a wide range of molecules, but they have in common that they are smaller than 0.45 µm and mainly consist of carbon (Bolan et al. 2011). The molecules have a wide variety of chemical functional groups (Leenheer and Croué 2003). DOC often gives the water a yellow to brown colour due to a specific compound often present in DOC: humic substances (HS). HS usually make up 60-90% of the total DOC in natural waters and mostly originate from plant- or animal material from which readily bioconsumable parts have been removed (Frimmel 2005). Since the 1990s DOC concentrations are increasing in European and North American rivers and lakes (Monteith et al. 2007), and the relative contribution of HS also becomes larger (Kellerman et al. 2014). This can negatively affect macrophyte growth, since HS decrease the amount of light available to macrophytes (Thrane et al. 2014). The exact effects of this 'brownification' of the water on macrophytes are still poorly understood. There are studies that found a negative effect on primary production (Szmeja and Bociąg 2004, Karlsson et al. 2009), but there are only a few experimental studies on macrophytes. HS may also directly affect macrophytes by entering the plant's cells and causing damage by production of reactive oxygen species (Grigutytė et al. 2009) or by interfering with photosynthesis (Pflugmacher et al. 2006). An overview of effects of DOC on macrophytes is still lacking, which makes it difficult to predict how macrophytes will respond if concentrations of DOC are rising.

When DOC is degraded by microorganisms, CO_2 is released (Sobek et al. 2005). CO_2 is a form of inorganic carbon which exists as a gas in the atmosphere but can dissolve in water. Dissolved CO_2 is not the only form of inorganic carbon: it forms an equilibrium with bicarbonate (HCO_3^-), carbonate (CO_3^{2-}) and carbonic acid (H_2CO_3), although carbonic acid is unstable and is only present as a negligible small fraction (Stumm and Morgan 1996). The abundance of the forms of inorganic carbon depends on the pressure of atmospheric CO_2, and the pH, alkalinity, temperature and salinity of the water (figure 1.2) (Stumm and Morgan 1996). For macrophytes this carbon equilibrium is relevant, as they need inorganic carbon for their photosynthesis. Some species only use CO_2, whereas other species have adapted to also use HCO_3^-, as CO_2 can be scarce because diffusion occurs 10^4 times slower than in air (Madsen and Sand-Jensen 1991).

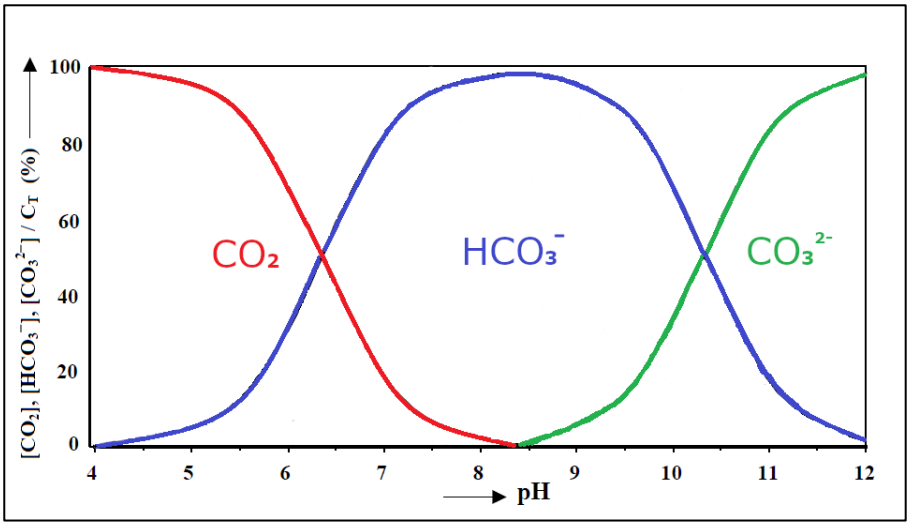

Figure 1.2 The inorganic carbon equilibrium (at 25 °C), with on the y-axis the concentration of CO_2, HCO_3^- and CO_3^{2-} as percentage of total inorganic carbon, and on the x-axis the pH.

In water bodies with low buffering capacity (e.g. softwater lakes), the concentration of CO_2 is usually in equilibrium with the atmosphere (Murphy 2002). If the CO_2 concentration in the air rises due to anthropogenic emissions, the concentration in the water rises as well, which can lead to the disappearance of isoetids: a type of macrophytes that has adapted to low CO_2

concentrations in the water column (Spierenburg et al. 2009). Most freshwater ecosystems, however, are already supersaturated with CO_2. The median for rivers and streams is 3100 ppm, for non-tropical freshwater lakes this is 1120 ppm and for tropical lakes 1910 ppm (Raymond et al. 2013), whereas in the air the concentration is around 400 ppm. Although current concentrations are relatively high, it is expected that CO_2 concentrations in freshwater ecosystems will increase. This is partly due to lower outgassing as atmospheric concentrations rise (Phillips et al. 2015), but another main driver of aquatic CO_2 concentrations is microbial respiration (Manahan 2000). Increased input of organic matter caused by climate change leads to increased CO_2 concentrations in aquatic systems (Hasler et al. 2016).

Studies on increased CO_2 in aquatic systems mostly focus on marine ecosystems. In those systems rising CO_2 concentrations lead to a drop in ocean pH (figure 1.2) and its effect on marine organisms is well documented (Boyd et al. 2016). In freshwater ecosystems this is less well documented and more complex because freshwater ecosystems are very heterogenic with regard to factors that determine the pH and CO_2 concentrations of the water, like alkalinity, substrate, number of autotrophs and heterotrophs, precipitation, source of the water and land use in the surrounding area (Hasler et al. 2016). Oceans have an alkalinity of around 2.3-2.6 meq kg^{-1} and a pH of around 8.1 (McCulloch et al. 2012). In Freshwater ecosytems, alkalinity can vary from <0.1 to > 4.0 meq kg^{-1}, and pH from <5 to >9 (Bloemendaal and Roelofs 1988). Because of processes like photosynthesis and respiration the pH fluctuates in freshwater systems, which means that species are adapted to this, in contrast to species in the more stable ocean. However, if the average CO_2 concentration is slowly increasing due to climate change, acidification can become a problem. There is no evidence that macrophytes are negatively affected by small drops in pH. In contrast, they often seem to benefit if there is a larger availability of CO_2 (Vadstrup and Madsen 1995). Acidification by increased CO_2 concentrations can lead to lower growth rates, changed behaviour, damage and even mortality in animals like fish and invertebrates (Hasler et al. 2017, Weiss et al. 2018).

It is important to keep in mind that some stressors will continuously act on macrophytes (e.g. temperature) and others will come in pulses (e.g. flow velocity and increases in nutrients. In this thesis all stressors will be tested in a

continuous way, to be able to tell how they affect macrophytes. In reality, most of the stressors tested will come in pulses, which is relevant as macrophytes have less time to adapt to sudden changes.

Research questions and aims of the thesis

When looking at studies focusing on effects of climate change on macrophytes, a couple of conclusions can be drawn. Firstly, many studies focus on effects of temperature and changes in discharge. Secondly, climate change often results in a complex combination of different stressors that sometimes have opposing or enforcing effects and possible interactions between those stressors are mostly neglected. In figure 1.3 an overview is shown of what is currently known about effects of climate change on macrophytes. Although separate effects have been studied, knowledge on interaction effects is limited, which makes it difficult to predict the net effect of different stressors. Besides, effects of DOC have not been studied on a large number of plant traits. Therefore, in this thesis there will be a focus on a relatively understudied, but important aspect of climate change (carbon) and secondly on interactions with other aspects of climate change. I chose to focus on the effects of aquatic carbon. As explained above, aquatic CO_2 concentrations are rising, but this cannot only explained by rising atmospheric concentrations: it is also caused by degradation of dissolved organic carbon DOC (Hasler et al. 2016). Concentrations of DOC are rising as well, and this highly impacts macrophytes (Steinberg et al. 2006). As the rise of CO_2 and DOC concentrations are linked, I decided to study the effects of both of them in this thesis, and to combine this with other relevant stressors. In this thesis effects of variables tested in the experiments, like CO_2 and DOC are called stressors in the text, but whether the macrophytes experience it as a stress will of course depend on the dose.

Although there are studies that have looked into the effects of elevated CO_2 and DOC concentrations on macrophytes, there are still many questions regarding those effects. Firstly, quite a number of studies have looked into the effects of CO_2 on macrophytes, but usually only two different concentrations were used e.g. (Cao and Ruan 2015, Eller et al. 2015), and those concentrations are often lower than current and predicted future concentrations in most natural waters.

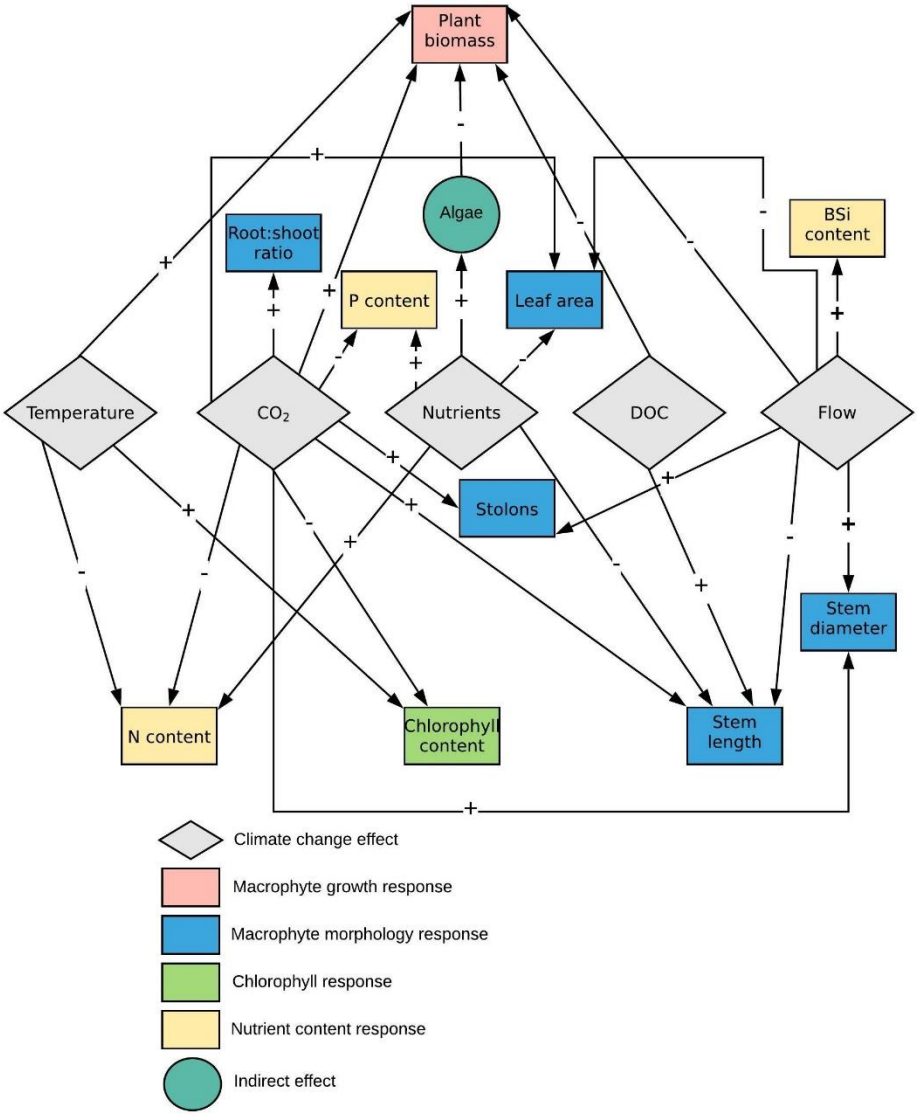

Figure 1.3 Overview of effects of climate change on submerged macrophytes. This is a general scheme and exact effect will depend on the magnitude of each climate change factor. Especially effects of DOC are not clear yet, and interaction effects are largely unknown. This makes it difficult to determine the net effect on each macrophyte trait.

Research on effects of DOC is more limited. Many studies are field studies that only look at a couple of effects, like colonisation depth (Chambers and Prepas 1988) or species diversity (Bociąg 2003). Studies on primary production often focus on algae and bacteria (Ask et al. 2009, Karlsson et al. 2009). Experimental studies looking at macrophytes often use charophytes and mainly test effects of artificial DOC (Pflugmacher et al. 2006, Pörs and Steinberg 2012, Choudhury et al. 2019), which is rather different from natural DOC. Lastly, as explained above, effects of CO_2 and DOC are often studied in isolation, whereas in reality they will often co-occur with stressors like increased discharge or eutrophication (Pagano et al. 2014). This study aims to address these scientific lacunas by combining field studies with greenhouse experiments in standing and flowing water. As explained above, I chose to mainly focus on one macrophyte species to be able to compare different aspects of climate change tested in this thesis. This leads to the following overarching research question: **What is the effect of climate change induced rising carbon concentrations (i.e. CO_2 and DOC) on growth and development of freshwater macrophyte species *Berula erecta* and how do those effects interact with other stressors related to climate change?**

Hypotheses

Based on literature I hypothesise that:

1. Elevated CO_2 will increase growth rate in macrophytes, increase root:shoot and the C:N ratio. Differences will be stronger in *B. erecta* (obligate CO_2 user) than in *M. spicatum* (which can also use HCO_3^- as an inorganic carbon source).
2. Elevated DOC will decrease macrophyte growth and increase plant stem length due to shading effects. Low DOC levels may stimulate macrophyte growth, as CO_2 is released when DOC is degraded and shading effects are relatively small.
3. Elevated flow velocity will decrease macrophyte growth due to drag forces and decrease plant stem length.
4. Elevated nutrient concentrations will decrease macrophyte growth rate due to competition with algae.

5. Combined effects of elevated CO_2, nutrients and flow velocity will decrease macrophyte growth, due to competition with algae and drag forces.
6. Combined effects of elevated CO_2, DOC and flow velocity will slightly increase macrophyte growth (if low levels of DOC are used). Shading effects due to DOC and drag forces due to increased flow velocity will decrease growth, but this will partially be compensated for by CO_2 and the extra CO_2 that is released when DOC breaks down.

Each chapter focuses on a different aspect of the research question, in figure 1.4 an overview is shown how the chapters are linked. **Chapter 2** is a field study on *B. erecta* where its development over a growing season in different plant densities is monitored. In this thesis there is a large focus on a trait-based approach. Therefore, it was decided to first study how macrophyte traits related to growth, morphology and nutrient stoichiometry develop in the field, to be able to compare this to the results from the experiments.

As knowledge on effects of DOC on macrophytes is still very limited and a general overview specifically about macrophyte is lacking, **Chapter 3** is a literature review describing the role of DOC in freshwater systems, how its quality and quantity are changing due to climate change, what is known about its effect on macrophytes and how macrophytes can defend themselves against effects of DOC.

Chapter 4 focusses on the effects of multiple concentrations of CO_2 and DOC in a wide range on macrophyte growth, morphology, chlorophyll content and nutrient stoichiometry. This was tested in a greenhouse experiment in standing water to which CO_2 or DOC was added. To give a more complete overview of those effects, it was decided to also work with a second macrophyte species: *M. spicatum*, which is able to also take up bicarbonate as inorganic source, in addition to CO_2.

Chapter 5 is about the combination of multiple stressors related to climate change. In a racetrack flume experiment in a greenhouse, macrophytes were exposed to different combinations of CO_2 concentrations, eutrophication and flow velocity to be able to test interactions between those three factors. This experiment was repeated in **Chapter 6**, but in this case effects of CO_2, DOC and

flow velocity were tested. Again, in both chapters responses of many traits of the macrophytes were investigated.

Chapter 7 is a synthesis of the results of all other chapters. Results from the experiments are compared to findings from literature, and integrated with the field study to be able to answer the research question and to be able to predict how macrophytes in rivers will respond to climate change and what consequences this may have for the rest of the ecosystem. Remaining knowledge gaps and suggestions for future research are addressed as well.

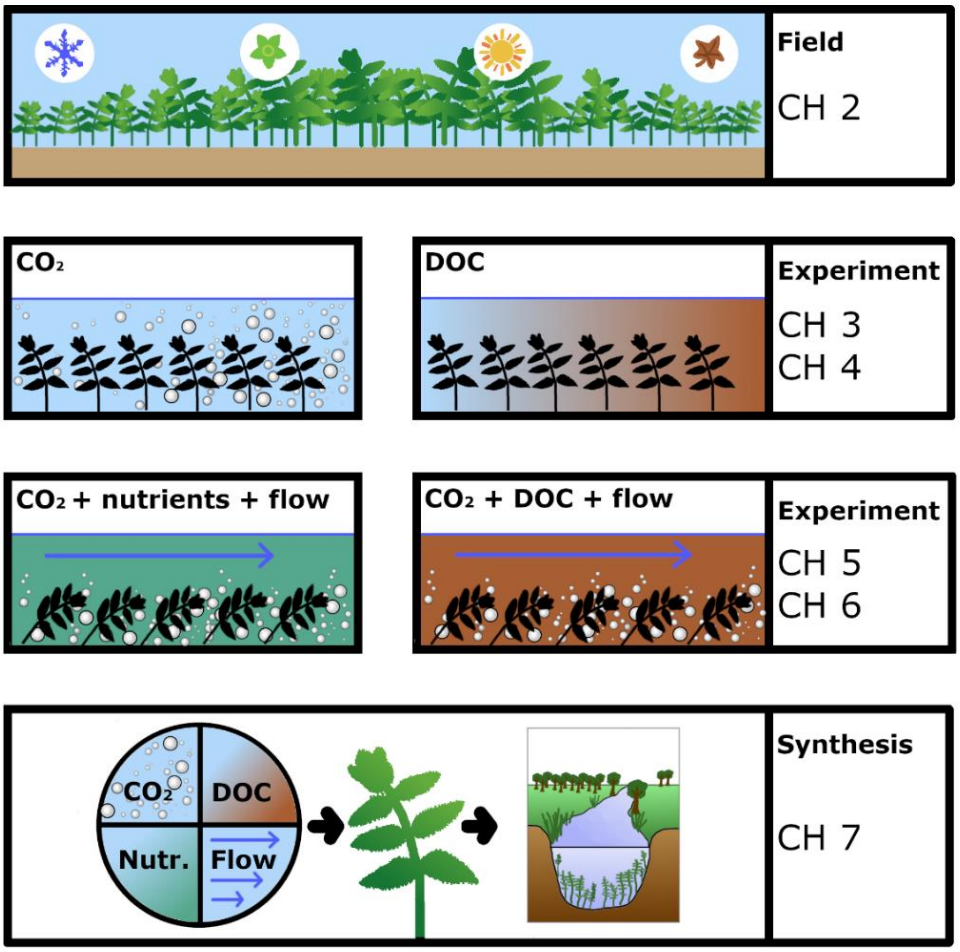

Figure 1.4 Overview of the chapters of this thesis and how they are linked. Chapter 2 describes a field study in which plant traits have been measured monthly to be able to describe seasonal and spatial variation. Chapter 3 is a literature review about DOC and CO_2 and their effects on macrophytes. Those effects have been tested in an experiment with different gradients in chapter 4. In chapter 5 and 6, interactions between multiple stressors have been tested: CO_2, nutrient stress and flow velocity in chapter 5 and CO_2, DOC and flow velocity in chapter 6. Chapter 7 is a synthesis where the results of all experiments are combined and compared to literature to answer the research question, moreover, predictions on consequences for the ecosystem are made.

Chapter 2.

Environmental control of macrophyte traits and interactions with metabolism and hydromorphology in a groundwater-fed river

Rosanne E. Reitsema, Stefan Preiner, Patrick Meire, Thomas Hein, Yanran Dai, Jonas Schoelynck

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Abstract

Macrophytes are important organisms in running water systems, having a decisive role in ecological processes and interactions. Their temporal and spatial distribution in streams can be highly variable, and this is often determined by flow velocity. In this study, macrophyte growth, morphology and nutrient stoichiometry were studied monthly during one growing season in river reaches with different flow velocity and flow velocity distribution and, as a result, different distributional plant patterns in an Austrian lowland stream, dominated by evergreen macrophyte species *Berula erecta*. Flow velocity, water depth, fine sediment layer depth and metabolism were measured and the correlation with plant biomass and morphological traits was tested. We aimed to study differences between reaches with different distributional plant patterns and whether common interactions between macrophytes and flow velocity can also be observed when vegetation is evergreen. Plant biomass showed seasonal variation, with the highest values in June and the lowest in February. In the reach with low flow velocity and homogeneous macrophyte distribution, biomass peaked in summer and plant morphology changed with the seasons, whereas biomass and morphology in the reach with high flow velocity and patchy distribution were more constant throughout the year. Plant carbon, nitrogen and phosphorus content were higher in spring and autumn than in summer, whereas biogenic silica accumulated over the course of the growing season. Stream metabolism was strongly correlated with macrophyte biomass, and this correlation was stronger in the reach with homogeneous macrophyte distribution than in the reach with a patchy distribution. Moreover, average leaf area and stem length were positively correlated with fine sediment layer depth, and negatively with flow velocity. The results stress the importance of macrophyte growth and morphology in river processes like metabolism, hydromorphology and nutrient dynamics: especially plant morphology plays an important role in macrophyte-flow interactions, rather than just plant biomass.

Keywords: aquatic ecology, *Berula erecta*, plant-flow interactions, river management

Introduction

Macrophytes are important species in the aquatic ecosystem, as they affect oxygen dynamics in the water and sediments (Uehlinger et al. 2000), and they affect the nutrient and carbon cycles (Clarke 2002). With their roots they can stabilise sediment and their shoots can locally slow down flow velocity, thereby providing a habitat for invertebrates and fish (Sand-Jensen 1998). As a result of metabolic processes of macrophytes and their associated epiphyte community, macrophytes can drive ecosystem metabolism (O'Brien et al. 2014, Preiner et al. 2020). All in all, by modifying the aquatic ecosystem, macrophytes can be seen as physical engineers in river systems (Gurnell 2014). Although effects of macrophyte growth on river processes are well studied, this is usually measured in conditions with homogeneous and well-developed vegetation growing in a seasonal pattern with maximum biomass in summer and a dying phase in autumn. There are many exceptions to this 'classical' view of macrophyte growth. Firstly, macrophyte distribution is not always homogeneous: due to flow velocity, macrophytes can organise themselves in patches (Cornacchia et al. 2020), and locally they affect their surroundings: there is reduced flow and increased sedimentation within the patches and high flow and erosion outside of the patches (Sand-Jensen and Mebus 1996, Schoelynck et al. 2012b). Macrophyte patches can consist of one or multiple species, they can be clearly or poorly delineated. More importantly: macrophyte patches can act as one hydrodynamic patch in their interaction with flow velocity, which increases their influence in river processes i.e. it is more than expected when looking at just plant coverage (Schoelynck et al. 2018). Macrophytes growing in self-organised patches may also be less vulnerable to sudden changes in discharge than homogeneous vegetation (Cornacchia et al. 2020). Secondly, some macrophyte species are evergreen and maintain or even increase their number of ramets in winter (Greulich and Bornette 2003).

Morphological macrophyte traits like the complexity of the growth form, leaf area and stem length can affect processes in rivers, like flow velocity reduction, nutrient uptake or sediment retention. Macrophyte species with a complex morphology have a larger effect on flow velocity reduction and nutrient uptake than macrophytes with a simple morphology (e.g., with strap-like leaves) (Sand-Jensen and Mebus 1996, Sand-Jensen and Pedersen 1999, Levi et al. 2015). Moreover, when macrophytes have a large leaf area they can take up

more nutrients, provide a larger substrate to epiphytes that take up nutrients (Wolters et al. 2019) and sediment retention increases (Clarke 2002). Between species, there can be large differences in leaf surface area and morphology (Levi et al. 2015), but differences can also exist within species,. Vári et al. (2010) found that the morphology of *Potamogeton perfoliatus* can differ based on their exposure to waves, depth of the water and trophic state of the water. In more sheltered conditions this species has longer stems and thinner leaves, and in nutrient rich waters it grows larger. Thus, the growth form of a species can also change due to interactions with the environment. It was found that *Nuphar lutea* plants (that have floating and submerged leaves) growing in flowing water developed more and larger submerged leaves and accumulated more sediment than plants in standing water (Schoelynck et al. 2014).

Another macrophyte trait that is subject to close interactions with the environment is nutrient stoichiometry. The nutrient composition of macrophytes firstly depends on the species (Demars and Edwards 2007), but there are also links between concentrations of elements like C, N, P and Si in the water or sediment and the concentrations of elements in plant tissue (Xing et al. 2013). Moreover, there are interactions between flow velocity and plant nutrient stoichiometry; it has been demonstrated that *Egeria densa* plants contain a higher concentration of biogenic silica (BSi) when they are exposed to hydrodynamic stress, probably to strengthen their tissue (Schoelynck et al. 2012a, Schoelynck et al. 2015). Altered macrophyte nutrient stoichiometry can also have consequences for the rest of the ecosystem, with regard to the nutritive value of the tissue (Elser et al. 2000) or the rate of decay (Schaller and Struyf 2013).

Although temporal changes in macrophyte biomass, morphology and nutrient stoichiometry are relatively well-studied, there are still several knowledge gaps. Firstly, it is poorly understood how a patchy river affects river functioning: are processes like metabolism only related to coverage, or is there a large influence of the patches? Secondly, many studies investigating seasonal development of macrophyte traits focus on rivers where macrophytes die off in winter; rivers with evergreen vegetation are relatively understudied. Some evergreen macrophytes have a relatively low growth rate in the growing season, and they compensate for this by maintaining growth in winter (Greulich and Bornette 2003). It is not clear how this affects interactions with flow

velocity, as compared to rivers where vegetation dies off in winter. The main aim of this study was to quantify variation in macrophyte biomass, coverage, and plant traits related to morphology and nutrient stoichiometry within one macrophyte species during one growing season. Secondly, it was aimed to study how evergreen vegetation interacts with flow velocity to be able to compare this to other studies about macrophyte species that die off in winter. Macrophytes were sampled in an oligotrophic groundwater-fed lowland river in Austria with a dominant evergreen macrophyte species in two different reaches close to each other, with similar and stable environmental conditions (temperature and nutrients) but with different flow velocities: 1) a river reach with high flow velocity, patchy vegetation cover and coarse sediment, and 2) a river reach with slower flow velocity, homogeneous vegetation coverage and dominant fine sediment. We studied macrophyte development (biomass and morphology) and correlations with other stream parameters like water depth, fine sediment layer depth, flow velocity and stream metabolism. We formulated the following two research questions: 1). Are there seasonal differences in macrophyte development, gross primary production and plant traits between macrophytes growing in reaches with different flow velocity? 2). Are patterns in macrophyte traits, coverage, metabolism and interactions between macrophytes and the environment present in rivers with evergreen vegetation and is this comparable to other studies focusing on rivers with vegetation that dies off in winter?

Materials and methods

Study area and sampling sites

Macrophyte samples were taken in a lowland branch of the Fischa River in Austria (figure 2.1a). This groundwater-fed river originates in the south east of Vienna and ends in the Danube River. The environmental conditions in the Fischa near the study area are stable (table 2.1 and 2.2; figure S2.1), with an annual average discharge of 1.2 ± 0.13 m^3 s^{-1} and an annual average water temperature of 13.3 ± 2.3 °C. Sampling was done close to the village of Pottendorf (47.91° N, 16.39° E) in two different areas (figure 2.1b and 2.1c): A 600 m reach with high macrophyte density (80-100% coverage) and a thick fine sediment layer (hereafter called 'homogeneous reach') and a 600 m reach with patchy macrophyte coverage (40-70% coverage) with sediments composed of gravel and sand and finer sediment within macrophyte patches (hereafter called 'patchy reach') (figure 2.1d and 2.1e). The homogeneous and patchy reach were similar in hydrology, nutrient and suspended solid values, but average flow velocity was almost twice as high in the patchy reach than the homogeneous reach (table 2.1 and 2.2; figure S2.1b). Both reaches were surrounded by riparian vegetation (deciduous trees and shrubs) and the estimated percentage of river surface covered by this vegetation was 60% in the homogenous and 70% in the patchy reach (calculated in ArcGIS, using aerial images). Aquatic vegetation in both reaches was almost exclusively dominated by the macrophyte species *Berula erecta* (Huds.) Coville, a homophyllous amphibious plant (Nielsen 1993) that can reproduce sexually (it flowers in summer) and vegetative with stolons (Oudot-Canaff et al. 2015). In some sections, occasional shoots of *Groenlandia densa* (L.) Fourr.*, Elodea Canadensis* Michx. and *Potamogeton crispus* L. were found. As the biomass of those species was negligible, they were not taken into account in the analyses.

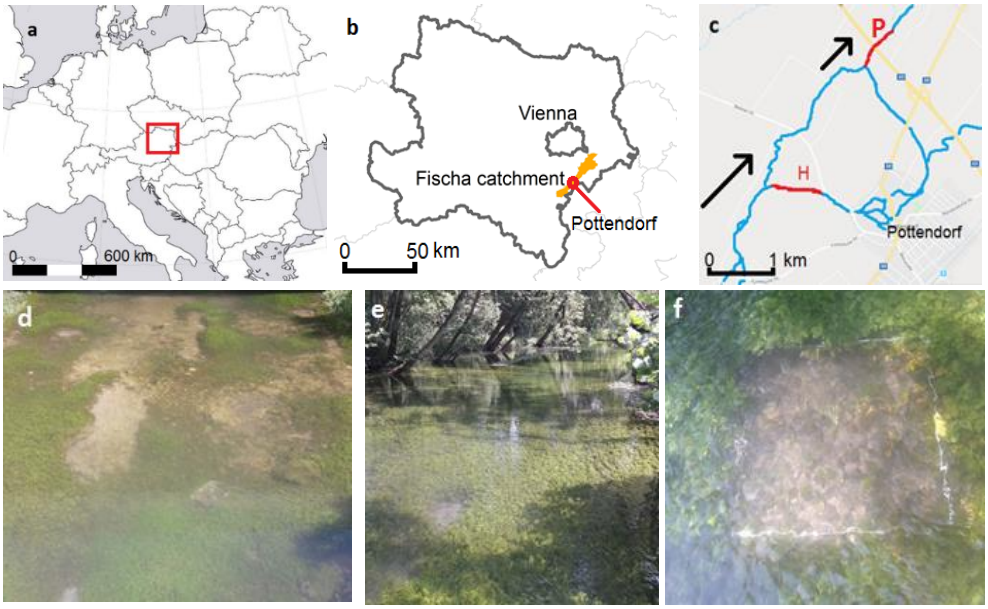

Figure 2.1 Locations of the study sites visited in Austria (a) in the Fischa catchment (b) near the village of Pottendorf (c). The patchy reach has clear patches of vegetation and bare sediments in between (d) and the homogenous reach has a higher coverage with homogeneous distribution (e). Macrophytes were harvested in a 50 × 50 cm square (f).

Table 2.1 Characterisation of the homogeneous reach and patchy reach.

	Homogeneous reach	**Patchy reach**
Area (m^2)	6323	6989
Length (m)	605	600
Mean width (m)	10.5	11.6
Mean flow velocity ($m\ s^{-1}$)	0.17 ± 0.12	0.36 ± 0.22
Mean water depth (m)	0.56 ± 0.26	0.44 ± 0.21

Table 2.2 Nutrients, suspended solids and dissolved organic matter in the water column in the homogeneous and patchy reach. Mean values and standard deviations are shown, measurements were taken in 2017-2018 (n=26 per reach). $N\text{-}NH_4$, ammonium; $N\text{-}NO_3$, nitrate; $P\text{-}PO_4$, phosphate; P-tot, total phosphorus; Si, Silicon; TSS, total suspended solids; RelPOM: relative proportion of organic particulate matter; DOC, dissolved organic carbon; Chl-a, chlorophyll-a

	Homogeneous reach H	**Patchy reach I**
Temperature (°C)	12.7 ± 2.5	12.8 ± 2.6
Conductivity (µS cm^{-1})	575 ± 8	583 ± 6
$N\text{-}NH_4$ (µg L^{-1})	6.7 ± 2.2	7.5 ± 2.7
$N\text{-}NO_2$ (µg L^{-1})	2.6 ± 0.6	2.8 ± 0.4
$N\text{-}NO_3$ (mg L^{-1})	3.1 ± 0.4	3.3 ± 0.2
$P\text{-}PO_4$ (µg L^{-1})	1.5 ± 0.4	1.1 ± 0.5
P-tot (µg L^{-1})	4.4 ± 1.2	5.9 ± 1.9
Si (mg L^{-1})	2.1 ± 0.1	2.3 ± 0.1
TSS (mg L^{-1})	1.8 ± 0.7	4.2 ± 1.1
RelPOM (%)	49.0 ± 10.9	38.4 ± 6.2
DOC (mg L^{-1})	0.52 ± 0.15	0.57 ± 0.20
Chl-a (µg L^{-1})	0.4 ± 0.1	0.4 ± 0.1

Sampling methods

Macrophytes were sampled from April 2017 until October 2017, twice in May and once in the other months. As macrophytes were still present in the river in winter, one extra sampling campaign was done in February 2018, which makes nine sampling campaigns in total. Sampling was done from downstream to upstream to prevent disturbance of the sediment. In the patchy reach, two sections of 50 metres each were selected and every time a vegetation map was drawn of those two sections to monitor the development of the vegetation. During each sampling campaign mapping was done only at those two river sections of 50 m. On the map, the two river sections of 50 m were divided into 1x1 m quadrats and the state of the vegetation was evaluated visually and noted down for each quadrat using three gradations: bare plots, partially vegetated plots and fully vegetated plots. For the analyses, the two reaches were each divided into ten subsections of 5 m, which were used as (pseudo)replicates. In the homogeneous reach, vegetation was not mapped in defined quadrats, but it was estimated for each subsection of 60 metres, so for the entire reach, as the coverage was very high and homogeneous.

In the patchy reach, both 50 m sections were divided into 10 subsections of 5 metres and in each subsection a macrophyte sample was taken (so 20 samples

in total in the patchy reach). The homogeneous reach (600 m) was divided into ten subsections of 60 metres each (10 samples in total); as the homogeneous reach was less heterogenous in terms of macrophyte distribution and sediment characteristics than the patchy reach, it was decided to take fewer sampling points there. In each subsection a random plot of 0.5 x 0.5 metres was selected (figure 2.1f). Within this plot, water depth, depth of the fine sediment layer and flow velocity (electromagnetic velocity meter HACH FH 950.0) were measured. All macrophytes within the plot were harvested using grass shears and collected in a fishing net, after which debris was removed and the sample was transferred into a ziplock bag, which was kept in a cooling box.

Measurements of plant traits

From each macrophyte sample, 20 representative, undamaged shoots of *B. erecta* were selected and weighed. The rest of the sample, so the main part of the macrophytes, was dried for five days at 70^{o}C and dry weight was measured. If species other than *B. erecta* were present in the sample they were dried and weighed separately. The 20 selected *B. erecta* shoots were used for further analyses: the number of leaves, stem length and fresh weight of the leaves and stems separately was measured. From one shoot in each sample of 20 shoots, the leaves were photographed on a white background, after which the leaf surface area per shoot was calculated using image processing programme ImageJ. From this value the average leaf area and total leaf surface in m^2 per m^2 of riverbed was calculated. For each plot, the average was taken of the measured parameters of the 20 shoots. This resulted in one value for each plot, to be able to correlate them to the hydromorphological measurements, which were also measured in every plot.

Nutrient stoichiometry analyses

The stems and leaves were dried separately in paper bags at 70^{o}C and the dry weight was determined. From each river section, three samples were randomly selected and both leaves and stems were ground with an Ultra Centrifugal Mill ZM 200 (Retsch, Germany), sieve size 0.5 mm. The ground material was analysed for %N and %C on a FLASH 2000 Organic Elemental Analyser, based on Flash Dynamic Combustion (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Plant P content was determined by nitric acid digestion (69 % HNO_3), after which the samples were measured on ICP-OES (iCAP 6300 Duo view, Thermo Fisher, Waltham, Massachusetts, USA). Biogenic silica (BSi)

was extracted by incubating 30 mg of ground plant material in 0.5 M NaOH at 80°C for 5 hours. After filtering the samples (Chromafil® Xtra MV-45/25, Macherey-Nagel, Düren, Germany), the BSi content was determined (SAN++, Skalar, Breda, The Netherlands).

Stream metabolism

Gross primary production (GPP) and ecosystem respiration (ER) in the studied reaches were estimated by a modified oxygen time curve analysis (Odum 1956). Daily rates were calculated in R package *streamMetabolizer* (Appling et al. 2018) and afterwards monthly averages were calculated to account for variation in light availability (Preiner et al. 2020). Oxygen measurements were obtained in 5-min intervals from automated data logging units (YSI 6-series v2, YSI Incorporated, USA) installed at the downstream site of each reach.

Statistics

In order to test whether plant traits differed between the two reaches and between the different sampling months, a linear mixed effects model was performed using package *nlme* (Pinheiro et al. 2018), with sampling month, plant density (high and intermediate) and their interaction as fixed factors and plot as random factor. Subsequently, ANOVA tests with type III sums of squares were performed. With package *lsmeans* (Lenth 2016) additional post hoc tukey tests were performed on the Month-Density interaction. For nutrient stoichiometry, first a three-way ANOVA test was performed on the mixed model to test for effects of month density and plant organ. Afterwards, the dataset was split and ANOVA tests and post hoc tukey tests were performed for plant leaves and stems separately. For correlation tests between macrophyte traits and the environment, normal distribution of the data was checked with the Shapiro-Wilk test, but as none of the variables had a normal distribution, Spearman correlation tests were performed in R (version 3.5.2) using package *ggpubr* (Kassambara 2019), after splitting the dataset based on macrophyte distribution (patchy and homogeneous reach). Additionally, Z-scores were calculated for the correlation coefficients for the patchy and homogeneous reach to check whether they differed significantly. As GPP and ER were only measured on reach scale, this was correlated to average values of plant traits per reach (one value per month), whereas all parameters that were measured on quadrat scale were correlated to plant traits measured in each plot (10 values for the homogeneous reach and 20 for the patchy reach.

Results

Macrophyte growth

Even though *B. erecta* is an evergreen species in this river, the development of biomass showed a seasonal pattern, but this was most pronounced in the homogeneous reach, which had a high biomass in May and June and significantly lower biomass in the other months; up to 6.5 times smaller. In the patchy reach dry biomass of *B. erecta* was more constant: there were only significant differences between biomass in summer (July) and autumn/winter (October and February), there was only 2.3 as much biomass in summer compared to winter (figure 2.2a). Dry annual biomass density of *B. erecta* in the river was, on average 47.65 ± 27.92 g m^{-2} in the patchy reach and 78.28 ± 31.75 g m^{-2} in the homogeneous reach. From April to June, dry biomass was significantly higher in the homogeneous reach than in the patchy reach (figure 2.2a).

There were significant differences in macrophyte coverage in the patchy reach between the sampling months (see table 2.3 for all statistics on macrophyte biomass and morphology). The number of fully vegetated plots was significantly lower (up to 2.1 times) in February than in almost all other months (figure 2.2b). The number of plots that were only partially vegetated (developing or decaying vegetation) was significantly higher in winter and spring than in summer, this was more than twice as high (figure 2.2c).

Macrophyte morphology

Most morphological traits followed a trend that was similar to the total biomass: the number of leaves (figure 2.2d), average leaf area (figure 2.2e), total leaf area per shoot and the total leaf surface (figure 2.2f) were all positively correlated to dry biomass ($p<0.001$). In the homogeneous reach, all leaf traits were higher than in the patchy reach and in the homogeneous reach there was a clear seasonal response, whereas traits in the patchy reach was again more constant throughout the year. Stem length was significantly higher in the homogeneous than the patchy reach in all months except February (figure 2.2g), but leaf:stem ratio was similar in both reaches in most months (figure 2.2h). In February for both reaches the leaf:stem ratio was significantly higher than in all other months: the stem biomass in February shows a relatively larger decline than leaf biomass, compared to the other months. In February, stems

were significantly shorter than in all other months (up to 4 times smaller), whereas the number of leaves was more constant. Specific leaf area (SLA) was constant throughout the seasons in both reaches.

Macrophyte nutrient content and stoichiometry

Nutrient content of *B. erecta* did not differ between the two reaches, except for BSi content, which was higher in the homogeneous than in the patchy reach in July and August and P content, which was higher in the homogeneous than in the patchy reach in September. In general, the concentration of all nutrients (C, N, P and BSi) was significantly higher in the leaves than in the stems (see table 2.4 for all statistics on nutrient content). For most elements there were significant effects of the sampling months: C, N and P were lower in summer than in autumn and winter, whereas BSi was higher in summer than in spring (figure 2.3 a-f). The C:N ratio was lower in the beginning of May than in rest of the months, but only in the patchy reach (figure 2.3g-h). For N:P and C:P ratio there were significant differences between some months, but there were no clear seasonal patterns. There were also effects of plant organ: the C:N and C:P ratio was significantly higher in the stems than in the leaves, whereas the N:P ratio was higher in the leaves than in the stems.

Correlations of plant traits to stream metabolism and hydromorphology

Although *B. erecta* is an evergreen species in this river, there were many significant correlations between plant traits and stream metabolism and hydromorphology. GPP was highly correlated with dry biomass of *B. erecta*, leaf:stem ratio, average leaf area and total leaf surface in m^2 per m^2 of river bed (hereafter called total leaf surface) (figure 2.4a, table 2.5), the correlation with dry mass was significantly stronger in the homogeneous than the patchy reach. There was also a strong positive correlation between GPP and the number of fully vegetated plots and a negative correlation between GPP and the number of partially vegetated plots. ER, however, was only positively correlated with *B. erecta* dry mass, average leaf area and total leaf surface in the patchy reach. For water depth there were larger differences between the reaches. Dry biomass was positively correlated with water depth in the homogeneous reach and negatively in the patchy reach. For specific leaf area (SLA) this was the other way around: it was positively correlated with water depth in the patchy reach and negatively in the homogeneous reach. In both reaches, there was a positive correlation between stem length and water depth (figure 2.4b). Although there

was no significant correlation between flow velocity and biomass, flow velocity was negatively correlated to stem length and average leaf area (figure 2.4c). Lastly, the depth of the fine sediment layer was positively correlated to average leaf area (figure 2.4d). There was no significant correlation between flow velocity and the depth of the fine sediment layer ($p=0.22$). Total leaf surface and the number of fully vegetated plots and negatively correlated to LDMC, but this was only significant in the patchy reach.

Macrophyte response to flow velocity in the field and in the lab

Apart from studying interactions between *B. erecta* and flow velocity, the response of this macrophyte species to flow velocity was also tested in a racetrack flume experiment. Plants were exposed to a high flow velocity (0.4 m s^{-1}) and a low flow velocity (0.04 m s^{-1}), see chapter 5 and 6 for more details. This high flow velocity is comparable to the average flow velocity measured in reach P. In the experiment a similar response of *B. erecta* to flow velocity was observed as in the field. When exposed to high flow velocity, macrophytes had shorter stems and a smaller average leaf area than when exposed to low flow velocity.

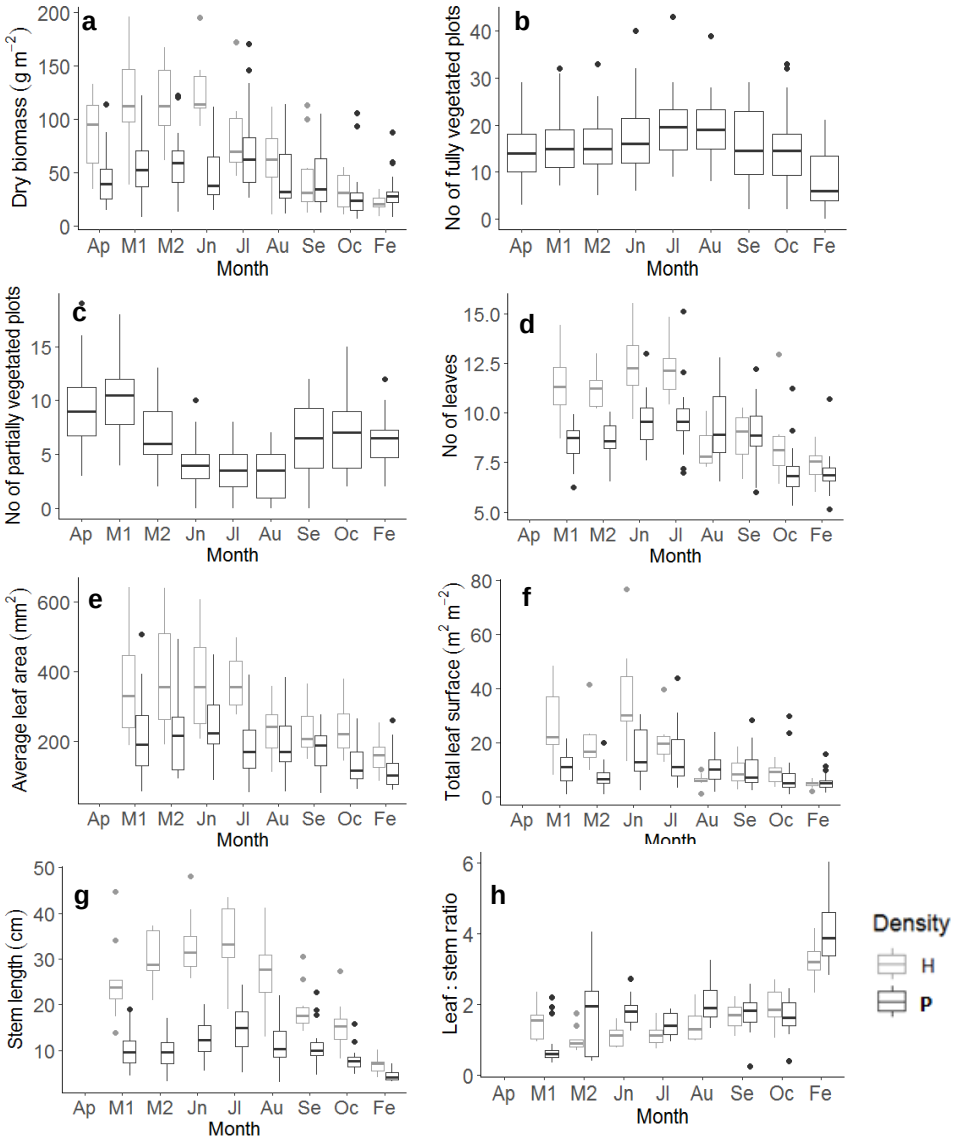

Figure 2.2 Boxplots for macrophyte coverage, biomass and morphology of *B. erecta* between April 2017 and February 2018 in a homogeneous and a patchy reach. Dry biomass of *B. erecta* per m² (a), Number of fully vegetated plots (1 × 1 m) per 5 m river section (b), number of partially vegetated plots (1 × 1 m) per 5 m river section (c), number of leaves per shoot (d), average leaf area (mm²) (e), total available leaf surface in m² per m² of river bed (f), average stem length (cm) (g), and leaf:stem ratio (h).

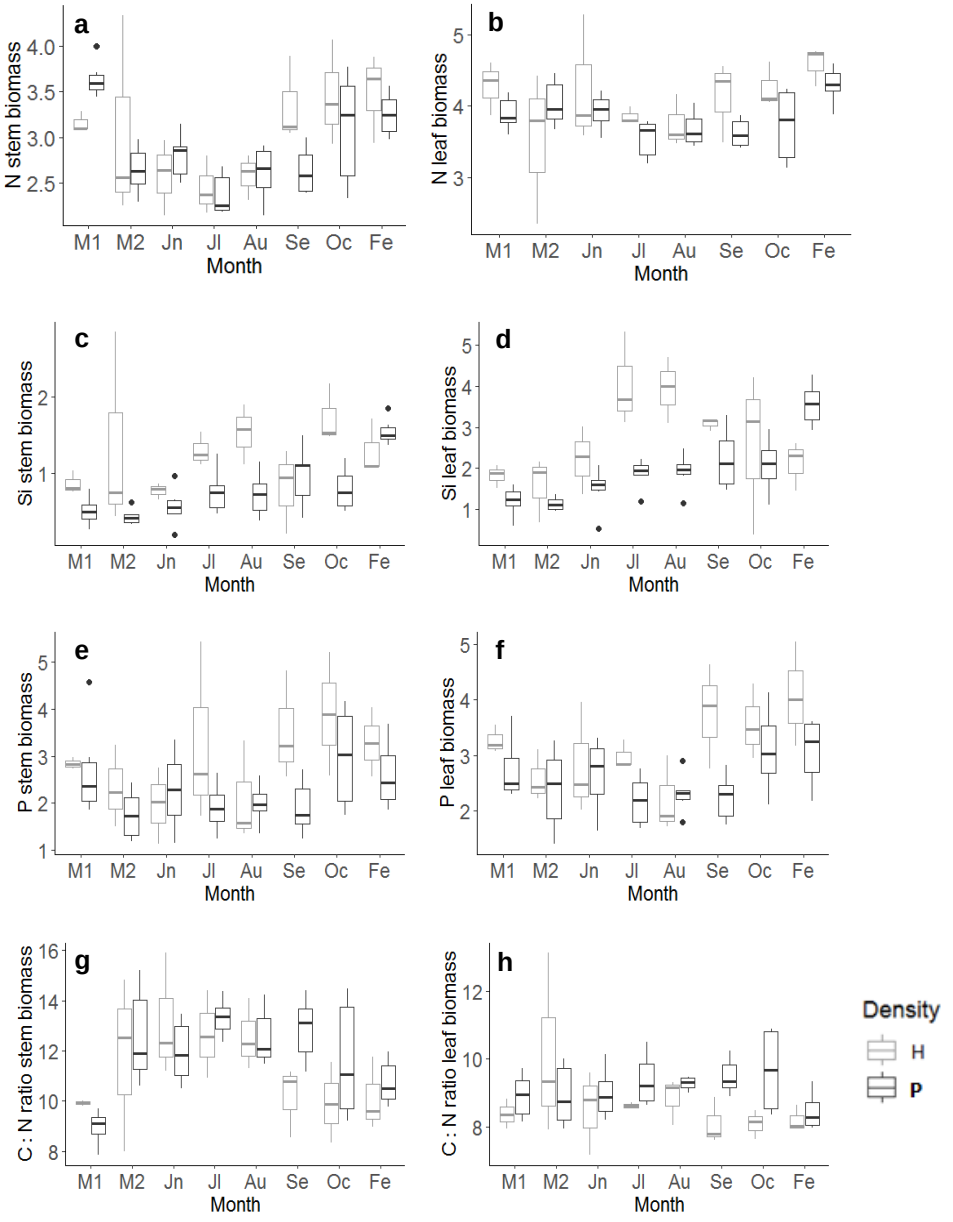

Figure 2.3 Boxplots for nutrient content and stoichiometry of *B. erecta* between May and February in a homogeneous and a patchy reach. % nitrogen (N) in stem (a) and leaf (b) biomass, % silica (Si) in stem (c) and leaf (d) biomass, % phosphorus (P) in stem (e) and leaf (f) biomass and carbon to nitrogen (C:N) ratio in stem (g) and leaf (h) biomass.

Table 2.3 F-values of the linear mixed effects model of plant traits. Total dry mass and macrophyte coverage (fully vegetated and developing plots) were measured from April until February, all other parameters were measured from May until February. Macrophyte coverage (fully and partly vegetated plots) was only measured in the patchy reach. DMC: dry matter content, NA = not available

	Month	**Density**	**Month*Density**
Total dry mass (g m^{-2})	17.96***	61.49***	8.00***
DMC of total dry mass	6.29***	29.93***	3.19**
Fully vegetated plots	5.49***	NA	NA
Partly vegetated plots	10.25***	1.22	2.10*
Dry mass per 20 shoots	31.07***	186.98***	14.30***
Dry mass per stem	31.04***	149.45***	14.44***
Dry mass per leaf	23.02***	171.09***	11.65***
DMC stems	0.74	21.03***	3.50**
DMC leaves	6.45***	2.48	5.09***
Leaf:stem ratio	15.01***	11.98***	5.28***
Stem length	35.43***	371.52***	12.76***
Number of leaves	16.94***	41.17***	7.22***
Total leaf area per shoot	14.23***	86.34***	5.27***
Average leaf area	8.70***	73.55***	2.64*
Specific leaf area	0.59	3.65	2.89**
Leaf surface per m^2	18.74***	30.16***	8.39***
Fine sediment layer	2.95*	9.33**	2.87*
Water depth	4.00***	NA	NA
Velocity	2.43*	NA	NA

Signif. codes: * <0.05, ** <0.01 *** <0.001

Table 2.4 F-values of the linear mixed effects model for nutrient stoichiometry. ns = not significant.

	Month	Density	Plant organ	Month:Density	Month:Organ	Density:Organ
N	8.9615***	1.67	214.51***	ns	ns	ns
C	12.42***	0	55.30***	ns	ns	ns
P	5.90***	5.46*	8.00**	3.03**	ns	ns
C:N ratio	1.26	0.13	189.38***	2.33*	3.98**	ns
N:P ratio	3.59**	1.98	19.51***	2.13*	ns	ns
C:P ratio	3.70**	0.8	9.83**	2.29*	ns	ns
Si	9.30***	23.35***	92.53***	8.77***	4.40**	2.85

Signif. codes: * <0.05, ** <0.01 ** <0.001

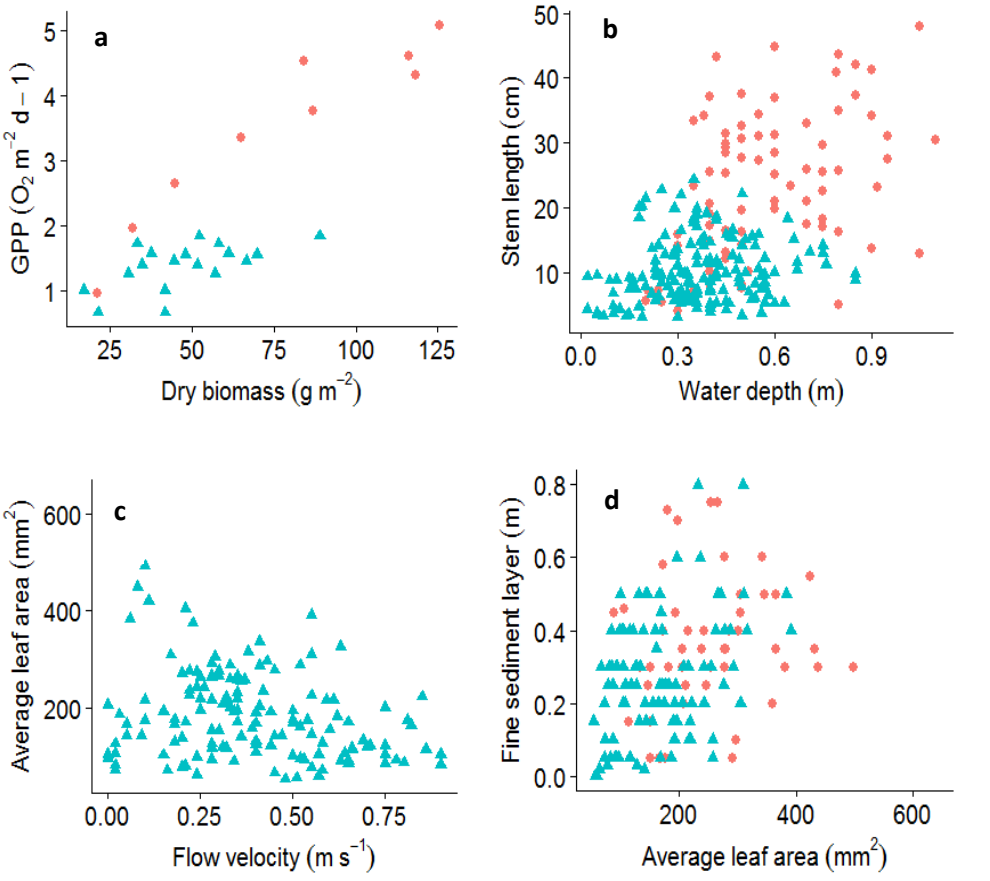

Figure 2.4 Scatter plots with spearman correlation tests for GPP (gross primary production) and dry mass of *B. erecta* (a), depth of the water column and stem length (b), flow velocity and average leaf area (c) and depth of the fine sediment layer and average leaf area (d). For the patchy and homogeneous reaches correlation coefficients and significance (* <0.05, ** <0.01, *** <0.001) are as following: (a) patchy (P): R = 0.38***, homogeneous (H): R = 0.77***, (b) P: R = 0.19*, H: R = 0.39***, (c) P: R = -0.24**, (d) P: R = 0.33***, H: R = 0.22. In figure a there is a significant difference between the reaches (p<0.001). Flow velocity was only measured in the patchy reach, therefore it cannot be shown for the homogeneous reach in figure c.

Table 2.5 Spearman correlation tests between macrophyte variables and stream characteristics for the homogeneous (H) and patchy (P) reach and, if both significant, the comparison between the slopes for both reaches (P-I) based on z-scores (significant differences in bold print). S/LDMC = stem/leaf dry matter content, NS = not significant, NA = not available.

		GPP	ER	Depth	Velocity	Fine sediment
Dry mass	H	0.92**	-0.37	0.37***	NA	0.04
	P	0.55*	0.54*	-0.15*	NS	0.19
	H-P	**0.02**		**0**	NA	
SDMC	H	-0.55	0.14	-0.22*	NA	-0.09
	P	-0.62*	-0.59*	-0.27***	NS	-0.15
	H-P			0.35	NA	
LDMC	H	-0.48	-0.07	-0.03	NA	-0.05
	P	-0.24	-0.22	-0.26**	NS	-0.35***
	H-P				NA	
Leaf:stem ratio	H	-0.91**	-0.38	-0.20	NA	0.02
	P	-0.25	-0.33	-0.03	NS	-0.14
	H-P				NA	
Stem length	H	0.90**	0.014	0.39***	NA	0.04
	P	0.74***	0.75***	0.19*	-0.24**	0.34***
	H-P	0.016		0.058		
Average leaf area	H	0.90**	-0.69	0.2	NA	0.22
	P	0.38	0.41	0.32***	-0.24**	0.33***
	H-P				NA	
SLA	H	-0.02	-0.05	-0.32**	NA	0.1
	P	0.08	0.12	0.21**	-0.18*	0.15
	H-P			**0**	NA	
Total leaf surface	H	0.83*	-0.45	0.24*	NA	0.19
	P	0.71**	0.77***	-0.14	NS	0.20*
	H-P	0.28			NA	
Fully vegetated plots	H	NA	NA	NA	NA	NA
	P	0.62**	0.61**	0.28***	0.16*	0.25*
	H-P	NA	NA	NA	NA	NA
Partly vegetated plots	H	NA	NA	NA	NA	NA
	P	-0.54*	-0.49*	NS	NS	NS
	H-P	NA	NA	NA	NA	NA

Signif. codes: * <0.05, ** <0.01 ** <0.001

Discussion

Seasonal changes in macrophyte growth

Despite the fact that *B. erecta* is an evergreen macrophyte, coverage and biomass changed substantially over the season, with the highest biomass in May and June and the lowest biomass in October and February. In the homogeneous reach, there was a clear seasonal pattern where summer biomass was 6.5 times higher than winter biomass, whereas the patchy reach was more stable throughout the year: this was only twice as high. Both values fall in the same order of magnitude as other studies in groundwater-fed streams (Riis et al. 2003, Willis et al. 2017). Although other studies have investigated seasonal patterns of biomass development in reaches, studies regarding seasonal effects on reaches with different macrophyte patterns are limited. Flow velocity appears to be an important factor in the seasonal pattern of macrophyte biomass. High flow velocity limits this: summer biomass climax was substantially lower in high flow conditions than in low flow conditions. In the homogeneous reach, macrophyte biomass increased with depth, plants were able to grow in the entire water column. In the patchy reach biomass decreased with depth, which may be explained by the adaptation strategy to hydrodynamic stress. *B. erecta* has a smaller growth form in high flow velocity environments (Puijalon et al. 2005). This may limit plant growth in deeper areas because of reduced light availability.

Seasonal changes in macrophyte morphology

Macrophyte morphology also changed with the season, similar to the development in biomass. This seasonal response was more pronounced in the homogeneous than in the patchy reach. There are studies that prove that biomass can be used as a proxy for traits like total available leaf surface area (Jamoneau et al. 2017) and in the current study this was observed as well: biomass and total leaf surface were highly correlated. As expected, leaf:stem ratio remained constant, except in winter, due to strongly reduced stem length. Probably, long stems died and decayed in autumn and they were replaced by new stems that remained small during winter. Being evergreen combined with sufficient vegetative reproduction can be a strategy of dominant plant species (Wiegleb et al. 2014), and this also seems to be applicable to the river in this study as *B. erecta* was dominant in both reaches. Overall, the relatively stable year-round availability of *B. erecta* leaf surface is important for stream

metabolism (O'Brien et al. 2014), epiphytic species (Blindow 1987) and other organisms that use macrophytes as a habitat and flow refugia (Hargeby 1990, Camp et al. 2014, Wolters et al. 2018).

Macrophyte nutrient content and stoichiometry

C, N and P content of *B. erecta* (all parts) were higher in spring and autumn than in summer. In other studies a peak in nutrient content in spring has been observed as well (Boyd and Vickers 1971, Baldy et al. 2007) and it has been suggested that due to this strategy macrophytes can acquire a sufficient amount of nutrients before reaching their maximum growth rate, which gives them a competitive advantage (Reddy et al. 1999). The observed increase in C, N and especially P content of the plants in autumn and winter was not expected. Concentrating the nutrients in leafs and stems that remain in the water after the growing season may be an overwintering strategy (Rong et al. 2015), which is a likely explanation in this river as the remaining biomass in winter is relatively high due to the constant temperature. In contrast to the other nutrients, the concentration of BSi in *B. erecta* was lowest in spring and accumulated during summer, which has also been found in wetland plants (Hou et al. 2010) and is probably related to the ageing of the leaves. The C:N ratio of the plants was lower in early spring than in the rest of the year. This can be explained by dilution effects of N relative to C in summer, which has also been observed in wetland plants (Rong et al. 2015).

Correlations between macrophytes and stream characteristics

As expected, GPP was positively correlated with macrophyte biomass, plant density and total leaf surface, and the correlation was significantly stronger in the homogeneous reach, which shows that macrophytes play an important role in stream metabolism. For ER significant correlations with plant traits were only found in the patchy reach, which might be explained by the homogeneity of the homogeneous reach: ER was more constant in the homogeneous than the patchy reach. Metabolism and biomass had similar seasonal patterns with the highest value in early summer and the lowest in winter and this temporal variation has also been found in other studies with evergreen vegetation (Riis et al. 2019). As macrophytes interact with their environment, we expected correlations between macrophytes and flow velocity and the depth of the fine sediment layer. Abundant macrophyte growth can slow down flow velocity and traps sediment, leading to an accumulation of sediment within macrophyte

beds (Clarke 2002, Wharton et al. 2006, Heppell et al. 2009, Schoelynck et al. 2012b). In the current study those correlations were found: in the patchy reach there was a positive correlation between the depth of the fine sediment layer and macrophyte average leaf area. In a laboratory experiment by Hu et al. (2018) it was found that, at least in large patches, the length of sediment deposition increased with leaf length, leading to a higher mass of deposited sediment. Based on the results from this field study it is difficult to make a strong conclusion on the effect of macrophytes on the fine sediment layer, as there was no significant correlation between flow velocity and the fine sediment layer. A negative correlation was found between flow velocity and average leaf area. Reduction of leaf size during high flow velocity is regarded as a common response to hydrodynamic stress in macrophytes, but other studies also found a reduction in plant size in *B. erecta* under high flow velocity (Puijalon and Bornette 2004). However, a large leaf area can also increase due to flow velocity: it has been found that the leaves of *Nuphar lutea* are bigger under high flow than under low flow conditions (Schoelynck et al. 2014). In the current study, the results suggest, based on the interaction with the environment, that macrophyte morphology is more important than macrophyte biomass, as no correlations were found between hydromorphology and *B. erecta* biomass. This is supported by literature: the importance of macrophyte morphology in the process of sedimentation (Sand-Jensen 1998) and flow velocity (Sand-Jensen and Pedersen 1999) has also been demonstrated in other studies. In the homogeneous reach no significant correlation was found between the fine sediment layer and macrophyte traits, which may be explained by the homogeneity of macrophyte coverage and fine sediment layer depth. For *B. erecta,* this homogeneous environment appears very suitable for macrophyte growth. However, this is not the case for all species: in Cotton et al. (2006), *Ranunculus* growth was monitored in a reach with homogeneous and a reach with heterogeneous vegetation and in the homogeneous reach macrophytes completely disappeared in winter and did not return, probably due to a lack of clean gravel, which was present in the heterogeneous reach.

Altogether, it appears that known patterns in interactions between vegetation and the environment are also applicable to rivers with evergreen vegetation. Still, it would be interesting to study this in more detail, by looking at different

evergreen plant species and rivers with different hydrogeomorphology. In this study, macrophytes were only studied in one river during one growing season, which makes it difficult to make general conclusions on the effect of macrophytes on river functioning, but specific aspects of evergreen macrophytes and the effects of different plant distribution and flow velocities were demonstrated in this study.

Conclusion

This study suggests that macrophytes are important in river processes like stream metabolism, hydromorphology and nutrient dynamics and that interactions between plant traits and hydromorphology are present when vegetation is evergreen. The dominant plant species *B. erecta* showed, despite being an evergreen species, a clear seasonal biomass patterns under low flow velocity conditions and a more stable amount of biomass under high flow conditions, so flow velocity has a profound effect on seasonal development. Whereas macrophyte coverage and density had a major effect on stream metabolism, stream hydromorphology was mainly correlated with macrophyte morphology, rather than just biomass, which suggests that morphological traits are highly important in interactions between macrophytes and their surroundings.

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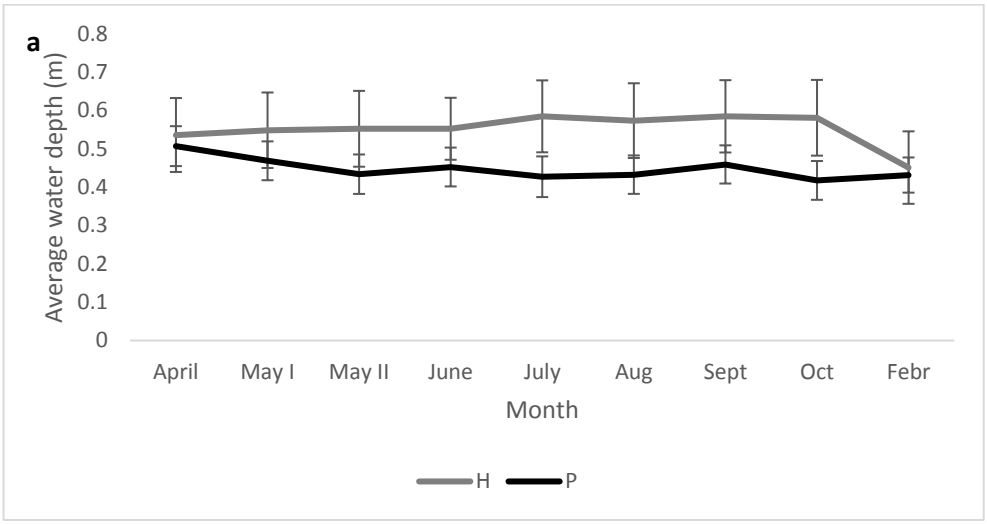
Supplementary figures



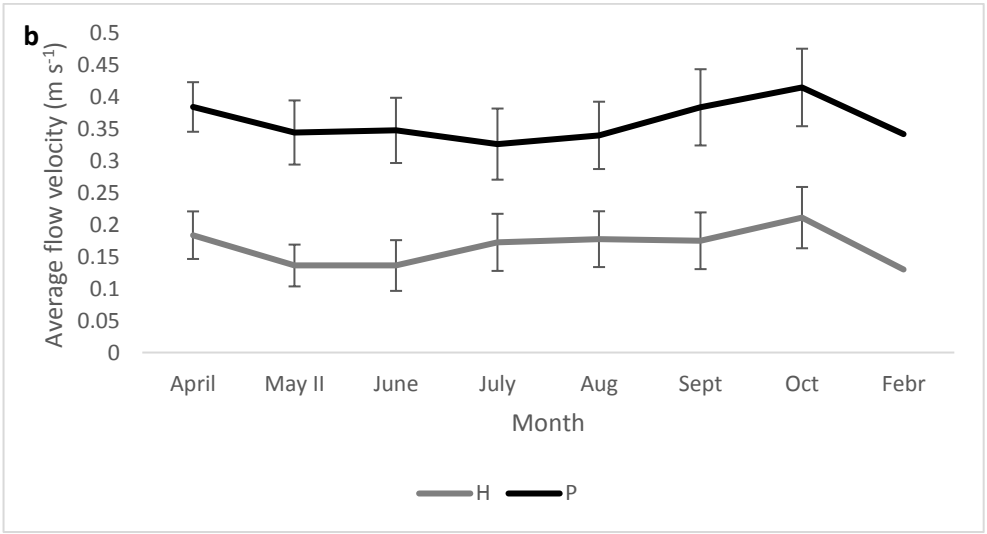


Figure S2.1 Average water depth (a) and average flow velocity (b) of the homogeneous (H) and the patchy (P) reach. Averages were calculated from cross-section measurements with intervals of one metre that were taken every month. Error bars show standard error

Chapter 3.

The future of freshwater macrophytes in a changing world: dissolved organic carbon quantity and quality and its interactions with macrophytes (literature review)

Rosanne E. Reitsema, Patrick Meire, Jonas Schoelynck

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Abstract

Freshwater ecosystems are confronted with the effects of climate change. One of the major changes is an increased concentration of aquatic carbon. Macrophytes are important in the aquatic carbon cycle and play as primary producers a crucial role in carbon storage in aquatic systems. However, macrophytes are affected by increasing carbon concentrations.

The focus of this review lies on dissolved organic carbon (DOC), one of the most abundant forms of carbon in aquatic ecosystems which has many effects on macrophytes. DOC concentrations are rising; the exact cause of this increase is not known, although it is hypothesised that climate change is one of the drivers. The quality of DOC is also changing; for example, in urban areas DOC composition is different from the composition in natural watersheds, resulting in DOC that is more resistant to photo-degradation.

Plants can benefit from DOC as it attenuates UV-B radiation, it binds potentially harmful heavy metals and provides CO_2 as it breaks down. Yet plant growth can also be impaired under high DOC concentrations, especially by humic substances (HS). HS turn the water brown and attenuate light, which limits macrophyte photosynthesis at greater depths. This leads to lower macrophyte abundance and lower species diversity. HS form a wide class of chemicals with many different functional groups and they therefore have the ability to interfere with nearly all biochemical processes that occur in freshwater organisms. Few studies have looked into the direct effects of HS on macrophytes, but there is evidence that HS can interfere with photosynthesis by entering macrophyte cells and causing damage. DOC can also affect reactivity of heavy metals, water and sediment chemistry. This indirectly affects macrophytes too, so they are exposed to multiple stressors that may have contradictive effects. Finally, macrophytes can affect DOC quality and quantity as they produce DOC themselves and provide a substrate to heterotrophic bacteria that degrade DOC.

Because macrophytes take a key position in the aquatic ecosystem, it is essential to understand to what extent surface water DOC quantity and quality are changing and how this will affect macrophyte growth and species diversity in the future.

Keywords: aquatic plants, DOC, climate change, humic substances, freshwater ecology, CO_2

Introduction

Like many ecosystems, freshwater ecosystems are confronted with the effects of climate change (Hossain et al. 2016). One of the major changes is an increased concentration of C in the water (Evans et al. 2005, Hasler et al. 2016, Williams et al. 2016). Research in this regard mostly focusses on ocean acidification: decreasing ocean pH caused by uptake of atmospheric CO_2, which is currently rising because of emission by human activities (Doney et al. 2009). The consequences for fauna and flora are well studied: e.g. coral diversity decreases at a lower pH, whereas non-calcareous algae benefit (Fabricius et al. 2011). Less research, however, has been done in freshwater ecosystems and consequences are less well understood. A recently published review paper concluded that the effects of elevated atmospheric CO_2 levels on freshwater CO_2 levels have not been clearly demonstrated (Hasler et al. 2016). 'Freshwater acidification' due to climate change is likely not comparable to acidification in oceans since CO_2 concentrations in most freshwater ecosystems are currently already several times higher than in the oceans. However, degradation of DOC (dissolved organic carbon) has been mentioned as a potential alternative driver of CO_2 concentrations in freshwater (Sobek et al. 2003). In addition, DOC can affect aquatic ecosystems in various ways; for example by attenuating light (Karlsson et al. 2009) and interfering with biochemical processes within aquatic organisms (Steinberg et al. 2008). Although DOC is not always taken into account when determining aquatic system characteristics (such as trophic status), DOC concentrations can provide information about how aquatic systems may react to contaminants and global warming (Williamson et al. 1999). An increased DOC concentration can have multiple effects on macrophyte productivity (Steinberg et al. 2006) and hence on the entire food web and ecosystem.

The goal of this review is to: (i) give an overview of CO_2 and DOC concentrations and origins in freshwater ecosystems and summarise possible explanations for the rise in DOC concentrations that is being observed in many waterbodies, (ii) summarize the direct and indirect effects of DOC on macrophytes, (iii) explain how macrophytes affect aquatic carbon themselves, (iv) discuss how C cycling and macrophytes are affected by the interaction between changing DOC and other effects of climate change, and (v) identify research gaps with regard to those four topics.

CO_2 and DOC in freshwater ecosystems

There are different forms and interactions of aquatic C (see box 3.1). Two of those forms, CO_2 and DOC have the most direct interaction with macrophytes and are therefore discussed in more detail.

CO_2

In 2017 the average atmospheric CO_2 concentration was 406 ppm (Tans and Keeling 2018) and it has been predicted that this value may increase to over 1000 ppm by the year of 2100 (IPCC 2013). However, the concentration of CO_2 in the atmosphere is lower than in most freshwater systems, which are supersaturated with CO_2 and act as CO_2 sources to the atmosphere (contrary to oceans which are sinks). Raymond et al. (2013) found that in 95 % of the over 6500 stream and river sampling points they studied, the median CO_2 partial pressure (pCO_2) was larger than atmospheric CO_2 levels. The average of the medians in rivers and streams was 3100 ppm and in freshwater lakes it was 1120 ppm.

Increasing atmospheric CO_2 concentrations will only have a small effect on the concentration of CO_2 in the water and will likely not lead to acidification on the scale observed in oceans. Phillips et al. (2015) calculated hypothetical pH decrease in freshwater lakes with different CO_2 concentrations under rising atmospheric CO_2 concentrations. If the CO_2 concentration in the air rises to 800 ppm, in an average lake with a CO_2 concentration of 1100 ppm, the pH will decrease by 0.14. The calculated changes in pH caused by increased CO_2 normally depend on the alkalinity; systems with low alkalinity may be more vulnerable to acidification caused by increased CO_2 concentrations and systems with a high alkalinity may be less vulnerable (Stets et al. 2017). However, the change in pH in the calculation by Phillips et al. (2015) was independent of alkalinity if this fell between 800 and 2500 meq m^{-3}, although the initial pH of the water was determined by alkalinity. The study by Phillips et al. (2015) focussed on the Laurentian Great Lakes, but in other freshwater systems the effect on pH may be different. Alkalinity may play a more prominent role and other factors can affect the pH, such as the sediment, photosynthesis and respiration in the water, water influx and land use. Since those factors can be highly variable both in time and space, the effect of increased CO_2 on pH is more difficult to predict than in oceans (Hasler et al. 2017). The IPCC (2007) predicted that the global average decrease of the pH in oceans will be 0.35 if the

concentration of CO_2 in the air rises to 800 ppm, a larger value than predicted for the Laurentian Great Lakes. For rivers, possible decreases in pH as a result of rising atmospheric CO_2 concentrations have not been calculated, but it can be expected that this will be even lower than in lakes, as rivers have on average a higher CO_2 concentration.

There can be substantial variation in the amount of CO_2 in freshwater systems, depending on, for example, characteristics of the catchment soil (Manahan 2000), discharge from the catchment (McDonald et al. 2013) and the season and time of the day. Seasonal variation is caused by high autotrophic productivity in summer and autumn compared to winter and spring (Dawson et al. 2009). Autotrophic organisms can also cause daily fluctuations in the concentration of CO_2. In productive lakes, the concentration of CO_2 can decrease to near zero during the day and is restored during the night, when no photosynthesis takes place (Maberly 1996). Another important driver of aquatic CO_2 concentrations is degradation of DOC. DOC is converted to CO_2 by photo-degradation caused by UV light and to a smaller extent by microbial respiration (Goulsbra et al. 2016). The rate of DOC degradation highly depends on the type DOC: chromophoric (coloured) structures in DOC are degraded most easily by UV light (Jones et al. 2015), even though microbes can degrade coloured DOC as well and mainly respire it instead of incorporating it into their biomass. Still, protein-like DOC is most readily degraded by microbes (Berggren and del Giorgio 2015). Moreover, the rate of microbial degradation depends on nutrient availability (Jones et al. 2015). Raymond et al. (2013) estimated that global inland freshwater ecosystem CO_2 emissions amount to 2.1 Pg C per year. In comparison, anthropogenic total emission of CO_2 was 8.03 Pg C per year (IPCC 2013).

During photosynthesis, macrophytes take up inorganic C, primarily CO_2. Even though freshwater systems are usually supersaturated with CO_2 (Raymond et al. 2013), photosynthesis may still be limited since i) diffusion of CO_2 in water occurs 10^4 times more slowly than in air (Maberly and Spence 1989), and ii) in highly productive environments with slow water flow velocity, the pH of the water is raised by photosynthesis, which reduces availability of CO_2 (Maberly and Spence 1983). In order to maintain net photosynthesis macrophytes have evolved four different strategies. First, there are submerged macrophyte species that can develop aerial leaves that can take up atmospheric CO_2 (Maberly and Spence 1989). Secondly, some species can take up CO_2 from the sediments if they have a suitable morphology i.e. sufficient root development

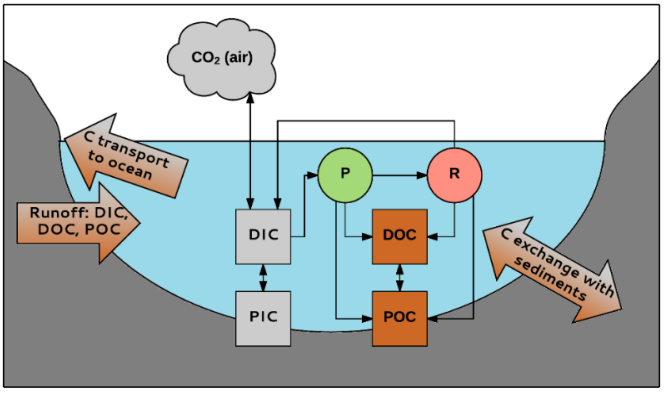
and high tissue porosity (Winkel and Borum 2009). The third strategy is utilising HCO_3^- instead of CO_2 as an inorganic C source; a strategy used by 50% of all macrophytes (Madsen and Sand-Jensen 1991). The fourth strategy to prevent photosynthesis limitation because of C deficit is using an alternative form of photosynthesis than the common C_3 pathway. There are macrophyte species with C_4 or CAM metabolism, although this is not widespread and can occur simultaneously with HCO_3^- use. Since both HCO_3^- and C_4 / CAM metabolism are a costly process, their use is often phenotypically plastic (Madsen and Sand-Jensen 1991).

DOC

The terms DOC (dissolved organic carbon) and DOM (dissolved organic matter) are often used interchangeably, but in fact, DOC is a quantification of C in DOM; approximately 67% of DOM consists of C (Bolan et al. 2011). In this review, the term DOC will be used. DOC consists of a diverse mixture of compounds with a molecular weight from 100 to 100,000 daltons. The compounds have wide variety of chemical functional groups like amide, carboxyl, hydroxyl and ketone groups (Leenheer and Croué 2003). The main part of DOC (60-90%) consists of humic substances (HS) (Sachse et al. 2005). HS consists of plant- or animal material from which readily bioconsumable parts have been removed (Frimmel 2005). HS are relatively complex molecules that do not have a standard chemical formula, in contrast to non-humic substances (such as carbohydrates, lipids and amino acids). There is a subdivision of HS into fulvic acid, humic acid and humin (Pettit 2004). This subdivision is based on solubility in water with different degrees of acidity. HS have a relatively high molecular weight and they have a yellow to black colour often causing brownification of the water (Findlay and Sinsabaugh 2003).

Other major classes of DOC are hydrophilic acids (high molecular weight) and compounds with a low molecular weight: carbohydrates, carboxylic acids and amino acids (Findlay and Sinsabaugh 2003). Although these substances can serve as food- (carbohydrates, amino acids) or information source (amino acids and carboxylic acids (Thomas 1997)) to aquatic organisms, there are no known effects on freshwater macrophytes, so the main focus of this review is on HS.

DOC in freshwater systems can originate from allochthonous and autochthonous sources, but usually there is a larger contribution of



Box 3.1 Different forms and interactions of aquatic carbon. Inland waters primarily receive C from terrestrial ecosystems (Thomas, 1997). This C (1.9 Pg C y^{-1}) is transported to oceans (0.9 Pg C y^{-1}), buried in the sediments (0.2 Pg C y^{-1}) or emitted as CO_2 (0.8 Pg C y^{-1}) (Cole et al. 2007). More recent estimations are different: Raymond et al. (2013) claims that CO_2 emission from inland waters can be as high as 2.1 Pg C y^{-1}. Aquatic C occurs in different forms. Firstly, a division is made between organic and inorganic C. Organic C is a mixture of organic compounds originating from detritus or primary producers. It can be divided into **POC** (particulate organic carbon; particles > 0.45 µm) and **DOC** (dissolved organic carbon; particles < 0.45 µm). DOC usually makes up 90% of the total amount of aquatic organic C. Its concentration ranges from 0.1 to > 300 mg L^{-1} (Sobek et al. 2007). Likewise, inorganic C also consists of a particulate (**PIC**) and a dissolved phase (**DIC**). PIC mainly consists of carbonates (e.g. $CaCO_3$), DIC consists of carbonate (CO_3^{2-}), bicarbonate (HCO_3^-), CO_2 and a negligibly small fraction of carbonic acid (H_2CO_3). The inorganic C compounds exist in equilibrium that depends on the pH of the water (Stumm and Morgan 1996). DIC concentrations in freshwater range from about zero in acidic waters to 60 mg C L^{-1} in areas with carbonate-rich sediments (Madsen & Sand-Jensen, 1991). POC can be degraded to form DOC; DOC can become POC by flocculation. Inorganic and organic C are linked through aquatic organisms. CO_2 is used in photosynthesis (P) by for instance macrophytes, produced by respiration (R), and exchanged with the atmosphere. Organic C is produced by organisms and is released during and after their lifespan; e.g. in rivers, 1-20% of the total amount of DOC is produced by macrophytes (Thomas, 1997). Carbon can enter the system from the catchment and is transported to the oceans by rivers and streams. There is also exchange with C in the sediments, e.g. burial of organic carbon, which is important for C sequestration in aquatic habitats (Regnier et al., 2013). Aquatic systems are very important in global C sequestration; e.g. when different European ecosystems are compared, inland aquatic systems form the second largest C sink (19 to 41 Tg C y^{-1}); only forests take up more C (125 to 223 Tg C y^{-1}) (Luyssaert et al. 2012).

allochthonous DOC (Thomas 1997). Allochthonous DOC mainly comes from terrestrial plant material (Steinberg et al. 2006) and enters rivers and lakes after precipitation has flowed through vegetation and / or the soil (Findlay and Sinsabaugh 2003). Autochthonous DOC is produced by algae (usually phytoplankton in lentic systems and periphyton in lotic systems) and macrophytes (Findlay and Sinsabaugh 2003) and is in general more labile than allochthonous DOC (Williamson et al. 1999). DOC concentrations can vary on different scales. On a large scale, DOC concentrations tend to be higher with more peatland area in the catchment, more precipitation and if water that enters a river or stream has flowed through organic-rich soil (Findlay and Sinsabaugh 2003). There are also differences on a smaller scale. DOC concentrations are usually highest in the pore water and lowest in the water column. At the air-water interface, intermediate concentrations are found; however, humic substances (HS) are degraded by UV-radiation, so its share in the DOC concentration is lower at the air-water interface. The higher concentrations of DOC in the pore water and at the air-water interface can be explained by the higher densities of detritivores and increased exposal to UV radiation, respectively, compared to the water column (Thomas 1997).

Increasing DOC concentrations and changing DOC quality

Since the 1990s an increase in DOC has been observed in European and North American rivers and lakes; between 1990 and 2004 concentrations increased by up to 0.15 mg L^{-1} y^{-1} (Monteith et al. 2007). The increases have been observed in acid sensitive rivers and lakes and appear to be present in both waters that already had a relatively high DOC concentration and waters that initially had a low DOC concentration (Evans et al. 2005). Data on long-term DOC trends in other parts of the world is scarce, but increasing DOC concentrations have also been reported, for example in Lake Jaisamand in India (Pandey and Pandey 2012) and it has been suggested that DOC concentrations have increased in Lake Paldang in South Korea (Kang et al. 2010). The exact cause of this rise in DOC has not been found, though it has been suggested that an interaction between several mechanisms is responsible (Sucker and Krause 2010). In figure 3.1 a graphical overview of current explanations for increased allochthonous DOC is shown. The main cause appears to be decreased atmospheric deposition of sulphur (acid rain) (Pagano et al. 2014). Anthropogenic SO_2 emissions led to acidification of the soil, which decreases solubility of organic matter in the soil pore water. When sulphur deposition

started to decline around 1990, DOC concentrations started rising, so DOC concentrations may be returning to pre-industrial levels (Monteith et al. 2007). A second possible cause of increasing DOC concentrations is altered land use. Worrall et al. (2012) studied DOC fluxes in the UK and showed that most DOC originated from organic soils (9.2 tonnes C $km^{-2} y^{-1}$), but urban (6.7 tonnes C $km^{-2} y^{-1}$) and grazed land (2.4 tonnes C $km^{-2} y^{-1}$) can also contribute significantly to DOC in rivers. Regnier et al. (2013) estimate that on top of the 1.9 Pg C y^{-1} (see Box 3.1), inland waters receive another 0.8 Pg C y^{-1} from terrestrial soils because of anthropogenic perturbations, which mainly leads to higher amounts of CO_2 emission, but also increased C storage and increased C transport to oceans. In a recent study by Noacco et al. (2017), a large data set (130 years) of DOC concentrations in the Thames basin was analysed and it was concluded that 90% of the increase in DOC was linked to effects of increased urbanisation, such as discharge of waste water, and land use changes like the conversion of grassland into farmland. However, changing land use can also decrease DOC concentrations. Around the Mississippi River, for instance, a significant part of wetlands, which could have released substantial amounts of DOC, have now been replaced with farmland. In the tributaries of the Mississippi River DOC subsequently decreased with 58%, leading to a lower downstream DOC concentration in the river (Duan et al. 2017). A third cause that could lead to increased DOC concentration is the effects of climate change such as: i) increased precipitation (Wu et al. 2007, Brothers et al. 2014), but in other cases also ii) decreased precipitation (Porcal et al. 2009) in combination with iii) increased temperature (Fenner and Freeman 2011), iv) rising CO_2 emissions, which can cause increased organic matter production by terrestrial (Zangerl and Bazzaz 1984) and aquatic (Song et al. 2013) primary producers, and v) increased nitrogen deposition (McElarney et al. 2010), although there are also studies that claim that DOC increases are caused by a decrease in nitrogen deposition (Musolff et al. 2017).

Altered land use and climate change can also change the quality of DOC. For example, DOC quality can change due to fragmentation of streams caused by drought. Vazquez et al. (2010) found that during fragmentation of a stream, the fluorescence index of DOC decreased, indicating that there was a higher contribution of autochthonous DOC. Moreover, they found that natural variation in DOC quality, like aromaticity, N content or biodegradability, at different locations in the stream became more pronounced after drought. Increased precipitation can also change DOC quality: there will be more

terrestrial DOC as the climate becomes wetter, which reduces light and oxygen availability in the water (Kellerman et al. 2014). Altered land use can also affect DOC quality: Butman et al. (2014) concluded from a global data set of DOC in which the age was determined by carbon-14 dating that in highly populated areas, DOC had a higher age. Sources of this older DOC are probably C released due to land use changes, human waste water or fossil C products such as petroleum products. Concentrations of other anthropogenic compounds such as biocides, pharmaceutical products and remains of genetically modified crops are also increasing and affecting DOC composition in the water (Stanley et al. 2012). Recreational use of freshwater systems, like camping and bathing during festivals can increase DOC concentrations. Substances like beer and urine can increase ecosystem respiration and the quality of DOC can highly change by usage of personal care products like sunscreen (Harjung et al. 2020). Urbanisation can also affect DOC quality: it was found that in urban watersheds with high population density, the composition of DOC was different from natural or agricultural watersheds (Williams et al. 2016). The exact chemical differences were not studied, but DOC from urban watersheds appeared to be more humic-like, probably of microbial origin and more resistant to photo-degradation and may therefore be less likely to be broken down. In a study by Hosen et al. (2014), this was found as well, and they also found an increase in labile, protein like DOC and a decrease in natural humic-like DOC. They found that this urban DOC is more likely to be degraded, as microbial bioavailability of urban DOC is higher than bioavailability of natural DOC.

The effects of DOC on macrophytes

The effect of humic substances on light availability to macrophytes

HS are the type of DOC that has the most pronounced effect on macrophytes and their effect has been studied most. HS are responsible for the brown colour of water with a high DOC concentration (Evans et al. 2005). HS attenuate UV radiation and photosynthetically active radiation (PAR), and can thereby limit benthic primary production (Karlsson et al. 2009, Thrane et al. 2014), see figure 3.2. DOC mainly attenuates the shorter wavelengths of PAR (the blue light) and the absorption coefficient decreases exponentially towards the longer wavelengths (Thrane et al. 2014). Although most studies about the effect of DOC on primary production focus on boreal lakes with limited macrophyte growth, there is evidence that macrophytes are affected as well by the effect of DOC on light quantity and quality; it can reduce their maximum colonisation depth

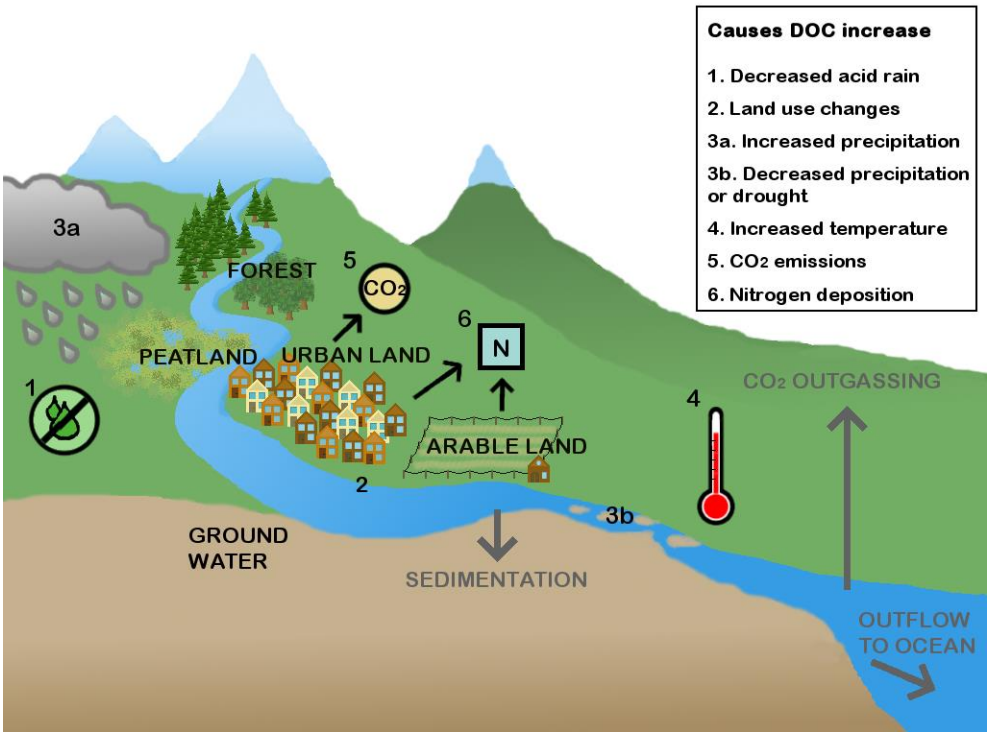


Figure 3.1 The DOC cycle in a river. DOC enters the rivers mainly from the terrestrial system, especially peatlands can release substantial amounts of DOC, but it can also come from other natural systems such as forests and through ground water seepage. Additionally, DOC also comes from urban and arable land. Six main causes of rises in DOC are shown. DOC leaves the system when it is incorporated in the sediments, degraded into CO_2, or transported to the oceans.

(Chambers and Prepas 1988). In oligohumic soft water lakes (<4 mg L^{-1} DOC), macrophytes can grow at a depth of 12 meters, whereas in meso- and polyhumic soft water lakes (4 to more than 40 mg L^{-1} DOC) this decreases to 1 meter (Bociąg 2003).This means that macrophytes are confined to the shallowest parts of the lake where additional disturbance from wave action may exclude some species (Szmeja and Bociąg 2004). Not all species are equally vulnerable to changes in light quantity and quality. In Polish lakes, for instance, habitat characteristics of two *Ceratophyllum* species were studied, and it was found that water transparency and water colour (mainly determined by HS) were important factors determining species occurrence. *C. demersum* was found in transparent waters, whereas *C. submersum* was found in more coloured waters (>100 mg Pt L^{-1}) (Nagengast and Gąbka 2017). As a more general phenomenon, charophyte abundance decreased and bryophytes and

vascular plants dominated during a wet period in a Polish lake in which conductivity decreased and DOC concentrations increased. DOC changed the colour of the water and thereby reduced visibility. However, charophytes generally do not have higher light requirements than vascular plants. There are two alternative explanation for the decreased charophyte abundance: it has been suggested that the altered colour of the water diminished the establishment of charophytes and provided an opportunity to competitors (Ejankowski and Lenard 2015). Middelboe and Markager (1997) suggested that the negative effect on charophyte growth can also be caused by the fact that coloured substances reduce the pH of the water. McElarney et al. (2010) found that DOC can reduce macrophyte abundance and diversity. In their study especially isoetids appeared to be sensitive to the change in water colour, but they argued that DOC may have increased sedimentation of organic matter which increases sediment alkalinity and nutrient concentration, which is unfavourable to some macrophyte species. Besides light, other examples of indirect effects of DOC on macrophytes are discussed in section 'indirect effects of DOC on macrophytes'. Effects on primary production in general and on macrophytes have been summarised in table S3.1. From this overview it can be concluded that most research on the effect of coloured DOC on macrophytes focusses on lakes in northern Europe.

Direct effects of humic substances: intracellular damage

Although macrophytes are probably mainly indirectly affected by HS by light attenuation, HS may also directly affect macrophytes. HS form a wide class of substances with many different functional groups. This gives them the ability to interfere with nearly all biochemical processes that occur in freshwater organisms (Steinberg et al. 2008). Only a few studies have looked into these effects. There is evidence that small particles (<3.5 kDa) can be taken up by macrophyte cells (Steinberg et al. 2006), but it has not been studied yet in great detail. Inside cells they can, for example, lead to the formation of reactive oxygen species (ROS) that can damage the cells (see figure 3.2). Production of oxidative stress enzymes by macrophyte cells significantly increased after exposure to DOC derived from decomposing beech leaves, which contain ROS (Grigutytė et al. 2009). Secondly, HS can interfere with photosynthesis (see figure 3.2). This was demonstrated in *Ceratophyllum demersum* and is caused by quinoid structures in HS that take up electrons and thereby inhibit photosynthetic oxygen production. It has been hypothesised that macrophyte

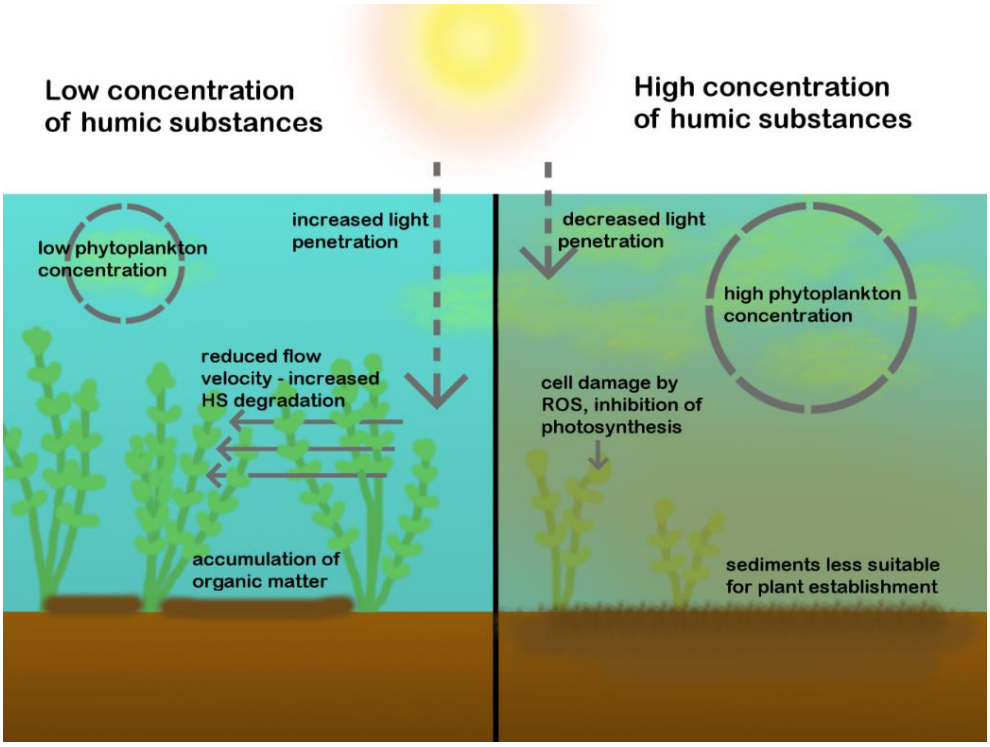


Figure 3.2 The effects of HS on macrophytes. On the left side, a scenario with low HS, high plant density and low phytoplankton density is shown. Plants receive enough light. Water flow is reduced which causes OM accumulation within macrophyte patches and HS degradation. On the right side, a scenario with high HS, low plant density and high phytoplankton density is shown. Plants are damaged by HS, receive less light and HS makes the sediments less suitable for plant establishment. The increase in phytoplankton is not a direct effect of DOC, but they can increase when macrophytes decrease, because of reduced competition for carbon and nutrients. Heterotrophic bacteria are expected to increase with higher DOC levels, as DOC can serve as a food source.

species may not be equally vulnerable to those quinoid structures (Pflugmacher et al. 2006), so species composition may be altered in very humic waters. Even though HS stress can cause damage to aquatic organisms, exposure to HS can also train the stress resistance of aquatic animals such as fish and nematodes. This improves their fitness in a fluctuating environment and can increase survivorship. Nematode *Caenorhabditis elegans* is even attracted to HS and actively seeks HS rich water (Steinberg et al. 2007). It is not known whether this intriguing phenomenon also applies to macrophytes, but Steinberg et al. (2008) suggest that mild HS stresses may be beneficial to specific plant organs

caused by increased expression of anti-stress genes, resulting in multistress resistance. The chemical composition of DOC can vary substantially, based on catchment characteristics such as vegetation type (Amiotte-suchet et al. 2007), the presence of wetlands (Singh et al. 2015) and the type of soil (e.g. peat, mineral soil, anthropogenic influences) (Sachse et al. 2005). Steinberg et al. (2006) tested the response of different aquatic primary producers to DOC from different origins and found that the primary producers were not equally sensitive. They suggest that primary producers may adapt to the DOC type from their native environment.

It is important to take into account that natural sources of DOC, like peat and tree leaves, can also be an important source of phosphorus; an element that often limits primary production (Wallace et al. 2008). This means that in aquatic ecosystems negative effects on macrophyte growths due to DOC may be partly compensated by the increased phosphorus availability.

Defence mechanisms against HS

Macrophytes have developed defence mechanisms against HS and other environmental stressors. Studying the production of defensive compounds may help to find the molecular mechanisms behind the cellular damage caused by HS, interaction with other stressors and the way macrophytes cope with this. Omic technologies may be a valuable technique to gain more understanding in this field (Van Aggelen et al. 2010) Although most studies using omics focus on terrestrial plants, omics are increasingly applied to marine macrophytes (Kumar et al. 2016) and there are also a few examples of studying stress tolerance using omics in freshwater macrophytes.

A general stress response is the production of defence proteins like HSP70, a heat shock protein. This protein, or similar proteins, are present in virtually all living organisms and aim to protect cells from thermal or oxidative stress (El Golli-Bennour and Bacha 2011). In macrophytes HSP70 expression in response to HS has not been tested, but in algae (Bierkens et al. 1998), fish and invertebrates (Steinberg et al. 2006) exposure to HSs leads to an increase of the concentration of HSP70. Other substances have been found to affect heat shock protein expression in macrophytes: Tukaj et al. (2011) found that HSP70 was induced in *Lemna minor* when it was exposed to different chemicals like heavy metals, polycyclic aromatic hydrocarbons and herbicides. This means that HSP70 may be used as biomonitor to see whether DOC causes stress in

macrophytes, provided that other parameters such as temperature are kept constant. Another way macrophytes protect themselves from oxidative stress is the use of detoxification mechanisms like antioxidant enzymes and ROS-scavenging proteins (Chalanika De Silva and Asaeda 2017). Acquired stress tolerance can be heritable to next generations, although heritability of HS tolerance has not yet been studied in macrophytes. In cladocerans, however, there is evidence of epigenetic inheritance: when they were exposed to HS, the percentage of methylated DNA increased. It has also been found that resistance to HS stress is transferred to the next generation, so it has been suggested that this may be caused by epigenetics (Menzel et al. 2011). Studying epigenetics in ecotoxicological research is relatively new but as it may explain inherited changes in the phenotype caused by environmental stress and stress adaptation it may be an interesting approach in aquatic ecology (Vandegehuchte and Janssen 2014).

Indirect effects of DOC on macrophytes

Although high concentrations of DOC are disadvantageous to macrophytes, DOC can also positively affect macrophytes by mitigating the effect of other stressors. DOC attenuates UV-B radiation (Scully et al. 1995) that can decrease growth rates and damage DNA of charophytes (de Bakker et al. 2005). DOC may also stimulate heterotrophic bacteria by attenuating UV-B radiation and providing a food source, leading to faster DOC degradation. Moreover, UV-B radiation can transform recalcitrant DOC and make it more accessible to bacteria (Karentz et al. 1994). Another positive effect of DOC, or more specifically, polyphenols (major building blocks of HS) is that they can inhibit cyanobacteria and thereby can contribute to controlling blooms (Steinberg 2014). DOC can also mitigate toxicity of anthropogenic pollutants like anthracene in macrophytes (Gensemer et al. 1999) and protect macrophytes against harmful heavy metals. Some chemical functional groups in humic acids, a subgroup of HS, have a negative charge, such as carboxylic and phenolic groups, which can bind to positively charged metal ions (Christl et al. 2001). Heavy metals such as copper, cadmium (Wang et al. 2010), lead (Kruatrachue et al. 2002) and zinc (Bunluesin et al. 2006) are taken up by macrophytes and can lower chlorophyll content. HS in the sediments bind to heavy metals and thereby significantly reduce accumulation of heavy metals in macrophytes (Wang et al. 2010). This appears to be beneficial to macrophytes, but others argue that especially binding of metals to allochthonous DOC, which has a higher binding capacity than autochthonous DOC due to the higher HS content, may be harmful to macrophytes. If there is a high concentration of

allochthonous DOC, heavy metals may, instead of being adsorbed by the sediments, be bound to DOC and stay in the water column. When DOC is degraded, the heavy metals are released in the water column and this may be detrimental to aquatic organisms in general (Zhang et al. 2013). DOC can also negatively affect primary producers by enhancing mercury accumulation in macrophytes and epiphytes. Mercury can bind to sulphide and precipitate, but when mercury binds to DOC, it will stay in solution. DOC may also stimulate mercury methylating bacteria. Methylated mercury can accumulate in the food chain; in most fish species more than 95% of the mercury is methylated (Ravichandran 2004). Both methylated and unmethylated mercury can accumulate in macrophytes, and in epiphytes even higher concentrations can be found. Water level fluctuations and higher temperatures also stimulate mercury uptake, so under climate change mercury concentrations in macrophytes and epiphytes are expected to rise (Hamelin et al. 2015).
DOC can also change soil properties, making the sediment more gelatinous and hydrated, which limits macrophyte establishment (Bociąg 2003). DOC concentrations often correlate with CO_2 concentrations in freshwater ecosystems (Sobek et al. 2003). DOC can be converted to CO_2 by biodegradation (bacteria break down DOC) or by photodegradation (DOC is broken down by UV radiation). With increasing DOC concentrations, the fraction of biodegradable DOC appears to be constant, but the proportion of photodegradable DOC is enhanced as the input of terrestrial DOC increases, leading to more CO_2 production (Lapierre et al. 2013). This can be beneficial to macrophytes as CO_2 is often limiting (see paragraph 2), but high levels of CO_2 in the water can lead to dominance of macrophyte species that only use CO_2 as their inorganic C source. Species adapted to low CO_2 concentrations such as isoetids lose their advantage and may disappear (Spierenburg et al. 2009). DOC can also bind to phosphorus (P) and iron (Fe), although it is not fully understood how binding of DOC to P and Fe affects their bioavailability, Findlay and Sinsabaugh (2003) conclude that reactivity of P and Fe is reduced if it is bound to DOC.

The concentration of DOC in water can also indirectly affect nitrogen availability to macrophytes. DOC serves as an energy source to denitrifying bacteria, so if there is a sufficient amount of nitrate in the water DOC can stimulate denitrification and therefore reduce nitrate availability (Taylor and Townsend 2010). However, microbes that carry out dissimilatory nitrate reduction to ammonium (DNRA) are stimulated by high C:N ratios in the water,

so this may favour conversion of nitrate to ammonium instead of denitrification (Tiedje 1988). Ammonium is by most macrophyte species preferred over nitrate as source of nitrogen (Feijoó et al. 2002).

Altogether, it appears that DOC can have various indirect positive and negative effects on macrophyte growth. The net effect on macrophytes does not only depend on the concentration of DOC but also, for example, on the quality of DOC (HS content), intensity of UV radiation in the water and presence of microbes that degrade DOC. Moreover, the net effect of DOC on primary production largely depends on the characteristics of the aquatic system. For example, in boreal lakes with low productivity that are supersaturated with CO_2, the net effect is negative: elevated CO_2 concentrations due to DOC degradation do not lead to increased productivity because DOC diminishes light availability (Hessen et al. 2017).

The effects of macrophytes on aquatic carbon

The relationship between carbon and macrophytes is not one-way; macrophytes increase sedimentation of organic C, they produce DOC, and take up inorganic C. Macrophytes contribute to sedimentation of carbon by taking C out of the water and sinking to the bottom after senescence (Flanagan et al. 2006). The physical structure of macrophytes also contributes to the removal of C from the water column: macrophytes reduce flow velocity and this causes accumulation of organic matter within macrophyte patches (Schoelynck et al. 2012b). Still, carbon burial efficiency can also be reduced by the presence of macrophytes. Brothers et al. (2013) found that in an algae-dominated shallow lake, 80% of the amount of carbon entering the lake was buried in the sediments, whereas in macrophyte-dominated lakes this was only 40%. This can be explained by the fact that macrophytes provide bacteria in the sediments with oxygen which leads to enhanced C mineralisation. DOC release and inorganic carbon uptake by macrophytes are explained in next paragraphs.

DOC release by macrophytes

When macrophytes grow, <1-10% of the amount of C they fix photosynthetically is released again as DOC (Carpenter and Lodge 1986). Macrophytes can therefore be an important DOC source, yet most studies on autochthonous DOC only focus on algae (Findlay and Sinsabaugh 2003). Søndergaard (1981) studied DOC release by several macrophyte species and concluded that it mostly consists of small (<1000 Daltons) and a smaller

fraction of large (>10000 Daltons) molecules, depending on the plant species. Small molecules that are released can include amino acids and simple sugars, especially glucose. DOC release appears to be related to photosynthesis; in dark conditions DOC production is only 1% of DOC production in light conditions (Søndergaard 1981). Moreover, in fast growing species, the rate of DOC release is higher than in slower growing species (Thomas and Kowalczyk 1997). The effect of nutrient availability on DOC production is not clear. Takamura et al. (2003) found that *Trapa japonica* only causes DOC enhancement in the water when nutrient concentrations are high. However, (Demarty 2009) did not find a correlation between DOC production by *Myriophyllum spicatum* and *Potamogeton* spp. and nutrient concentrations. Lastly, there is a relationship between the amount of inorganic C in the water and DOC release by the free floating macrophyte species *Lemna minor*. When there is a limited amount of inorganic C in the water, DOC release is higher than when there is an excess of inorganic C, even though macrophyte growth is impaired under low inorganic C conditions. It was suggested that the stress caused by the low inorganic C concentrations may have led to DOC leakage from the plants (Baker and Farr 1987).

The contribution of macrophytes to the total amount of DOC in the water varies. Especially in lotic systems, the DOC contribution by macrophytes is small (1-20% of the total amount of DOC), probably because of DOC degradation by epiphytic bacteria and algae (Thomas 1997), or insignificant (Hummel and Findlay 2006). This may also be caused by the relatively low abundance of macrophytes in rivers compared to e.g. wetlands. In wetlands (Briggs et al. 1993) and shallow lakes (Lapierre and Frenette 2009) macrophytes can contribute significantly to DOC concentrations in water and organic C release by emergent macrophytes can even be in the same order of magnitude as organic C input from the catchment. In Lake Frisksjön in Sweden for example, organic C input from the catchment is 9600 kg C y^{-1} and production by emergent macrophytes is 6000 kg C y^{-1} (Sobek et al. 2006). Macrophyte DOC production is dependent on the season: in summer, when macrophyte biomass reaches its climax, macrophytes can cause large increases in DOC concentrations. At this time they can also alter the composition of DOC, as they primarily release carbohydrates whereas allochthonous DOC contains more humic and protein-like material (Catalán et al. 2014).

It is not exactly known why macrophytes release DOC, but there are a few hypotheses. The first hypothesis is the overflow mechanism, a passive mechanism which has been demonstrated in planktonic algae. The algae excrete sugars they produce during photosynthesis, when nutrient availability is limited (Jensen 1984). It has also been hypothesised that macrophytes may actively release DOC. Some species excrete DOC from their roots to stimulate bacterial (Catalán et al. 2014) or endomycorrhizal (Wigand et al. 1998) growth and activity in the sediment in order to obtain more nutrients. It has also been suggested that DOC release serves as a C concentrating mechanism when CO_2 is limiting, which works as follows: Demarty (2009) found that DOC release is positively linked to HCO_3^- uptake, which is one of the strategies used by macrophytes to avoid inorganic C deficit. It has been suggested that the type of DOC released by the plant is carbonic anhydrase, an enzyme involved in HCO_3^- use. However, DOC released by macrophytes mostly consists of small compounds, whereas carbonic anhydrase is a nitrogenous high weight compound and only 10% of the DOC falls into that category. Another form of DOC released by macrophytes is allelochemicals that serve to inhibit phytoplankton growth. This topic has been reviewed by Hilt and Gross (2008) and it can be concluded that there are at least 37 macrophyte species that produce allelochemicals, like *Myriophyllum, Ceratophyllum, Elodea and Najas.* Most of the allelopathic compounds have not been identified, but at least part of them are polyphenols. It is hypothesised that those compounds are also involved in defence against herbivores and infections (Gutierrez and Mayora 2015). Diatoms and cyanobacteria appear to be more sensitive to the allelochemicals than chlorophytes. Epiphytes are targeted as well, but it has been suggested that they have developed resistance against allelopathic substances from macrophytes (Hilt and Gross 2008). Macrophytes can also diminish phytoplankton growth by limiting their nutrient availability (see 'the effect of macrophytes on DOC concentrations').

Dead macrophyte tissue also releases DOC. This can occur because of cell death, but also when cells are damaged by grazers or viral lyses (Findlay and Sinsabaugh 2003). The nature of this DOC depends on the macrophyte species; it can differ, for example, in colour and C:nutrient ratio (Cuassolo et al. 2011), amount of humic-like matter and photoreactivity (Cuassolo et al. 2015) and percentage of proteins, amino acids and carbohydrates (Qu et al. 2013). Macrophyte DOC is less aromatic than allochthonous DOC but has a similar or higher aromaticity than phytoplankton DOC (Qu et al. 2013). In general, DOC

released by macrophytes is relatively labile and rapidly decomposed by bacteria, compared to allochthonous DOC (Mann and Wetzel 1996). However, They et al. (2012) found that a significant part of the DOC produced by macrophytes in a subtropical shallow lake remained in the water as unreactive molecules with a low molecular weight.

The effect of macrophytes on DOC concentrations
Freshwater macrophytes can also diminish DOC concentrations in several ways. Some species primarily take up nutrients from the sediments and this can reduce nutrient exchange between water and sediments. The resulting reduction in water column nutrient concentration leads to diminished growth of DOC producing organisms without roots such as phytoplankton, bacteria and filamentous algae (Wigand et al. 2000). Moreover, macrophytes release oxygen from their roots, which stimulates bacterial decomposition of DOC (Mann and Wetzel 2000). Macrophytes also increase the residence time of the water and this leads to a longer exposure to photo- and microbial degradation (see figure 3.2). DOC forms an important food source for heterotrophic bacteria. Macrophytes serve as a substrate for those bacteria and epiphytic algae (together called epiphyton). The resulting communities of macrophytes and epiphyton can be very productive and highly efficient with regard to DOC degradation (Wetzel and Sondergaard 1998). Martin et al. (2005) found that the concentration of chromophoric DOC, the part of DOC that absorbs light in water, decreases as the water flows through macrophyte beds. A possible explanation may be the high abundance of epiphytic bacteria that degrade DOC. The interaction between heterotrophic bacteria and macrophyte-epiphytic algae complexes can also have implications for the aquatic food web. de Kluijver et al. (2015) found, by studying carbon isotope ratios ($\delta^{13}C$) in a Chinese lake, that carbon produced by macrophytes and epiphytic algae contributes to bacterioplankton (55%) and zooplankton (47%). Stimulation of zooplankton can, in turn, reduce abundance of phytoplankton and thereby maintain clear water.

Interactions with other effects of climate change on C cycling and macrophytes

The effects of DOC on macrophytes are complex and depend mainly on the characteristics of the environment (see paragraph 3.3). In addition to that, climate change can also have other effects on the aquatic carbon cycle. For

example, drought does not only affect DOC concentrations but also other dissolved compounds. This effect has been observed in Canadian lakes; decreased runoff rates lowered concentrations of iron, phosphorus, dissolved organic nitrogen (DON) and DOC in the water. This may have implications for the aquatic C cycle, more specifically the C sequestration in the sediments. One of the mechanisms of C sequestration is binding of DOC to amorphous iron. Since iron concentrations are even more reduced by drought than DOC concentrations, it has been suggested that in this way drought may lead to a decrease in C sequestration (Dillon and Mollot 2005). Temperature can also affect carbon cycling. When temperatures are raised, community respiration increases; e.g. in an Alpine river, benthic community respiration increased by 20% when temperature was raised by 2.5°C (Acuña et al. 2008). When exposed to extremely high temperatures, gross and net photosynthesis rates, as well as plant respiration can even be reduced, whereas heterotrophic respiration rates increase. This means that more C stored in the system is now emitted as CO_2 during warming (Moss 2010).

Climate change and altered land use also can have profound effects on macrophytes. Especially changing temperatures can form a substantial threat to macrophytes (Short et al. 2016), but storms (wave action, mixing of water layers and nutrient loading), water level fluctuations (Zohary and Ostrovsky 2011) increasing CO_2 concentrations, increases in UV-B radiation, increasing salinity (Short et al. 2016) and eutrophication (Hossain et al. 2016) all affect macrophyte growth and the distribution of species. It can be concluded that mainly submerged macrophytes will decline, as they suffer most from the increases in water turbidity caused by increased DOC (Karlsson et al. 2009) and they may be outcompeted by phytoplankton and floating macrophytes that benefit from higher temperatures and from eutrophication, (Short et al. 2016). Different effects of climate change can have contrasting effects on macrophytes. For example, rising temperatures can enhance productivity of macrophytes, whereas increased HS in the water decrease productivity (Rodríguez et al. 2015). In lakes, DOC may even act as a buffer against rising temperatures. As DOC attenuates light that heats up the water and enhances stratification of the water, so deeper parts of the lake are less exposed to higher temperatures (Read and Rose 2013).

Knowledge gaps

Although many studies have looked into DOC in aquatic systems, there still are a number of research gaps, especially with regard to the link between DOC and macrophytes. Firstly, production of DOC by living macrophytes and the effect of elevated CO_2 on DOC production are still poorly understood. It is still not clear why macrophytes produce DOC, what compounds it exists of and how much this process is affected by climate change. Secondly, there are research gaps with regard to the effects of DOC on macrophytes. Since the exact cause of the increase in DOC concentrations is not known, it is difficult to predict how DOC concentrations will develop in the future. Therefore, it is important that DOC concentrations are monitored over longer periods of time. Currently, most DOC research focusses on North America and Europe. Global monitoring campaigns are needed to provide more insight into the cause of DOC increases and role of freshwater ecosystems in the global carbon cycle.

DOC covers a wide class of substances with many chemical functional groups. In most studies, those substances are not identified and it is not known whether and how they affect macrophytes on the cellular level. The quality of DOC also varies, depending on its source. DOC quality also appears to be different in densely populated areas (Williams et al. 2016). The chemical characteristics of this 'anthropogenic DOC' are not completely known and the number of studies looking at the changing quality of DOC due to anthropogenic disturbances is low. Gaining more knowledge about the nature of this changed quality of DOC and its effect on freshwater organisms is crucial to understanding the stability of freshwater ecosystems. Anthropogenic DOC compounds such as pesticides, hormones and remains of genetically modified crops may pose a considerable threat to macrophytes although the exact consequences and scope of this problem are still poorly understood and require more research (Stanley et al. 2012).

It is also important to note that freshwater ecosystems are naturally heterogeneous systems. For example, rivers can be seen as a patchwork of different zones that vary in hydrogeomorphology and are affected by differences in the catchment and the climate. Those different patches may have different inputs of C and may vary in C processing rates (Thorp et al. 2006). In addition, drought may further increase those differences by decreasing connectivity between different parts of the river (Vazquez et al. 2010). This

needs to be taken into account when studying the effects of changed DOC quality and quantity on macrophytes.

There is also a lack of knowledge about the fate of allochthonous DOC from different origins; whether it is degraded or not, how it is degraded and to what extent abiotic factors like light and nutrients play a role (Evans and Thomas 2016). It was assumed that terrestrial, coloured DOC is relatively resistant to degradation by microbes. However, laboratory experiments have indicated that when organic C is added to stream water, it is rapidly broken down to CO_2 after it had entered the water during a storm (Goulsbra et al. 2016). It appears that this terrestrial, coloured DOC is degraded by microbes, but the carbon use efficiency is low, meaning that the main part is converted to CO_2 instead of microbial biomass (Fasching et al. 2014). Molecular characteristics are an important factor determining degradability of DOC. Oxidised, aromatic molecules are better degradable than reduced, aliphatic and N-containing molecules (Kellerman et al. 2015). Furthermore, DOC with a large molecular size and DOC originating from terrestrial plants appears to be more easily degraded than DOC from agriculture or wastewater (Bodmer et al. 2016). More knowledge on the degradability and residence times of DOC, and therefore also the degree of exposure to macrophytes can help to predict the effects on macrophytes. If DOC is degraded, this is not always beneficial to macrophytes. For example, photo-oxidation of DOC can lead to release of toxic trace metals that can be taken up by macrophytes (Porcal et al. 2009). Understanding the fate of DOC is also vital to understand the aquatic C cycle. If the quantity and quality of DOC is changed by climate change, this may have large effects on the extent of C sequestration in aquatic sediments and on aquatic CO_2 emissions. Although it is important to study the fate of DOC, DOC itself can also be regarded as C sink for anthropogenically emitted CO_2 as DOC production in algae increases under elevated CO_2 concentrations (Song et al. 2013).

To conclude, in order to gain improved understanding of the effects increased quantity and quality of DOC has on macrophytes and to be able to conserve stable macrophyte populations, the following points have priority: 1) detailed modelling covering a large spatial scale can contribute substantially to understanding the effects of increasing DOC and changing climate on the aquatic C cycle (Porcal et al. 2009). 2) More inclusion of DOC quality and quantity in river management, especially in relation to the potential for riparian zones to buffer DOC rises (Stanley et al. 2012) As most studies on DOC focus on lakes in northern Europe (see table S3.1), it is also important to study DOC in

other parts of the world and to include lotic systems. 3) Carrying out experimental studies to help predicting the morphological and physiological responses to changing DOC quantity and quality in freshwater organisms like macrophytes.

Supplementary tables

Table S3.1 Effects of DOC on primary production in general and on macrophytes

DOC concentration	DOC type	Macrophyte species	Effect	Location	Geology and climate	Type of system	Reference
Effect of light limitation on primary production in general							
2.4-16.8 mg L^{-1}	-	General effect on primary production was measured	Light attenuation and mean depth explained 73% of benthic PP	Northern Sweden	Boreal climate	Oligo- or mesotrophic lakes.	**Karlsson et al., 2009**
0.25-27.5 mg L^{-1}	-	General effect on primary production was measured	A doubling in CDOM can decrease PP/PAR by 32%	Norway and Sweden	Boreal climate	Oligo- or mesotrophic lakes. pH >5	**Thrane et al., 2014**
Effect of light limitation on macrophytes							
	-						**Chambers & Prepas, 1988**
Between <4.0 and >40.0 mg L^{-1}	Humic substances	Bryophyta, cormophyta, characeae	Macrophytes grow at 12 meter depth in oligohumic lakes and at 1 meter depth in humic lakes	Northwestern Poland	Temperate climate, landscape of moraines	Oligo- or mesotrophic soft water lakes	**Bociag, 2003**
Between <4.0 and >40.0 mg L^{-1}	Humic substances	*Isoëtes lacustris, Lobelia dortmanna, Sphagnum denticulatum, Fontinalis antipyretica*	Settlement and aggregation density indexes are lower in humic lakes, especially in deep central parts	Northwestern Poland	Temperate climate, landscape of moraines	Oligo- or mesotrophic soft water lakes	**Szmeja & Bociag, 2004**

Secchi disk transparency: 0.14-28.2 m Water colour was measured as well (results not mentioned)	Humic substances	Bryophytes, charophytes, caulescent angiosperms, rosette-type angiosperms, *Isoetes* spp.	Negative correlation between water colour light intensity, some species are more vulnerable than others	Denmark, Finland, Norway, Scotland, Canada, New Zealand, The Netherlands, Poland, US	Various	Danish lakes: Shallow and often eutrophic. Non-danish lakes differed in nutrient status	Middelboe & Markager
0.36-22.47 mg L^{-1}	Humic substances	*Ceratophyllum demersum* and *C. submersum*	*C. demersum* was found in relatively transparent water, *C. submersum* in more coloured water	Western Poland	Temperate climate	Oxbow lakes, post-exploitation ponds, periodical overflow areas and small lakes	Nagengast & Gabka, 2017
Water colour: 0.2-65.4 mg Pt L^{-1}	Humic substances	Bryophytes, charophytes and vascular plants	Charophyte establishment decreased when DOC concentrations were higher, which provided an opportunity to bryophytes and vascular plants	Eastern Poland	Temperate climate	Mesotrophic, hard water lake	Ejankowski & Lenard, 2015
Mean annual concentration: 14.81 mg L^{-1} ± 0.84 SE	Humic substances	Bryophyta, characeae, elodeids, isoetids, macroalgae, potamogetonaceae, callitrichaceae, cyperaceae, lemnaceae, nymphaeaceae, sparganiaceae	Increases in DOC have a negative impact on macrophyte abundance and diversity because of light limitation and effects on lake chemistry	Northern Ireland	Temperate oceanic climate	Softwater, humic lakes	McElarney et al., 2010

Chapter 4.

Effects of different gradients of CO_2 and DOC on growth, morphology, nutrient stoichiometry and chlorophyll content in two freshwater macrophyte species

Rosanne E. Reitsema, Stefan Preiner, Patrick Meire, Thomas Hein, Jonas Schoelynck

Abstract

Freshwater ecosystems are affected by climate change, and effects of elevated carbon concentrations on freshwater macrophytes are relatively understudied. Levels of dissolved organic carbon (DOC) are rising, leading to increases in water colour (brownification) that can decrease light availability. Another form of aquatic carbon affecting macrophytes is CO_2, which can affect plant growth and nutrient stoichiometry. Although a few studies have investigated those effects, often only effects of two concentrations are tested. In this study, macrophyte species *Myriophyllum spicatum* and *Berula erecta* were exposed to multiple CO_2 and DOC concentrations in a wide range. Plant growth, morphology, chlorophyll and nutrient content were tested using a trait-based approach. We found that CO_2 and DOC had strong effect on both species, with the strongest effects in the plants exposed to the higher CO_2 or DOC doses: there were no clear threshold levels. In the CO_2 treatment there were also notable differences between the two macrophyte species. *B. erecta* mainly showed responses in growth and biomass production, whereas *M. spicatum* showed changes in plant morphology. CO_2 stimulated growth in *B. erecta* and increased its C:N ratio, whereas in *M. spicatum* there were more but smaller leaves and longer and heavier stems in the higher CO_2 treatments. High levels of DOC mainly stimulated stem length and chlorophyll concentration in *M. spicatum*. Although the results suggest that macrophytes are highly affected, from comparing the current study to other research investigating the effect of elevated carbon concentrations on macrophytes it can be concluded that results not always consistent: they depend substantially on the plant species and water conditions (for example the DOC source). Therefore, we suggest that more studies should test effects of elevated carbon concentrations on dominant and / or ecologically important macrophyte species, as well as combinations with other aspects of climate change.

Keywords: Climate change, brownification, *Berula erecta*, *Myriophyllum spicatum*, aquatic ecology

Introduction

Freshwater ecosystems are affected by the effects of climate change (Short et al. 2016). Within those ecosystems, freshwater macrophytes are essential organisms. As primary producers, they contribute to a clear water state, play a role in the aquatic carbon and nutrient cycle (Uehlinger et al. 2000, Clarke 2002) and provide a habitat to many other organisms (Sand-Jensen 1998). As changes in macrophyte biomass and morphology can affect their functioning within aquatic ecosystems, it is important that those effects are studied. Still, research looking into the effects of climate change mostly focus on terrestrial ecosystems.

One of the effects of climate change that is relatively understudied is the effect of changing concentrations of carbon in the water (Evans et al. 2005, Hasler et al. 2016, Williams et al. 2016). Since the 1990s an increase in DOC (dissolved organic carbon) has been observed in many European and North American rivers and lakes; between 1990 and 2004 concentrations increased by up to 0.15 mg L^{-1} y^{-1} (Monteith et al. 2007). This often also leads to a change in water colour called 'brownification' (Kritzberg and Ekström 2012). Brownification 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 CO_2 concentrations, more terrestrial organic matter is produced and with increased precipitation intensity this material can be flushed into rivers (Pagano et al., 2014). 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 (HS) 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 a larger part of DOC will consist of HS in the future (Creed et al. 2018). HS can directly negatively affect macrophytes as they diminish light availability to primary producers (Szmeja and Bociąg 2004, Karlsson et al. 2009, Choudhury et al. 2019) and reduce macrophyte colonisation depth (Chambers and Prepas 1988). Moreover, some HS may directly affect macrophytes by entering the plant's cells and causing damage by production of reactive oxygen species (Grigutytė 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 (see chapter 3).

Another form of aquatic carbon is CO_2: a primary source of inorganic carbon for macrophytes. Most freshwater systems are supersaturated with CO_2 due to respiration in the sediment. Currently, the median CO_2 concentration of non-tropical freshwater lakes is 1120 ppm and in rivers and streams 3100 ppm (Raymond et al. 2013), which is substantially higher than the concentration of 400 ppm in the atmosphere. The concentration of CO_2 can vary substantially on temporal and spatial scale, so macrophytes may be exposed to peak concentrations that can amount to over 10000 ppm (Abril et al. 2015). Despite the fact that aquatic CO_2 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_2 efflux from the water as a result of higher atmospheric CO_2 concentrations, caused by anthropogenic CO_2 emissions (Phillips et al. 2015). It is difficult to predict future CO_2 levels in freshwater ecosystems because the exact factors controlling aquatic CO_2 concentrations and their response to climate change are not yet well understood. Moreover, current CO_2 and total inorganic carbon levels in rivers are highly variable and can depend on the catchment (Cole et al. 2007), and location within a water body (Maberly et al. 2015). As a consequence, it is hard to predict future CO_2 levels and how freshwater organisms will respond (Hasler et al. 2016). Research on the effects of CO_2 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_2 concentrations on freshwater macrophytes observed increased plant growth rates under high CO_2 concentrations (Pagano and Titus 2007, Eusebio Malheiro et al. 2013, Dülger et al. 2017), increased biomass production (Andersen et al. 2005, Cheng et al. 2010, Hussner et al. 2016), and an increase in root:shoot ratio (Madsen 1996, Hussner et al. 2016, Dülger et al. 2017). Moreover, the N content of macrophyte tissue was found to be lower (Titus and Andorfer 1996, Titus and Pagano 2002, Yan et al. 2006), the P content was higher (Yan et al. 2006), chlorophyll content was lower (Madsen 1996, Dülger et al. 2017), their dry matter content higher (Eusebio Malheiro et al. 2013) and specific leaf area (SLA) lower (Madsen 1996).

While the effects of CO_2 and DOC on macrophytes have been studied before, in experiments usually only two different concentrations of CO_2 (Cao and Ruan 2015, Eller et al. 2015) or DOC (Pflugmacher et al. 2006, Pörs and Steinberg 2012) are used, and especially in studies investigating effects of CO_2, those concentrations are lower than current and predicted future concentrations in most natural waters. Therefore, this study aims to fill this knowledge gap with an experiment where macrophytes were exposed to multiple concentrations of CO_2 and DOC in a wide range that more resembles the expected future situation. A trait-based approach was used, with analysis of growth rate, morphology, biomass allocation, chlorophyll production and C, N and P content of the plants. Most studies focus on biomass and plant nutrient content, but as morphological changes can be essential in macrophyte functioning (Levi et al. 2015), this was measured as well. For the CO_2 treatment, two different plant species were used, as not all macrophytes use CO_2 as their main inorganic carbon source (Allen and Spence 1981). Therefore, an obligate CO_2 user and a species that can both use CO_2 and bicarbonate (HCO_3^-) were chosen. We hypothesised that CO_2 would increase growth rate, especially in the obligate CO_2 user and that DOC would decrease growth rate due to shading. We also hypothesised changes in morphology: an increase in root:shoot ratio in the CO_2 treatment and longer stems in the DOC treatment, to compensate for shading effects. We expected that effects would be strongest in the highest CO_2 and DOC treatments, or that there would be threshold levels of CO_2 or DOC above which the value of a plant trait stops increasing or decreasing.

Materials and methods

Plant material

The experiment was carried out with macrophyte species *Myriophyllum spicatum* L., and *Berula erecta* (Hudson) Coville. *M. spicatum* is a submerged rooted macrophytes with feather-like leaves that is native to Eurasia (Aiken et al. 1979). This species can take up CO_2 and HCO_3^- as inorganic carbon source (Maberly and Madsen 1998). *B. erecta* is a homophyllous amphibious species that can only take up CO_2 as inorganic carbon source (Sand-Jensen et al. 1992). Plants were bought from a plant nursery (*M. spicatum*) or collected in the Fischa river in Austria (*B. erecta*), near the village of Pottendorf (47.91° N, 16.39° E). Shoots of equal length (approximately 10 cm) were placed in 9×9×10 cm square pots filled with 0-2 mm grainsize cleaned river sand (commercially bought: Cobo gardens, Niel, Belgium). The initial fresh weight of the plants was 0.53 ±

0.23 g (*M. spicatum*) and 0.85 ± 0.42 g (*B. erecta*) and initial stem diameter was 1.59 ± 0.40 mm (*M. spicatum*) and 1.19 ± 0.25 mm (*B. erecta*). In each treatment 20 (pseudo) replicates were used.

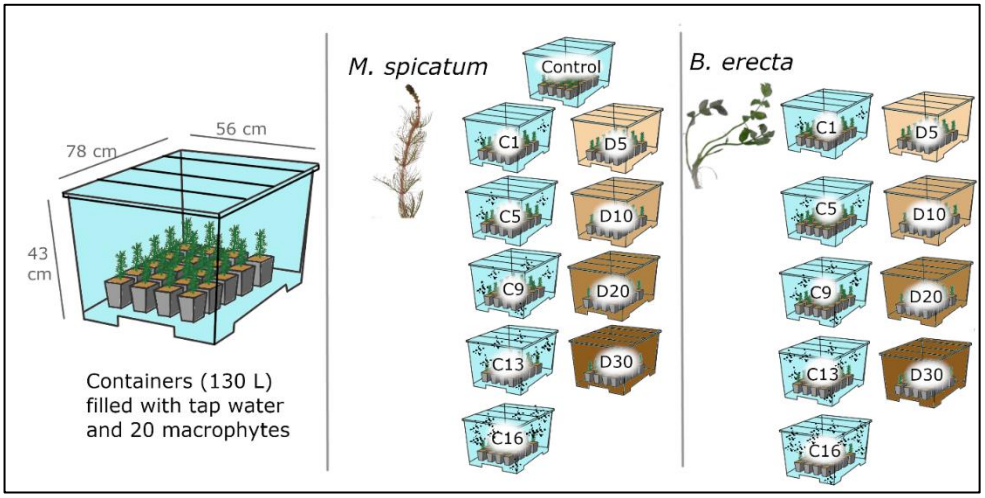


Figure 4.1 Experimental setup. Plants were kept in 130L containers filled with tap water and 20 macrophytes. Species *M. spicatum* and *B. erecta* were exposed to a control treatment (only *M. spicatum*), five CO_2 treatments (C1 – 1000 ppm, C5 – 5000 ppm, C9 – 9000 ppm, C13 – 13000 ppm and C16 – 16000 ppm) and 4 DOC treatments (D5 – 5 mg C L^{-1}, D10 – 10 mg C L^{-1}, D20 – 20 mg C L^{-1}, D30 – 30 mg C L^{-1}. The experiment with *B. erecta* was carried out between 10 May and 8 July 2019 and the experiment with *M. spicatum* was done between 11 July and 12 August 2019.

Experimental design

The experiment was carried out in a greenhouse at the University of Antwerp (Belgium), where the plans were exposed to the natural day/night cycle. Plants were divided over transparent plastic containers (78×56×43 cm; 130 L) that were filled with tap water (initial nutrient concentrations: 0.002 mg L^{-1} PO_4-P, 0.03 mg L^{-1} NH_4-N, 0.002 mg L^{-1} NO_2-N and 2.308 mg L^{-1} NO_3-N). The containers were covered with a plastic lid and they were placed in larger containers with cooled water in order to avoid large temperature fluctuations. Temperature in the containers was logged using DS1921GF5 Thermochron iButtons (figure S4.1). First, the experiment was carried out with *B. erecta*, from 10 May until 8 July 2019, a total of 60 days including an acclimatisation period of 20 days. After, the experiment was carried out with *M. spicatum*, from 11 July until 12 August 2019, a total of 33 days including an acclimatisation period of 4 days. It

was decided to harvest *M. spicatum* earlier than *B. erecta* as the plants grew faster. At the end of the experiment, plants reached the top of the container and this could reduce shading effects of DOC.

The treatments consisted of five different concentrations of CO_2 (1000, 5000, 9000, 13000 and 16000 ppm, hereafter called C1, C5, C9, C13 and C16), four different concentrations of DOC (5, 10, 20 and 30 mg C L^{-1}, hereafter called D5, D10, D20 and D30) and a control treatment without added CO_2 or DOC. For a complete overview of the treatments, see figure 4.1. In the CO_2 treatment, CO_2 was added from a commercial gas bottle and it was kept constant by using pH controllers (Milwaukee SMS122) and magnet valves (Velda) and the CO_2 was dispersed in the water by using small aquarium pumps (Prodac magic pump 350 L h^{-1}). The following pH values were used: 7.4 (C1), 7.1 (C5), 6.8 (C9), 6.5 (C13), 6.2 (C16), calculated based on the alkalinity of tap water using R package *AquaEnv* (Hofmann et al. 2010).

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 a container of approximately 500 litres of water, to which a 100 L bag of leaf litter (a mix of *Fagus sylvatica* and *Quercus robur*) and 7.5 L of peat (commercially bought: Aveve) was added. The container was covered with cloth to prevent photodegradation of the DOC. After measuring the C concentration in the stock, DOC was added to the containers with *M. spicatum* on day 5 and day 20 to establish the aimed concentrations. For *B. erecta* this occurred on day 21, 40 and 50. On those days half of the total amount of water in the containers was removed and replaced with new water and DOC. In the CO_2 treatments, half of the water was removed and replenished with tap water.

Water quality measurements

Water quality was measured regularly during the experiment: pH, conductivity and dissolved oxygen were measured on day 14, 20, 23 and 30 of the *M. spicatum* experiment on approximately the same time of the day in the morning (multiline F/set-3 multimeter). On day 27 and 40 (*B. erecta*) and day 5, 20 and 33 (*M. spicatum*) water samples were taken to analyse alkalinity, nutrients (PO_4-P, NH_4-N, NO_2-N and NO_3-N) and DOC (SAN++, Skalar, Breda, The Netherlands). For nutrients and DOC measurements water was filtered with a

0.45 µm filter. 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. The DOC in the sample 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. A DOC sample was also measured on a spectrophotometer in a wavelength range from 200-700 nm (figure S4.2). In the stock solution of DOC, the amount of nutrients was relatively high, especially phosphate and ammonium. In a DOC solution of 5 mg C L^{-1} there was 0.29 mg L^{-1} phosphate (PO_4-P) and 0.87 mg L^{-1} ammonium (NH_4-N).

The effect of DOC on photosynthetically active radiation (PAR) availability was measured as well. A light sensor (MQ-210 Apogee underwater quantum PAR meter) was mounted to a frame and a light profile was made in the middle of each container by measuring PAR at every 5 cm, starting at the bottom. This was done on day 9, 23 and 30 of the *M. spicatum* experiment. Light availability decreased in all containers with depth, in the control treatment the decrease was the smallest (at the bottom PAR was 71% of the value measured at the surface) and in the highest DOC treatment the largest (8%) (figure S4.3). Lastly, in order to investigate respiration in the DOC treatments, at the end of the experiment jars were filled with either DOC from the stock or tap water (n=3) and after a couple of days, water quality was tested (table S4.1); the low oxygen values in the jars with DOC compared to the jars with tap water suggest that respiration by microorganisms is high in the jars with DOC.

Plant growth and morphology measurements

After harvesting the plants, a subset of five *M. spicatum* plants per treatment was selected, and approximately 150 mg of fresh leaf material was placed in a fridge for chlorophyll analysis (for *B. erecta* there was not enough biomass). For all plants, the length and diameter of the main stem was measured, and the number of stems (*B. erecta*) or branches and their location on the main stem: for every branch it was measured at what height on the main stem it started; the average for all branches on one plant was divided by the length of the main stem to correct for this (*M. spicatum*). From each plant all leaves (*B. erecta*) or three representative leaves (*M. spicatum*) were selected and photographed on a white background, after which the surface area of the leaves was calculated using the image processing programme ImageJ. From each plant the stems,

leaves and roots were separated and weighed fresh, and after drying the plant material for 48 hours at 70 °C the dry mass was determined.

Chlorophyll analysis
Fresh leaf material of *M. spicatum* (150 mg per plant) 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):

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

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

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

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

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 of both plant species (leaves and stems separately) from each container was combined into five samples (2-3 plants per sample), in order to have enough material for the analyses. Those combined samples were ground with a MM 200 ball mill (Retsch, Germany). The total carbon and nitrogen concentrations were determined by dry combustion, based on the Dumas method using an elemental analyser (Model FLASH 2000, Thermo Fisher Scientific, Waltham, MA, USA). P content was determined by acid digestion and subsequently measured on ICP-OES (iCAP 6300 Duo view, Thermo Fisher, Waltham, Massachusetts, USA).

Statistical analyses
All statistical analyses were carried out in R version 3.4.3. The dataset was split, in order to test the effects of CO_2 and DOC separately. The effects of CO_2 and DOC gradients on growth and morphology plant traits, chlorophyll, C, N and P

content were tested with a one-way ANOVA test. 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. If significant, a Tukey HSD post hoc test was performed. If the normal distribution assumption was not met after transformation, a Kruskal-Wallis test was performed with a nemenyi post hoc test, using R package 'PMCMR' (Pohlert 2014). If the homogeneity of variance assumption was not met after transformation, a Welch test was performed with a games-howell post hoc test, using R package 'userfriendlyscience' (Peters 2018).

Results

Water quality

In the CO_2 treatment, the measured pH values were close to the aimed values, mentioned in the experimental design. In the control treatment, without added CO_2, the pH was higher than in the treatments (table 4.1, figure S4.4a). Alkalinity and conductivity increased with decreasing pH. In the CO_2 treatment with *B. erecta* the measured alkalinity was lower than in with *M. spicatum*. This can probably be explained by the fact that in the *B. erecta* treatment part of the water had just been replaced with new tap water (see materials and methods). Towards the end of the experiment alkalinity was probably higher in the *B. erecta experiment.* Dissolved oxygen was high (usually above saturation) (figure S4.4b-d and figure S4.5a) and nutrient values were low in all treatments (figure S4.4e-h and S4.5b-e). In the DOC treatment, all DOC values were close to the aimed values mentioned in the experimental design, but concentrations increased during the experiment (figure S4.6a). pH and conductivity slightly decreased with increasing DOC concentration, dissolved oxygen was mostly high (above saturation) (figure S4.6b-d) and PO_4-P and NH_4-N increased with DOC concentration (table 4.2, figure S4.6e-h).

Effects of CO_2 treatments

CO_2 had a substantial effect on growth and biomass production in *B. erecta*, in 24 out of 31 plant traits a significant effect was found (table 4.3). Although RGR was negative in all treatments, RGR was significantly higher in the higher CO_2 treatments (C9, C13, C16) than in the lower CO_2 treatments (C1, C5), see figure 4.2a. Consequently, plants exposed to the high CO_2 treatment had higher dry biomass (leaves, stems, roots and total) (figure 4.2b), thicker stems (figure 4.2c) and more and larger leaves (figure 4.2d-e) than in the low CO_2 treatment. DMCS

was also higher in the high CO_2 treatment than in the low CO_2 treatment, whereas SLA was lower in the high CO_2 treatment than in the low CO_2 treatment (figure 4.2f). There were no significant differences in leaf:stem or root:shoot ratio. There was a clear trend in nutrient content; C and N content was lower in the high CO_2 treatment than in the low CO_2 treatment (figure 4.2g) and C:N was higher in the high CO_2 treatments (figure 4.2h). N content was higher and C:N content lower in leaves than stems. In general, differences in traits were not significantly different between all treatments, but in most cases between one of the lower values (C1 or C5) and one of the higher values (C13 or C16). P content was not affected by CO_2 in *B. erecta.*

In contrast to what was observed for *B. erecta*, the CO_2 treatment relatively affected fewer traits in *M. spicatum* (27 out of 41 traits), and those traits were mainly related to plant morphology: there were no significant differences in relative growth rate (table 4.4). With higher CO_2 concentrations plants had more leaves (figure 4.3a), but also smaller leaves (figure 4.3b), stems were longer (figure 4.3c) and had a higher dry weight (figure 4.3d). Leaf:stem ratio (figure 4.3e) and root:shoot ratio were smaller under high CO_2 concentrations and stem, root and total dry matter content was higher. There were differences in the chlorophyll content, but there was no clear trend in the CO_2 gradient. In treatment C5, the chlorophyll a, b, a+b and carotenoids was significantly higher than in most of the other treatments (figure 4.3f). Both C and N tended to be higher in the high CO_2 treatments than in the low CO_2 treatments, but this was not always consistent. The C:N ratio was higher in C9 than in the control, but in treatment C16 it was lower than in C9 (figure 4.3g). Like in *B. erecta*, N content was higher and C:N content lower in leaves than stems. Similar to *B. erecta*, the differences were usually not significantly different between all treatments, but in most cases between one of the lower values (control, C1 or C5) and one of the higher values (C13 or C16). P content in the stems was significantly higher in the control treatment than in all CO_2 treatments, and as a consequence, C:P and N:P ratios were higher in nearly all CO_2 treatments, compared to the control treatment (figure 4.3h).

Table 4.1 Water quality in the CO_2 treatment (averages from three (*M. spicatum*) or two (*B. erecta*) measurements with standard deviations). High standard deviations can be explained by the large variation between the measurements, as some measurements have been taken after some of the water was replaced with new water or DOC. See figure S4.4 and S4.5 for graphs.

	Control	**C1**	**C5**	**C9**	**C13**	**C16**
M. spicatum						
Alkalinity (meq L^{-1})	1.634 ± 0.417	2.232 ± 0.118	2.521 ± 0.319	3.259 ± 0.706	4.563 ± 2.058	5.575 ± 2.843
pH	9.144 ± 0.112	7.591 ± 0.172	7.405 ± 0.158	7.015 ± 0.099	6.644 ± 0.132	6.379 ± 0.108
O_2 (mg L^{-1})	10.93 ± 0.71	9.40 ± 0.71	9.71 ± 0.85	9.61 ± 1.51	10.26 ± 1.51	9.38 ± 1.94
Conductivity (µS cm^{-1})	401 ± 16	476 ± 4	488 ± 14	577 ± 23	736 ± 48	855 ± 50
Temp (°C)	23.4 ± 2.9	24.9 ± 3.2	24.5 ± 2.8	24.6 ± 3.1	24.4 ± 2.7	25.0 ± 3.0
PO_4-P (mg L^{-1})	0.001 ± 0.001	0.000 ± 0.000	0.000 ± 0.000	0.002 ± 0.002	0.001 ± 0.001	0.001 ± 0.001
NH_4-N (mg L^{-1})	0.023 ± 0.020	0.021 ± 0.019	0.025 ± 0.022	0.022 ± 0.019	0.021 ± 0.019	0.020 ± 0.017
NO_2-N (mg L^{-1})	0.002 ± 0.002	0.001 ± 0.001	0.001 ± 0.001	0.002 ± 0.001	0.001 ± 0.001	0.000 ± 0.001
NO_3-N (mg L^{-1})	0.294 ± 0.467	0.225 ± 0.345	0.262 ± 0.419	0.243 ± 0.382	0.309 ± 0.486	0.258 ± 0.409
B. erecta						
Alkalinity (meq L^{-1})		1.556 ± 0.653	1.605 ± 0.584	1.765 ± 1.212	1.914 ± 1.352	2.579 ± 2.126
PO_4-P (mg L^{-1})		0.016 ± 0.006	0.014 ± 0.003	0.015 ± 0.007	0.013 ± 0.007	0.017 ± 0.004
NH_4-N (mg L^{-1})		0.047 ± 0.021	0.043 ± 0.025	0.032 ± 0.007	0.032 ± 0.008	0.042 ± 0.013
NO_2-N (µg L^{-1})		0.7 ± 0.2	0.2 ± 0.2	0.6 ± 0.7	0 ± 0	0.6 ± 0.8
NO_3-N (mg L^{-1})		0.309 ± 0.411	0.246 ±0.328	0.306 ± 0.411	0.138 ± 0.176	0.276 ± 0.369

Table 4.2 Water quality in the DOC treatment (averages from three measurements with standard deviations). High standard deviations can be explained by the large variation between the measurements, as some measurements have been taken after some of the water was replaced with new water or DOC. See figure S4.6 for graphs.

	Control	**D5**	**D10**	**D20**	**D30**
DOC (mg C L^{-1})	8.420 ± 3.154	14.280 ± 3.319	19.658 ± 5.326	22.452 ± 6.521	31.210 ± 5.972
pH	9.144 ± 0.112	9.139 ± 0.144	8.944 ± 0.165	8.977 ± 0.163	8.890 ± 0.152
O_2 (mg L^{-1})	10.93 ± 0.71	11.42 ± 1.00	9.82 ± 2.23	9.67 ± 2.56	8.97 ± 1.26
Conductivity (µS cm^{-1})	401 ± 16	455 ± 10	470 ± 15	490 ± 18	456 ± 14
Temp (°C)	23.4 ± 2.9	23.9 ± 3.3	24.0 ± 3.0	24.1 ± 3.3	23.7 ± 3.0
PO_4-P (mg L^{-1})	0.001 ± 0.001	0.051 ± 0.086	0.090 ± 0.154	0.121 ± 0.209	0.307 ± 0.529
NH_4-N (mg L^{-1})	0.023 ± 0.020	0.154 ± 0.236	0.341 ± 0.538	0.400 ± 0.655	0.756 ± 1.268
NO_2-N (mg L^{-1})	0.002 ± 0.002	0.001 ± 0.001	0.001 ± 0.001	0.001 ± 0.000	0.002 ± 0.001
NO_3-N (mg L^{-1})	0.294 ± 0.467	0.281 ± 0.440	0.254 ± 0.370	0.234 ± 0.361	0.209 ± 0.340

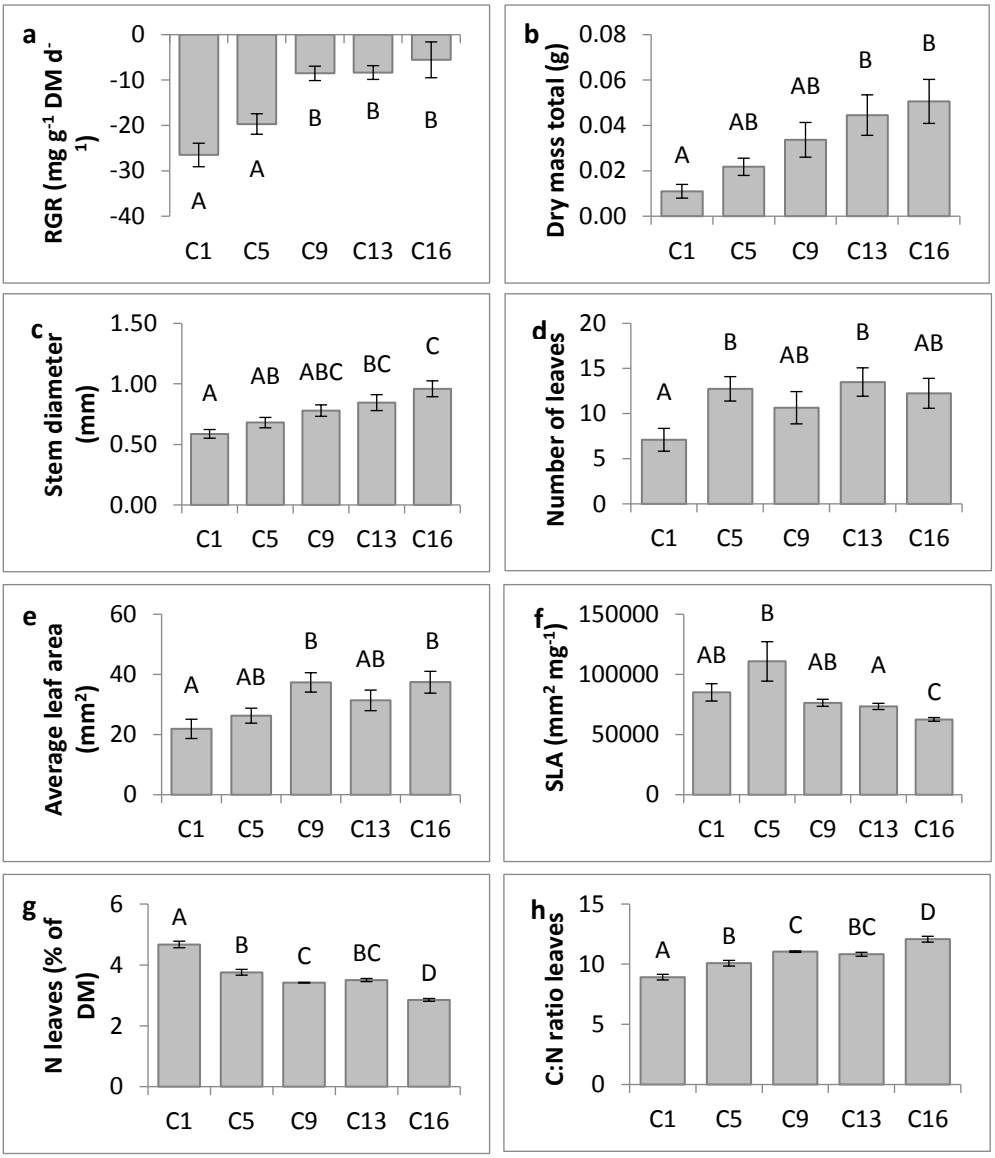


Figure 4.2 Results of the CO_2 treatments in plant species *B. erecta*. CO_2 Treatments: C1 – 1000 ppm, C5 – 5000 ppm, C9 – 9000 ppm, C13 – 13000 ppm, C16 – 16000 ppm. Results are shown for relative growth rate (RGR) (a), total dry mass (b), stem diameter (c), number of leaves (d), average leaf area (e), SLA (specific leaf area) (f), N content of leaves (g) and C:N ratio of leaves (h). Letters signify significant differences tested with one-way ANOVA tests, error bars show standard errors, N=20 for RGR, dry mass total, stem diameter, number of leaves, average leaf area and SLA, N=5 for N leaves and C:N ratio of leaves.

Table 4.3 F-values from ANOVA tests on plant traits affected by CO_2 treatments in *B. erecta*. Significance: *** p<0.001, ** p<0.01, * P<0.05. [1]measured with Welch test [2]measured with Kruskal-Wallis test. For C, N and C:N total the F-value signifies whether there is a difference between the stems and the leaves. Data transformations: x^(1/2) – fresh mass leaves, roots, total, dry mass leaves, stems, roots total, total leaf area, x^10 – C leaves.

	CO_2		**CO_2**
Number of stems	NS[2]	Total leaf area	4.57**
Number of leaves	3.23*	Specific leaf area	30.79***[2]
Length longest stem	NS	N total	443.47***
Stem diameter	6.82***	N leaves	86.78***
Fresh mass leaves	4.61**	N stems	37.82***
Fresh mass stems	NS	C total	91.28***
Fresh mass roots	6.1***	C leaves	85.25***
Fresh mass total	4.41**	C stems	5.07**
Dry mass leaves	6.28***	P total	12.99**
Dry mass stems	4.41**	P leaves	NS
Dry mass roots	6.19***	P stems	NS
Dry mass total	5.34***	C:N total	261.54***
Leaf:stem ratio	NS	C:N leaves	34.48***
Root:shoot ratio	NS	C:N stems	34.89***
Dry matter content leaves	14.00**[2]	C:P total	4.56*
Dry matter content stems	18.75***[2]	C:P leaves	NS
Dry matter content roots	NS	C:P stems	NS
Dry matter content total	NS	N:P total	NS
Relative growth rate	39.24***[2]	N:P stems	NS
Average leaf area	4.18**	N:P leaves	NS

Effects of DOC treatment

DOC also mainly affected plant morphology in *M. spicatum*: this treatment also did not affect relative growth rate (table 4.4). In total DOC affected 20 out of 35 traits. Similar to the CO_2 treatment, the number of leaves was higher in the high DOC treatment (figure 4.4a). Moreover, there was a strong positive effect of DOC on stem length (figure 4.4b). Stem diameter was higher in the high DOC treatment, but this was only significant between the D5 and D10 treatments. In the high DOC treatments the location of the side branches on the main stem, relative to the length of the main stem was higher than in the low DOC

treatments (figure 4.4c). Moreover, the dry matter content of the stems was lower in the high DOC treatments than in the low DOC treatments. Chlorophyll had a clear general trend, with higher chlorophyll a, b, a+b and carotenoids in the high DOC treatments than in the low DOC treatments (figure 4.4d). In the DOC treatment there were large differences in C content: in leaves this was higher in the high DOC treatments than the low DOC treatments (figure 4.4e), whereas in the stems it was the other way around (figure 4.4f). Like in the CO_2 treatment, N content was higher and C:N content lower in leaves than stems.

Again, differences were often not significant between all DOC treatments, but only between one of the lowest and one of the highest treatments. P content was both in leaves and stems significantly smaller in the D5 treatment than in the higher DOC treatments (figure 4.4g), and C:P and N:P ratios were higher in the D5 than in the higher treatments (figure 4.4h).

DOC had a strongly negative effect on *B. erecta* survival. Biomass was very low and did not differ significantly between the treatments (figure 4.5a). Many plants died (figure 4.5b), in the D5 treatment 20% of the plants survived and in the D20 treatment no plants survived. Survival in the D30 treatment was surprisingly high, although the water was very dark. No further analyses have been done on the effects of DOC on *B. erecta.*

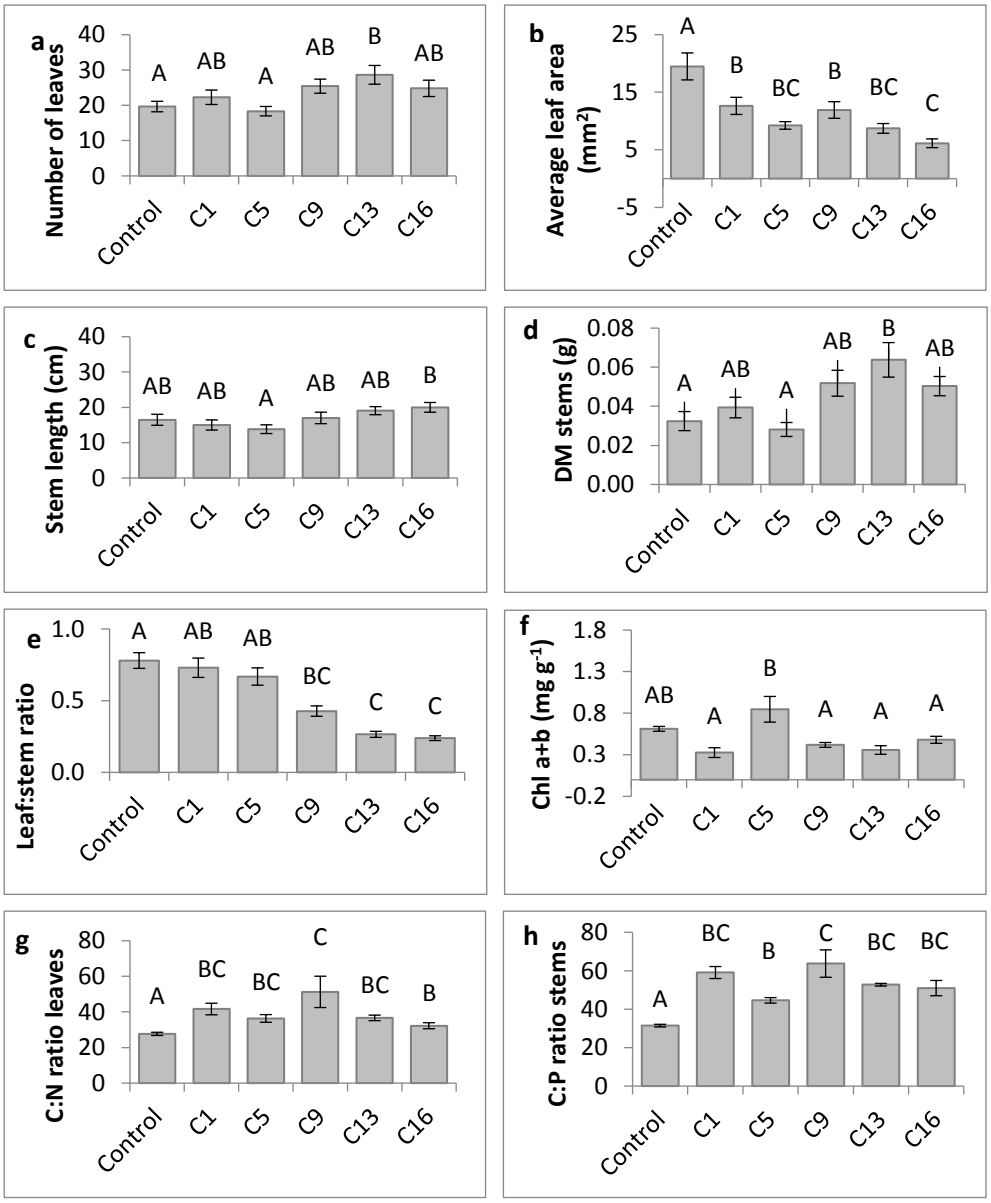


Figure 4.3 Results of the CO_2 treatments in plant species *M. spicatum*. CO_2 Treatments: Control – no CO_2 added, C1 – 1000 ppm, C5 – 5000 ppm, C9 – 9000 ppm, C13 – 13000 ppm, C16 – 16000 ppm. Results are shown for number of leaves (a), average leaf area (b), stem length (c), dry mass of stems (d), leaf:stem ratio (e), chlorophyll a+b (f), C:N ratio of leaves (g) and C:P ratio of stems (h). Letters signify significant differences tested with one-way ANOVA tests, error bars show standard errors, N=20 for number of leaves, average leaf area, stem length, dry mass of stems and leaf:stem ratio, N=5 for chlorophyll a+b, C:N ratio of leaves and C:P ratio of stems.

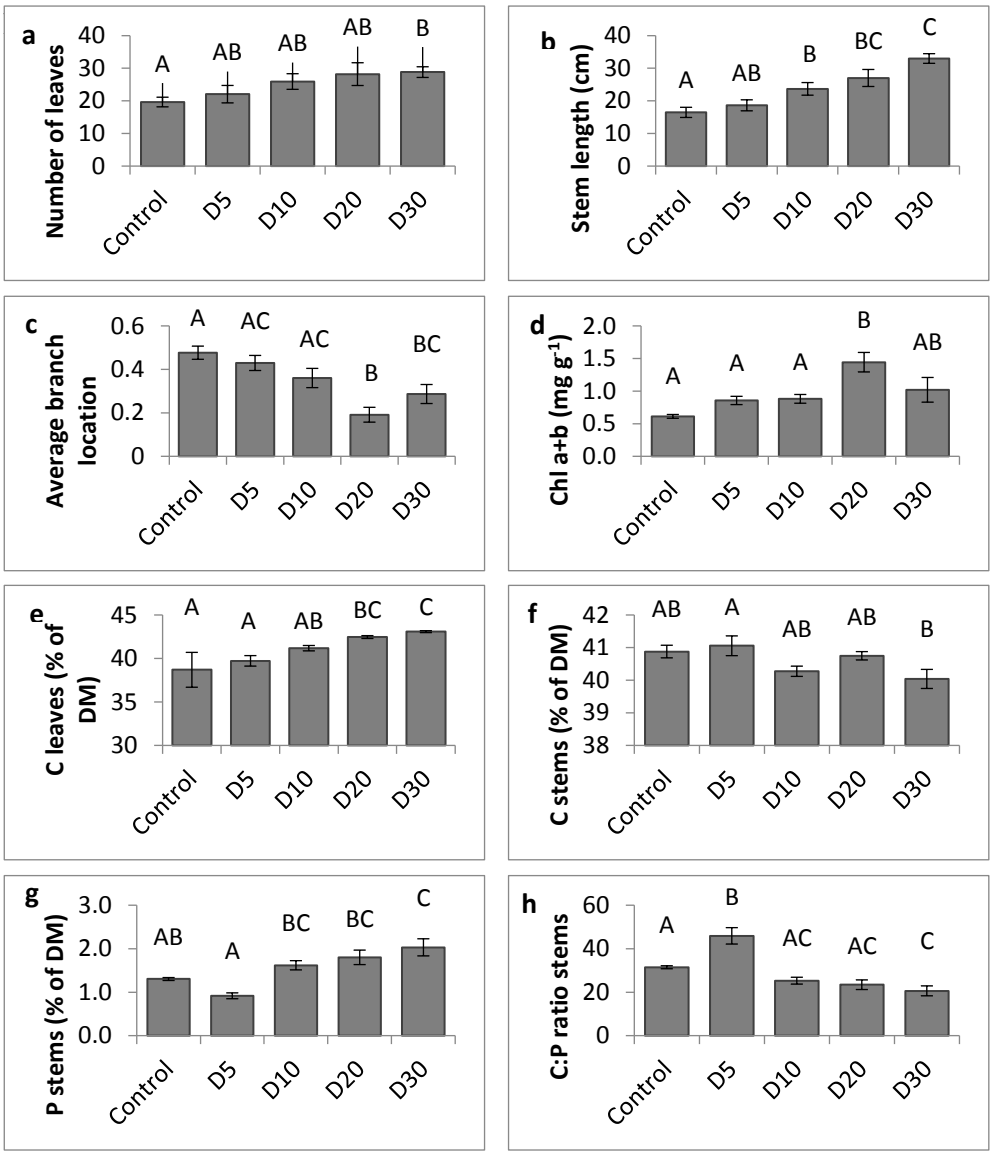


Figure 4.4 Results of the DOC treatments on plant species *M. spicatum*. DOC Treatments: Control – no DOC added, D5 – 5 mg C L^{-1}, D10 – 10 mg C L^{-1}, D20 – 20 mg C L^{-1}, D30 – 30 mg C L^{-1}. Results are shown for number of leaves (a), stem length (b), average location of branches relative to length of main stem (value between 0 and 1) (c), chlorophyll a+b (d), C content of leaves (e), C content of stems (f), P content of stems (g) and C:P ratio of stems (h). Letters signify significant differences tested with one-way ANOVA tests, error bars show standard errors, N=20 for number of leaves, stem length and average branch location, N=5 for chlorophyll a+b, C content of leaves and stems, P content of stems and C:P ratio of stems.

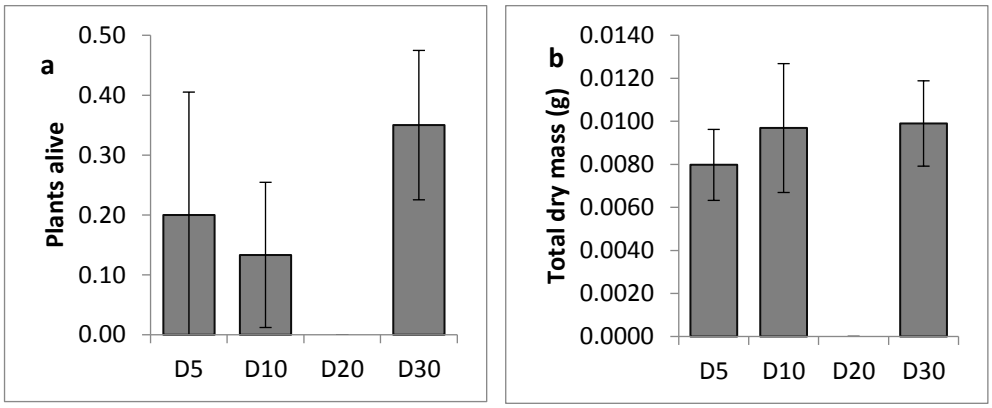


Figure 4.5 Results of the DOC treatments on plant species *B. erecta.* DOC treatments: D5 – 5 mg C L^{-1}, D10 – 10 mg C L^{-1}, D20 – 20 mg C L^{-1}, D30 – 30 mg C L^{-1}. Results are shown for total dry mass (a) and part of the plants that were alive at the end of the experiment, 1 signifies that 100% was alive (b). Error bars show standard errors, N=20.

Table 4.4 F-values from ANOVA tests on plant traits affected by CO_2 and DOC treatments in *M. spicatum*. Significance: *** p<0.001, ** p<0.01, * P<0.05. [1]measured with Welch test [2]measured with Kruskal-Wallis test. For C, N and C:N total the F-value signifies whether there is a difference between the stems and the leaves. Data transformations CO_2 treatment: x^(1/2) – Dry mass stems, leaf:stem ratio, dry matter content leaves, average and total leaf area, P stems, x^(1/4) – Stem length, x^(1/1.2) – root:shoot ratio, x^(1/0.6) – dry matter content roots, x^(1/1.1) – dry matter content total, x^20 – N stems, x^12 – C leaves, 1/x^1.5 – C:N total, 1/x – C:N leaves, log(x) – P total, C:P total, C:P stems, N:P total, N:P stems. Data transformations DOC treatment: x^(1/2) – stem length, average branch location, root:shoot ratio, dry matter content stems, roots, total, N total, x^(1/3) – number of leaves, stem diameter, x^2 – dry matter content leaves, relative growth rate, x^14 – C total, x^10 – C leaves. loc. = location, Chl = chlorophyll.

	CO_2	**DOC**		**CO_2**	**DOC**
Number of leaves	3.57**	3.29*	SLA	5.26***	14.08***
Stem length	2.75*	17.05***[1]	N total	211.95***	828.68***
Average stem diameter	NS	4.54**[1]	N leaves	7.19***	3.41*
Number of branches	NS	NS	N stems	12.59*	NS
Average branch length	NS	NS	C total	8.05**	26.00***
Total branch length	NS	NS	C leaves	7.54***	12.43***
Average branch location	NS	3.27*	C stems	5.82**	3.55*
Branch loc. - branch length	2.52*	9.92***	P total	119.63***	14.84***
Fresh mass leaves	NS	NS	P leaves	5.80**	6.53**
Fresh mass stems	NS	NS	P stems	14.18**	11.37***
Fresh mass roots	NS	NS	C:N total	229.53***	759.82***
Fresh mass total	NS	NS	C:N leaves	9.60***	3.44*
Dry mass leaves	NS	NS	C:N stems	5.82**	3.24*
Dry mass stems	4.7***	NS	C:P total	19.63***	22.33***
Dry mass roots	NS	NS	C:P leaves	5.80**	9.39***
Dry mass total	NS	NS	C:P stems	12.24***	18.73***
Leaf:stem ratio	33.62***[2]	NS	N:P total	NS	101.53***
Root:shoot ratio	7.25***[2]	2.84*[2]	N:P leaves	NS	6.90**
Dry matter content leaves	8.57***[2]	13.81***	N:P stems	4.51**	16.05***
Dry matter content stems	6.32***	10.8***	Chl a	7.67***	5.66**
Dry matter content roots	10.53***[2]	3.31*	Chl b	5.81**	5.96**
Dry matter content total	5.18**[2]	8.63***	Chl a+b	7.04***	6.16**
Relative growth rate	NS	2.96*	Carotenoids	5.65**	4.71**
Average leaf area	11.35***	NS	Chl a/b	4.94**	NS
Total leaf area	3.27*	NS	Total plant chl	NS	NS

Discussion

As hypothesised, CO_2 and DOC had strong effects on both macrophyte species, with the strongest effects in the plants exposed to the higher CO_2 or DOC doses: there were no clear threshold levels. In the CO_2 treatment there were also notable differences between the two macrophyte species. *B. erecta* mainly showed responses in growth and biomass production, whereas *M. spicatum* showed changes in plant morphology. Overall, in *B. erecta*, more traits were affected by the CO_2 treatment than in *M. spicatum*.

Effects of CO_2 on macrophytes

The RGR of *M. spicatum* was not affected by the CO_2 treatment, whereas the RGR of *B. erecta* was higher when exposed to high CO_2 concentrations. This can be explained by the difference in organic carbon uptake by the two species. *M. spicatum* has a relatively efficient carbon uptake, it can take up HCO_3^- and is able to take up a large percentage of available inorganic carbon (Maberly and Spence 1983), *B. erecta*, however, can only use CO_2 as inorganic carbon source, so it needs waters with high, or even oversaturated CO_2 levels to maintain a sufficient rate of photosynthesis (Sand-Jensen et al. 1992). Although it was expected that *B. erecta* would respond more strongly to increases in CO_2, it was not expected that there would be no response in *M. spicatum* growth rate. In most other studies where HCO_3^- users were exposed to elevated CO_2 levels, biomass production significantly increased (Eusebio Malheiro et al. 2013, Cao and Ruan 2015, Dülger and Hussner 2017). The alkalinity in the high CO_2 treatment was relatively high, which could imply that photosynthesis was saturated with regard to inorganic carbon. On the other hand, growth of *M. spicatum* strongly increased when exposed to very high CO_2 levels in other experiments (Hussner et al. 2016). It is not likely that the plants showed large uptake rates of HCO_3^-, as CO_2 uptake is energetically more efficient (Madsen and Sand-Jensen 1991). As the macrophytes grew in a greenhouse in summer, temperature or light stress may have played a role as well. Although the containers with the plants were cooled, temperatures were often above 20°C. *B. erecta* was sampled in a river shaded by trees and *M. spicatum* came from a plant nursery, so both species may not have been adapted to the high light levels in the greenhouse. High light levels can cause photoinhibition in macrophytes, reduce the chlorophyll content and photosynthesis rates (Hussner et al. 2010).

In both species there were responses of plant morphology to the CO_2 treatments, which suggest that this is an important effect of elevated CO_2 levels.

However, the morphological responses were not equal for the two species and sometimes even opposing: *B. erecta* had larger, but *M. spicatum* smaller leaves in the high CO_2 treatments. Few other studies have investigated the effect of elevated CO_2 on morphology, and results are not consistent, sometimes no effect on morphology was found (Xie et al. 2004, Eusebio Malheiro et al. 2013). A response that has been reported several times is an increase in root:shoot ratio (Yan et al. 2006, Pagano and Titus 2007, Cao and Ruan 2015) and it has been suggested that increasing root surface may be a way to improve nutrient uptake, as inorganic carbon is no longer a limiting factor in high CO_2 conditions, or the roots can serve as storage for starch (Dülger et al. 2017). Surprisingly, in *B. erecta* no effect of CO_2 on root biomass was found and in *M. spicatum* root:shoot ratio was smaller in high CO_2 concentrations. *M. spicatum* appeared to invest more in stem length and weight instead. Another common response to elevated CO_2 concentrations observed by macrophytes is an increase in C:N ratio (Titus and Pagano 2002, Cheng et al. 2010, Hussner et al. 2016), but in the current study this was only observed in *B. erecta* and (less clearly) in *M. spicatum* stems. In *M. spicatum* leaves, C:N ratio was higher in treatment C9 than the control, but in treatment C16 it was lower than in C9. It has been demonstrated that HCO_3^- users have a higher C:N ratio under elevated CO_2 levels (Hussner et al. 2019), but this has not been tested before with high CO_2 values (above 1000 ppm), so in this case there may be a threshold CO_2 level above which the C:N ratio does not further increase. In *M. spicatum* stem P content was lower when CO_2 increased, whereas in other studies the opposite has been found (Yan et al. 2006).

Effects of DOC on macrophytes

DOC did unexpectedly not significantly affect RGR of *M. spicatum*, although in other studies exposure to high DOC levels decreased plant growth (Périllon and Hilt 2015, Choudhury et al. 2019), though it should be taken into account that in the current study DOC from leaf and peat material was used. This leachate did not only contain carbon but also a substantial amount of phosphate and ammonium, which may have compensated the negative effect of shading. Most treatment effects observed were related to plant morphology. Most other studies focusing on effects of DOC on macrophytes focus on charophytes. Those studies found no effect on stem length when a low dose (2 mg DOC L^{-1}) of DOC was used (Pörs and Steinberg 2012) or an increase in stem length until a threshold level of DOC had been reached after which stems declined (Choudhury et al. 2019). In *M. spicatum* no threshold value was found, which could signify that *M. spicatum* is better able to cope with increasing levels of

DOC, but it should be taken into account that in the charophyte experiment by (Choudhury et al. 2019) mesocosms of 1 metre depth were used, whereas in the current study they were only 40 cm, and when the plants reached the water surface at the end of the experiment, the shading effect was smaller. The shading effect caused by elevated DOC levels can also affect nutrient stoichiometry: according to the light:nutrient hypothesis, in autotrophs like algae or phytoplankton the C:nutrient ratio decreases when light is limiting; light is in this case the limiting factor, whereas nutrients are available and are taken up (Sterner et al. 1997). This relationship between light and C content has also been found in macrophytes (Xing et al. 2013) and it has been hypothesised that it will also occur due to shading by DOC (Creed et al. 2018). In the current study, however, lower C content due to shading by DOC was only found in the stems of *M. spicatum*, in the leaves C content was higher in the high DOC treatment than in the low DOC treatment. Total chlorophyll concentration was higher in the high DOC treatments than the low DOC treatments, which can be explained by shading effects (Barko and Filbin 1983).

The P content of *M. spicatum* stems was higher when exposed to DOC, which can probably be explained by the fact that the DOC source had a high P concentration. The high P concentration in the DOC source should also be taken into account when interpreting the other results. Although some of the results are typical plant responses to shading (longer stems, higher plant chlorophyll content), the effects of DOC and P cannot be disentangled. In many plant traits no significant effect was found of DOC. Possibly, negative effects of DOC and positive effects of increased P cancelled out each other. Besides, humic substances in DOC can also directly affect macrophytes by damaging cells (Grigutytė et al. 2009) and interfering with photosynthesis (Pflugmacher et al. 2006). We expect this did not play a major role, as macrophyte growth was not impaired at high DOC leaves, but it cannot be excluded that those direct effect of DOC took place. Although not being able to disentangle direct and indirect effects of DOC and effects of P is a limitation of this study, in natural systems plants will probably also be exposed to those effects at once when DOC concentrations in the water rise. In order to improve understanding of the mechanisms, we suggest that doing experiments that focus on each effect separately (shading, direct effects of DOC and P loading) could be a valuable addition to this study.

The high mortality in *B. erecta* in the DOC was not expected. This may be explained by competition with periphytic algae, and by the high temperatures

in the containers. Although the containers were cooled, this was not sufficient to keep the temperatures below 20°C.

Implications for macrophytes under elevated concentrations of carbon
To summarise, in *B. erecta,* a macrophyte species that only uses CO_2 as inorganic carbon source, increased CO_2 levels stimulated growth and increased C:N ratio. In *M. spicatum*, a macrophyte species that also uses HCO_3^- as inorganic carbon source, morphology changed. This can have consequences for the plants themselves, e.g. obligate CO_2 users can gain competitive advantage over HCO_3^- users under elevated CO_2 levels (Spierenburg et al. 2009), but it can also affect other aquatic organisms. Although more biomass may stimulate species that depend on macrophyte biomass as a food source, the C:N ratio increases, which makes the biomass less nutritive which can cause problems for aquatic herbivores (Elser et al. 2000). Changes in macrophyte morphology can also affect other organisms: many functions of macrophytes depend on their morphology. When leaves become smaller due to elevated CO_2, sediment retention may decrease (Clarke 2002). High levels of DOC mainly stimulate stem length in *M. spicatum*, which, together with the increased concentration of chlorophyll, can be seen as a response to shading caused by DOC. However, the taller plants may be more vulnerable to disturbances like wind waves or increases in flow velocity (Puijalon et al. 2011). This also means that plants that cannot grow fast enough may not be able to adapt to high DOC levels. They will be confined to shallower parts of the system.

It should also be taken into account that climate change has many aspects. Although it is valuable to measure stressors in isolation, in ecosystems many stressors, like changes in temperature, nutrients, carbon and light will act simultaneously, so the conclusions drawn from this study cannot be directly applied to natural situations. From comparing the current study to other research investigating the effect of elevated carbon concentrations on macrophytes it can be concluded that results are not always consistent: they depend substantially on the plant species, plant traits and water conditions (for example the DOC source). Therefore, we suggest that more studies should test effects of elevated carbon concentrations on dominant and / or ecologically important macrophyte species, as well as combinations with other aspects of climate change.

Acknowledgements

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Supplementary tables

Table S4.1 water quality in jars with DOC (filtered from stock with tree leaves and peat) and tap water (n=3), average values with standard deviations.

	TA meq L^{-1}	**DOC (mg C L^{-1})**	**pH**	**O_2 mg L^{-1}**	**O_2 (% sat)**	**Cond. (µS cm^{-1})**	**Temp (°C)**
DOC	2.88 ± 0.06	64.61 ± 1.66	7.38 ± 0.03	1.96 ± 0.40	21.83 ± 4.51	538 ± 2.1	20.77 ± 0.06
Tap water	3.17 ± 0.15	4.52 ± 1.11	8.31 ± 0.04	8.62 ± 0.10	95.80 ± 1.31	585 ± 23.6	20.50 ± 0.1

Supplementary figures

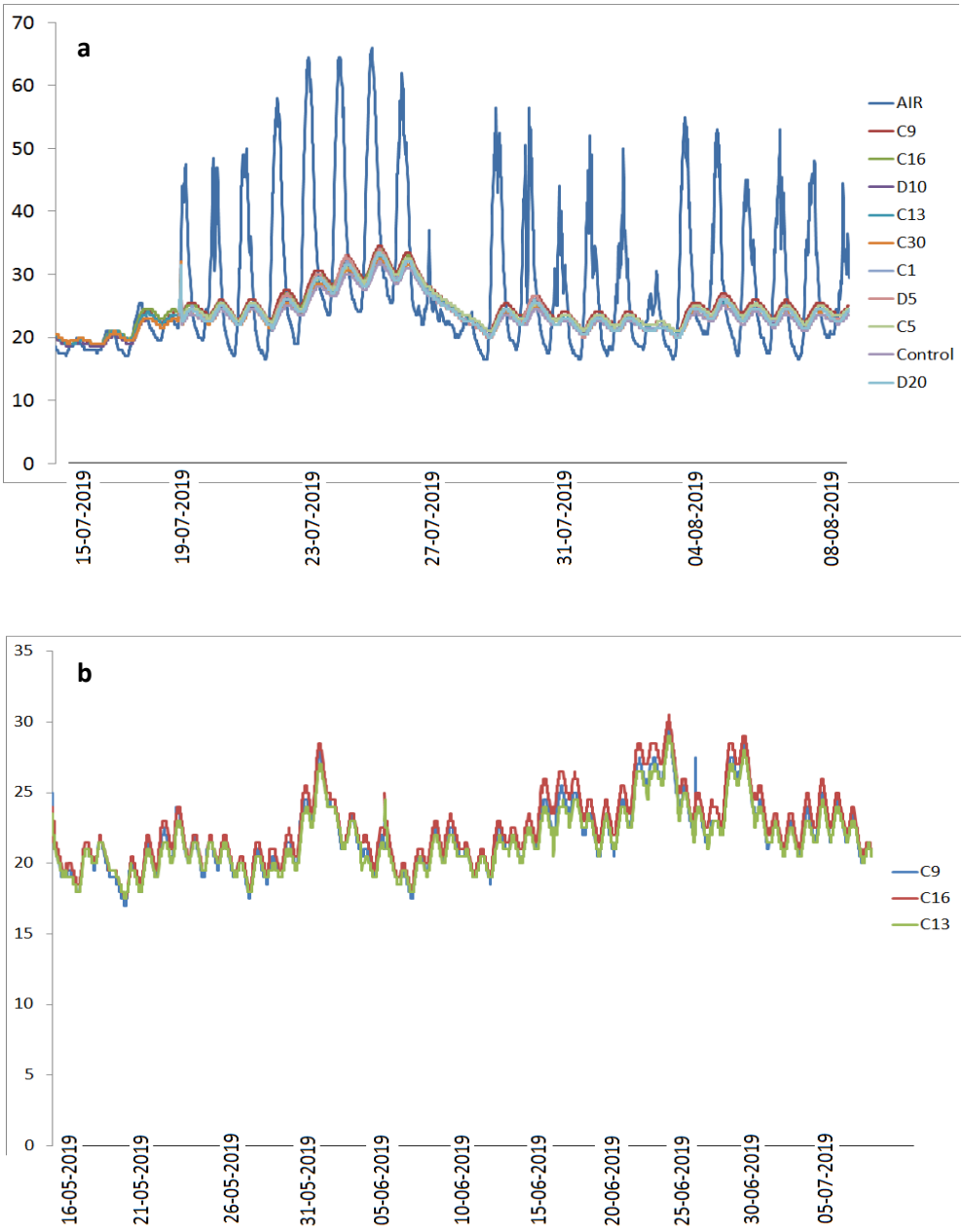

Figure S4.1 Temperature (°C) measured in the containers during the experiment with *M. spicatum* (a) and *B. erecta* (b). Air temperature in figure a was measured on top on the containers, exposed to full sunlight.

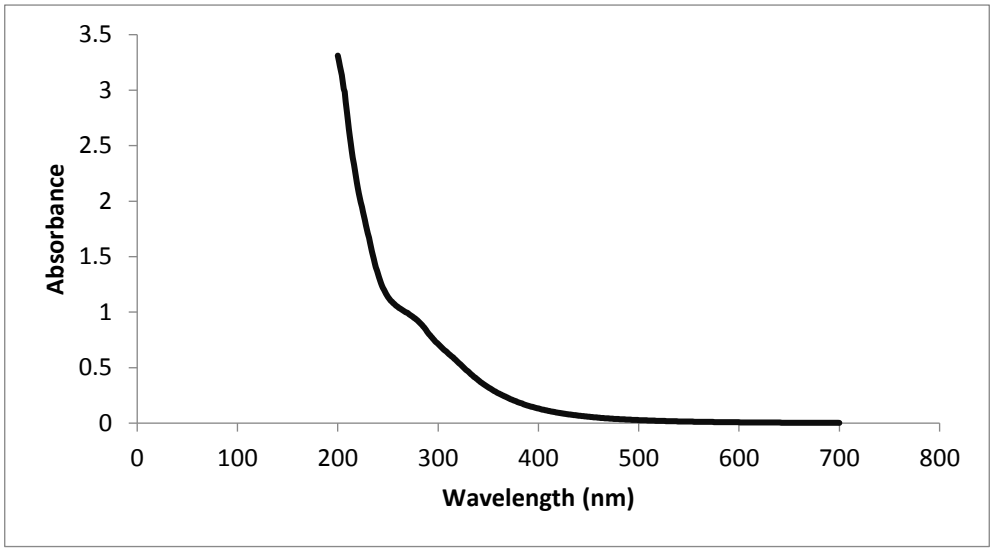

Figure S4.2 Absorbance (cm^{-1}) of DOC sample (99.37 mg C L^{-1}) for wavelengths between 200 and 700 nm.

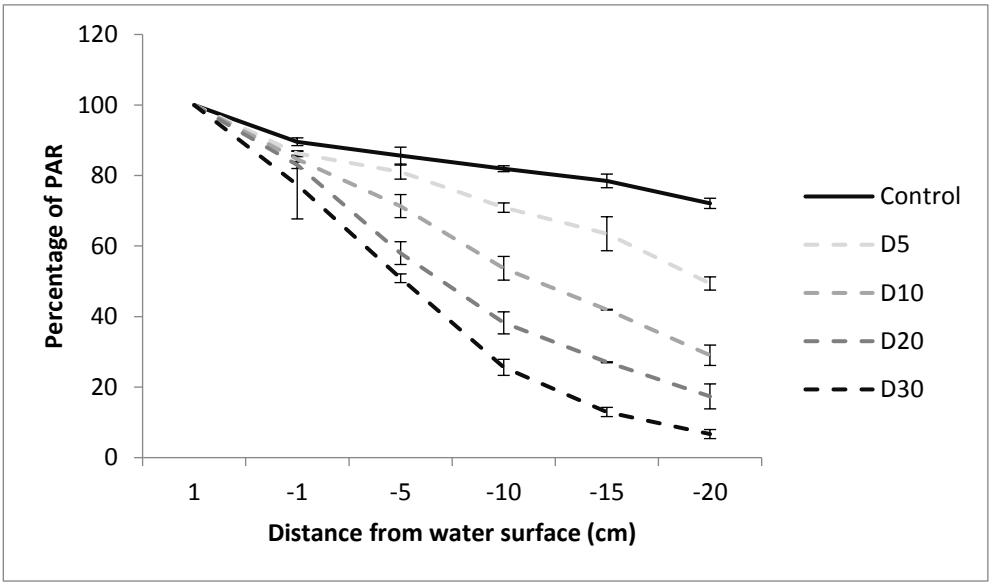

Figure S4.3 Percentage of available photosynthetically active radiation (PAR) measured at different depths, relative to availability at the water surface. Measured for different DOC Treatments: Control – no DOC added, D5 – 5 mg C L^{-1}, D10 – 10 mg C L^{-1}, D20 – 20 mg C L^{-1}, D30 – 30 mg C L^{-1}. Average values are taken from measurements at two different days (day 23 and 30), error bars signify standard deviations.

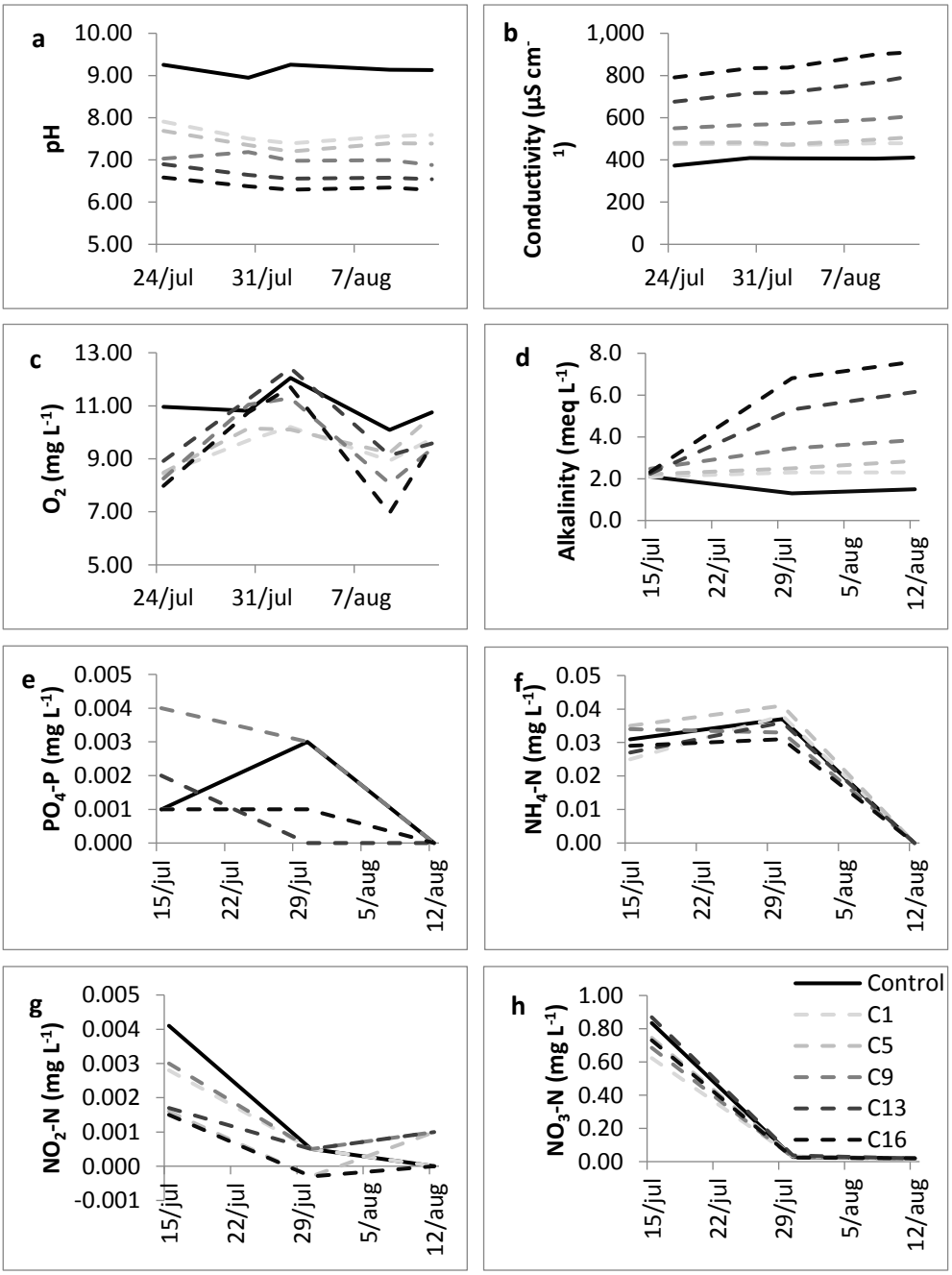

Figure S4.4 Water quality in the CO_2 treatment with plant species *M. spicatum*: pH (a), conductivity (b), dissolved oxygen (c), alkalinity (d), phosphate concentration (e), ammonium concentration (f), nitrite concentration (g) and nitrate concentration (h).

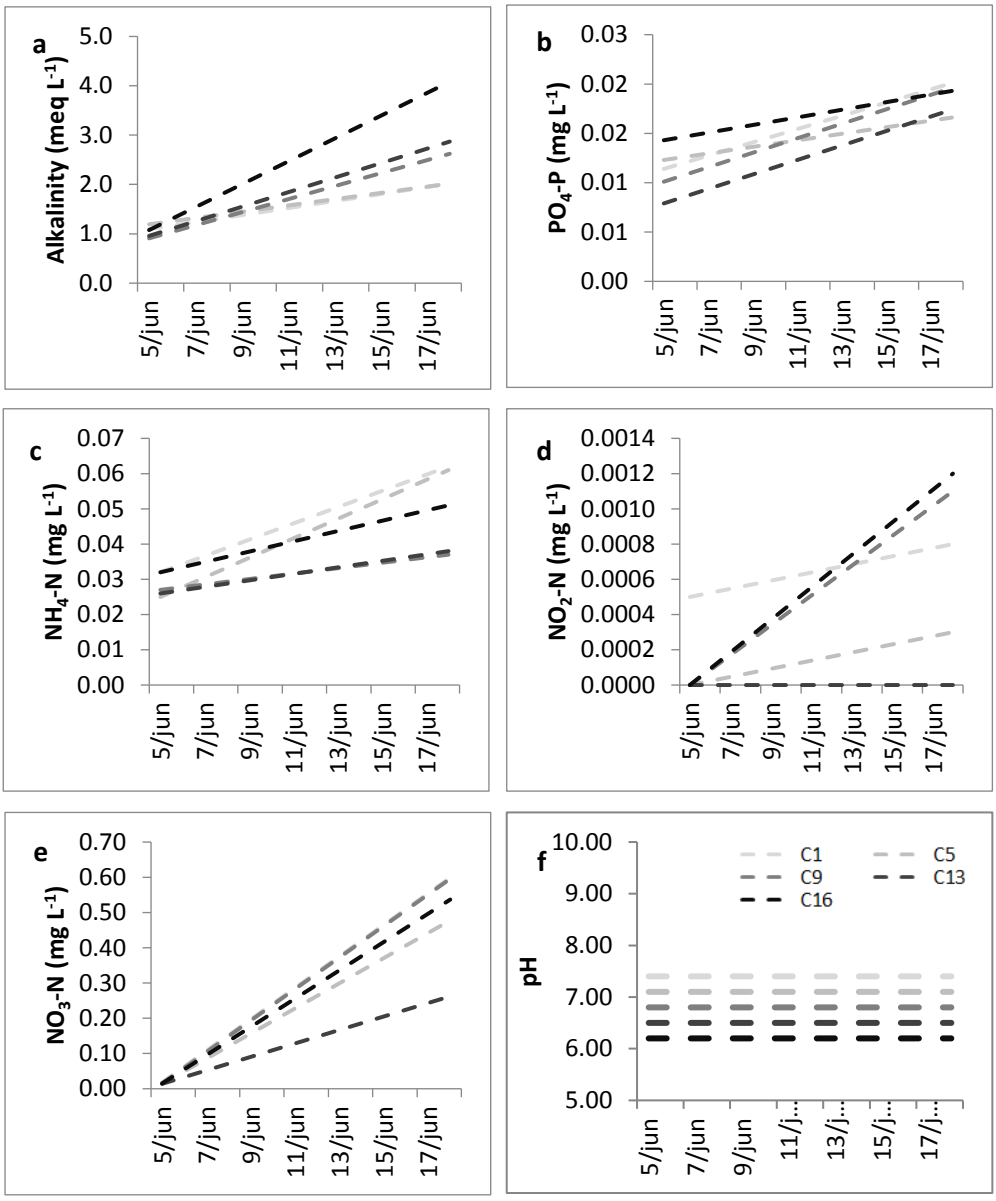

Figure S4.5 Water quality in the CO_2 treatment with plant species B. erecta: alkalinity (a), phosphate concentration (b), ammonium concentration (c), nitrite concentration (d) nitrate concentration (e) and pH (f). Higher values toward the end of the experiment can be explained by the fact that some of the water had just been replaced with new water.

Chapter 5.

Implications of climate change for submerged macrophytes: effects of CO_2, flow velocity and nutrient concentration on *Berula erecta*

Rosanne E. Reitsema, Stefan Preiner, Patrick Meire, Thomas Hein, Gudrun De Boeck, Ronny Blust, Jonas Schoelynck

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Abstract

Climate change can result in multiple indirect alterations of the environment in riverine ecosystems, due to changes in precipitation and runoff. Flow velocity, concentrations of CO_2 and nutrients are thereby expected to change, and consequences of the combination of those effects for macrophytes, a key organism group, are still poorly understood. This was tested in a racetrack flume experiment on macrophyte species *Berula erecta*, an amphibious species growing fully submerged in the experiment. In a full-factorial design plants were exposed to two different CO_2 concentrations, two nutrient concentrations (N, P and Si) and two flow velocities. 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 CO_2 and high nutrient concentrations and a dry scenario simulating evapotranspiration with low flow velocity, high CO_2 and high nutrient concentrations. Growth rate, biomass, morphology, chlorophyll and nutrient content were measured. *B. erecta* responded strongly to both scenarios. Biomass and relative growth rate increased, leaf BSi content decreased and especially in the wet scenario macrophytes had shorter stems and formed stolons with new ramets: the plants invested in horizontal growth to avoid hydrodynamic stress. Moreover, the C:N ratio was higher, leading to lower quality of macrophyte tissue as food source, and chlorophyll concentration was lower in the high CO_2 treatment. It can be concluded that combined stressors caused by climate change strongly affect macrophytes, which may indirectly have consequences for other organisms of the aquatic ecosystem that depend on macrophytes.

Keywords: aquatic plants, carbon dioxide, eutrophication, silica, flow velocity, multiple stressors

Introduction

As a result of global warming, it is expected that precipitation patterns will change: the frequency of both heavy precipitation and droughts will increase. For example, it has been predicted for Europe (especially in the north) that precipitation intensity will increase in winter and (especially in the south) decrease in summer (Hoegh-Guldberg 2018). The maximum of consecutive 5-day precipitation would increase by 5-10% in most European countries, and in some regions even up to 25% when average temperatures would rise by 1.5°C, whereas in some periods precipitation may decrease by 5-15%, especially in the Mediterranean area (Jacob et al. 2018). Accordingly, river discharge and flow velocity are expected to show more extremes, which can have profound effects on the aquatic ecosystem (van Vliet et al. 2013). Aquatic macrophytes play an important role within those aquatic ecosystems, for example by affecting nutrient cycling, sedimentation (Clarke 2002), oxygen dynamics (Uehlinger et al. 2000) and organizing stream structure and functioning (Schoelynck et al. 2012b). Changed precipitation patterns and altered river discharge can affect them in multiple ways.

Firstly, aquatic macrophytes are directly affected by decreasing or increasing river discharge. During drought, flow velocity is reduced, leading to increased diffusive boundary layer thickness, which can limit resource uptake and can eventually decrease macrophyte biomass (Riis et al. 2017). Increased discharge can result in pulling forces in the water that can cause uprooting or breakage of macrophytes (Schutten et al. 2005). Moreover, due to the stress the plants experience, photosynthesis can decrease by 30-60% (Madsen et al. 1993). Plant morphology may change to avoid or tolerate hydrodynamic forces. Plants become more streamlined or develop a smaller growth form (avoidance) or improve their resistance to breakage by increasing cross-sectional area or developing stronger tissue (tolerance) (Puijalon et al. 2011). Macrophyte tissue is mainly strengthened by cellulose, lignin and biogenic silica (BSi) and it was found that lignin and BSi concentrations in macrophyte species were higher under hydrodynamic stress in the model species *Egeria densa* (Schoelynck et al. 2012a, Schoelynck et al. 2015), but it has not yet been tested in *B. erecta*.

A second consequence of changing precipitation patterns is the effect on the amount of nutrients in the water. During drought, elevated nutrient concentrations have been reported due to increased evapotranspiration (Jeppesen et al. 2011). With increased precipitation intensity, surface run-off

increases, which can, despite dilution effects, lead to increased nutrient loading and eutrophication as well (Jeppesen et al. 2011, Coffey et al. 2019). So both high precipitation events and drought can lead to a eutrophic situation where periphyton (Sand-Jensen and Borum 1991) and, when drought results in stagnant conditions, non-rooted macrophytes (Hough et al. 1989) limit submerged macrophyte growth due to shading (Hilton et al. 2006). Macrophytes can respond to shading by adapting their morphology. Guan et al. (2018) found that *Potamogeton malaianus* grown in eutrophic, turbid waters formed a distinctive ecotype with longer leaves and more biomass accumulation close to the water surface, compared to the plants of the same species in clear water. There is also evidence that increased nutrient concentrations can directly affect macrophytes. Increased N and P concentrations in the water can reduce length and volume of roots and lower the number of chloroplasts (Wang et al. 2012).

With increased precipitation and surface run-off, concentrations of other elements relevant to macrophyte growth can increase as well. It has been found that concentrations of silicon (Si) in rivers increase during precipitation events (Smis et al. 2011). Increased Si concentrations can affect the nutrient stoichiometry of macrophytes: mainly submerged macrophytes use Si as a substitute for carbon, as this is more energy-efficient, leading to a decreased C:P and C:N ratio (Schaller et al. 2016). This can have consequences for nutrient- and carbon cycling, as microbial degradation of plant material with high Si content occurs faster than plant material with low Si content (Schaller and Struyf 2013).

Thirdly, altered precipitation patterns can also indirectly lead to increased organic carbon concentrations in the water. Both increased run-off due to increased precipitation intensity (Brothers et al. 2014) and lower water levels due to droughts (Porcal et al. 2009) may be contributors to the rising trend in dissolved organic carbon (DOC) concentrations in surface waters in Europe and North America (Monteith et al. 2007). DOC degradation can be an important source of CO_2 (Sobek et al. 2005). Although the natural CO_2 concentration in rivers and streams (3100 ppm) (Raymond et al. 2013) is usually higher than the current concentration in the atmosphere, (400 ppm), a further rise in aquatic systems is generally expected. This can occur, because of degradation of extra DOC, as explained above, and because the efflux of CO_2 from the water will be reduced as atmospheric CO_2 concentrations rise (Phillips et al. 2015), and the

global atmospheric CO_2 concentration currently rises with 20 ppm per decade (Allen et al. 2018).

It is difficult to predict future CO_2 levels in freshwater ecosystems because the exact factors controlling aquatic CO_2 concentrations and their response to climate change are not yet well understood. Moreover, current CO_2 and total inorganic carbon levels in rivers are highly variable and can depend on the catchment, river type (Cole et al. 2007), and location within the river (Maberly et al. 2015). As a consequence, it is hard to predict future CO_2 levels and how freshwater organisms will respond (Hasler et al. 2016). Most research devoted to the effects of elevated aquatic CO_2 concentrations has been done in marine ecosystems: the resulting drop in ocean pH and its effects on marine organisms are well documented (Boyd et al. 2016). Still, there are studies that have analysed the effect of elevated CO_2 concentrations and pH drop on freshwater macrophytes. Those studies mainly conclude that under elevated CO_2 concentrations plants have a higher relative growth rate (Pagano and Titus 2007, Eusebio Malheiro et al. 2013, Dülger et al. 2017), an increased biomass production (Andersen et al. 2005, Cheng et al. 2010, Hussner et al. 2016) and an increase in root:shoot ratio (Madsen 1996, Hussner et al. 2016, Dülger et al. 2017). Furthermore, tissue N content was found to be lower (Titus and Andorfer 1996, Titus and Pagano 2002, Yan et al. 2006), P content was higher (Yan et al. 2006), chlorophyll content was lower (Madsen 1996, Dülger et al. 2017), dry matter content (DMC) was higher (Eusebio Malheiro et al. 2013) and specific leaf area (SLA) was lower (Madsen 1996). There is no evidence that the drop in pH due to increased CO_2 concentrations negatively affects macrophytes, but when the pH drops to very low values (5.0 or lower), in some species sexual reproduction is inhibited (Hasler et al. 2017). Still, pH levels lower than 5 are rare in freshwater ecosystems and only occur under extreme conditions, for example in bogs with high concentrations of organic matter (Hasler et al. 2017).

Although several studies have investigated the effects of elevated CO_2, eutrophication and increased flow velocity on macrophytes, knowledge about their combined effects is still limited. Yet, it is exactly a combination of effects that will affect macrophyte species in riverine ecosystems in the future. Important ecological drivers such as climate change are often tested in a simplified way in experiments (Knapp et al. 2018), so by testing interactions we attempt to approach a more realistic situation, as for example macrophytes often develop an avoidance or tolerance strategy to withstand increased flow velocities (Puijalon et al. 2011). However, increased nutrient loading can lead

to a reduction in root length and volume (Wang et al. 2012) and increased biomass close to the water surface (Guan et al. 2018), and when exposed to elevated CO_2 concentrations growth rate (Dülger et al. 2017) and clonal growth increases (Yan et al. 2006). Those adaptations can make macrophytes more vulnerable to sudden increases in flow velocity. When more aspects of climate change are studied together, more accurate predictions can be made about the response of macrophytes to climate change effects.

The aim of this study is to test the responses of macrophytes to CO_2, nutrient loading and flow velocity and their interactions, in order to be able to make predictions about the consequences for other components of the ecosystem (figure 5.1). More specifically, the effects of two climate change scenarios were tested. With regard to the two scenarios that were chosen, it should be taken into account that the effects of climate change are complicated and the exact future conditions in rivers are not known yet. We chose scenarios that have enough support from literature and that allow to test interactions between different aspects of climate change. Firstly, a wet scenario was chosen with: 1) increased flow velocity caused by increased precipitation intensity as is predicted for the following decades, if global warming is not decreased (Hoegh-Guldberg 2018), 2) increased CO_2 concentrations (1000 ppm), which is predicted for the year 2100, according to the worst-case scenario (IPCC 2013), and 3) increased nutrient concentrations based on average values for eutrophic streams (Smith et al. 1999). Secondly, a dry scenario was chosen with 1) low flow velocity caused by decreased summer precipitation, which is predicted for the following decades, especially in southern Europe, if global warming is not decreased (Hoegh-Guldberg 2018), 2) increased CO_2 concentrations (1000 ppm) and 3) increased nutrient concentrations (eutrophication). It was decided to choose a relatively high concentration of CO_2, the worst-case scenario for 2100. We did this to also include increases in CO_2 concentration caused by instream processes like DOC degradation, rather than only increases due to changes in the atmospheric concentration. For reasons of consistence and clarity, we will call effects of flow velocity, nutrients and CO_2 climate change effects in the rest of this manuscript. Still, it should be taken into account that changes in flow velocity, nutrients and CO_2 concentrations can also be affected by other factors, like land use change or pollution. The response of several plant traits related to growth rate, morphology, biomass allocation, chlorophyll production and C, N, P and Si content of the plant tissue were measured. These traits are a key aspect of the analysis. 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 due to the increased CO_2 concentration. In contrast, we hypothesised that nutrient loading would partially counteract the effect of elevated CO_2, resulting in decreased plant growth (due to increased competition with periphytic algae), smaller plant roots, and that increased levels of N, P and Si in the water would lead to lower plant C:N and C:P ratios. We also hypothesised that plants would be shorter, with thicker stems and increased BSi in plant tissue in the wet scenario. Lastly, we hypothesised that there would be interaction effects between CO_2 and nutrient concentrations and flow velocity, as described in the previous paragraph.

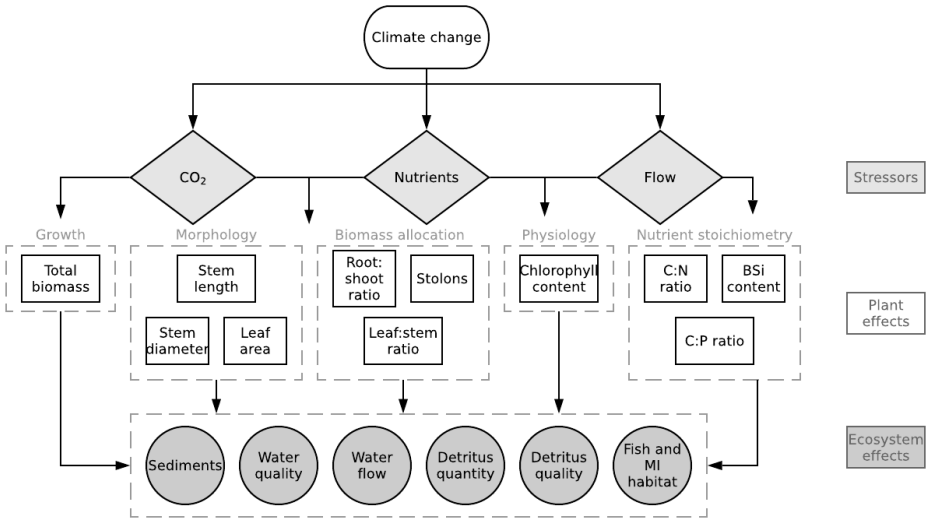

Figure 5.1 Conceptual model of the hypotheses to be tested in this study. Due to climate change levels of nutrients, CO_2 and flow velocity are altered. In this study the effects on plant growth, morphology, biomass allocation, physiology and nutrient stoichiometry will be tested. Based on those results, the effects on the rest of the ecosystem can be predicted. MI = macroinvertebrates

Materials and methods

Plant material

In this experiment *Berula erecta* (Hudson) Coville was chosen as a 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

widespread in streams (Baattrup-Pedersen et al. 2003). *B. erecta* is not a floating species, which makes it relatively vulnerable to the effects of climate change (Short et al. 2016). Although it has not been investigated whether *B. erecta* takes up nutrients with their shoots or roots, evidence has been found for both uptake through shoots (Baldy et al. 2007) and roots (Preiner et al. 2020). *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 (HCO_3^- and CO_2), *B. erecta* can only take up CO_2 (Sand-Jensen et al. 1992), so we expected that this species would respond strongly to changes in CO_2 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 (11.4 ± 2.4 cm) were selected with an initial mean dry mass of 0.14 ± 0.07 g (determined on 12 representative individuals that were not used in the experiment) and 384 plants (48 pseudo replicates per treatment) were each placed in 9×9×10 cm square pots filled with 0-2 mm grainsize 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 (see table S5.1a for nutrient concentrations) 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^{-1}) and the other two flumes to low flow velocity (0.04 m s^{-1}). Flow velocity was 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, afterwards the average was calculated. CO_2 gas from a commercial bottle was added through an airstone to two flumes at approximately 2 L h^{-1} (gas pressure 2 bar). Gas flux was regulated with a Skalar GT1355 Sho-Rate G flowmeter. The other two flumes had a gas concentration which was in equilibrium with the atmosphere. This resulted in four different treatments: high CO_2 (1000 ppm) with high flow velocity (HC-HF), high CO_2 with low flow velocity (HC-LF), low CO_2 (400 ppm) with high flow velocity (LC-HF) and low CO_2 with low flow velocity (LC-LF). The experiment was performed between 19 May and 24 July 2017 and the experiment was repeated afterwards (2 August until 9 October), but with extra nutrients added to the flumes to simulate stream eutrophication conditions

according to (Smith et al. 1999), so the experiment was carried out once with low nutrient concentrations (LN) and once with high nutrient concentrations (HN). On day 3, 8 and 24 of the second experiment, 400, 120 and 400 mL, respectively, of a commercial water plant fertilizer was added to each flume (Prodac Nutronflora). As this did not lead to eutrophic conditions, it was decided to add a more concentrated dose of N and P: on day 34, 44, 52, 59 and 65 KNO_3 (37.5 g per flume) and KH_2PO_4 (2.5 g per flume) were added. The concentration of nutrients added was based on average values for eutrophic streams: 1.5 mg L^{-1} N and 0.075 mg L^{-1} P (Smith et al. 1999). As nutrients were taken up relatively quickly, a higher concentration was added to the flumes. On day 8 and day 34 of the second experiment, SiO_2 was added (6 g per flume; Merck, Darmstadt, Germany, DAB certificated). CO_2, pH, alkalinity and nutrient concentrations in the water were measured throughout the experiment. Periphytic algae were removed twice by carefully taking them off the leaves by hand (supplementary materials and methods), but often started growing again within a few days.

Plant 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 on day 1, 30, 55 and 70 (experiment 2) plant growth characteristics were measured in order to test whether the plants would immediately respond to the treatments or later in the experiment. The number of stems and leaves was counted on each individual and length and stem diameter of the longest stem were measured. Stem diameter was measured at the pale ring that is visible on the stem. Moreover, the number of stolons (if visible) and the number of stems and leaves on the new ramets was counted (all are non-disturbing measurements). After harvesting the plants at the end of the experiment, stems, leaves and roots were separated and weighed fresh, and after drying the plant material for 48 hours at 70˚C the dry mass was determined. Prior to this drying, 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. The subset of 10 plants was further used for chlorophyll and nutrient stoichiometry analyses (see supplementary material and methods for more detailed information). In total 24 traits were measured: number of stems, number of leaves, length of the longest stem, diameter of the longest stem, total dry mass, leaf:stem ratio, root:shoot ratio, number of stolons, average stolon length, relative growth rate,

dry matter content (DMC), % N of leaves, % N of stems, % C of leaves, % C of stems, C:N ratio of leaves, C:N ratio of stems, total leaf area, average leaf area, specific leaf area, chlorophyll a+b content, total plant chlorophyll, BSi of leaves and BSI of stems.

In total, eight different treatments were tested (table 5.1), with the LN-LC-LF treatment as 'no climate change scenario', and the HN-HC-HF and HN-HC-LF treatments as two climate change scenarios; in both scenarios increased CO_2 and increased nutrient loading were tested, with heavy precipitation and drought simulated in the HF and LF scenario respectively (see the last paragraph of the introduction for a more detailed explanation of the scenarios). The other five treatments help in understanding the relative contribution of the three tested aspects of climate change to the response of the macrophytes.

Table 5.1 The different treatments and their labels. LN – low nutrients, LC – low CO_2, LF – low flow, HN – high nutrients, HC – high CO_2, HF – high flow. The first treatment is the control treatment without climate change (CC) effects, the second treatment is the wet climate change scenario and the third treatment the dry climate change scenario. Other treatments serve to understand the different interactions between the three tested stressors.

Treatment	**Nutrients**	**CO_2**	**Flow velocity**
LN-LC-LF (no CC)	Tap water, no nutrients added	400 ppm	0.04 m s^{-1}
HN-HC-HF (CC wet)	NO_3-N > 1.5 mg L^{-1}, PO_4-P > 0.075 mg L^{-1}	1000 ppm	0.4 m s^{-1}
HN-HC-LF (CC dry)	NO_3-N > 1.5 mg L^{-1}, PO_4-P > 0.075 mg L^{-1}	1000 ppm	0.04 m s^{-1}
LN-LC-HF	Tap water, no nutrients added	400 ppm	0.4 m s^{-1}
LN-HC-LF	Tap water, no nutrients added	1000 ppm	0.04 m s^{-1}
LN-HC-HF	Tap water, no nutrients added	1000 ppm	0.4 m s^{-1}
HN-LC-LF	NO_3-N > 1.5 mg L^{-1}, PO_4-P > 0.075 mg L^{-1}	400 ppm	0.04 m s^{-1}
HN-LC-HF	NO_3-N > 1.5 mg L^{-1}, PO_4-P > 0.075 mg L^{-1}	400 ppm	0.4 m s^{-1}

Statistical analyses

All statistical analyses were carried out in R version 3.4.3. The effects of elevated CO_2, nutrients and flow velocity on growth and morphology parameters, chlorophyll, C, N, P and Si content were tested with a three-way ANOVA (CO_2*Nutrients*Flow) with type III sums of squares. Normal distribution of the residuals was tested with Shapiro-Wilk tests and checked visually with QQplots, homogeneity was tested with Levene's tests, and if necessary, data was transformed to meet the assumptions. For significant results, 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 stolon length and leaf, stem and root dry matter content) were analysed with gamma regression. For the P content, not enough material was available to test samples from all treatments (HN-LC-HF and HN-LC-LF were completely missing so a three-way ANOVA was not possible). Therefore, two-way ANOVA tests were carried out with a part of the data: CO_2*Flow interaction in the LN treatment and nutrients*flow interaction in the HC treatment.

In order to test the relative importance of the treatments and their interactions omega squared (ω^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 ω^2 values. To test how the plants responded to the treatment over time, the Principal Response Curve (PRC) method 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' package in R (Oksanen et al. 2019). In a PRC plot the effect of the different treatments is shown over time, relative to a control treatment that had been assigned before the analyses. The control treatment that was chosen is the 'no climate change' scenario, with low CO_2, low nutrients and low flow velocity.

Results

In this section the main results are described, for more detailed results and discussion of each parameter individually, see supplementary results and discussion. *B. erecta* was affected by flow velocity, CO_2 and nutrient treatments. Out of the 24 plant traits measured, in 16 of them there was a significant effect of CO_2, in 19 there was a significant effect of nutrients and in 15 a significant effect of flow (table 5.2). For the interaction terms, in 18 a significant effect of the CO_2*nutrients interaction, in 11 a significant effect of the CO_2*flow interaction, in nine a significant effect of the flow*nutrient interaction and in three a three-way interaction was found (table 5.2). When looking at the relative importance of the treatments (the omega squared values), in most cases CO_2 had the greatest effect, relative to the other treatments, followed by flow, nutrients, CO_2*flow and CO_2*nutrients (table 5.3).

Table 5.2 F-values of the three-way ANOVA tests and z values of the generalized linear models of growth and morphological parameters (n=48), nutrient stoichiometry, leaf area SLA and chlorophyll (n=10). Interaction effects that were not significant have been removed from the model (ns). Number of stems, number of stolons and average stolon length and DMC have been tested with a GLM. Some variables have been transformed: number of leaves: x^(1/8), length of the longest stem: x^(1/3), stem diameter: x^(1/3), DM total: x^(1/6), leaf:stem ratio: log(x)^(1/3), root:shoot ratio: log, % N leaves: $x^{(1.1^{-1})}$, % N stems: log, % C leaves: x^8, % C stems: x^2, C:N leaves: x^{-1}, C:N stems: $\log(x^{0.5})$, total leaf area, mean leaf area, SLA, BSi leaves, BSi stems: $x^{0.5}$. Signif. codes: * <0.05, ** <0.01 ** <0.001. C=CO_2, N=nutrients, F=Flow velocity.

	CO_2	Flow	Nutrients	CO_2*Flow	CO_2*Nutrients	C*N*F
Number of stems	-8.654***	2.003*	1.826	-3.619***	2.154*	ns
Number of leaves	109.1925***	0.5015	23.6800***	22.1759***	6.4645*	ns
Length longest stem	55.701***	240.037***	65.714***	25.391***	29.989***	ns
Diameter longest stem	8.3569**	0.5598	372.4467***	5.6645*	38.7297***	ns
Dry mass total	151.0648***	5.2830*	299.6663***	23.8321***	5.5215*	ns
Leaf : stem ratio	0.0113	0.0367	11.5671***	ns	9.3922**	ns
Root : shoot ratio	0.2771	9.3104**	17.1442***	12.0603***	49.5382***	20.1917*
Number of stolons	-10.587***	-0.261	2.967***	ns	ns	ns
Average stolon length	-9.291***	-2.589**	13.233**	ns	-3.533***	ns
Relative growth rate	122.6992***	341.9385***	2.8144	10.8597**	44.7598***	4.6245*
DMC	0.095	-4.55***	1.02	ns	-5.00***	ns
% N Leaves	1.0845	35.8489***	67.9707***	ns	102.7910***	ns
% N Stems	77.8146***	15.8111***	153.5192***	ns	56.1184***	ns
% C Leaves	6.8005*	2.1096	27.8960***	ns	29.8561***	ns
% C Stems	65.0685***	16.1302***	19.1423***	6.2195*	ns	ns
C:N leaves	11.936***	53.201***	88.400***	ns	102.317***	ns
C:N stems	51.531***	10.671**	354.549***	ns	126.519***	ns
Total leaf area	12.64.52***	8.7812**	40.5743***	13.7817***	ns	ns
Mean leaf area	21.3110***	9.3932**	48.7321***	ns	ns	ns
SLA	1.0967	1.5811	36.8267***	ns	8.1865**	ns
Total chlorophyll	1.3591	0.4076	6.6712*	5.8083*	9.1063**	ns
Plant chlorophyll	28.9038***	2.1586	12.0513***	9.1807**	9.5486**	ns
BSi Leaves	0.323	15.8554***	1.3417	1.6483	0.9187	5.2540*
BSi Stems	0.0688	0.4344	0.5348	ns	ns	ns

Growth and morphology

The relative growth rate (RGR) appeared to be mainly affected by CO_2 and flow velocity (table 5.3) and it was significantly higher in plants exposed to HC compared to LC (figure 5.2a). 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 LC conditions (see table 5.2 for statistics). In all treatments with LC and HN the average RGR was negative. Biomass allocation was also highly affected by the treatments: the root:shoot ratio was mainly affected by CO_2, flow and the interaction between CO_2 and nutrients (table 5.3, figure 5.2b and figure 5.3b). Furthermore, increased CO_2 and low nutrient concentrations both resulted in more and longer stolons (figure 5.2c). Most other plant organs responded in the same way: especially the number of leaves was higher in the HC treatment than the LC treatment (figure 5.2d and table 5.3). The number of stems, stem length, stem diameter (figure 5.2e) average leaf area and total dry mass they were also significantly more numerous or larger under HC than under LC conditions. In contrast, under HN conditions there were smaller and fewer leaves, a higher SLA (figure 5.4a), shorter and thinner stems and lower total dry mass than under LN conditions (table 5.3). Flow velocity also affected morphology: plants exposed to HF had more leaves and more and thicker stems than plants growing under LF, but this was only significant under LC (figure 5.2d+e). There was also a strong effect of flow velocity on stem length, with the longest stems in the LF treatment (figure 5.2f and 5.3a).

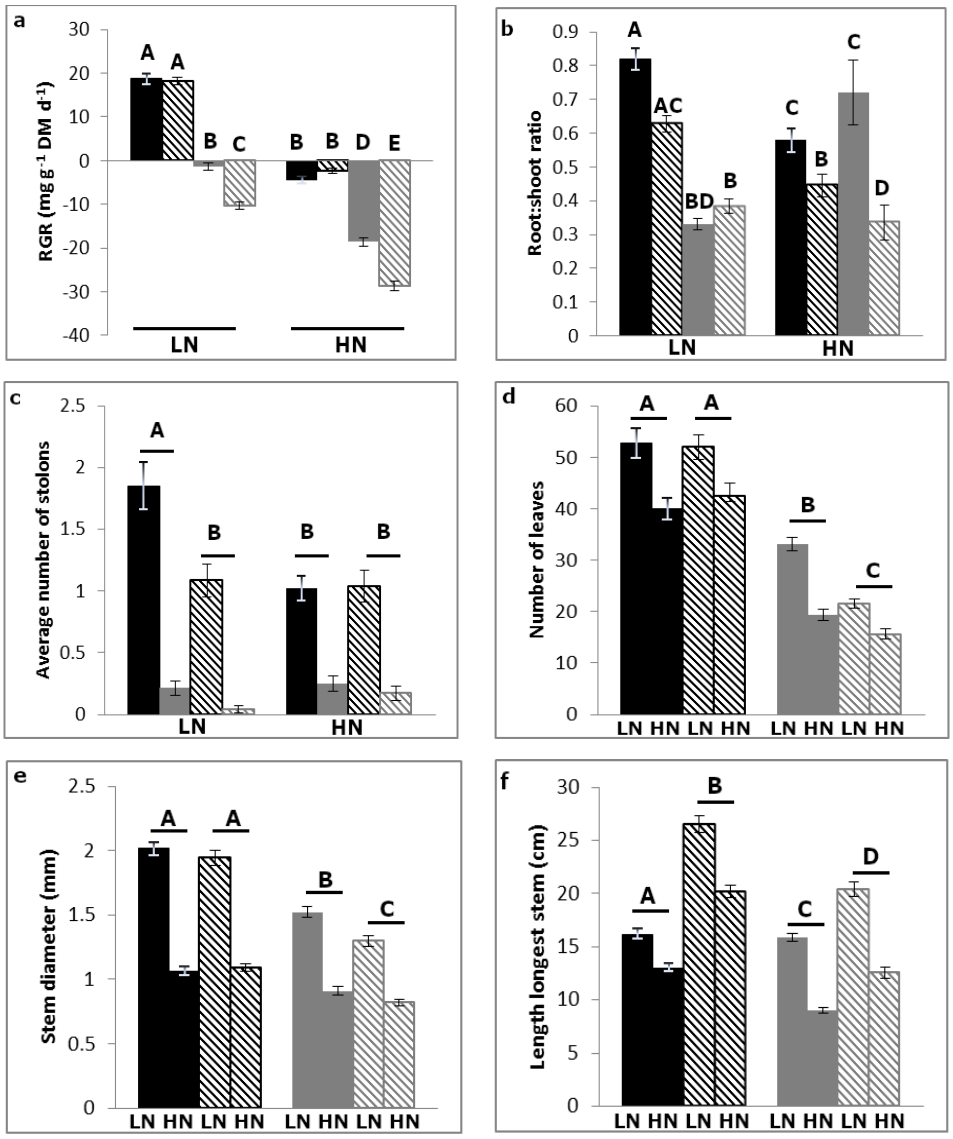

Figure 5.2 Relative growth rate (a), root:shoot ratio (b), number of stolons (c), number of leaves (d), stem diameter (e), and length of the longest stem (f). The letters above the graph indicate significant differences ($p<0.05$, n=48). In figure a and b, three-way interactions are shown, in figure c Flow*Nutrient interaction and in figure d, e and f CO_2*Flow interactions.

Chlorophyll and nutrient stoichiometry

Plants exposed to LC had a higher chlorophyll (a+b) concentration than plants exposed to HC, but this was only significant if plants grew at HF and LN regimes (figure 5.4b). However, when looking at the total chlorophyll content per plant, the plants exposed to HC appeared to have the highest chlorophyll content (figure 5.4c), as those have more biomass. The high CO_2 treatment also resulted in higher C:N ratios in leaves and stems (table 5.3, figure 5.3d+e and 5.4d). This difference can be mainly explained by the lower leaf and stem N content in the high CO_2 treatment under LN conditions (figure 5.4e). Leaf and stem P content was highly affected by flow velocity, with the highest values in the HC-HN-LF treatment, which also implied that the C:P ratios were lowest in that treatment. Differences in BSi content were also to a large extent caused by flow velocity (table 5.3 and figure 5.3c). Plants grown under LF had a significantly higher leaf BSi content than plants under HF, except in the treatment with HN and LC (figure 5.4f). The highest BSi leaf content was found under LF, LC and LN; under this treatment the BSi content was significantly higher than in all other treatments. In the stems, in plants grown under LF the BSi content was higher than for the HF as well, but this was only significant under LN.

Differences over time

In the PRC diagram (figure S5.5) the effect of the treatments over time is shown for eight plant traits that have been measured four times during the experiment: 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. 18% of the treatment variance could be explained by the model ($F=47.625$, $p=0.001$). All plant traits had negative weights, indicating a negative relationship with the treatments. This means that, especially towards the end of the experiment, all plant traits (especially the number of leaves) were favoured by most treatments except for the LC-LF-HN and LC-HF-HN treatments. The differences between the treatments become more pronounced at the end of the experiment.

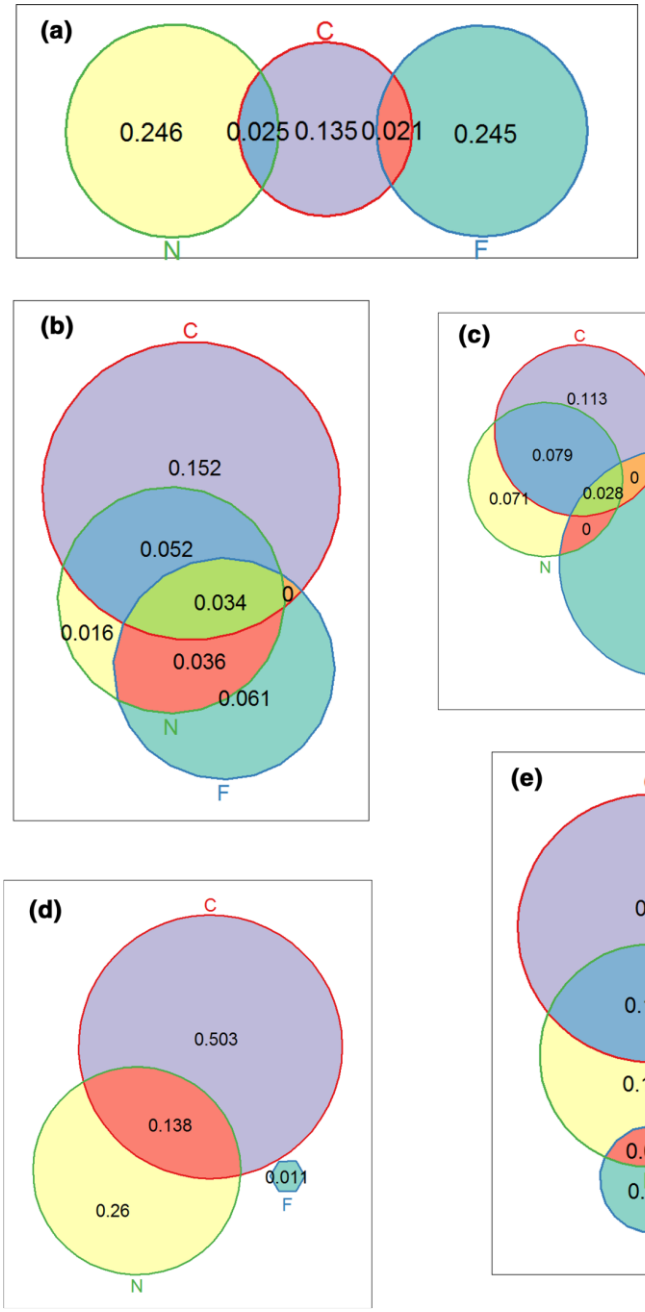

Figure 5.3 Venn diagrams showing the proportion of variance explained by CO_2 (C), flow (F), nutrients (N) and their interactions for the variables length of the longest stem (a), root:shoot ratio (b), leaf BSi content (c), stem C:N ratio (d) and leaf C:N ratio (e).

Table 5.3 Omega squared values for the growth and morphological parameters (n=48), nutrient stoichiometry, leaf area, SLA and chlorophyll (n=10). C=CO_2, N=nutrients, F=Flow velocity.

	CO_2	**Flow**	**Nutrients**	**C*F**	**C*N**	**F*N**	**C*F*N**
Number of leaves	0.445	0.014	0.097	0.023	0.006	0	0
Length longest stem	0.135	0.245	0.246	0.021	0.025	0	0
Diameter longest stem	0.145	0.007	0.508	0.004	0.03	0.003	0
DM total	0.418	0.002	0.367	0.012	0.002	0	0
Leaf:stem ratio	0.035	0	0.01	0	0.035	0	0
Root:shoot ratio	0.152	0.061	0.016	0	0.052	0.036	0.034
Relative growth rate	0.442	0.367	0.021	0.002	0.021	0	0.001
% N Leaves	0.326	0.042	0.165	0	0.243	0.038	0
% N Stems	0.482	0.009	0.337	0	0.067	0.008	0
% C Leaves	0.019	0.012	0.028	0	0.254	0	0
% C Stems	0.411	0.048	0.095	0.027	0	0	0
C:N leaves	0.4	0.064	0.192	0	0.178	0.029	0
C:N stems	0.503	0.011	0.26	0	0.138	0	0
Total leaf area	0.295	0	0.229	0.076	0	0	0
Mean leaf area	0.064	0.071	0.36	0	0	0	0
SLA	0	0.005	0.276	0	0.07	0	0
Total chlorophyll	0.05	0.079	0	0.047	0.085	0	0
Plant Chlorophyll	0.369	0	0.242	0.035	0.038	0	0
BSi leaves	0.113	0.364	0.071	0	0.079	0	0.028
BSi stems	0	0.077	0.02	0	0	0.082	0

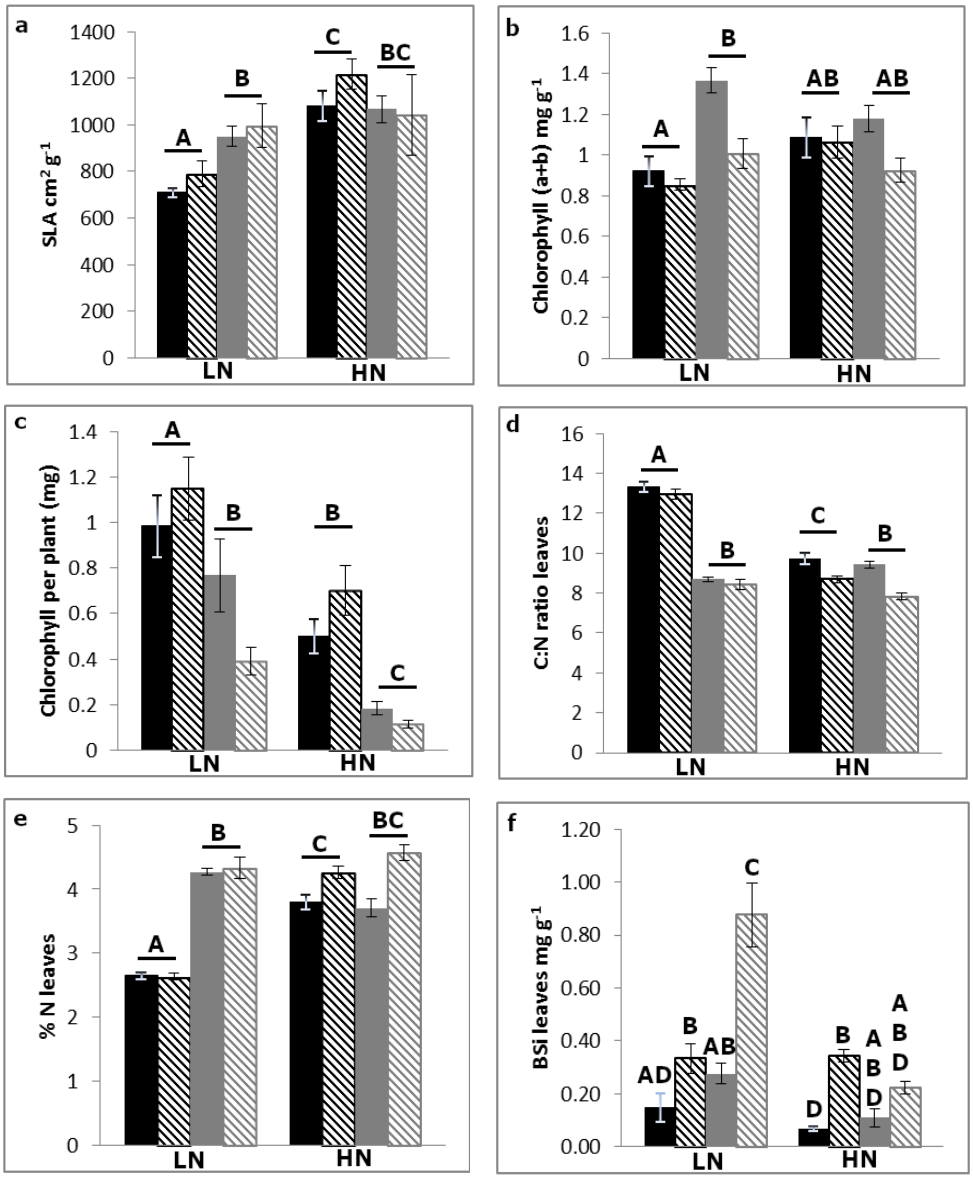

Figure 5.4 Specific leaf area (a), total chlorophyll concentration (b), total amount of chlorophyll per plant (c), C:N ratio of the leaves (d), % N in the leaves (e), and BSi concentration of the leaves (f). The letters above the graph indicate significant differences ($p<0.05$, $n=10$). In figure a, b, c, d and e, CO_2*Nutrient interactions are shown and in figure f a three-way interaction.

Discussion

It can be concluded that *B. erecta* responds strongly to CO_2, eutrophication and increased flow velocity, which has also been found for other macrophyte species (Hough et al. 1989, Chambers et al. 1991, Cao and Ruan 2015). Compared to the reference situation (the LN-LC-LF treatment, figure 5.5a), the macrophytes growing in the wet climate change scenario with increased heavy precipitation intensity (HN-HC-HF) have a higher root:shoot ratio, a lower N content, a lower leaf BSi content, more total biomass and a higher RGR, than the plants in the no climate change scenario (figure 5.5b). Still, the difference in RGR with the no climate change scenario is relatively small, as the positive effect of CO_2 is partially compensated by the negative effect of eutrophication (probably caused by shading by periphytic algae). The total plant chlorophyll content followed the same pattern as the plant biomass, which may imply that the total oxygen production by the macrophyte community increases under higher CO_2 concentrations but decreases under eutrophication (again probably due to shading effects from periphyton), as oxygen levels are lower with reduced macrophyte biomass (Kaenel et al. 2000).

The macrophytes in the HN-HC-HF scenario have more stems and leaves, which is mainly caused by the increased number of stolons on which new ramets have been formed, which may be explained as a strategy to increase the plants' potential nutrient uptake, induced by increased CO_2 levels (Yan et al. 2006). Moreover, the plants were shorter than in the no climate change scenario, suggesting that they invest more in horizontal than vertical growth. This can be seen as adaptations to avoid hydrodynamic stress: by developing a more compact growth form, the macrophytes can avoid breaking or uprooting caused by the higher flow velocity, which has been observed before with *B. erecta* (Puijalon et al. 2005). This may also explain the lower leaf BSi concentration: as this macrophyte species avoids hydrodynamic stress, a higher tissue BSi content will not necessarily improve their fitness. Although this horizontal growth strategy can enable the plants to colonise more habitats and strengthen their population, under eutrophication the RGR was negative. So eventually, the combined effect of eutrophication and increased flow velocity can result in a positive feedback loop with small and short plants that are limited by light and cannot keep up with periphyton growth, which may lead to the disappearance of macrophytes (Hilton et al. 2006, Hilt 2015).

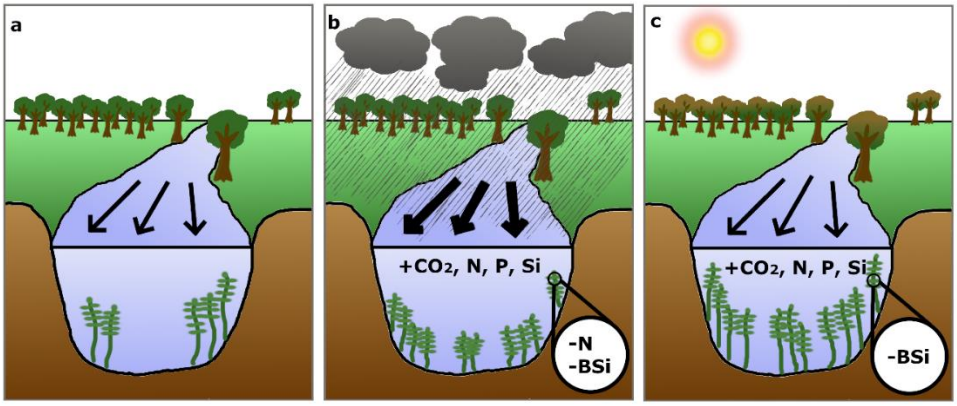

Figure 5.5 The appearance of macrophytes in the no climate change scenario (a) with a low CO_2 concentration, low flow velocity and low nutrient concentrations. In figure b macrophytes are shown in the wet climate change scenario with high CO_2 concentration, high flow velocity and high nutrient concentrations. Here the plants show more horizontal growth with stolons and new ramets and the plants are shorter and contain less N and BSi. In figure c macrophytes are shown in the dry climate change scenario with high CO_2 concentration, low flow velocity and high nutrient concentrations. In this scenario there is less pronounced horizontal growth, although new ramets are formed as well and plants contain less BSi.

In the climate change scenario with increased drought (HN-HC-LF) results were in most cases comparable to the heavy precipitation scenario (HN-HC-HF). This was not entirely expected as due to increased boundary layers, biomass production would normally decrease with lower flow velocity (Riis et al. 2017). This effect seemed to be compensated for by the high CO_2 treatment. The main differences between HN-HC-LF and HN-HC-HF could be found in the leaf N content, plant length and root:shoot ratio, which, in contrast to the plants in the heavy precipitation scenario, did not significantly differ from the plants in the no climate change scenario (figure 5.5c). Although the plants in the drought scenario also invested in horizontal growth, this was relatively less pronounced than in the heavy precipitation scenario, suggesting that this horizontal growth is mainly caused by increased flow velocity, which is supported by other studies (Puijalon et al. 2007). For most plant traits CO_2 appears to be the main driver explaining the differences between the climate change and the no climate change scenarios. However, in most rivers and streams CO_2 concentrations are currently higher than the control conditions in this experiment and rivers often act as a CO_2 source rather than a CO_2 sink (Raymond et al. 2013). This means

that extra CO_2 entering rivers due to climate change may be relatively small, and the effect on macrophytes more limited (Andersen and Pedersen 2002). Therefore, the relative contribution of CO_2 to the growth and morphology of macrophytes in rivers may be smaller than in this experiment. This implies that altered flow velocity and nutrient concentration may be more relevant under natural conditions. So even though climate change may seem to benefit *B. erecta* in this experiment, the RGR in the two climate change scenarios is negative due to eutrophication, and climate change can be expected to form a considerable threat to macrophytes. This observation stresses the importance of studying multiple aspects of climate change simultaneously, as although some studies suggest that especially invasive macrophytes may be favoured by increased CO_2 levels (Eusebio Malheiro et al. 2013, Cao and Ruan 2015), studying multiple factors and interactions can lead to different and possibly opposite results.

Even though this study only involves one macrophyte species, it may be expected that other rooted obligate CO_2 users respond similarly to climate change. In other species (among Hydrocharitaceae, Haloragaceae, Ceratophyllaceae and Potamogetonaceae) comparable responses to CO_2 (Xie et al. 2004, Hussner et al. 2016) and eutrophication (Hilt 2006, Olsen et al. 2015) have been observed. However, this needs to consider that some strategies of *B. erecta* are not shared by all other submerged macrophyte species. Other species may, for example, respond to hydrodynamic stress by increasing tolerance through enhancing their cross-sectional area or developing stronger tissue (Puijalon et al. 2011), not responding by a horizontal growth strategy. Moreover, *B. erecta* is an homophyllous amphibious plant that is unable to take up other forms of inorganic carbon than CO_2, so plants that can also take up bicarbonate are expected to respond less strongly to increased CO_2 concentrations (Eller et al. 2015).

The two climate change scenarios used in this experiment have been compared to a reference situation with low flow velocity, a low CO_2 concentration and a low nutrient concentration. However, rivers and streams may have different conditions, which have implications for the interpretation of the results. Flow velocity in small streams dominated by macrophytes can be higher than 0.04 m s^{-1} (Sand-Jensen and Pedersen 1999), which is the reference situation in this study. If flow velocity increases to values over 0.5 m s^{-1}, this can be highly stressful to macrophytes (Puijalon et al. 2005). Another problem is a sudden increase in flow velocity. If macrophytes are used to low flow velocities and they have long shoots, they do not have time to adapt if flow velocity suddenly

increases, leading to breaking or uprooting (Puijalon et al. 2005). The reference situation for CO_2 in streams may also be different from the one used in this experiment: in many streams the concentration of CO_2 is higher than in the atmosphere (Raymond et al. 2013). Photosynthesis rates in macrophytes do not show a linear response to CO_2 concentration; when CO_2 reaches a threshold level the photosynthesis rate does not further increase and for *B. erecta* this occurs above concentrations of 0.25 mM CO_2 or 7000 ppm (Sand-Jensen et al. 1992). If CO_2 concentrations amount to higher values, macrophyte biomass may not further increase as the saturation has been reached. However, as current average CO_2 values in streams are around 3000 ppm (Raymond et al. 2013), it is not likely that this saturation point is reached at a large scale. Lastly, in this experiment the reference situation for nutrients was tap water, which contained a relatively low phosphate concentration and a high nitrate concentration, which gradually decreased during the experiment. Some streams already contain high nutrient concentration due to pollution from agriculture, and increased nutrient loading due to climate change can even lead to disappearance of macrophytes (Jeppesen et al. 2011).

The changes in macrophytes due to climate change can indirectly affect the riverine ecosystem. In figure 5.6 the most important effects of CO_2, nutrients, flow velocity and their interactions on the measured plant traits are shown, as well as predicted effects on the ecosystem. When the root:shoot ratio increases, due to increased root- and stolon biomass, the stabilising effect of macrophytes on the sediments will be enhanced (Clarke 2002). On the other hand, macrophytes in rivers slow down flow velocity, fostering fine sediment accumulation and play an important role in nutrient cycling between the sediments and water column (Madsen et al. 2001, Clarke 2002). These effects may be reduced if macrophytes are shorter and have less biomass. Less biomass also means that the habitat for other aquatic organisms declines. Due to their physical structure in the water, flow velocity within macrophyte patches is slowed down and outside of the patches it is accelerated (Schoelynck et al. 2012b), and as a result, macrophytes serve as flow refugia, especially during events of high flow velocities (Lancaster and Hildrew 1993, Wolters et al. 2018). When macrophytes develop more horizontally instead of vertically, these flow refugia for macroinvertebrates may become smaller in the future. When macrophytes disappear and are replaced by filamentous algae, macroinvertebrate and small fish diversity decreases, which has further consequences for higher trophic levels (Camp et al. 2014). Macrophytes also play an important role in nutrient cycling (Clarke 2002) and if their biomass

decreases, their capacity to perform this key ecosystem function may decrease as well. As the current study concluded that macrophyte biomass is the main factor determining chlorophyll content of the plant, biomass decrease can lead to oxygen decrease in the water layer and the rhizosphere (Carpenter and Lodge 1986, Desmet et al. 2011). Changes in biomass also can have consequences for detritivores that rely on macrophyte detritus as food source, and moreover, the higher C:N ratio implies that the macrophytes have less nutritive value to aquatic herbivores. In terrestrial plants often C:N ratios are higher than in aquatic plants, and terrestrial herbivores often specialise with regard to plant species, plant part and time of consuming. Due to the lower C:N ratios in macrophytes compared to terrestrial plants, aquatic herbivores tend to have a more generalist approach (Elser et al. 2000). If C:N ratios rise due to increased CO_2 in the water, herbivores may need to adapt to acquire a sufficient amount of nutrients. Lastly, the reduced BSi content of the plant under climate change can also affect other organisms as it decreases macrophyte litter decay by microbes, but increases decay by macroinvertebrate shredders (Schaller and Struyf 2013).

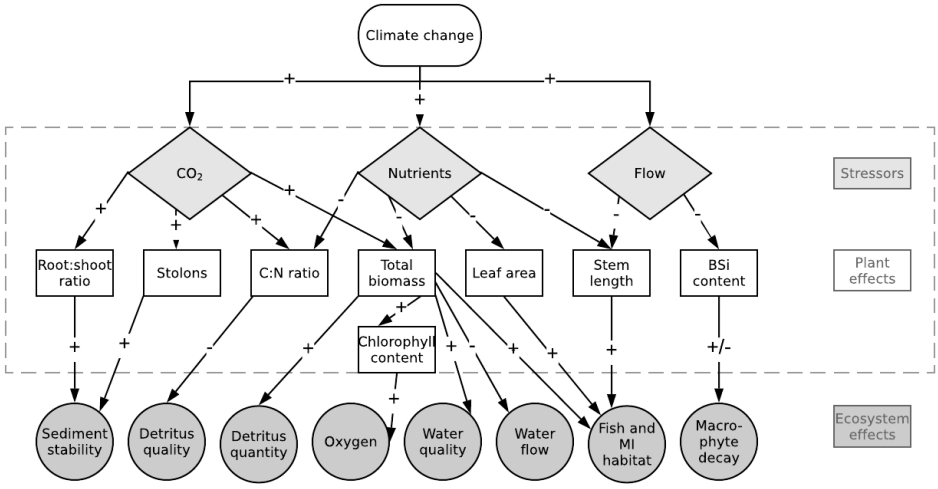

Figure 5.6 Conceptual model of the effects of climate change on submerged macrophytes and the predicted effects on the rest of the ecosystem. This scheme does not show the two climate change scenarios, but all three parameters tested and all relevant plant variables. The part within the dashed grey square is what was specifically tested in this study. The predicted effects on the ecosystem are based on literature. MI = macroinvertebrates.

It can be expected that macrophyte species *B. erecta* will strongly respond to climate change. Their growth rate is stimulated by CO_2, and limited when the water is eutrophic and when flow velocity is reduced to almost lentic conditions. Mainly due to effects of increased CO_2 and flow velocity, the macrophytes develop in a more horizontal way and stay shorter. The combined effects may lead to a decrease in macrophyte abundance, or even disappearance of macrophytes, due to the negative growth rate and shading effects by other organisms like periphytic algae. As macrophytes play an important role in the uptake of inorganic nutrients (Madsen and Cedergreen 2002), this can aggravate eutrophication problems and shifts to algal dominated conditions.

How macrophytes in rivers respond to climate change will of course depend on the extent of the increase in CO_2, nutrients and flow velocity. Other abiotic stressors such as increased temperature and UV-B radiation and in some locations increasing salinity may affect macrophytes, too (Short et al. 2016), as well as altered interactions with other aquatic organisms, such as competition with algae and other (invasive) macrophyte species, may increase in importance. We suggest that in future experiments the effects of those other stresses, especially competition with other primary producers under climate change scenarios are taken into account as well.

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Supplementary materials and methods

Periphytic algae

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 two times during each experiment when the plant traits were measured. Between the first and the second experiment the entire flumes were cleaned. The amount of algae growing in the flumes was not quantified, but on photographs it can be seen that in the HN treatment there appear to be more algae than in the LN treatment (see figure S5.1). The dissolved CO_2 pattern in the water also suggests that there were more algae in the HN treatment, as the day-night fluctuations were more pronounced than in the LN treatment (see figure S5.2), despite a lower plant biomass.

Water quality

The concentration of CO_2 in the water was measured continuously with a Pro-Oceanus Digital Mini CO_2 probe, which alternated between the flumes (see figure S5.2). In addition, pH was measured weekly on approximately the same time of the day (early afternoon) (multiline F/set-3 multimeter), see figure S5.3a+b and alkalinity (SAN++, Skalar, Breda, The Netherlands) was measured four times during the experiment. Nutrient concentrations in the water were measured on day 6, 20, 50 and 67 of experiment 1 and on day 15, 20, 30, 42, 48, 57 and 66 of experiment 2, see figure S5.3c-f and table S5.1c+b). Water samples were filtered with 0.45µm filters (Chromafil® Xtra MV-45/25, Macherey-Nagel, Düren, Germany) and the concentration of PO_4-P, NH_4-N, NO_2-N, NO_3-N and dissolved SiO_2-Si was measured (SAN++, Skalar, Breda, The Netherlands).

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 once centrifuged at 4000 rpm and then 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 plant material of the subsample was dried in the same way as the other material and the dry weight was measured. Beside chlorophyll concentration, the total chlorophyll content per plant was calculated as well by multiplying the total chlorophyll concentration with the total fresh weight of the leaves of each plant.

Plant carbon, nitrogen, phosphorus and biogenic silica analysis
The dried plant material (leaves and stems separately) from the subset of 10 plants used for the determination of leaf surface area was ground with an Ultra Centrifugal Mill ZM 200 (Retsch, Germany), sieve size 0.5 mm. The ground material was analysed for %N and %C on a FLASH 2000 Organic Elemental Analyser, based on Flash Dynamic Combustion (Thermo Fisher Scientific, 2014). Plant P content was determined by nitric acid digestion (69 % HNO_3), after which the samples were measured on ICP-OES (iCAP 6300 Duo view, Thermo Fisher, Waltham, Massachusetts, USA). Biogenic silica was extracted by incubating 30 mg of ground plant material in 0.5 M NaOH at 80°C for 5 hours. After filtering the samples (Chromafil® Xtra MV-45/25, Macherey-Nagel, Düren, Germany), the BSi content was determined (SAN++, Skalar, Breda, The Netherlands). If the 10 samples selected for the elemental analyses did not contain enough dry material for all analyses, extra samples of neighbouring plants were ground and added to the samples (this happened for most of the samples from the high nutrient treatment).

Supplementary results and discussion

Water quality
The averaged CO_2 concentration in the high CO_2 (HC) treatment was 1494 ± 299 ppm (62 ± 12 µM) in the low nutrients treatment (LN) and 834 ± 595 ppm (34 ± 24 µM) in the high nutrients treatment (HN) and in the low CO_2 (LC) treatment it was 449 ± 51 ppm (19 ± 2 µM) in the LN treatment and 162 ± 112 ppm (7 ± 5 µM) in the HN treatment. The concentrations followed a day-night rhythm with the most pronounced fluctuations in the high nutrients treatment (see figure S2). As a result of the CO_2 treatment, the pH was lower in the HC treatment: on average 7.95 ± 0.11 (low nutrients treatment) and 8.13 ± 0.34 (high nutrients treatment) and in the LC treatment it was 8.50 ± 0.09 (low nutrients treatment) and 8.66 ± 0.26 (high nutrients treatment), see figure S3a+b. The alkalinity in the HC treatment was 2.9 mM HCO_3^- (low nutrients treatment) and 2.8 mM HCO_3^- (high nutrients treatment) and in the LC treatment it was 3.1 mM HCO_3^- (low nutrients treatment) and 2.5 mM HCO_3^- (high nutrients treatment), in table

S5.2 the complete dissolved inorganic carbon equilibrium is shown for all treatments. Nutrient concentrations declined throughout the first experiment (see figure S3c+e and table S5.1b). Especially nitrate had a high concentration at the start due to the high background concentrations in tap water. In the second experiment, nutrients were added regularly but disappeared rapidly from the water (see figure S3d+f and table S5.1c). The concentration of DSi in the HC treatment was 0.16 mg L^{-1} (low nutrient treatment) and 0.21 mg L^{-1} (high nutrients treatment) and in the LC treatment it was 0.14 mg L^{-1} (low nutrient treatment) and 0.24 mg L^{-1} (high nutrients treatment), see table S5.1b+c.

Growth rate and biomass allocation

The relative growth rate (RGR) appeared to be mainly affected by CO_2 and flow velocity (see table 5.3). RGR was significantly higher in plants exposed to HC compared to LC, see figure 2a, which is consistent with other studies (Xie et al. 2004, Hussner et al. 2016). Plants exposed to HC produced four times as much biomass (DM) as plants exposed to LC. Eutrophication had an (indirect) negative effect on the RGR, cancelling out the positive effect of CO_2, which makes the difference between the no climate change and the climate change scenarios relatively small. The low RGR in the HN treatment may be explained by light limitation, since the macrophytes were covered by periphytic algae which probably caused shading. In both lakes and rivers shading by periphytic algae is an important cause of a regime shift from a macrophyte to phytoplankton dominated system (Hilton et al. 2006). Although both the LN and HN treatment received the same constant flow of CO_2, in the HN treatment the measured concentration was lower and the day/night fluctuations were larger, which indicates that probably the periphytic algae consumed a substantial amount of CO_2 during the day, which means that less CO_2 was available to the macrophytes. Additionally, periphyton growth may have increased the boundary layer which made it more difficult for the plants to take up CO_2.

There was a three-way interaction between the treatments for the RGR, but as this interaction is weakly significant ($p=0.03$) and explains only a negligible part of the variance (0.1%) it will not be discussed. In all treatments with LC and HN the average RGR was negative, which may indicate carbon limitation. *B. erecta* is an homophyllous amphibious plant that is unable to take up other forms of inorganic carbon than CO_2 and therefore it needs a high concentration of CO_2 to sustain photosynthesis (Nielsen 1993). Surprisingly, the relative growth rate was also highly affected by flow velocity. 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 LC conditions (see table 5.1 and 5.2 for statistics). It seemed that the carbon limitation was more pronounced in the LF treatment than the HF treatment. This may be explained by the larger boundary layer at lower flow velocities, causing a reduction in CO_2 supply and therefore slower growth rate (Westlake 1967).

Plants exposed to HC had a lower leaf:stem ratio than plants exposed to LC, but this was only significant for plants exposed to LN. The root:shoot ratio was mainly affected by CO_2, flow and the interaction between CO_2 and nutrients (see table 5.3 and figure 5.3b). In the HN treatment the plants exposed to HC had a significantly lower root:shoot ratio than plants grown under LC. As expected, in the LN treatment, plants grown under HC had a significantly higher root:shoot ratio than plants grown under LC, (see figure 2b). This has also been observed in other studies (Madsen 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 LN treatment and the HC treatment.

Plants exposed to HF had a higher root:shoot ratio than plants exposed to LF, both in the HC and LC treatment, which can be explained by the fact that roots enable plant anchoring (Schutten et al. 2005) and thereby prevent uprooting of the plants. Still, this difference may also have been caused by increased biomass of stolons, as stolons and roots were not weighed separately. It has been hypothesised that by producing stolons and new ramets (horizontal growth) instead of elongating their stems (vertical growth), macrophytes can avoid hydrodynamic stress and improve their fitness (Puijalon et al. 2005). This idea is also supported by the fact that the stems were shorter under HF than LF and that the average leaf area in the HF treatment was smaller than in the LF treatment. This can be seen as adaptations to avoid hydrodynamic stress: by developing a more compact growth form, the macrophytes can avoid breaking or uprooting caused by the higher flow velocity, which has been observed more often in *B. erecta* (Puijalon et al. 2005).

For root:shoot ratio there was again a significant three-way interaction: under HF, plants exposed to LN showed a significant ($p<0.001$) positive effect of HC,

compared to plants exposed to HN, where there was no difference, this interaction was not present in the LF treatment (see figure S4a). Plants exposed to HC had more stolons than plants exposed to LC and plants exposed to LN had more stolons than plants exposed to HN (see figure 2c). Under HF plants grew more stolons than under LF, but this was only significant under LN. Stolons were also longer under HC than LC, longer under LN than HN and longer under HF than LF, both in average stolon length and total stolon length per plant.

In leaf, stem and root dry matter content (DMC) there was a significant interaction between CO_2 and nutrients: in the leaves and roots, plants in the LC treatment had a higher DMC than the plants in the HC treatment, but this was only significant under HN. In the stems the opposite was found: plants in the HC treatment had a higher DMC than the plants in the LC treatment, but this was only significant under LN.

Plant morphology

Most plant organs were significantly more numerous or larger under HC conditions: the number of stems and leaves, stem length, stem diameter and total dry mass were larger than under LC. In contrast, under HN conditions there were fewer leaves, shorter and thinner stems and lower total dry mass than under LN conditions. The number of leaves was most affected by CO_2, the stem length by flow and nutrients and the stem diameter by nutrients and CO_2 (see table 5.3 and figure 3a). Plants exposed to HF had more leaves and more and thicker stems than plants growing under LF, but this was only significant under LC (see figure 2d+e). There was also a strong effect of flow velocity on stem length, with the longest stems in the LF treatment (see figure 2f). The average leaf area was significantly larger in plants exposed to HC compared to LC, larger in LN compared to HN and larger in LF compared to HF, but it was most affected by nutrients (see table 5.4). The total leaf area per plant was significantly larger in HC than LC and larger in LN than HN, CO_2 and nutrients both had a relatively large effect (see table 5.4). Plants exposed to LF had a larger total leaf area in the HC treatment, whereas there was no difference in the LC treatment. Specific leaf area (SLA) was strongly affected by nutrient concentration (see table 5.4), with a higher SLA in the HN treatment than in the LN treatment (see figure 4a), which has been reported in other studies, both in aquatic (Puijalon et al. 2007) and terrestrial plants, and may be driven by competition for light (Lusk et al. 1996), in the current study this was possibly caused by periphytic algae covering the plants. Plants had a higher SLA in the LC treatment than the HC treatment, but this was only significant under LN. This

has also been found in other studies and it may occur to stimulate CO_2 uptake by increasing leaf surface area and at the same time lowering the density of photosynthetic organs (Madsen 1996).

Chlorophyll content

For chlorophyll a, chlorophyll b and total chlorophyll (a + b) concentration (mg g^{-1} FM) there were similar results. In the HFLN treatment, plants exposed to LC had a higher chlorophyll concentration (a, b and a+b) than plants exposed to HC (see figure 4b), which is consistent with many other studies (Madsen 1996, Eusebio Malheiro et al. 2013, Dülger et al. 2017). In terrestrial plants it has been hypothesised that the reduction in chlorophyll content under high CO_2 levels is caused by accumulation of starch which can damage the photosynthetic unit (Delucia et al. 1985). This may lead to a reduction in chlorophyll and Rubisco, which is an important nitrogen sink in plant leaves (Dülger et al. 2017). In the current study the plants exposed to HC had a lower N content when grown under LN, moreover in the LCLN treatment dry matter content in the stems was lower than in the other treatments, suggesting that there may be starch accumulation in the HCLN treatment. However, there was no significant difference in plant C content between HC and LC. It is remarkable that the observed high chlorophyll concentrations in the LC treatment only occurred under HF conditions and LN conditions, one of the treatments that might have given a substantial stress to the plants.

The total amount of chlorophyll per plant showed a different pattern with significantly more chlorophyll in plants exposed to HC, but only in the LF treatment (see figure 4c). Chlorophyll concentration appeared to be mainly affected by the CO_2*Nutrient interaction, but the effect was relatively small. Total plant chlorophyll was greatly affected by CO_2 and nutrients (see table 5.4). The concentration of carotenoids was significantly higher in the LC treatment, but only in plants exposed to HF.

Nutrient stoichiometry: C, N and P

The C:N ratio in the leaves was mainly affected by CO_2 (see table 5.4 and figure 5.3e) and it was significantly higher in the HC treatment than the LC treatment and this was more pronounced in the LN treatment (see figure 4d). C:N ratio also was significantly higher in the HF treatment than the LF treatment, but this was only significant in the HN treatment. Although there were significant differences in leaf C content, with significantly more C in the LC treatment than the HC treatment, but only under LN concentrations, the differences in leaf N

content were more pronounced than leaf C content. The leaf N content was highly affected by CO_2 as well (see table 5.4) and it was higher in the LC than the HC treatment (see figure 4e), which is consistent with other studies (Titus and Pagano 2002, Cheng et al. 2010, Hussner et al. 2016). A higher C:N ratio implies that the macrophytes have less nutritive value to aquatic herbivores. In terrestrial plants often C:N ratios are higher than in aquatic plants, and terrestrial herbivores often specialise with regard to plant species, plant part and time of consuming. Due to the lower C:N ratios in macrophytes, aquatic herbivores tend to have a more generalist approach (Elser et al. 2000). When C:N ratios rise due to increased CO_2 in the water herbivores may have more trouble to acquire a sufficient amount of nutrients. Still, in this experiment, higher C:N ratios were not observed in the eutrophication treatment, so the fact that there was no effect of CO_2 in the eutrophication treatment shows that the eventual effect will depend on the extent of eutrophication and CO_2 increase.

Under LF the N content was higher as well, but this was only significant under HN. Other studies looking into this found an effect of flow velocity on plant N content as well, but both in freshwater (Bal et al. 2013) and marine (Morris et al. 2008) macrophytes they found that N content was lower when plants were exposed to low flow velocity compared to high flow velocity.

In the stems, results look similar; C:N ratio is mainly affected by CO_2, but flow and nutrients had relatively more effect than on the leaves (see table 5.4 and figure 5.3d). The stem C:N ratio was significantly higher in the HC treatment, but this was more pronounced in the LN treatment. Stem C content was higher in the HF treatment, but only significant under HC. Differences in stem N content were more pronounced than stem C content; the N content in plants exposed to HC was higher than plants exposed to LC and the N content in plants exposed to LF was higher than in plants exposed to HF, but only in the HN treatment.

In contrast to what was expected there was no effect of CO_2 or flow on leaf and stem P in the LN treatment (HN could not be tested due to missing data, see M&M section). However, in the HC treatment plants exposed to LF and HN had more leaf P content than plants exposed to HF and LN, respectively. In the stems there was a similar pattern with a significant positive effect of the HN treatment, but the effect of the LF treatment was only significant under HN. The positive effect of the HN treatment on the plant P content has also been observed in other studies (Xie et al. 2005). Both in leaves and stems in the HC treatment

plants exposed to LF and HN had a lower C/P ratio than plants exposed to HF and LN, respectively.

BSi concentration

The BSi concentration in the plants in this study were about 10 times lower than what has been reported for *B. erecta* growing in the wild (Schoelynck et al. 2010), which may be explained by the relatively low Si values in the water. As was expected, flow velocity had a major effect on the BSi content (see table 5.4 and figure 5.3c), but it was remarkable that plants growing in the LF treatment had the highest BSi concentrations (except in the treatment with HN and LC, see figure 4f). There is also a three-way interaction for leaf BSi (see figure S4b): in the low CO_2 treatment, in plants exposed to low flow velocity there was a negative effect of nutrients on leaf BSi, whereas in the high CO_2 treatment, there was no effect of nutrients in both flow velocity treatments. In the stems, in plants grown under LF the BSi content was higher than under LF as well, but this was only significant under LN. In other studies plant BSi concentrations were higher under hydrodynamic stress and it was thought that this increased their tolerance to tensile forces (Schoelynck et al. 2012a, Schoelynck et al. 2015). It may be hypothesised that the different results found in the current study is caused by *B. erecta's* response to high flow velocities. As earlier explained, the plants allocate their biomass in a horizontal way, which may be a form of stress avoidance (Puijalon et al. 2011). This may imply tissue with a higher BSi content will not necessarily improve their fitness. Alternatively, it may be hypothesised that the LF treatment rather than the HF treatment was a stress to the plants due to the increased boundary layer which decreased their CO_2 and nutrient supply; since BSi can help plants to cope with stress (Schoelynck and Struyf 2016) this may be the explanation for the higher amount of BSi.

In the HN treatment, SiO_2 was added to the water and although it has been found that BSi concentrations in plants increase if there is more dissolved Si in the water (Schoelynck et al. 2012a), BSi concentrations were lower in the HN treatment than the LN treatment. Another striking result is the relatively large BSi concentration in the leaves in the LCLFLN treatment and this is difficult to explain. It may be argued that as Si can mitigate a wide range of abiotic stresses (Liang et al. 2007), so plants may have taken up more Si to cope with carbon limitation, which was probably more severe than in the other treatments due to increased boundary layers. Still, this does not explain why the plants in the HN treatment (which were on top of the other stresses also exposed to

eutrophication stress) had lower BSi concentrations. On the other hand, in the LN treatment nutrients levels decreased to low levels; Emsens et al. (2016) also found that wetland plants have a lower Si concentration when exposed to eutrophication and they suggest that this may be caused by nutrient stress relief.

Differences over time

In the PRC diagram (see figure S5) the effect of the treatments over time is shown on eight plant traits that have been measured four times during the experiment: 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. 18% of the treatment variance could be explained by the model (F=47.625, p=0.001). All plant traits had negative weights, indicating a negative relationship with the treatments in the diagram. This means that, especially towards the end of the experiment, all plant traits were favoured by most treatments except for the LCLFHN and LCHFHN treatments. The differences between the treatments become more pronounced at the end of the experiment.

Supplementary tables

Table S5.1 Nutrient concentrations in the water at the start of the experiment (a); nutrients and D-Si concentrations in the first experiment with low nutrient concentrations (b) and in the second experiment with high nutrient concentrations (c).

a	PO4-P	NH4-N	NO2-N	NO3-N
	(mg P L^{-1})	(mg N L^{-1})	(mg N L^{-1})	(mg N L^{-1})
	0.002	0.031	0.002	2.308

b	HCHF	PO4-P	NH4-N	NO2-N	NO3-N	D-Si
Day	Date	(mg P L^{-1})	(mg N L^{-1})	(mg N L^{-1})	(mg N L^{-1})	(mg L^{-1})
6	24/05/2017	0	0.13	0.001	2.259	na
20	7/06/2017	0	0	0	2.109	na
50	7/07/2017	0.007827	0.005435	0	0.404355	0.16
67	24/07/2017	0.005	0.01	0	0.03	na

	LCHF	PO4-P	NH4-N	NO2-N	NO3-N	D-Si
Day	Date	(mg P L^{-1})	(mg N L^{-1})	(mg N L^{-1})	(mg N L^{-1})	(mg L^{-1})
6	24/05/2017	0	0.11	0.004	2.756	na
20	7/06/2017	0	0	0	2.4	na
50	7/07/2017	0.00587	0.008541	0	1.01405	0.14
67	24/07/2017	0	0.03	0	0.26	na

	HCLF	PO4-P	NH4-N	NO2-N	NO3-N	D-Si
Day	Date	(mg P L^{-1})	(mg N L^{-1})	(mg N L^{-1})	(mg N L^{-1})	(mg L^{-1})
6	24/05/2017	0	0.08	0.001	2.269	na
20	7/06/2017	0	0	0	2.14	na
50	7/07/2017	0.005218	0.009318	0	0.401644	0.16
67	24/07/2017	0	0.01	0	0	na

	LCLF	PO4-P	NH4-N	NO2-N	NO3-N	D-Si
Day	Date	(mg P L^{-1})	(mg N L^{-1})	(mg N L^{-1})	(mg N L^{-1})	(mg L^{-1})
6	24/05/2017	0	0	0	2.77	na
20	7/06/2017	0	0.06	0	2.39	na
50	7/07/2017	0.006849	0.010094	0	1.015857	0.14
67	24/07/2017	0	0.01	0	0.26	na

c	HCHF	PO4-P	NH4-N	NO2-N	NO3-N	D-Si
Day	Date	(mg P L^{-1})	(mg N L^{-1})	(mg N L^{-1})	(mg N L^{-1})	(mg Si L^{-1})
15	16/08/2017	0.00	0.00	0.00	0.01	0.23
20	21/08/2017	0.01	0.02	0.01	0.03	0.21
30	31/08/2017	0.03	0.00	0.00	0.81	0.20
42	12/09/2017	0.00	0.03	0.00	0.00	0.19
48	18/09/2017	0.00	0.00	0.00	0.24	0.21
57	27/09/2017	0.00	0.00	0.00	0.00	0.21
66	6/10/2017	0.06	0.04	0.00	2.36	0.18

	LCHF	PO4-P	NH4-N	NO2-N	NO3-N	D-Si
Day	Date	(mg P L^{-1})	(mg N L^{-1})	(mg N L^{-1})	(mg N L^{-1})	(mg Si L^{-1})
15	16/08/2017	0.00	0.00	0.00	0.01	0.24
20	21/08/2017	0.00	0.02	0.01	0.00	0.22
30	31/08/2017	0.00	0.00	0.00	0.82	0.20
42	12/09/2017	0.00	0.00	0.00	0.00	0.23
48	18/09/2017	0.00	0.00	0.00	0.85	0.26
57	27/09/2017	0.00	0.00	0.00	0.47	0.26
66	6/10/2017	0.09	0.07	0.00	3.22	0.23

	HCLF	PO4-P	NH4-N	NO2-N	NO3-N	D-Si
Day	Date	(mg P L^{-1})	(mg N L^{-1})	(mg N L^{-1})	(mg N L^{-1})	(mg Si L^{-1})
15	16/08/2017	0.00	0.00	0.00	0.02	0.24
20	21/08/2017	0.01	0.01	0.02	0.02	0.22
30	31/08/2017	0.01	0.00	0.00	0.83	0.21
42	12/09/2017	0.00	0.00	0.00	0.00	0.19
48	18/09/2017	0.00	0.00	0.00	0.26	0.2
57	27/09/2017	0.00	0.00	0.00	0.00	0.21
66	6/10/2017	0.06	0.05	0.00	2.49	0.18

	LCLF	PO4-P	NH4-N	NO2-N	NO3-N	D-Si
Day	Date	(mg P L^{-1})	(mg N L^{-1})	(mg N L^{-1})	(mg N L^{-1})	(mg Si L^{-1})
15	16/08/2017	0.00	0.00	0.00	0.01	0.24
20	21/08/2017	0.00	0.04	0.01	0.00	0.23
30	31/08/2017	0.00	0.00	0.00	0.82	0.20
42	12/09/2017	0.00	0.00	0.00	0.00	0.23
48	18/09/2017	0.00	0.00	0.00	0.87	0.26
57	27/09/2017	0.00	0.00	0.00	0.49	0.26
66	6/10/2017	0.09	0.06	0.00	3.51	0.24

Table S5.2 Inorganic carbon concentrations in the different CO_2 and nutrients treatments expressed in total dissolved organic carbon concentration (DIC), CO_2, HCO_3 and CO_3, and the pH of the water. All values are averages of the total time the experiment was running. DIC is lower in the HC-LN treatment than in the LC-LN treatment. This may have been based on a wrong measurement, as it is based on alkalinity which was measured only once in the LN treatments. The CO_2 and the pH values are more reliable, because they are based on more data points.

	DIC (mmol L^{-1})	CO_2 (mmol L^{-1})	HCO_3 (mmol L^{-1})	CO_3 (mmol L^{-1})	pH
HC-LN	3.86	0.062	3.71	0.000089	7.95
LC-LN	4.39	0.019	4.03	0.35	8.5
HC-HN	3.22	0.034	3.08	0.11	8.13
LC-HN	2.42	0.007	2.14	0.27	8.66

Supplementary figures

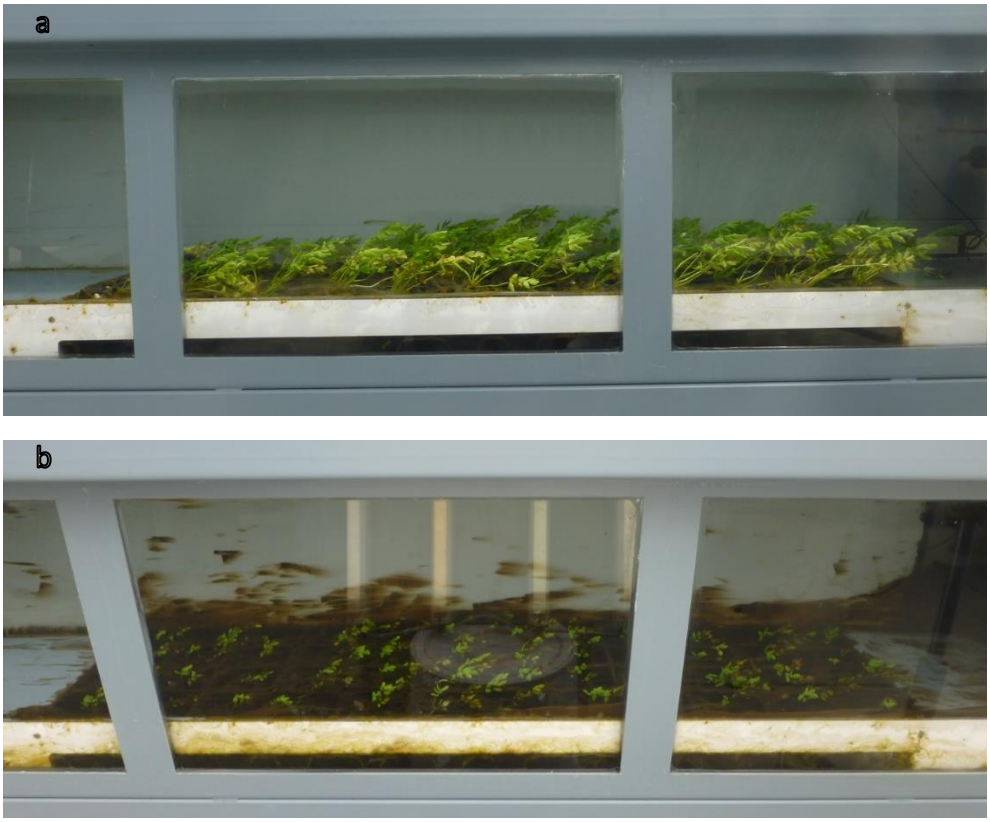

Figure S5.1 the LCHFLN treatment on day 57 of the experiment (a) and the LCHFHN treatment on day 52 of the experiment (b). Note the difference in algae growth.

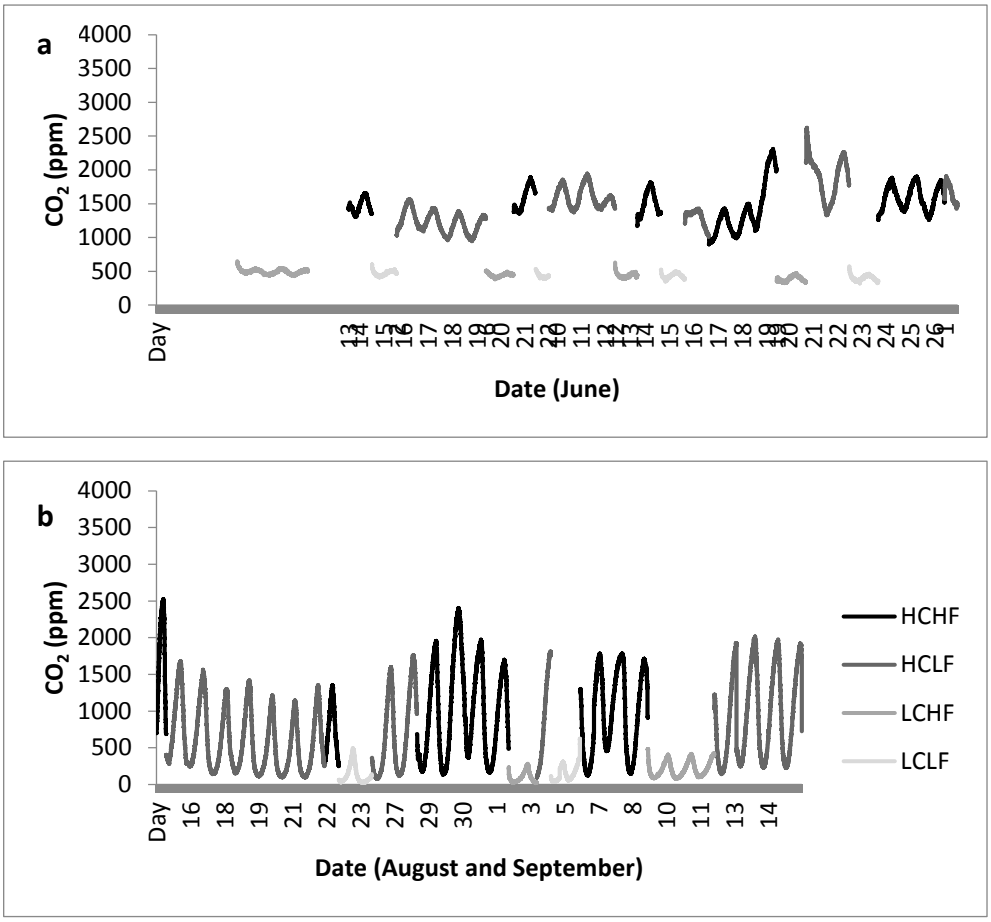

Figure S5.2 CO_2 concentrations in the flumes in the first experiment with the low nutrient treatment (a) and in the second experiment with the high nutrient treatment (b). Data are discontinuous because the probe alternated between the 4 different flumes.

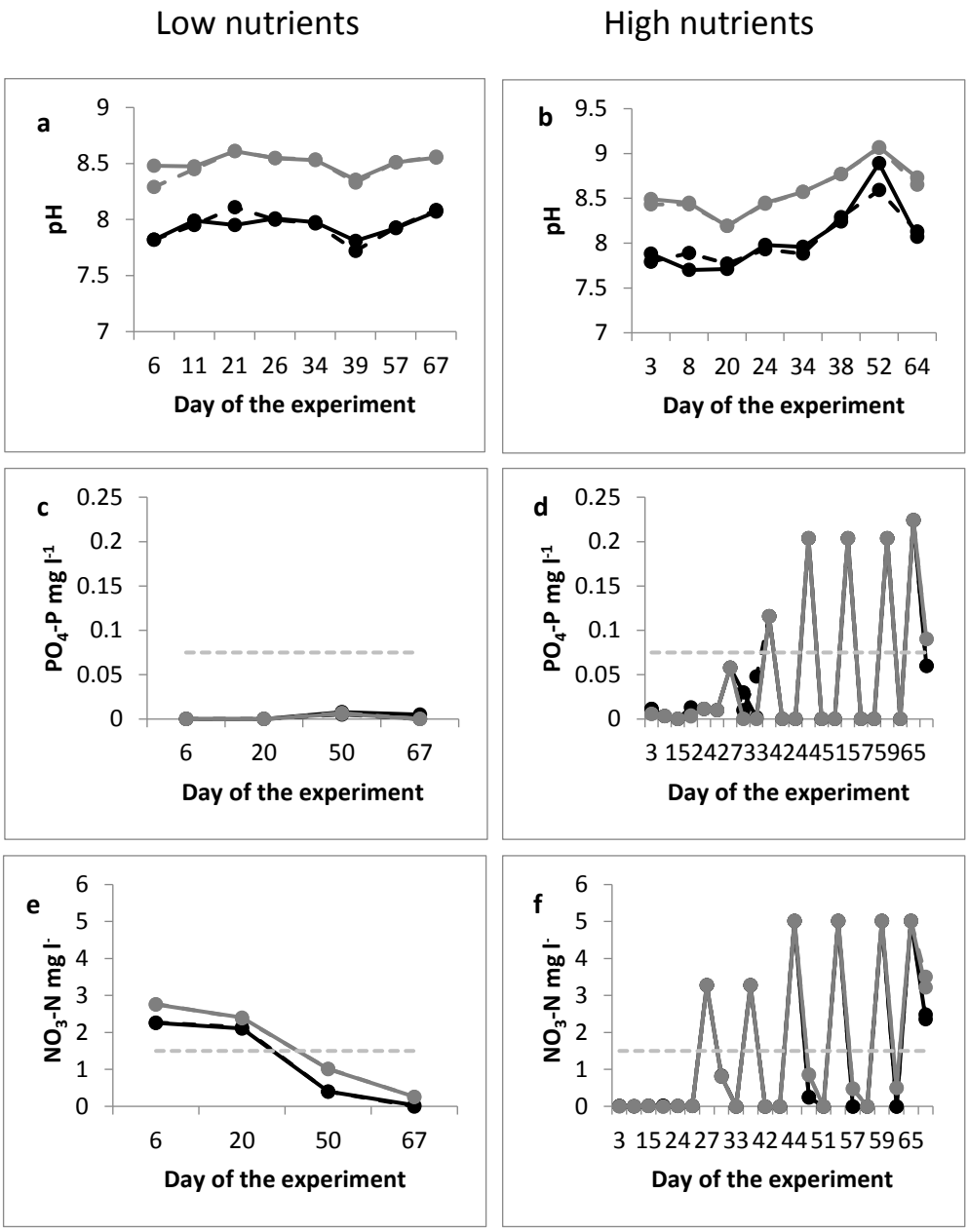

Figure S5.3 pH (a+b), PO_4-P (c+d) and NO_3-N (e+f) in the first experiment with low nutrient concentrations (a+c+e) and in the second experiment with high nutrient concentrations (b+d+f). The dashed line shows P eutrophication (c+d) and N eutrophication (e+f)(Smith et al. 1999).

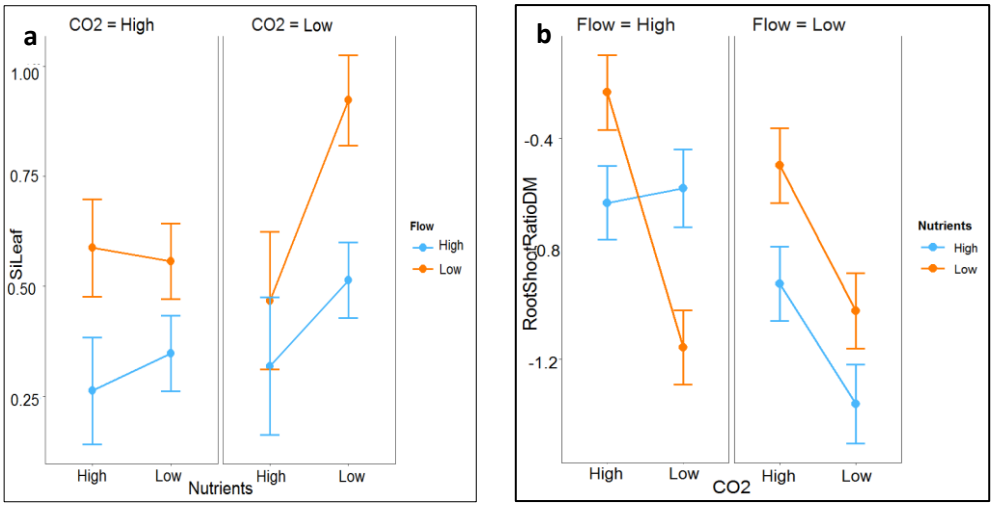

Figure S5.4 Three-way interactions between CO_2, flow and nutrients for the log of the root:shoot ratio (a) and the log of the leaf BSi content (b).

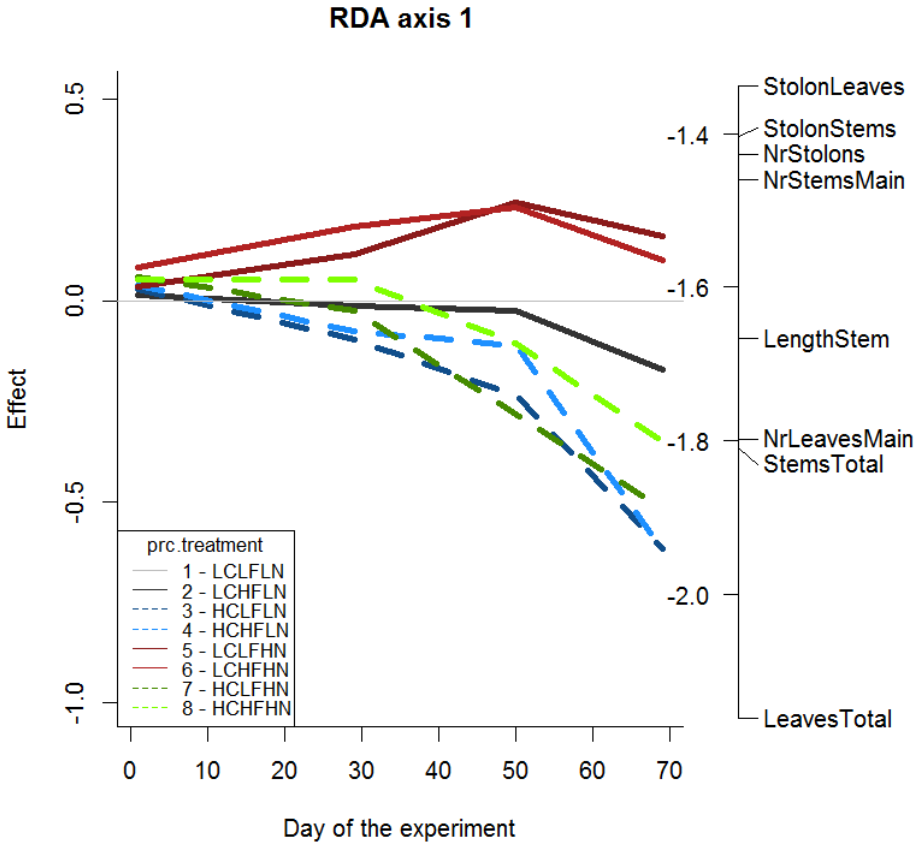

Figure S5.5 PRC with the effects of the treatments on eight plant traits. On the right vertical axis the response variables are shown (number of stems and leaves on the main plant, stems and leaves that grew on the new ramets on the stolons, total number of stems and leaves, number of stolons and length of the longest stem) and their relative contribution to the observed pattern. The treatment with low CO_2 concentration, low flow velocity and a low nutrient concentration is set as a reference situation (the horizontal grey line) and the effects of the other treatments are compared to this reference.

Chapter 6.

Response of submerged macrophyte growth, morphology, chlorophyll content and nutrient stoichiometry to increased flow velocity and elevated CO_2 and dissolved organic carbon concentrations

Rosanne E. Reitsema, Jan-Willem Wolters, Stefan Preiner, Patrick Meire, Thomas Hein, Gudrun De Boeck, Ronny Blust, Jonas Schoelynck

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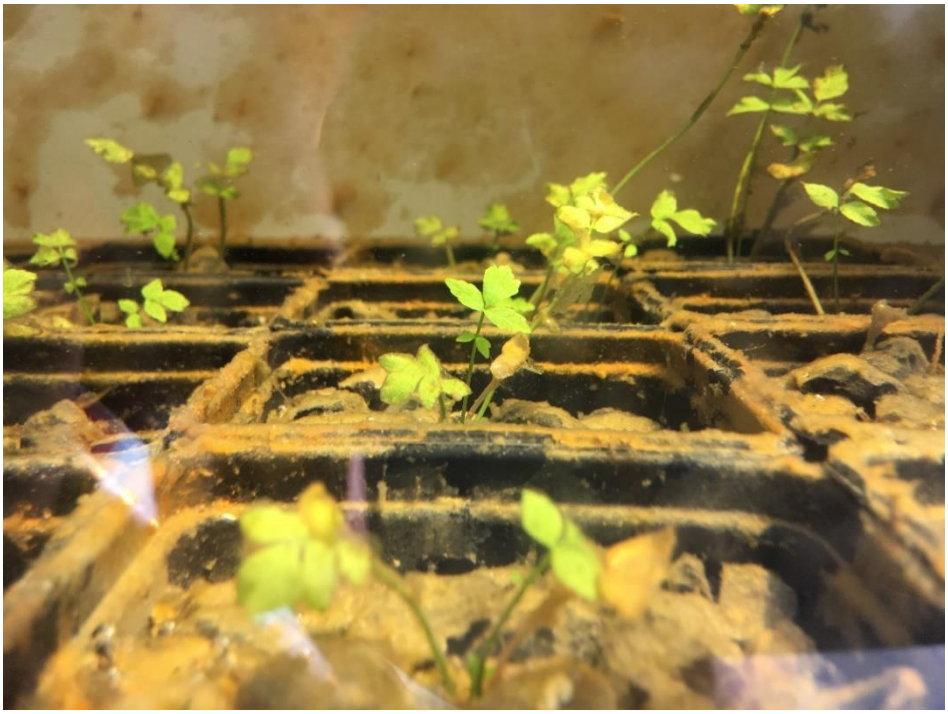

Abstract

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_2 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_2 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_2 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_2 concentrations and a dry scenario simulating evapotranspiration with low flow velocity, high DOC and high CO_2 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 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 (Puijalon 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' (Kritzberg 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_2) 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 yr^{-1}, and this is increasing with 0.3 Pg yr^{-1} (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 (Grigutytė 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 (see chapter 3).

Upon degradation, DOC can also be a source of CO_2 (Sobek et al. 2005). Mainly due to the high quantity of carbon entering from terrestrial soil or wetlands the world average CO_2 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_2 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_2 efflux from the water as a result of higher atmospheric CO_2 concentrations, caused by a rise in CO_2 emissions (Phillips et al. 2015). It is difficult to predict future CO_2 levels in freshwater ecosystems because the exact

factors controlling aquatic CO_2 concentrations and their response to climate change are not yet well understood. Moreover, current CO_2 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_2 levels and how freshwater organisms will respond (Hasler et al. 2016). Research on the effects of CO_2 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_2 concentrations on freshwater macrophytes observed increased plant growth rates under high CO_2 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 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 1996, Dülger et al. 2017), their dry matter content higher (Eusebio Malheiro et al. 2013) and specific leaf area (SLA) lower (Madsen 1996).

Although the separate effects of varying flow velocity, increased DOC and increased CO_2 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 (Szmeja and Bociąg 2004), whereas macrophytes exposed to high CO_2 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 (Puijalon 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_2 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_2 concentrations, and a dry scenario with low flow velocity, high DOC and high CO_2 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_2 concentration. In contrast, we hypothesised that DOC would partially counteract the effect of elevated CO_2 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_2 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_3^-) and CO_2), *B. erecta* can only take up CO_2 (Sand-Jensen et al. 1992), so we expected that this species would respond strongly to changes in CO_2 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 grainsize 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^{-1} phosphorus (PO_4^{3-}-P), 0.03 mg L^{-1} ammonium (NH_4^+-N), 0.002 mg L^{-1} nitrite (NO_2^--N) and 2.308 mg L^{-1} nitrate (NO_3^--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^{-1}) and the other two flumes to low flow velocity (0.04 m s^{-1}), 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, afterwards the average was calculated. Moreover, CO_2 gas from a commercial bottle was added to two flumes with an airstone at approximately 2 L h^{-1} (gas pressure 2 bar). Gas flux was regulated with a Skalar GT1355 Sho-Rate G flowmeter. This resulted in four different treatments: high CO_2 (1000 ppm) with high flow velocity (HC-HF), high CO_2 with low flow velocity (HC-LF), low CO_2 (400 ppm) with high flow velocity (LC-HF) and low CO_2 with low flow velocity (LC-LF). The experiment was done between 19 May and 24 July 2017 without any added DOC (low DOC or LD treatment) and the experiment was repeated the year after between 24 May and 31 July 2018, this time DOC was added to all treatments (high DOC or HD treatment), see figure 6.1 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^{-2} (224 W m^{-2} d^{-1}); in 2018 this was 4.65 MW m^{-2} (237 W m^{-2} d^{-1}).

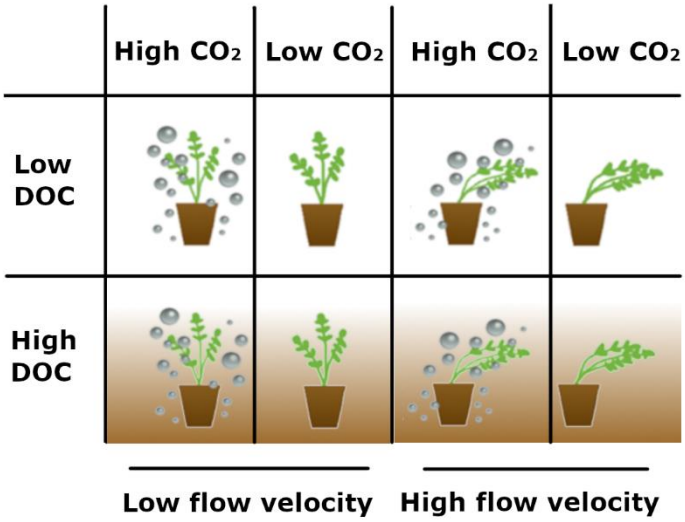

Figure 6.1 Schematic overview of the experimental setup. The experiment was carried out in four flumes with each their own combination of treatments: high CO_2 × low flow velocity, low CO_2 × low flow velocity, high CO_2 × high flow velocity, and low CO_2 × high flow velocity. This experiment was then repeated with the same treatments, but this time DOC was added to all flumes.

DOC

In this study it was decided to use leaf and peat leachate as DOC source, like in chapter 4. 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. It was chosen to use 5 mg C L^{-1}, as in chapter 4 *B. erecta* did not grow well in high concentrations of DOC. 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^{-1}.

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_2 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_2 in the water was measured continuously with a Pro-Oceanus Digital Mini CO_2 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, The 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 0.45µm filters (Chromafil® Xtra MV-45/25, Macherey-Nagel, Düren, Germany) and the concentration of PO_4^{3-}-P, NH_4^+-N, NO_2^--N, NO_3^--N was measured (SAN++, Skalar, Breda, The 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 clouded 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 hours 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 figure S6.1), 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 (figure S6.1). The dissolved CO_2 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 (figure S6.2), 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 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 (9 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, Massachusetts, USA). P content was determined by acid digestion and subsequently measured on ICP-OES (iCAP 6300 Duo view, Thermo Fisher, Waltham, Massachusetts, USA).

Statistical analyses

All statistical analyses were carried out in R statistics version 3.4.3. The effects of elevated CO_2, DOC and flow velocity on growth and morphology parameters, chlorophyll, C, N and P content (35 traits in total, table 6.1 and 6.2) 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 (ω^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 ω^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_2, low DOC and low flow velocity.

Results

CO_2 and DOC concentrations

The average CO_2 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 (figure S6.2). 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^{-1}), whereas in the second experiment, where DOC was added regularly, it reached a reasonably constant value of 5.9 ± 0.8 mg L^{-1} (figure S6.3a). In the stock solution of DOC, the amount of nutrients was relatively high, especially phosphate and ammonium. In a DOC solution of 5 mg C L^{-1} there was 0.29 mg L^{-1} phosphate (PO_4^{3-}-P) and 0.87 mg L^{-1} ammonium (NH_4^+-N). However, in the flumes the measured concentrations were far lower (figure S6.4), 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 ± 28.5 µmol m^{-2} s^{-1} just below the water surface to 163 ± 22.7 µmol m^{-2} s^{-1} at the bottom of the flumes (figure S6.3b). PAR availability decreased with 23.8 % in water with increased DOC concentrations (measured in a 20 cm deep bucket, see materials and methods). For more details on water quality (pH, alkalinity and nutrients) see supplementary results and discussion and figure S6.4.

Effects of the treatments and interactions

Flow velocity, 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 CO_2, in 13 traits a significant effect of flow, in 20 a significant effect of the CO_2*DOC interaction, in 14 a significant effect of the CO_2*flow interaction, in 9 a significant effect of the flow*DOC interaction and in 9 traits a three-way interaction (table 6.1 and 6.2). When looking at the relative importance of the treatments (omega squared values), in most cases DOC and CO_2 had the greatest effect, relative to the other treatments, followed by the CO_2*DOC treatment. Flow velocity and the other interactions had lower omega squared values in most traits (table 6.3 and 6.4).

Macrophyte growth and morphology

In the following paragraphs the main results will be highlighted, for a more detailed overview of the results, see supplementary results and discussion. CO_2 and DOC had a pronounced effect on the relative growth rate (RGR), (table 6.3) which was significantly higher in plants exposed to HC compared to LC and higher in LD compared to HD (figure 6.2a and figure 6.3a). 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 (table 6.1 and 6.2). 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 CO_2, and to a smaller extent by the CO_2*DOC interaction (table 6.3, figure 6.2b). This can be seen in the LD treatment, where there is a positive effect of CO_2 on root:shoot ratio, whereas there is no CO_2 effect in the HD treatment. Moreover, the high flow, high CO_2 and low DOC treatments resulted in more and longer stolons (figure 6.2c). In most morphological traits a positive effect of CO_2 and a negative effect of DOC was observed. This was most pronounced in the number of leaves, (figure 6.2d and table 6.3). The number of stems, stem length, stem diameter (figure 6.2e), 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 (figure 6.2d+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 6.4a). The clearest effect of flow velocity was observed in the stem length, with the longest stems in the LF treatment (figure 6.2f and figure 6.3b).

Table 6.1 F-values of the three-way ANOVA tests and z values of the generalized linear models of growth and morphological parameters (n = 48). 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.

	CO_2	Flow	DOC	C*F	C*D	F*D	C*F*D
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	-0.1	0.45	-2.03*	ns	ns	ns	ns

Signif. codes: * <0.05, ** 0.01 <0.01 *** <0.001

Table 6.2 F-values of the three-way ANOVA tests of morphological parameters, chlorophyll and nutrient stoichiometry parameters (n = 5 to 10). Interaction effects that were not significant have been removed from the model (ns). Some variables have been transformed: % N leaves: x^{-1}, % N stems: logx^(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: $logx^{1/3}$, 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.

	CO_2	Flow	DOC	C*F	C*D	F*D	C*F*D
% 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***	1.7	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

Signif. codes: * <0.05, ** <0.01 *** <0.001

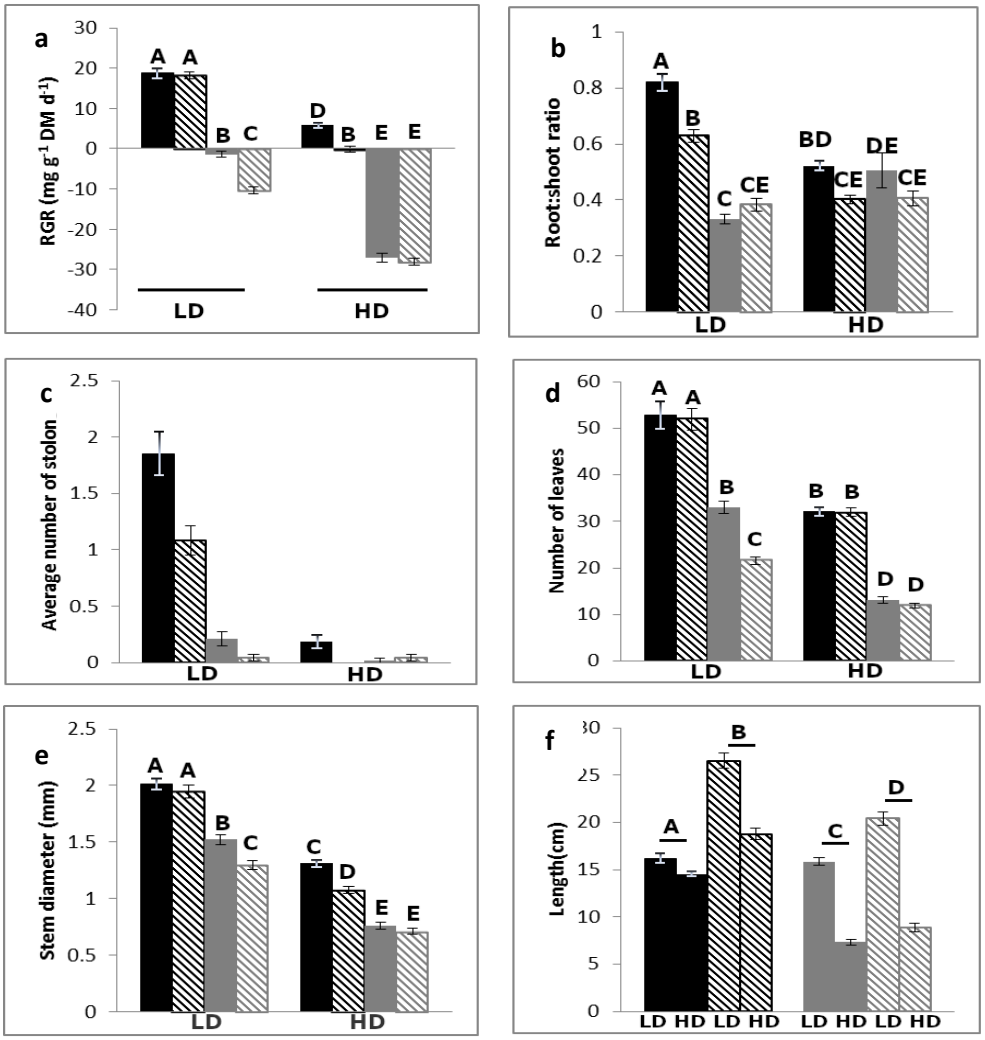

Figure 6.2 Relative growth rate (a), root:shoot ratio (b), number of stolons (c), number of leaves (d), stem diameter (e), and length of the longest stem (f). The letters above the graph indicate significant differences ($p < 0.05$, n = 48), tested with three-way ANOVA. In panel a, b, d and e three-way interactions are shown, in panel c there were no interactions, just main effects of flow velocity, carbon and DOC, and in panel f a CO_2*Flow interaction.

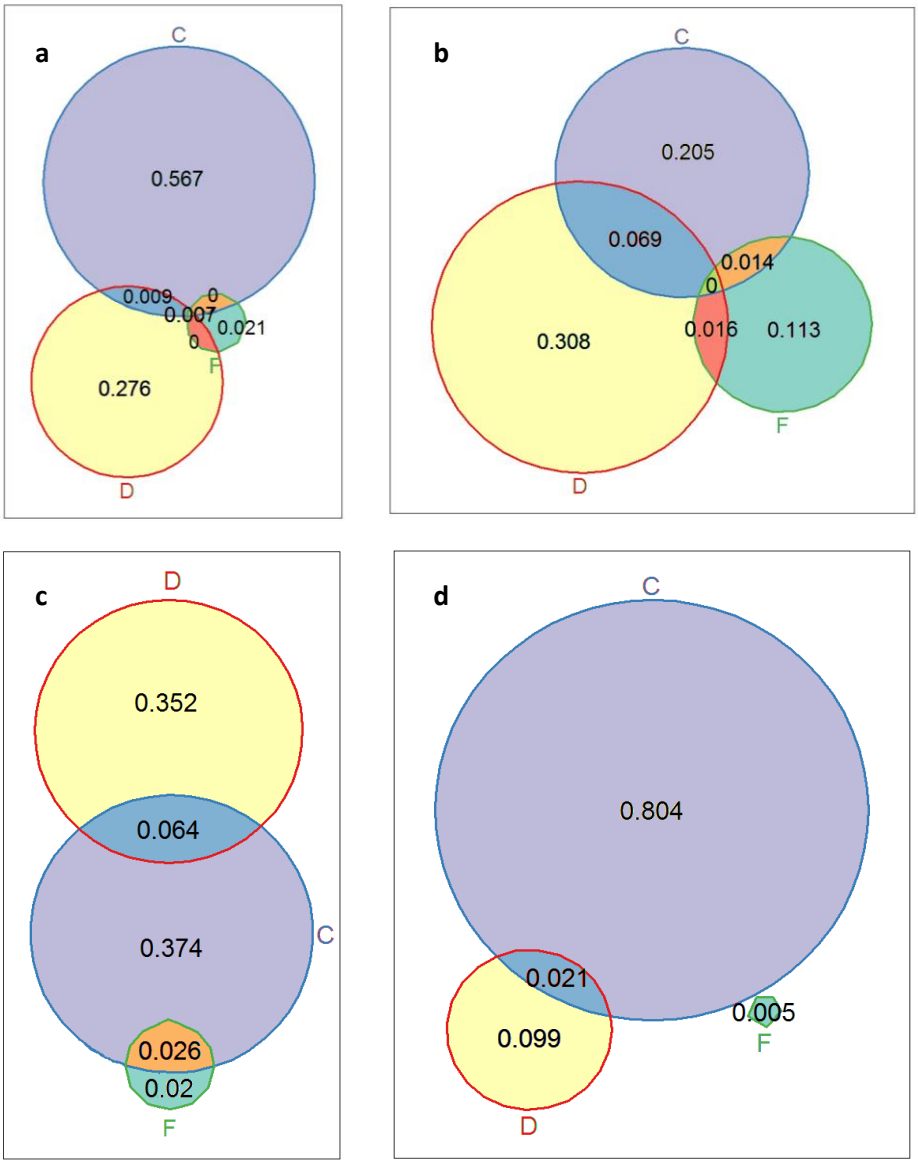

Figure 6.3 Venn diagrams showing omega squared values representing the proportion of variance explained by CO_2 (C), flow (F), DOC (D) and their interactions for the variables relative growth rate (a), length of the longest stem (b), total plant chlorophyll (c) and leaf C:N ratio (d).

Chlorophyll and nutrient stoichiometry

For chlorophyll a, chlorophyll b and total chlorophyll (a + b) concentration (mg g^{-1} FM) similar results were observed. Plants growing in the LC-HF treatment had a higher chlorophyll concentration than plants growing in other treatments (figure 6.4b). 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 (figure 6.4c and figure 6.3c), as the plants in the HC-LD 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 6.4d & table 6.2). The 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 6.4, figure 6.3d and figure 6.4e+f), especially in the LD treatment.

Table 6.3 Omega squared values for the growth and morphological parameters (n=48)

	CO_2	**Flow**	**DOC**	**C*F**	**C*D**	**F*D**	**C*F*D**
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 6.4 Omega squared values for the morphological parameters, chlorophyll and nutrient stoichiometry parameters (n = 5 to 10)

	CO_2	**Flow**	**DOC**	**C*F**	**C*D**	**F*D**	**C*F*D**
% 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
TotalArea	0.401	0	0.413	0.009	0.03	0	0
MeanArea	0.271	0.002	0.451	0	0.089	0.013	0
SLATotal	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

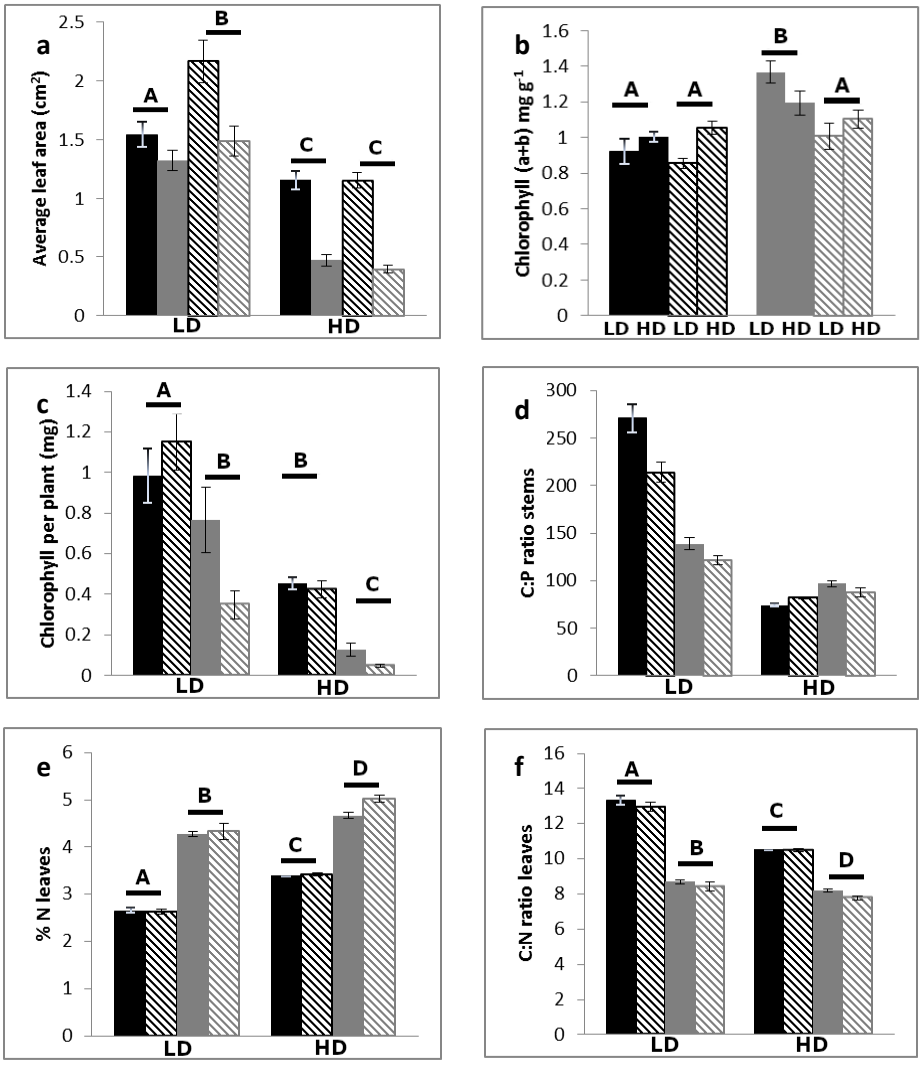

Figure 6.4 Average leaf area (a), total chlorophyll concentration (b), total amount of chlorophyll per plant (c), C:P ratio of the stems (d), % N in the leaves (e), and C:N ratio of the leaves (f). The letters above the graph indicate significant differences ($p < 0.05$, $n = 5$-10), tested with three-way ANOVA. In panel A, C, E and F, CO_2*DOC interactions are shown, in panel B a CO_2*flow interaction and in panel D there were no interactions.

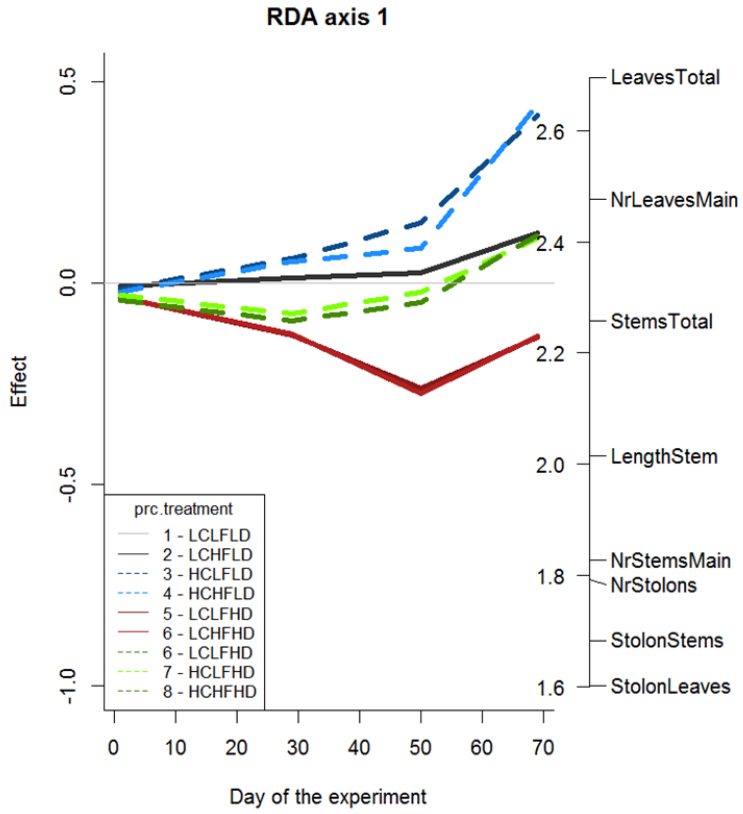

Figure 6.5 Principal response curve (PCR) with the effects of the treatments on eight plant traits. On the right vertical axis the response variables are shown (number of stems and leaves on the main plant, stems and leaves that grew on the new ramets on the stolons, total number of stems and leaves, number of stolons and length of the longest stem) and their relative contribution to the observed pattern. The treatment with low CO_2 concentration, low flow velocity and a low DOC concentration is set as a reference situation (the horizontal grey line) and the effects of the other treatments are compared to this reference.

Differences over time

For eight plant traits that have been measured four times during the experiment a PRC diagram was made (figure 6.5) 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 towards 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 towards the end of the experiment. While 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 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. 2008, McElarney et al. 2010, Cao and Ruan 2015). 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_2 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_2 concentrations in the HD-HC treatment were lower than in the LD-HC, possibly caused by growth of epiphytic algae on the macrophytes (figure S6.1). *B. erecta* is an homophyllous amphibious plant that is unable to take up other forms of inorganic carbon than CO_2 and therefore it needs a high concentration of CO_2 to sustain photosynthesis (Nielsen 1993).

The higher root:shoot ratio under HC has also been observed in other studies (Madsen 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* (Puijalon 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_2 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_2 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 (figure S6.4d).

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_2 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_2. However, it should be taken into account that most rivers and streams are supersaturated with CO_2 (Raymond et al. 2013), so *in situ* concentrations are likely always higher than the ones used in this experiment. Aquatic CO_2 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_2 production (Sobek et al. 2005), this was not observed in this experiment: CO_2 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_2 was consumed fast. The macrophytes may have taken up this CO_2, 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 (figure S6.1). 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.

DOC 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 natans* (Lour.) 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_2 and limited by DOC, and there were strong interaction effects between those two stressors. As CO_2 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_2 and DOC. However, in this study the combined effects of CO_2 and DOC on total dry biomass 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_2. 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_2 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.

Addendum

DOC used in this study had a high phosphate concentration, the same source was used as in chapter 4. It is possible that the results were affected by this high phosphate level. When looking at the P concentrations within the plants, both in the high nutrient treatment in chapter 5 and in the high DOC treatment in this chapter there is a higher P content than in the treatment without nutrients or DOC added, but this difference is bigger in the DOC treatment. Most plant traits responded similarly to increased nutrients and increased DOC. Possibly, the mechanisms were similar: both nutrients and DOC stimulated periphytic algae that shaded the macrophytes and probably competed with them for

nutrients and inorganic carbon. In figure S6.4 it can be seen that nutrients were taken up very quickly. It is likely that periphyton played an important role in this, and that macrophytes even experienced nutrient limitation. Even though effects of DOC and nutrients were similar, some responses were only observed in the DOC treatment. The largest difference was the occurrence of stolons. In the DOC treatment plants produced a far lower number of stolons than in the nutrients treatment in chapter 5. In other studies, it was found that combined high N and P levels can reduce stolon biomass (Wersal and Madsen 2011), but no effects are known of high P levels on stolon formation. Another difference was observed in the plant N content. Increased CO_2 had a negative result on plant N content, but this effect was greatly reduced when the plants were exposed to high nutrient levels. In the DOC treatment this was not the case, which can be explained by the lower nitrate concentration in the DOC compared to the nutrient treatment.

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Supplementary results and discussion

Water quality

As a result of the CO_2 treatment, the pH was lower in the HC treatment: on average 7.95 ± 0.11 (low nutrients treatment) and 7.97 ± 0.25 (high DOC treatment) and in the LC treatment it was 8.50 ± 0.09 (low nutrients treatment) and 8.34 ± 0.27 (high DOC treatment), see figure S4a+b. The alkalinity in the HC treatment was 2.9 mM HCO_3^- (low DOC treatment) and 2.5 mM HCO_3^- (high DOC treatment) and in the LC treatment it was 3.1 mM HCO_3^- (low DOC treatment) and 2.2 mM HCO_3^- (high DOC treatment). Nutrient concentrations declined throughout the experiment (see figure S4c-e). Especially nitrate had a high

concentration at the start due to the high background concentrations in tap water.

Plant morphology

Plant morphology was mainly affected by CO_2 and DOC (number of leaves, stem length and diameter, average leaf area), whereas the effects of flow velocity were less pronounced, except for stem length (see table 3 and 4 and figure 3b). Most plant organs were significantly more numerous or larger under HC conditions: the number of stems and leaves (see figure 2d), stem length, stem diameter (see figure 2e) and total dry mass were larger than under LC. In contrast, under HD conditions there were fewer leaves, thinner stems and lower total dry mass than under LD conditions, which has also been found in other studies (Szmeja and Bociąg 2004). In contrast to what was expected, stem length was shorter under HD conditions, whereas increased stem length has been reported as response to shading in macrophytes (Olesen et al. 2002). High flow velocity had a negative effect on stem length (see figure 2f). The average leaf area was significantly larger in plants exposed to HC compared to LC, larger in LD compared to HD and larger in LF compared to HF in the LD treatment (see figure 4a), but overall, the average leaf area was most affected by DOC (see table 4). The total leaf area per plant was significantly larger in HC than LC and larger in LD than HD, CO_2 and DOC both had a relatively large effect (see table 4). Statistical tests for the specific leaf area (SLA) could only be carried out for the flow*DOC interaction in the HC treatment and for CO_2*flow interaction in the LD treatment as there was not enough material in the LC-HD treatment. SLA was mainly affected by DOC (see table 4), with a higher SLA in the HD treatment than in the LD treatment, which is regarded as a plastic response to shading (Olesen et al. 2002). In the LD treatment, plants had a higher SLA in the LC treatment than the HC treatment. This has also been found in other studies and it may occur to stimulate CO_2 uptake by increasing leaf surface area and at the same time lowering the density of photosynthetic organs (Madsen 1996).

Chlorophyll content

For chlorophyll a, chlorophyll b and total chlorophyll (a + b) concentration (mg g^{-1} FM) there were similar results. In the HF-LD treatment, plants exposed to LC had a higher chlorophyll concentration (a, b and a+b) than plants exposed to HC (see figure 4b), which is consistent with many other studies (Madsen 1996, Eusebio Malheiro et al. 2013, Dülger et al. 2017). In terrestrial plants it has been hypothesised that the reduction in chlorophyll content under high CO_2 levels is caused by accumulation of starch which can damage the photosynthetic unit

(Delucia et al. 1985). This may lead to a reduction in chlorophyll and Rubisco, which is an important nitrogen sink in plant leaves (Dülger et al. 2017). In the current study the plants exposed to HC had a lower N content when grown under LD, however, there was no evidence of starch accumulation; there was no significant difference plant C content between HC and LC. When macrophytes are exposed to shading, this often results in a decreased chlorophyll a/b ratio (Andersen et al. 2005, Eller et al. 2015), in this experiment this was only observed as well: the chlorophyll a/b ratio was lower in the HD treatment compared to the LD treatment, but this was only the case in the LC treatment, in the HC treatment it was the other way around. The total amount of chlorophyll per plant showed a different pattern with significantly more chlorophyll in plants exposed to HC and less chlorophyll in plants exposed to HD (see figure 3c and figure 4c and table 4).

Nutrient stoichiometry: C, N and P

The nutrient stoichiometry was strongly affected by the treatments: N and C mainly by the CO_2 treatment and P mainly by the DOC treatment (see table 3 and 4). DOC had a relatively large positive effect on the P content, especially in the stems, leading to reduced C:P and N:P ratios in the HD treatment (see figure 4d). In literature the opposite was found: high DOC causes light limitation which results in elongated plant stems with a relatively high C and low P content (Su et al. 2016). The high amount of P in the HD treatment may be explained by phosphate leaching from the DOC that was added to the flumes. After adding DOC a large phosphate peak was observed (see figure S4d).

Leaf and stem N content were higher in the LC treatment than in the HC treatment (see figure 4e), which is consistent with other studies (Titus and Pagano 2002, Cheng et al. 2010, Hussner et al. 2016). This also resulted in a higher C:N ratio in the high CO_2 treatment compared to the low CO_2 treatment (see figure 3d and figure 4f), which makes the plant tissue less nutritive and this can have consequences for aquatic herbivores that consume macrophytes. C:N ratios in terrestrial plants are usually higher than in aquatic plants, and terrestrial herbivores have adapted to this by specialising with regard to which species and plants parts they consume and their timing of consumption. Since in aquatic plants C:N ratios tend to be lower, aquatic herbivores often show a more generalist approach (Elser et al. 2000), which implies that it may become more difficult for aquatic herbivores to acquire enough nutrients under increased CO_2 levels.

Supplementary figures

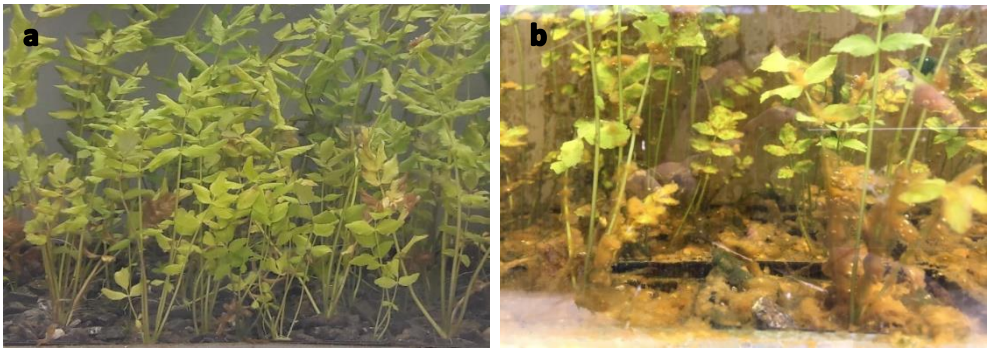

Figure S6.1 the HCLFLD treatment on day 67 of the experiment (A) and the HCLFHD treatment on day 65 of the experiment (B). Note the difference in algae growth.

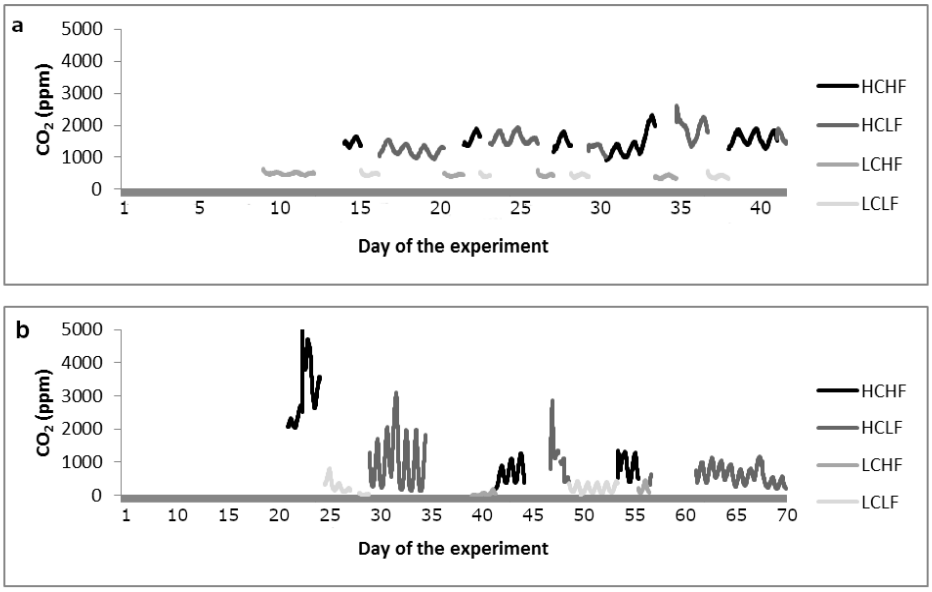

Figure S6.2 CO_2 concentrations in the flumes in the first experiment with the low DOC treatment (a) and in the second experiment with the high DOC treatment (b). Data are discontinuous because the probe alternated between the 4 different flumes.

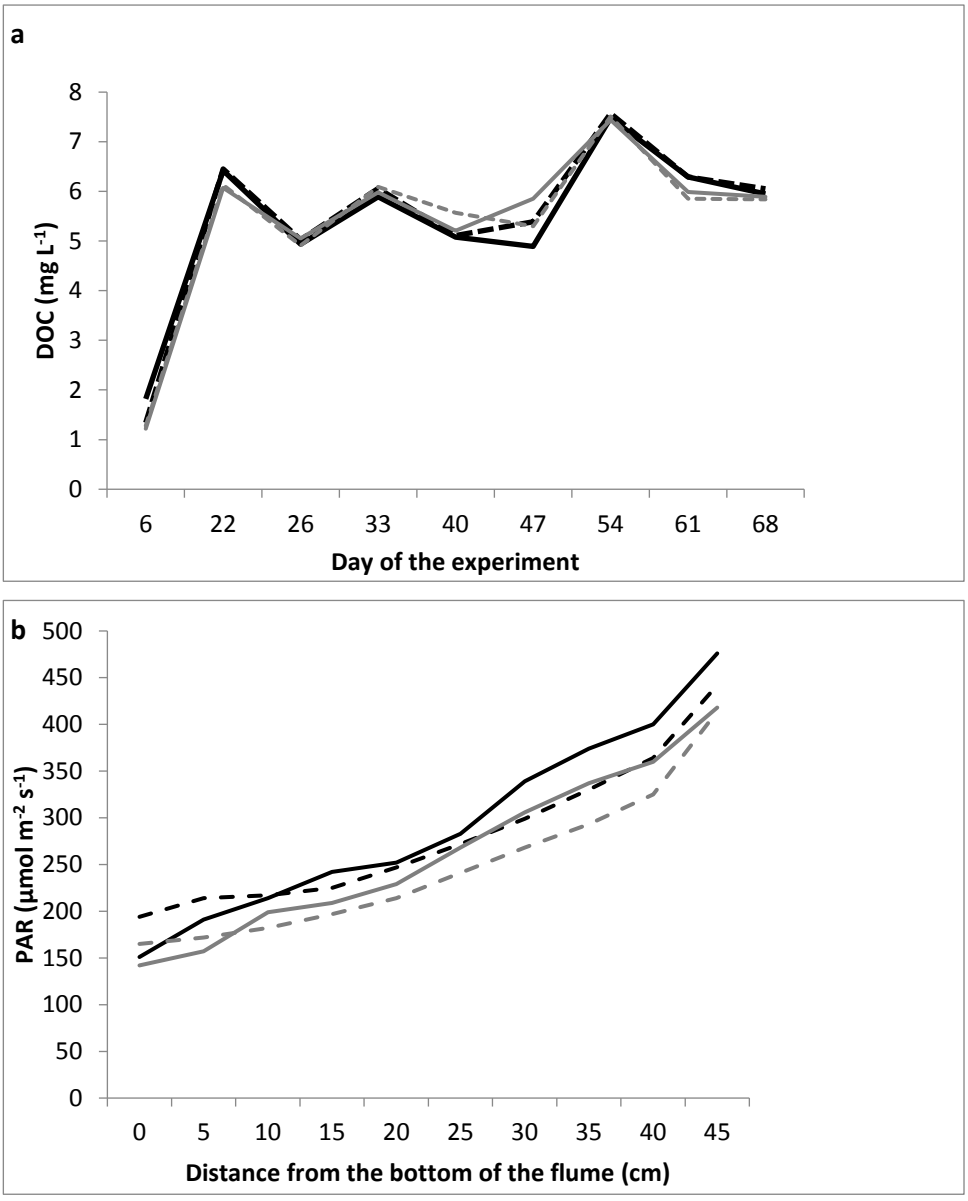

Figure S6.3 DOC concentration in the HD treatment (a). DOC was added to the flumes at day 21, 30 and 54. Light profile in the flumes during the HD treatment, measured on day 63 (b).

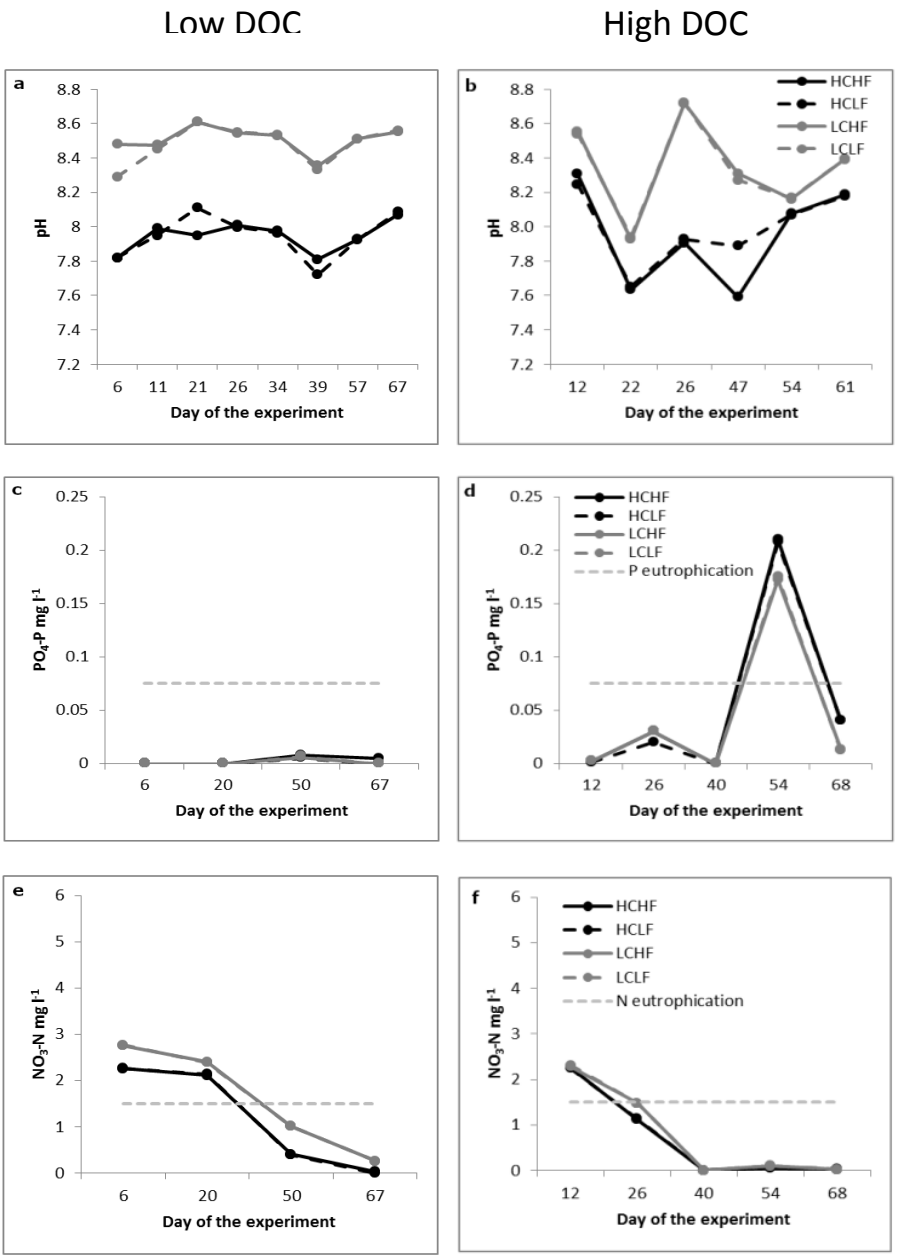

Figure S6.4 pH (a+b), PO_4-P (c+d) and NO_3-N (e+f) in the first experiment with low DOC concentrations (a+c+e) and in the second experiment with high DOC concentrations (b+d+f). The dashed line shows P eutrophication (c+d) and N eutrophication (e+f)(Smith et al. 1999).

Chapter 7.

Synthesis

Freshwater ecosystems are expected to change in the future due to many different direct and indirect effects of climate change, and organisms within those ecosystems will have to adapt. What will freshwater ecosystems look like in the coming decades? How do macrophytes respond to these changes and can they adapt to the new environment? How will those changes in macrophytes affect river functioning? How does this affect other species that depend on macrophytes by using it as a habitat or food source? In this chapter results from this thesis will be combined with studies from literature in order to make predictions for the future of freshwater ecosystems and macrophytes and attempt to answer those questions. Additionally, the strengths and limitations of the research in this thesis will be discussed and I will suggest some applications for management and ideas for future research.

Glimpse into the future: the fate of freshwater ecosystems

At the moment of writing this thesis, effects of climate change are already visible in freshwater ecosystems. Despite attempts to reduce CO_2 emissions, to reduce eutrophication and to restore rivers, the environment of macrophytes will probably continue to change even more in the future. According to the IPCC, within 20 years (around 2040) the earth will have warmed 1.5°C compared to pre-industrial levels, which is approximately 0.5 °C warmer than now. Depending on mitigation measures that might be implemented, by 2100 temperatures might have risen by 3-4°C above pre-industrial levels (Allen et al. 2018). The increase in temperature is not equal around the globe: in the warm season at mid-latitude and in the cold season at high latitudes average warming is expected to be strongest (Hoegh-Guldberg 2018). Due to changes in air temperature, the global mean temperature in rivers is expected to rise by 0.8-1.6°C in 2071-2100 relative to 1971-2000. Like air temperature, water temperature does not globally rise at the same rate: the strongest increases are expected in the United States, Europe, eastern China, southern Africa and Australia (van Vliet et al. 2013). The increase in temperature also leads to changes in precipitation, with increased frequency of extreme events like heavy precipitation and drought (Jacob et al. 2018). This causes changes in river discharge, which become more extreme as the global temperature rises (Döll et al. 2018). Like temperature, changes in discharge also differ locally: mean annual discharge is expected to increase in the high northern latitude and in the tropical regions, whereas it is expected to decrease in the mid northern and southern latitudes (which is also the region with the highest temperature

increases) (van Vliet et al. 2013). On a regional scale, in small streams, the main ecosystems of interest in this study, it is also expected that flow velocity will be affected by increased events of high flow and drought (Mimikou et al. 2000, Verdonschot and van den Hoorn 2010). During summer, some parts of small rivers and streams in Belgium and The Netherlands have been completely dry in the dry years 2018, 2019 and 2020 (figure 7.1).

Precipitation changes also indirectly affect nutrient and organic matter quantities in aquatic ecosystems. During droughts evapotranspiration increases, leading to higher concentrations of nutrients, whereas increased runoff during events of high precipitation intensity can increase nutrient loading (Jeppesen et al. 2011). In addition, global warming increases terrestrial organic matter production, so during high rainfall events there is more material that can end up in rivers (Pagano et al. 2014). After entering the water, organic matter can start leaching DOC and nutrients and eventually organic matter and DOC can be degraded and form CO_2 (Sobek et al. 2005). Increased organic material and nutrients also indirectly increase turbidity of the water by stimulating growth of algae, which decreases light availability (Hilton et al. 2006). In some areas salinity in freshwater ecosystem increases due to intrusion of seawater as a result of sea level rise or evapoconcentration during drought (Schallenberg et al. 2003).

Figure 7.1 This reach of the Boven Slinge (The Netherlands) completely dried during the dry summer in 2020

In summary, as a result of climate change, freshwater ecosystems in the future will be warmer, with a higher frequency of extreme changes in flow velocity (flooding or drought), nutrients, dissolved and particulate organic matter, increased CO_2 concentrations, increased salinity and more turbidity. In addition, other stresses may also increase in the future: new hydropower dams are built, reducing the number of free-flowing rivers by 21% (Zarfl et al. 2015). Moreover, due to high use of fertilisers in agriculture and high livestock density, nitrogen and phosphorus input is are still increasing in many aquatic ecosystems (Beusen et al. 2016). In industrialised countries, nutrient concentrations are decreasing, but this goes slowly and may not be sufficient to prevent algal blooms in the future (Blaas and Kroeze 2016). Besides nutrients, rivers are increasingly polluted with pesticides, metals and other contaminants due to expanding urbanisation (Paul and Meyer 2001). Other major problems in aquatic ecosystems are invasion by non-native plants (Hofstra et al. 2020) and habitat degradation within rivers or in the catchment, leading to changes

in runoff and erosion (Dudgeon et al. 2006). This combination of different stressors can be dangerous for abundance and diversity of freshwater organisms (Dudgeon et al. 2006, Hofstra et al. 2020).

Effects of climate change on macrophytes

Results from this study and from literature suggest that there will be major changes in macrophytes in the future due to climate change (warming of the water, extreme changes in flow velocity, increased nutrient levels, increased DOC levels and increased CO_2 concentrations). Effects of climate change on several major plant traits will be discussed in the following paragraph: macrophyte growth, morphology (root:shoot ratio, leaf area, stem length, stolon formation), chlorophyll content and nutrient content (C, N, P and Si). An overview of those effects is given in figure 7.2. This is the overview given in chapter 1 (figure 1.3), but results from the thesis have been added to it. In next paragraphs, it will be explained what the predictions in figure 7.2 are based on. It is important to note that the expected macrophyte responses mainly apply to submerged macrophytes. Secondly, in experiments, including the ones from this thesis, effects of stressors are usually measured continuously (continuous high flow velocity or high nutrient levels), whereas in reality, most stressors related to climate change (except temperature) will come in pulses.

Macrophyte growth

Effects of climate change on macrophyte growth are complex and often contrasting. When studying the effects separately, it appears that temperature (Zhang et al. 2016) and CO_2 (Hussner et al. 2016) promote growth, whereas extreme changes in flow velocity (Chambers et al. 1991) and increased nutrient (Hough et al. 1989) and DOC levels (Choudhury et al. 2019) decrease growth. In general, increased temperatures lead to increased biomass production when tested in experiments, but only if sufficient amounts of light, nutrient and inorganic carbon are available (Barko et al. 1982, Zhang et al. 2020). In many cases climate change is not only about general warming, but also about fluctuations in temperatures and seasons. Fluctuating temperatures can reduce the number of flowers and decrease sexual reproduction in macrophytes (Li et al. 2017, Xu et al. 2020). When winters are less severe due to climate change, macrophytes can develop faster in spring (Barko et al. 1982), which gives macrophytes and advantage over phytoplankton, but when warming is combined with high nutrient concentrations, phytoplankton can outcompete macrophytes (Moss et al. 2011). On itself, increased nutrient concentration can

also form a problem for macrophyte growth. Phosphorus and nitrogen are essential for macrophyte growth, but high concentrations can be disadvantageous as periphyton (Sand-Jensen and Borum 1991) and non-rooted macrophytes (Hough et al. 1989) become more dominant and may outcompete submerged macrophytes (Hilton et al. 2006). In chapter 5 increased nutrient concentrations had a large effect on *B. erecta* by limiting macrophyte growth and the size of plant organs. This was probably caused by the increased growth of periphytic algae covering the macrophytes, leading to shading and reduced availability of inorganic carbon and nutrients. When studying effects of climate change on macrophytes, it is also important to take into account effects on algae. Increased nutrient concentration can also have positive effects on macrophytes: under P enrichment, *Potamogeton crispus* had increased seed setting, which can improve its sexual reproduction (Xu et al. 2020).

Elevated CO_2 concentrations often increase the growth rate of macrophytes (chapter 4, 5 and 6), both in species that only use CO_2 as inorganic carbon source and in species that also use bicarbonate (Hussner et al. 2016, Dülger et al. 2017). However, in some studies CO_2 only affects plant morphology and not plant growth (chapter 4) (Eller et al. 2015). The total increase in biomass also depends on the amount of extra CO_2 in the water. In experiments often the CO_2 concentration in equilibrium with the atmosphere (±400 ppm) is taken as control, and this is compared to higher (>1000 ppm) concentrations. However, many freshwater ecosystems are already supersaturated with CO_2 (Raymond et al. 2013), so although it is expected that CO_2 concentrations will rise due to increased respiration, this increase may not be that extreme. It is also important to take into account that the response of macrophytes to CO_2 is non-linear. Photosynthesis increases with the CO_2 level until saturation is reached at a threshold level that differs between macrophyte species. For *B. erecta* this occurs around 0.25 mM CO_2 or 7000 ppm (Sand-Jensen et al. 1992), so above that concentration it is not expected that macrophyte biomass will further increase due to CO_2. At high CO_2 concentrations there is also the risk of weak acidification. Although this does not appear to have negative consequences on macrophyte growth, at very low pH values (<5.0), sexual reproduction can be impaired (Hasler et al. 2017).

As rises in CO_2 are often caused by increases in organic matter, it is relevant to study interactions with DOC, as those stressors will probably co-occur. Due to browning of the water (Karlsson et al. 2009, Thrane et al. 2014) and stimulation of algae, DOC often has a negative effect on macrophyte growth (chapter 6).

Humic substances are mainly responsible for this shading effect. They are often the main part of DOC and they give the water a yellow to brown colour (chapter 3). When studying effects of CO_2 and DOC in chapter 6, there appeared to be a positive antagonistic interaction (Piggott et al. 2015): the combined effects of

CO_2 and DOC on total dry biomass are less positive than the sum of the two effects separately. So, in the experiment CO_2 had a large positive effect on macrophyte growth and DOC a smaller negative effect, the combination was still negative for macrophyte growth. Even though this has only been tested in one experiment, it is important to keep in mind that these kinds of interactions can occur when multiple stressors act simultaneously, which makes it difficult to study effects of climate change. Another aspect to take into account is the source of DOC. In experiments often artificial DOC is used, which only causes shading, whereas in aquatic ecosystems DOC originates from organic matter which may be rich in nutrients, especially phosphorus levels can be high in DOC. Extra nutrients may partly compensate negative effects on biomass production by shading (chapter 4). However, in chapter 5 and 6 both DOC and nutrients had a negative effect on macrophytes and a positive effect on periphytic algae growth, so if plants are both exposed to high DOC and high nutrient levels (eutrophication), this will probably have a negative effect on plant growth. In addition, there is evidence that the combined effect of DOC and nutrients has a larger negative effect on water clarity than the two factors acting separately, causing a more severe limitation to macrophyte growth (Kritzberg et al. 2019). It is likely that in many cases rises in DOC and nutrients will co-occur, as dissolved organic matter is a potential source of nutrients that has been previously overlooked (Mackay et al. 2020). Of course, this depends on the source of organic matter, but for example, the DOC source used in chapter 4, 5 and 6 had a relatively high phosphate concentration.

In chapter 4, growth of *M. spicatum* was not affected by DOC, which may be explained by the fact that it increased its stem length and chlorophyll levels in order avoid the shading effects. However, from chapter 5 and 6 and other studies, e.g. Puijalon et al. (2011), it appeared that some plant species develop a more compact growth form when growing under hydrodynamic stress, which can happen after periods of high rainfall. In that case, macrophytes that have this avoidance strategy may have less chances to withstand the combination of DOC and flow velocity as they cannot increase their stem length without risking breakage. On the other hand, lentic periods during drought can also be a problem for macrophytes. If the water does not flow, diffusive boundary layer

thickness increases, which can limit resource uptake and limit macrophyte growth (Riis et al. 2017). So even if CO_2 levels rise, during drought this may not lead to biomass increase as it may not be available to the macrophytes. In the future it is expected that periods of droughts will be alternated with short periods of heavy rain; leading to low base flow in rivers with short periods of high peak flow. This can cause additional problems to macrophytes: if they are adapted to low flow with high temperatures, nutrients and CO_2 and they have a high biomass, this makes them vulnerable to hydrodynamic stress. They may not have enough time to adapt to the high flow velocity and they may uproot or break and they can even disappear (Bornette and Puijalon 2011).

When considering the effect of climate change on the macrophyte community is important to keep into account that not all species respond in the same way to climate change, which means that warming can change community composition (Short et al. 2016) and may favour invasive plant species (Hussner et al. 2014, You et al. 2014, Calvo et al. 2019), depending on the severity of each stressor and the response of the macrophytes. **To draw a general conclusion: temperature and CO_2 promote growth, whereas extreme changes in flow velocity and increased nutrient and DOC levels decrease growth. Effects of climate change, especially increased nutrient concentrations stimulate algae, that compete with macrophytes for light, carbon and nutrients. When all of those factors act simultaneously, it can be expected that in many cases macrophyte growth will be reduced** (chapter 5 and 6). This was also predicted by a review on the effect of climate change on submerged macrophytes (Short et al. 2016), so in figure 7.2 the expected net effect on macrophyte biomass is negative.

Morphology

In many studies the effects of climate change on plant morphology are not investigated in detail, but one of the main findings of this thesis is that climate change can have a profound effect on macrophyte morphology (chapter 4, 5 and 6). Still, it is difficult to draw general conclusions as morphological effects often differ between species, for temperature (Barko et al. 1982), flow velocity (Puijalon et al. 2011) DOC (chapter 4 and 6) and CO_2 (chapter 4). Still, there are some general trends. It has been suggested that CO_2 leads to increases in the size of plant organs that help collecting limiting resources like nutrients and light (Eller et al. 2015). Depending on the limiting factor and plant species, macrophytes can invest more in traits like roots and stolons (chapter 5) (Cao and Ruan 2015), or stem length and side branches (chapter 4) (Eller et al.

2015). Even though morphological differences caused by CO_2 are not the same in every macrophyte species, the underlying mechanism may be similar. Although in chapter 5 eutrophication did not have a clear result on root:shoot ratio, in other studies it has been observed that nutrient loading can decrease root:shoot ratio (Madsen and Cedergreen 2002). This is the opposite of effects of CO_2, which increases root:shoot ratio (Cao and Ruan 2015).

Less is known about general patterns caused by DOC, but in chapter 6, *B. erecta* had fewer stolons when exposed to DOC, which can form a limitation to its vegetative reproduction. One of the main effects of DOC is its shading effect. A common morphological response to shading is increased stem length (Barko et al. 1982), and this was also observed in chapter 4 in *M. spicatum.* However, when concentrations of DOC reach a threshold level, stem length no longer increases (Choudhury et al. 2019) and macrophytes are no longer able to avoid shading.

Most profound changes in morphology are probably caused by increased flow velocity. There are two main responses in macrophytes: tolerance and avoidance (Puijalon et al. 2011). By increasing cross-sectional area (e.g. thicker stems) and tissue strength, macrophytes can better tolerate hydrodynamic stress, or macrophytes can avoid hydrodynamic stress by decreasing their area exposed flow with smaller leaves, developing a more compact growth form and becoming more stream-lined (Puijalon et al. 2011). The species used in this thesis, *B. erecta*, shows a clear avoidance strategy, both in experiments and in the field: in chapter 2, 5 and 6 it had a more compact growth form with shorter stems when exposed to high flow velocity. In chapter 5, the combination of flow velocity and CO_2 led to more horizontal plant growth, with lower stems and more stolons and new ramets. This happened both in the treatment with eutrophication and without eutrophication, however in chapter 6, when DOC was added, the number of stolons decreased dramatically. So, for plants with an avoidance strategy to hydrodynamic stress, the combination of high flow velocity and DOC can be particularly dangerous for vegetative reproduction. When flow velocity decreases or when streams fall dry during drought, this also highly affects macrophyte morphology. It has been found that specific leaf area decreases to reduce evapotranspiration and plant species that can survive through seeds or belowground organs will be favoured (Manolaki et al. 2020). **As a general conclusion it can be said that climate change has a large effect on plant morphology. Plants respond to increased flow velocity with a more compact growth form or larger cross-sectional area. When those**

compact plants are exposed to light limitation caused by DOC, they may disappear. At low flow velocity, high DOC levels can lead to longer stems, which makes plants more vulnerable to sudden increases in flow velocity. When exposed to high CO_2 levels, some plants start investing more in organs that harvest nutrients or light, like roots or side branches. In figure 7.2 it is predicted that climate change has a negative effect on macrophyte morphology, meaning that plants are expected to be shorter with smaller leaves, mainly caused by increased flow velocity and increased nutrient loading. This prediction may not be applicable to plants that have a different response to flow velocity or systems that are not (much) affected by eutrophication. Exact effects are heavily dependent on species and the magnitude of each climate change effect.

Chlorophyll

Plant chlorophyll content is affected by different aspects of climate change. In some species, chlorophyll content increases when temperature increases (Barko and Filbin 1983). When plants are exposed to increased CO_2 concentrations, chlorophyll content is lower (chapter 5 and 6) (Madsen 1996, Dülger et al. 2017), which means that photosynthetic capacity is reduced. On the other hand, plant growth usually increases due to CO_2 (chapter 5 and 6), so when looking at total plant population biomass there is more chlorophyll in the plants exposed to high CO_2 than in plants exposed to low CO_2. There is also an interaction between CO_2 and nutrients: the negative effect of high CO_2 concentrations on plant chlorophyll content was smaller (Dülger et al. 2017) or not present at all (chapter 5) when nutrient concentrations were high. Still, this does not necessarily mean that photosynthetic capacity of the plant population is larger when exposed to high nutrient concentrations, since this also often decreases macrophyte growth due to competition with algae (chapter 5). When plants are exposed to high DOC concentrations, the opposite of occurs of what was observed for CO_2: plant chlorophyll content is higher under high DOC conditions (chapter 4). This may be caused by shading, which has a positive effect on chlorophyll content (Barko and Filbin 1983). However, DOC can decrease plant growth (chapter 3 and 6), leading to less chlorophyll in the plant population. **To conclude: temperature, CO_2, nutrients and DOC influence chlorophyll content, but the total amount of chlorophyll in the plant population often primarily depends on the amount of macrophyte biomass, which is highly affected by climate change.** In figure 7.2, it is predicted that chlorophyll concentration in the plants is expected to increase due to climate change, but it should be taken into account that when the total

amount of biomass decreases, the amount of chlorophyll in the population decreases as well, despite the higher concentration.

Nutrient content and stoichiometry

A last important macrophyte trait that is affected by climate change is nutrient content and stoichiometry. As a result of rising temperatures, N content decreases and C:N ratio increases, which may indicate a higher nutrient use efficiency in macrophytes (Zhang et al. 2016). There are also interactions between temperature and nutrient availability. When *Vallisneria spiralis* was exposed to increased temperatures and nutrient-poor sediments, plant C content decreased and P content increased, which decreases the C:P ratio (Zhang et al. 2020). When there is nutrient loading, N and P content in macrophytes increases (Zhang et al. 2020), but in chapter 5 this effect was only observed in the high CO_2 treatment: in the low CO_2 treatment there was no effect of nutrient loading on plant nutrient content. When exposed to high CO_2 concentrations, both plant P and N content are lower; leading to increased C:N and C:P ratios (chapter 4, 5, 6) (Titus and Pagano 2002, Hussner et al. 2016). DOC does not appear to affect nutrient stoichiometry by itself, but it depends on the amount of nutrients leaching from the DOC. In chapter 6, DOC only had a small effect on C and N, but a large positive effect on stem P content. The DOC stock had a large concentration of P, which is likely the cause of the increased plant P content. BSi is mainly affected by flow velocity: BSi concentrations increases in some macrophyte species when they are exposed to hydrodynamic stress. This is a more cost-effective way for plants to increase tolerance to hydrodynamic stress than increasing carbon-based molecules associated with strength, like cellulose or lignin (Schoelynck et al. 2012a). This does not occur in all species, as some macrophyte adapt their growth form in order to avoid hydrodynamic stress instead of strengthening their tissue (Puijalon et al. 2011) (chapter 5). BSi content is also related to other stressors like herbivory, nutrient loading or nutrient shortage (Schoelynck and Struyf 2016). **To conclude: climate change has opposite effects on nutrient stoichiometry. Rising temperature and CO_2 lead to increased C:nutrient ratios, whereas nutrient loading decreases C:nutrient ratios. However, there are interactions between the different stressors, which makes the effects more difficult to predict.** In figure 7.2 the net effect of climate change on biomass quality is predicted to be negative. However, this depends to a great extent on the surroundings of the macrophytes: the exact nutrient concentrations in the water and sediments, the availability of inorganic carbon and competitors like other macrophytes, phytoplankton and epiphytes.

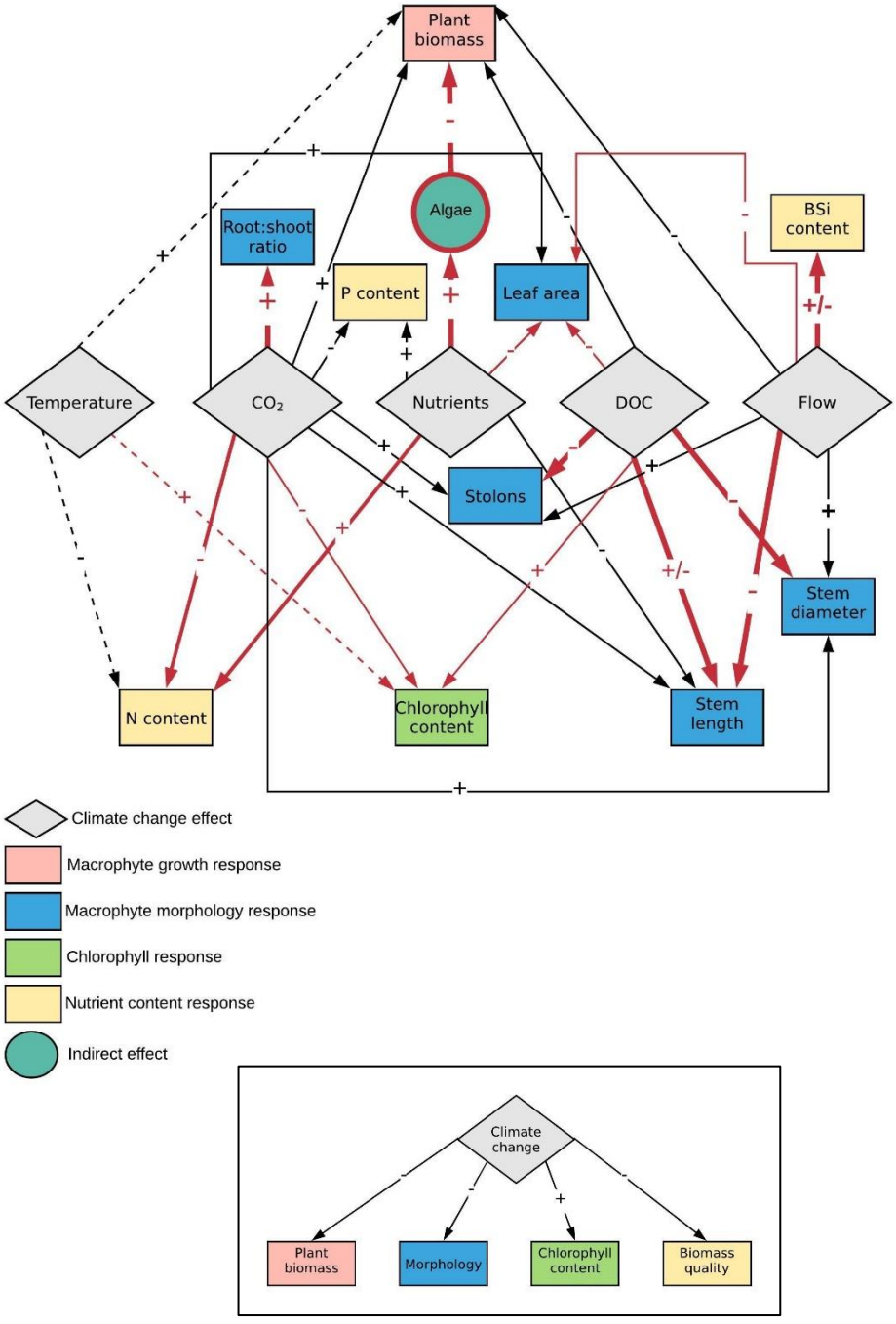
Plant biomass
Algae
Root:shoot ratio
P content
Leaf area
BSi content
Temperature
CO_2
Nutrients
DOC
Flow
Stolons
Stem diameter
N content
Chlorophyll content
Stem length
Climate change effect
Macrophyte growth response
Macrophyte morphology response
Chlorophyll response
Nutrient content response
Indirect effect
Climate change
Plant biomass
Morphology
Chlorophyll content
Biomass quality

Figure 7.2 Overview of major climate change effects on macrophyte growth, morphology, chlorophyll and nutrient content, as shown in the introduction (chapter 1, figure 1.3), but this time including results found in this thesis. Firstly, effects of DOC on macrophytes have been added to the scheme. In this thesis it was found that DOC can have a negative effect on leaf area and the number of stolons. In *B. erecta*, stem length decreased, but in *M. spicatum,* stem length increased due to DOC. Secondly, this thesis focussed on interaction effects among factors of climate change. This has been added to the scheme as red arrows, showing which of the climate change factors is expected to be dominant when they all act simultaneously. One thick arrow means that we expect that factor to be dominant (e.g. the negative effect of nutrients on macrophyte growth through increased competition with algae), although other factors also contribute. In other cases, several factors may be of importance (e.g. the effects of CO_2 and nutrients on the N content). Dotted lines represent factors that have not been tested in this thesis (temperatures) and effects on macrophytes are based on literature review. This means that temperature has not been taken into account in the interaction effects, which means that its effect may be different than depicted in this scheme. Below, a simplification of the scheme is shown, summarising effects of climate change on growth, morphology, chlorophyll and biomass quality. It is expected that there is a net negative effect on plant biomass and a negative effect on morphology, meaning that plants will be more compact with shorter stems and smaller leaves. Chlorophyll concentration is expected to increase, but if plant biomass decreases, the total amount of chlorophyll in the plant population will decrease as well, despite the higher concentration. Biomass quality is expected to decrease, as N and P will decrease. This scheme mainly applies to native, rooted macrophytes. Climate change can under some circumstances and for some species (e.g. floating or invasive species) be beneficial, so this scheme is not applicable to all situations. It also assumes that all factors increase, which may not always be the case. It should be taken into account that this scheme is a simplification of the real situation in aquatic systems. Exact changes depend on the circumstances in the system and the magnitude of changes.

Answers to hypotheses

When looking at the hypotheses made in chapter 1, the following conclusions can be drawn:

1. *Elevated CO_2 will increase growth rate in macrophytes, increase root:shoot shoot ratio and the C:N ratio. Differences will be stronger in* B. erecta *(obligate CO_2 user) than in* M. spicatum *(which can also use HCO_3^- as an inorganic carbon source).* This hypothesis is partially accepted. Increased growth rate, root:shoot ratio and C:N ratio have been observed in *B. erecta*, but *M. spicatum* mainly responded by changing its aboveground morphology.
2. *Elevated DOC will decrease macrophyte growth and increase plant stem length due to shading effects. Low DOC levels may stimulate macrophyte growth, as CO_2 is released when DOC is degraded and shading effects are relatively small.* In *B. erecta*, growth was decreased and in *M. spicatum* stem length increased. We found no evidence that macrophyte growth increased due to CO_2 release from DOC degradation.
3. *Elevated flow velocity will decrease macrophyte growth and decrease plant stem length.* Flow velocity used in this experiment was probably not enough to induce a significant stress. It was the other way around: growth was lower in the low flow velocity treatment, probably because of increased boundary layers.
4. *Elevated nutrient concentrations will decrease macrophyte growth rate due to competition with algae.* This hypothesis is accepted, algae were a significant problem.
5. *Combined effects of elevated CO_2, nutrients and flow velocity will decrease macrophyte growth.* In the experiment, macrophyte growth increased slightly when all factors were combined, but it can be expected that effects of nutrients will be more pronounced than CO_2 in reality, so then macrophyte growth will decrease. Competition with algae is important when ecosystems are exposed to high nutrient concentrations, so when studying macrophytes algae should also be taken into account.
6. *Combined effects of elevated CO_2, DOC and flow velocity will slightly increase macrophyte growth (if low levels of DOC are used).* This hypothesis can be accepted based on the results, but it can be expected that macrophyte growth will decrease in reality, when the three factors

act together. The effect of DOC will probably be more pronounced than the effect of CO_2, and we found no evidence that macrophytes benefitted from CO_2 release when DOC was degraded.

Effects on the aquatic ecosystem

Macrophyte biomass, morphology, chlorophyll content and nutrient stoichiometry are expected to change due to climate change. As macrophytes play an important role in the aquatic ecosystem and many organisms depend on them, it is expected that the ecosystem will change as well: riverine processes like the nutrient cycle and stream metabolism will be affected, and subsequently other organisms like macroinvertebrates and fish. Next paragraphs discuss indirect effects of climate change on aquatic ecosystems, caused by changes in macrophytes. However, it should be taken into account that climate change also directly affect ecosystems processes like nutrient cycling and metabolism of other organisms (Jankowski and Schindler 2019), but this is beyond the scope of this thesis.

Nutrient cycle

Macrophytes have a large effect on the sediments and the interaction between the water column and the sediments. They stimulate nutrient retention by taking up nutrients for their growth, by capturing organic particles in their shoots and by stabilising the sediments and prevent resuspension of nutrients (Clarke 2002). Moreover, macrophytes improve circumstances for denitrification, which leads to permanent removal of nitrate from the system (Veraart et al. 2011). Changes in macrophyte biomass and morphology can therefore also indirectly affect aquatic nutrient cycles. When exposed to increased flow velocity and increased CO_2 concentrations, macrophytes have an increased root:shoot ratio and a horizontal development, with a large number of stolons and new (short) ramets. This could enhance the stabilising effect of macrophytes on the sediments (Clarke 2002). On the other hand, if macrophytes are shorter and have less biomass, their ability to foster fine sediment accumulation and maintain nutrient cycling between the sediments and the water column may decrease (Madsen et al. 2001, Clarke 2002). Exact effects will probably depend on macrophyte biomass. In most scenarios of climate change this is expected to decrease, which means that nutrient retention decreases as well, giving an opportunity for growth of algae blooms.

A second issue is the effect of climate change on plant nutrient stoichiometry. As climate change (in particular rising CO_2 concentrations) affects plant nutrient stoichiometry, it can be expected that this also affects nutrient cycling. In an experiment by Titus and Pagano (2002), it was found that when plants were exposed to high CO_2 concentrations, plant decay was limited, but only in the first few weeks of decomposition. They also suggest that nitrogen cycling depends more on biomass quantity than biomass quality: although macrophyte tissue exposed to high CO_2 has a decreased N content, total N in the plant biomass was still higher in the high CO_2 than in the low CO_2 treatment because in the high CO_2 treatment there was more biomass. N and C cycling are expected to accelerate in high CO_2 conditions because of this increased plant growth (Titus and Pagano 2002). However, there are many other aspects of climate change that limit macrophyte growth, so in that case it is more likely that N and C cycling decreases, as less nutrients are taken up from the sediments and water column by macrophytes.

Macrophytes are not only affected by CO_2, it is also the other way around: they partly control fluxes of greenhouse gases CH_4 and CO_2 from aquatic ecosystems. When the water warms, macrophyte abundance increases and this reduces emissions of greenhouse gases from the water. However, if there is both warming and eutrophication, macrophyte biomass decreases and phytoplankton is the main primary producer, which means that greenhouse gas fluxes will increase (Davidson et al. 2015).

Stream metabolism

Macrophytes have a large effect on stream gross primary production (GPP) (O'Brien et al. 2014), and to a smaller extent on ecosystem respiration (Preiner et al. 2020). If macrophyte biomass is altered due to climate change, this is likely to indirectly affect stream metabolism. Firstly, as GPP is strongly correlated to the amount of macrophyte biomass (chapter 2), it is expected that effects of climate change on macrophytes indirectly affect GPP. In a mesocosm study, warming had a positive effect and humic runoff had a negative effect on GPP in a littoral habitat with macrophytes, whereas those effects were not observed in pelagic or benthic habitats (Rodríguez et al. 2015). Combined effects of rising temperatures and eutrophication often lead to macrophyte biomass decrease (Moss et al. 2011). This effect is also observed when studying plant metabolism: at low nutrients levels and high temperatures, macrophyte abundance increases, which promotes GPP; whereas GPP decreases when nutrient levels are high (Davidson et al. 2015). Results from chapter 2 suggest an indirect effect

of flow velocity on primary production: in a reach with high flow velocity, biomass and primary production were relatively stable, whereas in a homogeneous reach there was a large biomass peak in summer and a peak in primary production. In chapter 5, there was no effect of flow velocity on plant biomass. This is an important difference between experiments and the field situation: in the field flow velocity has a large effect on plant coverage which decreases macrophyte biomass and primary production.

Macroinvertebrates and fish

Macroinvertebrates and fish are directly affected by climate change, especially by increased nutrient loading and warming (Mantyka-Pringle et al. 2014), however they can also be indirectly affected by climate change through changes in macrophytes, which some species heavily depend on. Firstly, as discussed in in earlier paragraphs, submerged macrophyte biomass is expected to decline. Arzel et al. (2020) found a correlation between brownification of the water and decreasing abundance of macroinvertebrates in Finnish lakes, and it is suggested that one of the reasons for this effect is the decline of macrophyte biomass. Secondly, increases in flow velocity can lead to a more compact growth form in some species. Due to interactions between vegetation and flow velocity, macrophytes form patches with low flow velocity within patches and accelerated flow velocity outside of the patches (Schoelynck et al. 2012b). As a result, macrophytes serve as flow refugia, especially during events of high flow velocities (Lancaster and Hildrew 1993, Wolters et al. 2018). When macrophytes have less biomass and a more compact growth form, these flow refugia for macroinvertebrates and fish may become smaller in the future. When macrophytes disappear and are replaced by filamentous algae, macroinvertebrate and small fish diversity decreases, which has further consequences for higher trophic levels (Camp et al. 2014).

From the experiments in this thesis is can be concluded that macrophyte biomass is a main factor determining chlorophyll content of the macrophyte population, so macrophyte biomass decrease can lead to lower oxygen concentrations in the water layer and the rhizosphere (Carpenter and Lodge 1986, Desmet et al. 2011). Algal blooms can occur, which have more extreme oxygen dynamics than macrophytes with oversaturated and hypoxic peaks (Sabater et al. 2000), which makes the environment less suitable for heterotrophs that depend on macrophytes as an oxygen source.

Changes in biomass also can have consequences for herbivores and detritivores that rely on macrophyte tissue and detritus as food source. Due to climate change food quantity is diminished, but it can also decrease food quality. Both due to increasing temperature and CO_2 concentrations the C:N ratio increases, which implies that macrophytes have less nutritive value to aquatic herbivores (Elser et al. 2000). In terrestrial plants usually C:N ratios are higher than in aquatic plants, and terrestrial herbivores often specialise with regard to plant species, plant part and time of consuming. Due to the lower C:N ratios in macrophytes compared to terrestrial plants, aquatic herbivores tend to have a more generalist approach (Elser et al. 2000). If C:N ratios rise due to increased CO_2 in the water, herbivores may need to adapt to acquire a sufficient amount of nutrients. Lastly, the changes in BSi content of the plant under climate change can also affect other organisms. Increased BSi content increases macrophyte litter decay rates by microbes, but decreases decay rates by macroinvertebrate shredders (Schaller and Struyf 2013).

Strengths and limitations

In the field study in chapter 2, macrophyte growth, morphology and nutrient stoichiometry was followed over the course of one growing season. This gives valuable information as often macrophytes are only studied during biomass climax in summer and usually there is no detailed information on macrophyte morphology. On the other hand, a limitation of this study is the fact that only one river was studied for one year. This makes it difficult to draw strong conclusions, as some patterns may only be applicable to specific conditions in the river and not translatable to other situations.

Other chapters in this thesis mainly involve semi-controlled greenhouse experiments with a high number of treatments, pseudo-replicates, and a trait-based approach. This has several advantages, but there are also limitations. As described earlier, in many studies only a couple of different CO_2 and DOC concentrations are studied, so the wide range of concentrations in this study provides new insights. Due to the high number of treatments and difficulties with maintaining high CO_2 concentrations in small volumes of water, it was decided to work with large aquaria with pseudo-replicates. Although this is disadvantageous as the plants in the same treatment are not entirely statistically independent, the focus on gradients rather than replicates also has advantages. When only testing two concentrations, the shape of the response (e.g. linear, exponential, trigonometric, power, etc.) which gives information

about the ecological driver behind the response, cannot be revealed, whereas a gradient design is better suited for this purpose (Kreyling et al. 2018).

In chapter 5 and 6 it was also decided to work with pseudoreplicates, but for different reasons. Complex research infrastructure (racetrack flumes) was used to test effects of flow velocity. It was decided to use a full-factorial experimental design with three different stressors, but due to the limited number of flumes not all experimental treatment combinations could be performed simultaneously and there was no true replication. Although all experiments were carried out in summer and temperatures were kept constant, the amount of sunlight was not equal during all experiments: the second experiment done in 2017, with increased nutrient concentrations had less light than the other two experiments as it was carried out at the end of summer when the days were starting to get shorter. The two experiments carried out in the beginning of summer received the minimum amount of light needed for photosynthesis 65% of the time, whereas for the experiment at the end of summer this was only 53%. This difference in timing and the use of pseudo-replicates are a disadvantage of this approach, but testing multiple stressors and their interactions through a full-factorial design gives more new insights, and more closely resemble reality than testing all stressors separately with replication (Knapp et al. 2018). In all experiments, algae were a huge problem. While competition with algae can also be expected in rivers, it may not be as extreme as in the experiments. Perhaps adding nutrients to the sediments could have helped to reduce competition between macrophytes and algae.

A last aspect of the experimental design I would like to discuss is the choice of macrophyte species and the measurements. It was chosen to use *B. erecta* as main macrophyte species, so all chapters could be linked to each other. At the field site *B. erecta* was the dominant species and it was easy to use in the experiments. However, it is an amphibious instead of obligate submerged species and it is an obligate CO_2 user, leading to a negative growth rate in some treatments with a low CO_2 concentration. This effect can partially be explained by *B. erecta*'s growth strategy. In the treatments with negative growth rate, the plants grew and developed new leaves throughout the entire experiments, but the new leaves were smaller than the old leaves they replaced. Climate change will probably not lead to continuously negative growth rates, as macrophytes would entirely disappear. This makes it difficult to translate experimental results to the real situation. It was also decided to work with a trait-based approach: macrophytes were thoroughly measured with regard to growth,

morphology and nutrient content. This gave valuable information; those traits were highly affected by the treatments. On the other hand, some other studies focus more on (cellular) mechanisms, to unravel the drivers behind changes in macrophyte change, whereas in this thesis the exact drivers are not completely elucidated.

All chapters have been subjected to peer-review and most chapters have already been published in international scientific journals.

Protecting rivers against climate change

The questions addressed in this thesis are of fundamental nature, which makes it difficult to directly translate conclusions from this thesis into practical measures for (river) management aimed at protection against climate change. However, macrophytes are involved in important functions in many freshwater ecosystems (Sand-Jensen 1998, Clarke 2002) and if negative effects from climate change can be mitigated, this can help to improve and maintain ecological quality of the water. Observations from this thesis can contribute to sustainable river management.

Climate change is a global problem, and on a river scale many problems related to climate change like CO_2 emissions, warming and input of nutrients and DOC are difficult to solve. From chapter 2, 4 and 6 it can be concluded that especially DOC can pose a threat to macrophyte growth and vegetative reproduction. DOC may not affect all freshwater ecosystems to the same extent, but managers should be aware of it. Extreme events of heavy precipitation that often lead to elevated DOC concentrations in the water by flushing organic matter into the water (Pagano et al. 2014) cannot be prevented, but management measures in the river and its surroundings can diminish the amount of organic ending up in rivers. Measures in rivers themselves can be implemented to increase the residence time of the water, for example by placing boulders or wood in the water, there is more time for DOC degradation (Frainer et al. 2018). In the surroundings of rivers, measures can be taken to prevent DOC from ending up in rivers. One important measure that is relatively easy to implement is increasing riparian zones, which are often small or absent (figure 7.3a), but have been restored in some rivers (figure 7.3b). This zone can contain various vegetation types, such as trees, shrubs, herbs, grasses and sedges and its soil characteristics and water availability are influenced by the river (Pert et al. 2010). Riparian zones also have a large influence on rivers: they can regulate

disturbances such as floods and they can have a large effect on water quality (Tockner and Stanford 2002). They act as a filter and prevent nutrients in the surrounding areas from entering rivers (Hill 1996). Riparian plants remove nutrients by direct uptake and they stimulate nutrient transforming processes like denitrification (Tabacchi et al. 2000). When organic matter first accumulates in riparian ecosystems before entering the river, microbial decomposition takes place. When this organic matter from the riparian zone enters the river, DOC and nutrient fluxes are smaller and the biodegradability of C is lower than when organic matter directly enters the river (del Campo et al. 2020). Restoring riparian zones can improve the ecological quality of rivers, but if a large extent of degradation has occurred it will also take a long time to recover, as the river bank may have eroded and important plant species have disappeared (Dosskey et al. 2010). Restoring riparian zones may also stimulate biodiversity: the decline of riparian zones in European rivers is thought to be responsible for 19% of threatened freshwater species (Gozlan et al. 2019). In this thesis it was not tested whether riparian zones can help to protect macrophytes from the consequences of climate change, but this new hypothesis can be tested in future studies on the effects of climate change on macrophytes.

Macrophytes themselves can also help to mitigate effects of climate change, they can play an important role in nature-based solutions. In addition to riparian vegetation, macrophytes can help to make aquatic systems more resilient to climate change. In shallow lakes, introducing or translocating macrophytes is used as a restoration measure to combat algal blooms (Triest et al. 2016). Emergent macrophytes like *Phragmites australis* L. and *Schoenoplectus lacustris* L. (Nikolakopoulou et al. 2020) and submerged species like *Potamogeton* spp. (Forshay and Dodson 2011) and *B. erecta* (Preiner et al. 2020) can enhance nutrient retention by taking up nutrients and stimulating processes like denitrification (Forshay and Dodson 2011). It has been suggested that the hyporheic zone (the zone alongside the stream bed, where groundwater and surface water are mixed) is an important zone for restoration practices. Vegetation could be restored there and form a buffer for nutrient loading associated with heavy rainfall (Nikolakopoulou et al. 2020). Moreover, macrophytes can also be efficient at carbon sequestering (Bernal and Mitsch 2012), so restoring macrophytes may help to reduce CO_2 emissions from inland waters. As macrophytes can be important when mitigating effects of climate change, it is also important to adjust management practices in order to make aquatic systems more robust to climate change (see box 7.1).

Box 7.1 Minimising negative effects of macrophyte mowing

Because of their hydraulic resistance in the water, macrophytes can slow down flow velocity and raise the water level, which can increase the risk of flooding (Bal and Meire 2009). Especially in agricultural areas this can lead to damage, so macrophytes are often mowed, which can have negative consequences for riverine flora and fauna (Baattrup-Pedersen et al. 2003). Moreover, mowing can stir the sediments, leading to increased turbidity, concentrations of nutrients and dissolved and particulate organic matter in the water may increase. In the Eefse Beek in The Netherlands the effect of an alternative mowing strategy was tested: only a subsection of the cross-section was mowed. In a three-kilometre river reach, a field experiment was carried out. First a stretch with a width of two metres was mowed, then this was expanded to four metres and later to six metres. After each mowing event, an upstream weir was lowered to create a peak discharge. Before, during and after mowing, the water quality was measured, and the water level and flow velocity were monitored. This streamline mowing strategy showed that in this river reach mowing a stretch with a width of four metres is most effective: increasing to six metres did not result in further reductions in water levels, so it is not necessary to remove all macrophytes (Berends et al. 2020). Mowing had a negligible effect on water quality. Oxygen, nutrients and concentrations of carbon were constant throughout the experiment. After lowering the weir there was a small peak in turbidity, but altogether there were no significant effects. Macrophytes were mowed using a mowing boat with a cutting device. With this method macrophytes could be cut without stirring the sediments or uprooting plants, so the sediments remained undisturbed.

Other effective but less feasible measures to mitigate effects of climate change include avoiding large areas of arable land next to rivers. This can help to keep DOC levels low, as converting grassland to farmland can be a major cause of DOC increase (Noacco et al. 2017). Surrounding forests can also be managed to diminish DOC in rivers and streams: more DOC leaches from coniferous forests than from deciduous forests (Camino-Serrano et al. 2014), so replacing coniferous with deciduous trees in forest surrounding rivers may help to lower DOC concentrations. Locally, this is already implemented to increase biodiversity in forests, so it can serve other restoration purposes as well (Kritzberg et al. 2019).

From chapter 5 and 6 it can be concluded that a combination of stressors can be harmful to macrophytes. There are several factors of climate change acting on macrophytes, but many freshwater ecosystems are already under stress and may be affected by climate change more negatively than expected. Many

freshwater ecosystems are already under threat by overexploitation, water pollution, modification of flow, habitat degradation and invasion by exotic species, and climate change can aggravate those problems (Dudgeon et al. 2006). Therefore, it is important for managers to be aware of those interacting stressors and to aim to make freshwater ecosystems more robust, so they can better withstand effects of climate change. After periods of droughts or severe flooding, sometimes engineering solutions like dams or dredging are implemented to protect cities and agriculture and to improve water security (Strayer and Dudgeon 2010). When those structures are built in a rush, often ecological impacts are poorly studied and as a consequence, engineering solutions can severely affect biodiversity and even enhance climate change impacts (Strayer and Dudgeon 2010). Therefore, managers should look ahead and take action to make rivers robust to drought or flooding, in a more holistic way (Dudgeon et al. 2006), taking into account safety, biodiversity, water quality and sustainability.

Figure 7.3 examples of (a) a stream without riparian vegetation (Desselse Nete in Belgium) and (b) a restored stream with a large riparian zone (Elsenerbeek in The Netherlands). Pictures by Jan-Willem Wolters.

Future research

It can be concluded from this thesis that CO_2, DOC, flow velocity and eutrophication can have large effects on macrophytes and that there are often complex interactions between different climate change factors. In this thesis new results have been obtained with regard to the effect of CO_2 and DOC on several macrophyte traits. This thesis also sheds new light on interactions between different aspects of climate change. However, as the effect of climate change on macrophytes is a complex problem, several new questions can be raised based on the results of this thesis.

Experiments testing effects of climate change on macrophytes provide opportunities for further research. Effects of single aspects climate change, like temperature or nutrients have been tested in many experiments. In this thesis, effects of a wide range of CO_2 and DOC concentrations were tested on several macrophyte traits (chapter 4). After that, interactions between different aspects of climate change were tested (chapter 5 and 6), which better resembles the reality. In next steps in this research, this translation from lab tests to the real situation can be further expanded. This can be achieved by conducting long-term experiments where more elaborate climate change scenarios are tested, also involving temperature rises. To make the experiment more realistic, it would be useful to test effects of pulses of flow, DOC and nutrients, rather than stable flow, DOC and nutrient conditions. This would simulate effects of heavy rain and subsequent increases in runoff, which could give different results as the macrophytes have less time to respond to changing conditions. This experiment can then be carried out with other macrophyte species as well, to see whether this differs from the response of *B. erecta*.

It would also be interesting to study how climate change affects different species. Response to climate change can depend on the plants' growth form: e.g. plants showing an avoidance strategy (more compact growth form) to hydrodynamic stress can be more vulnerable to light stress caused by DOC. With this kind of information, and other plant characteristics like inorganic carbon use and reproduction strategy, a risk-analysis can be made to find out which species are expected to be more vulnerable to specific combination of climate change aspects. This can teach us which macrophytes are most resistant to climate change, and what consequences this may have for the rest of the ecosystem. When there is sufficient knowledge about the response of macrophytes in different situations, this than can be modelled to predict how

macrophytes will change when exposed to climate change scenarios. With the combination of experiments and models important new questions can be answered like: which macrophyte species are most vulnerable to climate change? Which climate change aspect / combination of climate change aspects is most dangerous in which freshwater ecosystem? What is the maximum amount of climate change stress that aquatic ecosystems can cope with? With answers to those questions the final step can be made: protection of macrophytes. Macrophyte conservation in times of climate change can become more targeted if there is more detailed knowledge on the response of macrophytes. This can then be combined with studies focussing on possible management solutions, like increasing riparian zones (see 'protecting rivers against climate change').

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With this defence not only my PhD research comes to an end, but also my time in Belgium. More than five years ago I was looking for an interesting PhD topic anywhere in Europe when I found an advertisement at the University of Antwerp. The topic was about aquatic plants, and although I like studying plants a lot, aquatic plants were totally new to me. Still, the aquatic world looked very intriguing to me, so I decided to dive into this new world: studying macrophytes in Antwerp. I had visited Antwerp before and I really loved the city. It's also close to the Netherlands and people speak Dutch, so it can't really be that different from the Netherlands, can it? About this I was very wrong, Belgium is a very different country and I really had to adjust, but was a great experience to live there, to get to know the fantastic Flemish vocabulary and to meet many new people who helped me to feel at home and most importantly, to finish my PhD.

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Curriculum vitae

Rosanne Reitsema was born on 21 December 1991 in Winterswijk, the Netherlands. As she has always been interested in a wide range of topics, she moved to Middelburg to study Liberal Arts & Sciences at University College Roosevelt. Here she combined music (studying the harp and theoretical music courses) with scientific courses from other disciplines. She discovered she loved ecology and decided to continue in this field. In Wageningen she did the master study Biology. During this study, she specialised in bio-interactions and did her thesis on inbreeding effects in plants. For her Master internship at the NIOO-KNAW she investigated how plant growth and stress resistance are affected by biodiversity.

Doing greenhouse experiments and scientific research appealed to her very much, so in 2016 Rosanne started a PhD at the Ecosystem Management Research Group (ECOBE) at the University of Antwerp. In this PhD study, she investigated how macrophytes are affected by climate change under the supervision Prof. dr. Jonas Schoelynck and Prof. dr. Patrick Meire. Currently, she is employed as an aquatic ecologist at Witteveen+Bos.

International peer-reviewed publications

Reitsema, R. E., Preiner, S., Meire, P., Hein, T., Dai, Y., & Schoelynck, J. (2020). Environmental control of macrophyte traits and interactions with metabolism and hydromorphology in a groundwater-fed river. *River Research and Applications, 37* 294-306.

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Berends, K., Penning, E., Lenssen, J., Schoelynck, J., & **Reitsema, R** (2020). De effecten van stroombaanmaaien proefondervindelijk onderzocht in de Eefse Beek. H_2O.

Symposia

8-13 September, 2019, 6th Biennial Symposium of the International Society for River Science, Vienna (Austria). Oral presentation: Macrophyte growth in a future world: Effects of elevated CO_2 and DOC concentration on growth, biomass allocation, chlorophyll content and nutrient stoichiometry of submerged macrophytes.

6 March 2019, Centre for Wetland Ecology symposium: future of rivers and streams: ecology and restoration under global change, Antwerp (Belgium). Oral presentation: Effects of macrophytes on ecosystem metabolism and nutrient cycling in a groundwater-fed lowland river.

8 February 2018, 23rd National Symposium for Applied Biological Sciences, Brussels (Belgium). Oral presentation: Effects of climate change on macrophyte growth, biomass allocation, physiology and nutrient stoichiometry.