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Trophic and non-trophic interactions between macrophytes and macroinvertebrates in lowland streams

Trofische en non-trofische interacties tussen macrofyten en macroinvertebraten in laaglandbeken

Proefschrift voorgedragen tot het behalen

van de graad van doctor in de Wetenschappen

te verdedigen door Jan-Willem Wolters

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"*We snatch in vain at Nature's veil,*
She is mysterious in broad daylight,
No screws or levers can compel her to reveal
The secrets she has hidden from our sight."

Faust, Part One
Johann Wolfgang von Goethe (1808)

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Abstract

Through their form and ecosystem functions, aquatic macrophytes can have a great impact on their environment and also on the macroinvertebrate community present within macrophyte stands. The effects of macrophytes range from purely abiotic, such as the impact of growth form on water flow, to biotic, such as a food source for herbivorous macroinvertebrates. Although many effects of macrophytes on their environment, including the relationship with macroinvertebrate presence, have been studied before, there is still much unknown about the role of macrophytes within the aquatic food web. This thesis aims to further elucidate the role of non-trophic and trophic interactions between macrophytes and macroinvertebrates in temperate lowland streams, whereby special attention is paid to the role of macrophytes within the aquatic food web, including both their direct (e.g. direct consumption of living macrophytes and macrophyte-derived organic matter) and indirect role (e.g. influence on other food sources, such as epiphytic algae and bacteria).

Macrophytes were observed to have a significant effect on the macroinvertebrate community through a variety of mechanisms. The physical structure provided by aquatic macrophytes was found to influence habitat complexity and water flow velocity, both of which have an effect on the associated macroinvertebrate community as well. Furthermore, an increase in macrophyte complexity led to an increased cover of epiphytic algae, an important food source for many herbivorous macroinvertebrates. However, the net effect of macrophytes on their epiphytic biofilm was mixed, as a lower amount of biofilm was observed on living macrophytes compared to artificial analogues, possibly due to the exudation of chemicals that inhibit algal growth. However, this biofilm was found to have a higher concentration of nutrients, probably caused by nutrient exudation of living macrophytes.

Through the use of stable isotope measurements certain macroinvertebrate taxa, that are expected to feed on this epiphytic biofilm, were observed to assimilate macrophyte tissues. The fact that taxa of the scraper functional group, that feed close to the macrophyte leaf surface, were calculated to assimilate more macrophyte derived compounds than taxa classified as gatherers, which feed further away from the leaf surface, led to the hypothesis that accidental leaf erosion during grazing was the cause for the observed macrophyte consumption patterns. Additionally, certain macroinvertebrate shredders were observed to directly consume macrophyte tissues, which can in some cases lead to

a drastic reduction in macrophyte populations, especially when these are already subjected to other stressors. In addition to the consumption of living macrophytes, macroinvertebrate filter-feeders were also observed to consume macrophyte-derived organic matter, after it has been broken down to fine particulate organic matter (FPOM). This consumption pattern was found to be especially prevalent at the end of the growing season, when large amounts of macrophyte tissue died off and particulate organic matter was generated.

The observations in this thesis indicate that macrophytes have a significant effect on the functioning of the aquatic systems in which they occur, and that this effect is broader than the purely structural role that is often focussed upon, as macrophytes were also found to play an important role within the aquatic food web.

Samenvatting

Door hun vorm en functioneren kunnen waterplanten een grote invloed uitoefenen op hun omgeving en op levensgemeenschappen van aquatische macroinvertebraten die op de waterplanten voorkomen. De effecten van waterplanten variëren van puur abiotisch, zoals het remmen van waterstroming, tot biotisch, zoals het vormen van een voedselbron voor herbivore macroinvertebraten. Hoewel veel effecten van waterplanten op hun omgeving bekend zijn, is er nog steeds veel onbekend over de rol van waterplanten in het aquatische voedselweb. Het onderzoek dat in dit proefschrift wordt gepresenteerd heeft als doel om de non-trofische en trofische interacties tussen waterplanten en macroinvertebraten in gematigde laaglandbeken verder op te helderen. Hierbij wordt specifiek aandacht besteed aan de rol van waterplanten in het aquatische voedselweb, waarbij zowel naar hun directe (bijvoorbeeld directe consumptie van levende waterplanten en het daaruit ontstane organisch materiaal) als indirecte rol (bijvoorbeeld hun invloed op andere voedselbronnen zoals epifytische algen en bacteriën) wordt gekeken.

Waterplanten bleken, op verschillende manieren, significante effecten op de macroinvertebratengemeenschap te hebben. De fysieke structuur van de waterplanten had hierbij invloed op habitatcomplexiteit en de waterstroomsnelheid, die op hun beurt weer invloed uitoefenen op de geassocieerde levensgemeenschap van macroinvertebraten. Verder leidde een

toenemende complexiteit in de groeivorm van waterplanten tot een hogere bedekking van de plant met epifytische algen, die een belangrijke voedselbron vormen voor veel herbivore macroinvertebraten. Het uiteindelijke effect van waterplanten op hun epifytische biofilm was echter gemengd, omdat levende waterplanten een lagere algenbedekking hadden dan kunstmatige replica's, hetgeen mogelijk veroorzaakt werd door de uitscheiding van chemicaliën die de groei van algen remmen. Deze biofilm bevatte echter weer meer nutriënten dan die op kunstmatige waterplanten, wat waarschijnlijk werd veroorzaakt door de uitscheiding van nutriënten door levende waterplanten.

Door het meten van stabiele isotopen in het voedselweb bleek dat sommige soorten macroinvertebraten, waarvan verwacht werd dat ze van epifytische algen zouden leven, ook waterplanten consumeerden. Hierbij werd berekend dat macroinvertebraten uit de functionele voedingsgroep van de schrapers, die hun voedsel dicht op het bladoppervlak verzamelen, meer waterplantweefsel assimileren dan macroinvertebraten uit de functionele voedingsgroep van de verzamelaars, die hun voedsel minder dicht op het bladoppervlak verzamelen. Dit leidde tot de hypothese dat de assimilatie van waterplanten in deze groepen werd veroorzaakt door het onopzettelijk consumeren van de waterplantdelen tijdens het grazen op epifytische algen. Daarnaast werd bij sommige macroinvertebraten uit de functionele voedingsgroep van de knippers directe consumptie van waterplanten gemeten, hetgeen in sommige gevallen kan leiden tot een drastische achteruitgang van natuurlijke populaties waterplanten, zeker als deze al op andere manieren onder druk staan. Naast de consumptie van levende waterplanten werd er bij sommige macroinvertebraten, die hun voedsel uit de waterlaag filteren, ook de consumptie van organisch materiaal, ontstaan na de afbraak van dode waterplanten, waargenomen. Deze consumptie was het hoogst aan het einde van het groeiseizoen, wanneer grote hoeveelheden waterplanten afsterven en afgebroken worden tot organisch materiaal.

Het onderzoek dat in dit proefschrift beschreven staat laat zien dat waterplanten een significant effect hebben op de aquatische systemen waar ze in voorkomen en dat dit effect meer is dan de louter structurele rol, die vaak wordt bestudeerd, maar dat waterplanten ook een belangrijke rol spelen in het aquatische voedselweb.

Chapter 1.

General introduction

Wherever they co-occur, plants and animals affect each other. These relationships may take the form of trophic interactions in which plant tissue is consumed by herbivorous or omnivorous animals, but also include non-trophic interactions, such as insects pollinating plants or plants providing habitat to animals, by for example providing a substrate for attachment, shelter or oviposition sites. Both trophic and non-trophic interactions can drive the species composition and species diversity of ecosystems, community patterns and productivity, or even act as the foundation for the presence and persistence of communities (Jones et al. 1994, Dunne 2006, Kefi et al. 2012). The nature, strength and diversity of these interactions can be expected to vary strongly among different ecosystems. In this PhD thesis, I will focus on the different types of interactions between aquatic macrophytes and macroinvertebrates in temperate lowland streams and on the implications of these interactions on the functioning of this ecosystem. Aquatic macrophytes are hereby defined as plants that have adaptations to live in aquatic environments and that grow in the water or along the waterline. This definition includes both lower (e.g. bryophytes) and higher plants and encompasses submerged, floating and emergent growth forms (Hickey and King 2001). Also included are the helophytes; plants that root underwater but have emergent upper parts. Because of their dominance in temperate lowland streams, this thesis will exclusively deal with higher plants that grow completely submerged (*Callitriche obtusangula* Le gall, *Myriophyllum spicatum* L., *Egeria densa* Planch, *Vallisneria spiralis* L. and the submerged form of *Sparganium emersum* Rehmann) or have a submerged growth form with floating leaves (*Potamogeton natans* L.). Furthermore, macroinvertebrates are defined as all aquatic invertebrates that are larger than 0.5 mm, with the exclusion of typical planktonic taxa such as Cladocera or Ostracoda that occasionally grow over 0.5 mm (e.g. Werkgroep Ecologisch Waterbeheer 2016). Animals smaller than 0.5 mm, but larger than 40 µm, are classified as meiofauna (Higgins and Thiel 1988). Compared to macroinvertebrates, much less is known about this taxonomically varied group, which includes animals such as nematodes, rotifers and turbellarians. It is nevertheless assumed that these animals have a significant ecological role in stream ecosystems, by affecting important food web metrics, such as complexity and connectance (Schmid-Araya et al. 2002a, Schmid-Araya et al. 2002b). Due to the difficulties associated with studying this group, meiofauna will not be included in this thesis.

The lowland stream environment

Temperate lowland streams are low-energy rivers that are characterised by a channel width of 3 - 8 m and a slope of less than 1 m km^{-1} (Van der Molen et al. 2012). In Belgium, these lowland streams can be found in the northern part of the country, but they are common across the lowlands of the Northwestern European plain, which also includes The Netherlands, the northern part of Germany, Denmark, the southern part of Sweden and the Northwestern and central parts of Poland (Eekhout 2014). Under natural conditions, these streams are characterised by a meandering profile and by a high structural heterogeneity and diversity of mesohabitats, including sand bars, patches of dead woody debris and extensive depositions of fine and coarse particulate organic matter (FPOM and CPOM respectively) (Van der Molen et al. 2012). This variety in habitats also leads to a high diversity in plant and animal life (Van der Molen et al. 2012). However, lowland streams have undergone significant modification and degradation due to anthropogenic disturbances, which include processes such as land use change, channel modification and channelisation, eutrophication, pollution, intensive management to retain their drainage function (e.g. weed cutting) and fragmentation (Allan 2004, Friberg 2010). These anthropogenic impacts resulted in a decrease in stream biodiversity, among others, due to a homogenisation of the stream habitat and water flow patterns, loss of specific mesohabitats and an increased dominance of (non-native) disturbance tolerant species at the cost of more sensitive species (e.g. Johnson and Hering 2009).

Conditions within undisturbed lowland river systems generally change over the longitudinal course of the river, from the headwaters to the river mouth, as described by the River Continuum Concept (Vannote et al. 1980). Within this continuous and connected system, which ranges from narrow and shallow headwaters to broad and deep river mouths and estuaries, the lowland streams studied in this thesis generally occupy a position at the beginning of the continuum (Vannote et al. 1980). Under natural conditions, these streams are shaded by riparian forests, so that little light can penetrate the canopy and reach the stream (Vannote et al. 1980, Julian et al. 2011). Because macrophytes and benthic algae are light limited under these conditions, the base of the food web generally consists of allochthonous organic matter, resulting in a net heterotrophic system (Vannote et al. 1980). Due to deforestation and the transformation of riparian forests to open

meadows suitable for agriculture, many modern lowland streams are no longer light-limited. The increased light availability in these shallow streams, especially when combined with high anthropogenic nutrient inputs, led in turn to the dominance of macrophytes in the present lowland streams (Bloemendaal and Roelofs 1988, Allan and Castillo 2007). Macrophyte occurrence and growth are primarily influenced by a number of factors that include flow velocity, light availability and water chemistry (e.g. pH, pollutants and the availability of dissolved inorganic carbon (DIC), N and P) (e.g. Bloemendaal and Roelofs 1988). In addition to being greatly influenced by their environment, macrophytes can also significantly modify their own habitat by their form and function. In this sense they perform a role as ecosystem engineer in the stream ecosystem because they, by their various influences, "directly or indirectly modulate the availability of resources (other than themselves) to other species, by causing physical state changes in biotic or abiotic materials. In doing so they modify, maintain and/or create habitats" (Jones et al. 1994, 1997).

Effects of macrophytes on their environment

The ways in which macrophytes influence their environment, and also that of many other aquatic organisms, are varied. First of all, the physical structures (i.e. stems and leaves) of macrophytes can provide hydrodynamic resistance to water flow, which in turn leads to the creation of areas of low flow velocity within vegetation stands, thus providing a habitat for more limnophilous macroinvertebrate species (Sand-Jensen and Mebus 1996, Bell et al. 2013). The strength of this flow-attenuating effect is related to the density and growth form of the macrophytes, with denser macrophyte stands and more complex growth forms having a greater hampering effect on flow velocity than less dense stands and simple growth forms (Madsen et al. 2001, Bell et al. 2013, Verschoren 2017). Furthermore, the particle intercepting effect of macrophyte structures, combined with the lower flow velocity within the macrophyte stands facilitates the precipitation of suspended sediment and organic matter within vegetation stands, thus leading to a greater availability of nutrients and an increase of food for detritivorous macroinvertebrates (Sand-Jensen and Mebus 1996, Horppila and Nurminen 2003, Schoelynck 2011). By reducing the amount of suspended particles in this way, macrophytes can also have a positive effect on water clarity (Madsen et al. 2001,

Horppila and Nurminen 2003). The physical structures of aquatic macrophytes can also directly form a habitat for macroinvertebrates, whereby the structures significantly increase structural habitat complexity (i.e. the heterogeneity of physical structures and interstitial spaces), which in turn results in a higher amount of colonisable microhabitats (McNett and Rypstra 2000, McAbendroth et al. 2005). In doing so, the macrophytes also provide shelter opportunities for macroinvertebrates (e.g. high flow refuges (Lancaster and Hildrew 1993, Winterbottom et al. 1997)) and act as a refuge against predation by, for example fish (Crowder and Cooper 1982, Warfe and Barmuta 2004, 2006). Finally, the structures formed by the macrophytes create a large area suitable for colonisation by epiphytic algae and bacteria (i.e. the epiphyton), which forms an important food source for a wide array of herbivorous grazing macroinvertebrates. It has been shown that complex macrophytes (i.e. macrophytes with a complex growth form, consisting of finely dissected structures and a high diversity in the scale of interstitial spaces between those structures) support more epiphyton compared to plant species with a simple growth form, despite similar surface areas (Warfe and Barmuta 2006). In the already heterogeneous lowland stream habitat, the structures formed by macrophytes can thus add to the variation in environmental conditions, resulting in a more heterogeneous habitat. In addition, the large surface area created for colonisation, results in a significantly larger and more diverse macroinvertebrate community compared to non-vegetated areas in the same stream (Heck and Crowder 1991, O'Hare and Murphy 1999).

In addition to the effects created by the macrophytes' structure, the aquatic environment is also strongly influenced through plant metabolic processes such as photosynthesis and nutrient exchange. Underwater photosynthesis by submerged macrophytes is a process that influences the concentrations of both dissolved oxygen and DIC, and the associated inorganic carbon equilibrium. During photosynthesis, macrophytes preferably take up DIC in the form of CO_2. Some species can also utilise HCO_3^-, in which way they shift the inorganic carbon equilibrium, between CO_2, HCO_3^- and CO_3^{2-}, towards a dominance of CO_3^{2-} (e.g. Pedersen et al. 2013). This shift in the inorganic carbon equilibrium, and the production of OH^--ions following the use of HCO_3^- as a carbon source, also causes the pH of the water layer to increase (e.g. Pedersen et al. 2013). Although this increase in water layer pH has no significant direct effect on other aquatic

organisms, it has been shown that some pesticides are broken down at a faster rate under conditions of a higher pH (Brogan and Relyea 2015). Photosynthesis also produces oxygen. Besides transferring dissolved oxygen to the water layer, where it can be used for animal respiration, macrophytes also exude oxygen into the sediment through their roots (e.g. Sand-Jensen et al. 1982). The proportion of oxygen that is transported to either the water layer or the sediment depends on the plant species. For example, 28 to 100% of the oxygen production of macrophytes with an isoetid growth form, which features a large root system combined with large internal lacunae, is transported to the sediment, while this is only between 2 and 4% for some *Potamogeton* species (Sand-Jensen et al. 1982). Providing oxygen to the sediment could be beneficial for burrowing macroinvertebrates and sediment-inhabiting micro-organisms and it also accelerates the breakdown of toxic substances like sulphide (Bloemendaal and Roelofs 1988). This oxidation may however also mobilise sulphide-bound metals, leading to an increase in their bioavailability (Teuchies et al. 2011, De Jonge et al. 2012). A special case of oxygen acquisition from macrophytes is observed in larvae of some Diptera (e.g. *Coquillettidia*) and Coleoptera (e.g. *Noterus* and *Donacia*), which are able to pierce macrophyte roots and stems with an adapted respiratory organ in order to utilise this oxygen, which is transported through the aerenchyma, for their own respiration (Houlihan 1969a, b).

Macrophytes also exude a wide variety of chemicals to the water layer, including allelochemicals, nitrogen (N), phosphorus (P) and dissolved organic carbon (DOC), which can all have a significant effect on the growth of epiphytic algae (Sondergaard 1981, Wetzel 1983, Blindow 1984, Carpenter and Lodge 1986, Burkholder and Wetzel 1990, Wigand et al. 2000, Gross 2003). The excretion of N, P and DOC has been demonstrated to have a positive influence on the productivity and nutritious quality (i.e. lower C:N and C:P ratios) of epiphytic algae and bacteria (Kirchman et al. 1984, Theil-Nielsen and Sondergaard 1999, Bowman et al. 2005). On the other hand, allelopathic substances excreted by macrophytes can have a significant negative impact on planktonic and epiphytic algae by inhibiting their growth (e.g. Wigand et al. 2000, Gross 2003). Whether the combination of these various effects has a net positive or negative effect on the development of epiphyton is still not clear, as some authors report positive effects (Theil-Nielsen and Sondergaard 1999, Bowman et al. 2005), negative effects (Wium-Andersen

1987, Wigand et al. 2000) or assume macrophytes to be a neutral substrate (Shelford 1918, Sozka 1975).

Macrophytes in the aquatic food web

The various ways in which macrophytes influence their environment, and the variety of resources provided to aquatic macroinvertebrates, have of course a great effect on the composition and complexity of the aquatic food web. The non-trophic interactions between the plants, epiphytic algae and the macroinvertebrates have a significant positive effect on stream food web complexity (cf. Kefi et al. 2012, Borst et al. 2018). Regarding the trophic interactions between macrophytes and macroinvertebrates, it is often assumed that macrophytes are not directly consumed by macroinvertebrates, but instead enter the aquatic food web as detritus (Polunin 1984), which may serve as food source for resident macroinvertebrates or for animals living further downstream (c.f. Vannote et al. 1980). Earlier researchers even went so far as to assume that macrophytes only played a structural role in the aquatic food web and that the direct contribution of macrophytes to the food web was negligible (Shelford 1918, Whitton 1975, Fisher and Carpenter 1976). Shelford (1918) for example quoted “One could probably remove all the larger plants and substitute glass structures of the same form and surface texture without greatly affecting the immediate food relations”. Later research has however demonstrated that the nutritive quality of aquatic macrophytes does not differ significantly from that of terrestrial plants, as freshwater macrophytes generally have lower C:N ratios and relatively easy to digest carbon-rich structural compounds, such as lignin and cellulose, in comparison to many terrestrial plant species (Lodge 1991, Bakker et al. 2016). Instead, the reason for the relatively low herbivory rates on aquatic macrophytes may be sought in the presence of inhibitory secondary metabolites, such as alkaloids, glucosinolates and polyphenolics, which can act as a chemical defence against herbivory (Sotka et al. 2009, Gross and Bakker 2012). Indeed, only a few macroinvertebrate taxa are actually known to directly consume living macrophyte parts, including specialist Lepidoptera, Coleoptera and Diptera taxa and generalist omnivorous crabs and crayfish (Newman 1991, Olsen et al. 1991, Cronin et al. 1998). However, despite the presence of these inhibitory compounds, numerous cases of significant amounts of invertebrate- and fish-induced herbivore damage

on aquatic macrophytes have been observed under natural conditions (Bakker et al. 2016, Wood et al. 2017).

Research question and outline of the thesis

As stated in the previous section, there already exists a large body of information on the trophic and non-trophic interactions between macrophytes, their abiotic environment, epiphyton and aquatic macroinvertebrates. However, much of this information has been collected from lentic water bodies (McAbendroth et al. 2005, Verdonschot et al. 2012), making it difficult to apply to lotic temperate lowland streams. Additionally, most research on stream food webs has been performed on the role of terrestrial leaf litter or periphytic algae as a food source (e.g. Karouna and Fuller 1992, Descroix et al. 2010, France 2011, Crenier et al. 2017), while the direct and indirect role of living or decomposed submerged macrophytes has to a large extent been ignored. This thesis addresses these scientific lacunas, by integrating the different effects of macrophytes on their environment and on the macroinvertebrate assemblages inhabiting macrophyte stands in lowland streams in a series of field studies and greenhouse experiments. Special attention will be devoted to the macrophytes' direct role in the aquatic food web as a food source for invertebrates, but also on their indirect role by influencing the epiphyton growing on the plants. The focus on these subjects can be integrated into the main research question: '**To what extent are macroinvertebrate assemblages in temperate lowland streams influenced by the presence of living macrophytes?**'.

A schematic overview of the contents of this thesis, and which topics will be addressed herein, is given in Figure 1.1. **Chapter 2** will examine the structural role that macrophytes play in lowland streams by increasing habitat complexity and by creating low-flow-sections inside macrophyte patches. In order to quantify these effects, a two-year correlative field study is carried out, in which macroinvertebrate community structure is linked to the environmental factors water flow velocity and macrophyte structural complexity. These structural effects of macrophytes, and the impact this has on macroinvertebrate community structure, will be studied for three different plant species with different growth forms. In this way, it will be possible to study the importance of macrophyte complexity on the macrophytes' habitat providing and flow-attenuating role.

The direct role of macrophytes in the aquatic food web will be explored in **Chapters 3 and 4**. The focus of **Chapter 3** will hereby lie on the direct consumption of living macrophytes by herbivorous and omnivorous macroinvertebrates and fish. This will be done by reconstructing the stream food web using stable isotope measurements of the different basal resources and consumers of the stream ecosystem. On the other hand, **Chapter 4** will be more focussed on the role of macrophytes and macrophyte-derived organic material in the stream food web throughout the year. Special attention will hereby be paid to the role of macrophyte-derived matter in the system's CPOM and FPOM pool and in the diet of shredding and filtering macroinvertebrates. This will be studied by measuring the fatty acid composition of the different basal resources and consumers in the stream ecosystem throughout the year, in order to track the relative importance of the different food sources in the diet of the invertebrate over the course of the growing season.

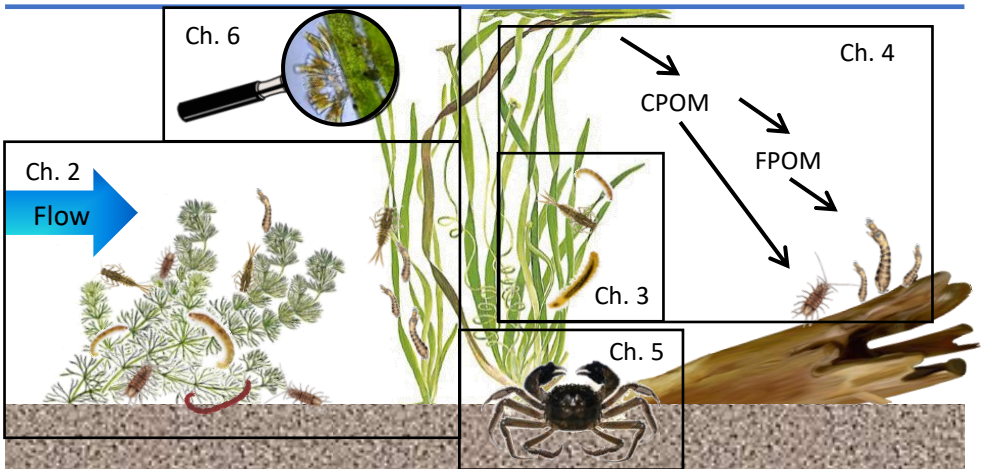


Figure 1.1 Overview of the different trophic and non-trophic interactions between macrophytes and macroinvertebrates that are studied in this thesis. These include the effects of macrophyte habitat complexity on water flow velocity patterns, and the associated macroinvertebrate assemblages (Chapter 2), the role of living macrophytes in the diet of herbivorous and omnivorous macroinvertebrates and fish (Chapter 3), the role of macrophyte-derived organic matter in the stream food web (Chapter 4), the impact of herbivory on aquatic vegetation in a multi-stressed environment, illustrated in a case study with the Chinese mitten crabs (*Eriocheir sinensis*) (Chapter 5) and the role of macrophytes as a substrate for epiphyton (Chapter 6).

In **Chapter 5**, the impact of macroinvertebrate herbivory in a multi-stressed environment, illustrated by a case study on the Chinese mitten crab, on the survival and growth of macrophytes will be studied in a greenhouse mesocosm experiment. By introducing different crab densities in mesocosms containing plants grown under chemical or light stress, I provide evidence for the recent disappearance of macrophytes in several Belgian rivers.

The indirect role of macrophytes on the stream food web will be examined in **Chapter 6**. In this chapter, the effect of the macrophytes' exudation of nutrients, DOC and allelopathic substances on epiphytic algae and bacteria will be studied in a greenhouse microcosm experiment. Additionally, the nutritive quality of the epiphyton will be measured and the effect of the macrophytes' impact on the epiphyton on the grazing macroinvertebrates will be assessed in a controlled macroinvertebrate growth experiment.

Finally, a synthesis of all these results will be given in **Chapter 7**. Here, an overview of the results of the research presented in this thesis is given and integrated with previous findings to answer the main research question. Furthermore, the remaining knowledge gaps and opportunities for further research will be explored.

Study sites

The research carried out in this thesis consisted of a combination of both greenhouse experiments and field studies. While the greenhouse experiments described in Chapter 5 and 6 were performed in the University of Antwerp mesodrome facilities, featuring natural light conditions and temperature that followed the outdoor conditions, the field studies described in Chapter 2, 3 and 4 were performed in two neighbouring lowland streams in the North-East of Belgium (Figure 1.2). The two studied streams are the Desselse Nete (studied in Chapter 2 and 3) and the Zwarte Nete (studied in Chapter 4), both situated close to each other in the Nete catchment. The Nete catchment in a sub-basin of the Scheldt catchment and has an area of 1673 km^2 over a predominantly sandy soil. With an average width of 5.4 m at the studied section and a discharge 0.3 – 0.7 $m^3 s^{-1}$, the Desselse Nete is slightly larger than the Zwarte Nete, with an average width of 4.4 m and a discharge between 0.2 - 0.5 $m^3 s^{-1}$ (Chapter 2; Verschoren et al. 2017). Due to their relative small size (i.e. low discharge) and low water level slope (Verschoren et al.

2017), both streams would under natural conditions follow an anastomosing fluvial style (Makaske 1998). Both streams are predominantly fed by rainwater runoff and subsurface seepage. The confluence where these two streams join together is very close to the studied sections of both streams, with the section in the Desselse Nete being only 300 m from the confluence. Land use surrounding the streams is mainly agricultural, which limits the amount of overhanging or other riparian vegetation. Because of the intensive agriculture in the catchment surrounding the streams, the anthropogenic impact on the stream is substantial, with stressors including hydromorphological degradation and water quality issues (i.e. pollution and agricultural nutrient inputs). Summer nutrient concentrations in both streams are within the same range, although they were slightly higher in the Zwarte Nete (187.5 ± 69.1 µg $N\text{-}NH^{4+}$ l^{-1}, 1022.5 ± 103.0 µg $N\text{-}NO^{3-}$ l^{-1} and 56.25 ± 11.7 µg $P\text{-}PO4^{3-}$ l^{-1}) compared to the Desselse Nete (95.0 ± 35.7 µg $N\text{-}NH^{4+}$ l^{-1}, 672.5 ± 60.5 µg $N\text{-}NO^{3-}$ l^{-1} and 17.25 ± 4.0 µg $P\text{-}PO4^{3-}$ l^{-1}) (Vlaamse Milieu Maatschappij 2016, 2017a).

Both streams are characterised by extensive instream plant growth, consisting of *Callitriche obtusangula* Le gall (Plantaginaceae), *Myriophyllum spicatum* L. (Haloragaceae), *Potamogeton pectinatus* L. (Potamogetonaceae), *Ranunculus peltatus* L. (Ranunculaceae), *Sagittaria sagittifolia* L. (Alismataceae), *Sparganium emersum* L. (Sparganiaceae) and *Potamogeton natans* L. (Potamogetonaceae). Despite the proximity of the two streams, *P. natans* has only been found in the Desselse Nete, before and after the confluence, and the reason for its absence in the Zwarte Nete is unknown. The macrophyte growing season occurs from March to August, with aboveground instream vegetation remaining present until October in the Zwarte Nete, while the majority of the macrophytes remain green throughout the winter in the Desselse Nete. Again, the reason for this difference remains unknown.

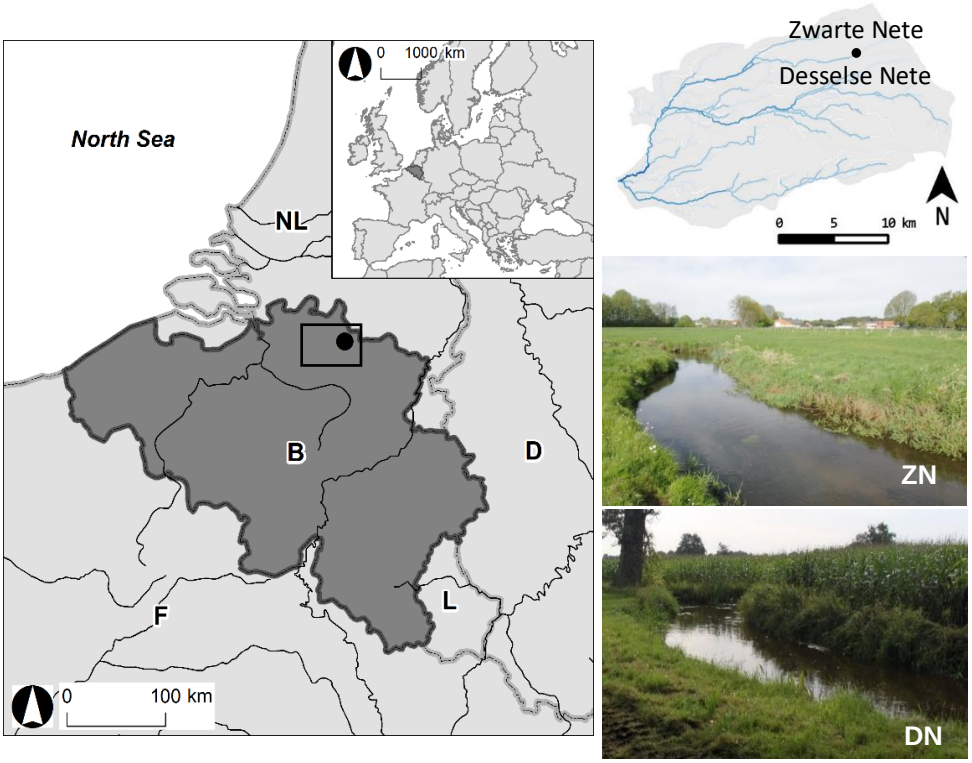
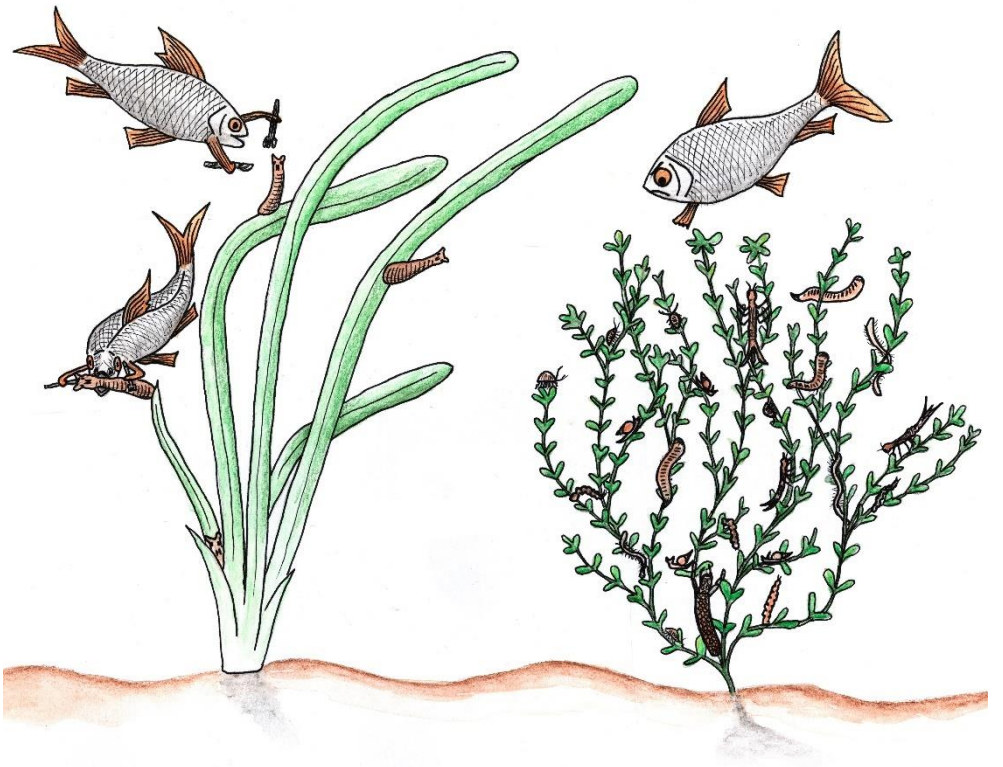


Figure 1.2 Location of the study sites visited during the research carried out in Chapter 2, 3 and 4 of this thesis. The location of the study sites in Belgium (left panel) is shown, together with the location of the Zwarte Nete (ZN) and Desselse Nete (DN) streams within the Nete catchment.



Chapter 2.

The role of macrophyte structural complexity and water flow velocity in determining the epiphytic macro-invertebrate community composition in a lowland stream

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Abstract

Habitat structural complexity provided by aquatic macrophytes in lowland streams affects the associated epiphytic macroinvertebrate assemblages in both direct (increased microhabitat diversity, refuge against predation) and indirect ways (e.g. flow attenuation by physical structures). In a correlative field study carried out in two different years in a Belgian stream, we investigated the effects of the factors macrophyte identity, macrophyte complexity (represented as fractal complexity) and water flow velocity on the composition of the macroinvertebrate community associated with monospecific macrophyte patches, consisting of plants with differing structural complexity; *Sparganium emersum* Rehmann (least complex), *Potamogeton natans* L. (intermediate) and *Callitriche obtusangula* Le Gall (most complex). In addition to significantly lower within-patch flow velocity being observed, vegetation stands consisting of complex macrophytes also harboured significantly richer macroinvertebrate communities than stands of simpler macrophytes. A significant part of the variation in the macroinvertebrate community composition could be explained by plant identity, macrophyte complexity and flow velocity. However, it was not possible to determine the relative importance of these three factors, because of their high degree of intercorrelation. Additionally, the explanatory power of these factors was higher under conditions of high flow velocity, suggesting a role of macrophyte patches as instream flow refugia for macroinvertebrates.

Keywords: Habitat complexity, phytomacrofauna, flow velocity, functional groups

Introduction

Habitat structural complexity has long been recognised as an important determining factor for biodiversity in community ecology, whereby more complex habitats typically support more diverse communities (e.g. MacArthur and Wilson 1967). In many freshwater ecosystems, ranging from stagnant ponds and ditches to slowly flowing lowland streams and rivers, macrophytes generally comprise the majority of habitat complexity in the ecosystem and support a significantly more diverse community of aquatic macroinvertebrates than the non-vegetated areas in these waters (Heck and Crowder 1991, O'Hare and Murphy 1999). The effect of aquatic macrophytes on their associated macroinvertebrate community depends to a large degree on their structural complexity, and therefore growth form (e.g. Ferreiro et al. 2011, Bell et al. 2013).

Macrophyte structural complexity influences a number of processes in freshwater ecosystems, which may in turn affect the macroinvertebrate community. First of all, the finely dissected stems and leaves of complex macrophytes can increase structural habitat complexity, increasing the amount of colonisable microhabitats (McNett and Rypstra 2000, McAbendroth et al. 2005) and providing a better refuge against predation (Crowder and Cooper 1982, Warfe and Barmuta 2004, 2006). This increased diversity in the scale of interstitial spaces can also lead to an increased diversity in macroinvertebrate body-size distributions; a product of size-specific interactions between organisms and the physical environment (Schmid et al. 2002, McAbendroth et al. 2005, Ferreiro et al. 2011). Secondly, in lotic systems, the physical structure of aquatic macrophytes provides resistance to water flow and is very efficient in decreasing flow velocity, creating areas of low water flow velocity within the vegetation stand and thus providing a habitat for more limnophilous macroinvertebrate species (Sand-Jensen and Mebus 1996, Bell et al. 2013, Schoelynck et al. 2013). Plant complexity and the density of structural elements hereby both positively affect the flow-attenuating potential of the macrophyte stands (Madsen et al. 2001, Bell et al. 2013, Schoelynck et al. 2013). This flow-attenuating effect can create a gradient within macrophyte stands, with higher flow velocities in upstream sections of the patch and lower velocities in downstream sections of the patch, further increasing environmental heterogeneity (Peralta et al. 2008). Finally, it has been shown that complex macrophytes support more epiphyton, an important food source for many macroinvertebrates,

compared to plant species with a simple growth form, despite similar surface areas (Chapter 6, Warfe and Barmuta 2006).

Although the importance of macrophyte complexity has been studied before, many of these studies were performed in lentic systems (e.g. McAbendroth et al. 2005, Verdonschot et al. 2012) or simply did not take the effect of flow velocity into account (Taniguchi et al. 2003, Warfe et al. 2008, Ferreiro et al. 2011). One study, performed by Bell et al. (2013), observed significant differences in macroinvertebrate community structure between macrophytes of a contrasting growth form and with consequent differences in within-patch flow velocities. However, this study attributed all variation in community structure solely to variations in flow velocity and did not attempt to distinguish between the separate effects of individual environmental variables (food availability, plant identity, plant complexity and flow velocity). To our knowledge, this is the first study that investigates not only the effect of plant complexity, but also the effect of plant identity and flow velocity on the macroinvertebrate community structure in two different seasons.

The aims of this study were twofold. First, to examine how different macrophyte species, varying in complexity, affected water flow velocity within their stands and how this had an effect on the associated macroinvertebrate community. To achieve this objective, the study attempts to disentangle the separate effects of macrophyte species, structural complexity and flow velocity. Second, it was attempted to distinguish between different locations within individual vegetation patches to discover if intra-patch variations in flow velocity had any effect on the macroinvertebrate community composition. These research questions were addressed in a correlative field study carried out in two different years in a slow-flowing lowland stream in the north of Belgium.

For the first research question, we expected that different macrophyte species would harbour distinct macroinvertebrate communities, and that this effect would be consistent for the separate time periods (sampling events). Furthermore, it was expected that the abundance of certain taxonomic or functional groups could be significantly explained by the environmental variables measured. For example, a positive correlation between the amount of passive filter-feeders, that depend on the water flow for their food supply, and flow velocity could be expected. However, it was also expected that a high degree of intercorrelation among the different

variables would hinder our ability to separate the effects of individual variables. For the second research question, we expected that the zonation in flow velocity within macrophyte patches, with a decreasing velocity towards the centre and distal end of the patch, would significantly affect the associated macroinvertebrate community, with a decreasing portion of rheophilous taxa towards the low flow areas of the patch and vice versa an increase of limnophilous taxa.

Material and methods

Study site

Fieldwork was performed on the 20th and 21st of August 2014 and the 4th of June 2015 in the Desselse Nete, a slow-flowing sand bottom lowland stream in the north of Belgium (51°14'53" N, 5°4'53" E) that is predominantly fed by rainwater runoff and subsurface seepage. In the studied reach, stream width varied between 3.5 and 5.5 m with an average depth of 58 cm in August 2014 and 33 cm in June 2015. Average discharge was 0.69 m^3 s^{-1} in August 2014 and 0.43 m^3 s^{-1} in June 2015. The June 2015 lower water level and discharge can be attributed to the lower amounts of rainfall in the 2 months prior to sampling (189 mm in 2014 vs. 47 mm in 2015 (Vlaamse Milieu Maatschappij 2016)). Summer nutrient concentrations are 95.0 ± 35.7 µg $N\text{-}NH^{4+}$ l^{-1}, 672.5 ± 60.5 µg $N\text{-}NO^{3-}$ l^{-1} and 17.25 ± 4.0 µg $P\text{-}PO4^{3-}$ l^{-1}, with an average pH of 7.45 (Monthly measurements, VMM - Flemish Environment Agency 2016). Dominant macrophytes in the studied stream section included *Sparganium emersum* Rehmann (Sparganiaceae), *Potamogeton natans* L. (Potamogetonaceae) and *Callitriche obtusangula* Le Gall (Plantaginaceae). The macrophyte growing season is between March and August, with vegetation remaining present throughout the year, which is exceptional for lowland streams in temperate regions.

At each sampling event, macroinvertebrates and environmental variables were measured in and around nine monospecific, similar sized and fully submerged macrophyte stands of the three dominant plant species present. The growth form of the species (classification after Den Hartog & Van der Velde (1988)) differed: *S. emersum* was characterised by a relatively simple Vallesnerid growth form, *P. natans* by with a more complex Nymphaid growth form and *C. obtusangula* had the most complex Peplid growth form. Within each macrophyte patch, three locations were selected in which flow velocity was measured and samples were taken; the most upstream section (hereafter called 'front', located at the patch's upstream border), the most central section ('middle', situated in the middle of the patch) and the most downstream section ('back', located at the patch's downstream border) (Figure 2.1). Patch length varied between 1.4 and 7.5 m (with an exception of 13.5 m) and patch width ranged from 0.5 to 2.9 m (Table S2.1). The depth at which the patches were situated varied between 37 and 75 cm for 2014 and between 17 and 47 cm for 2015 (TableS2.1).

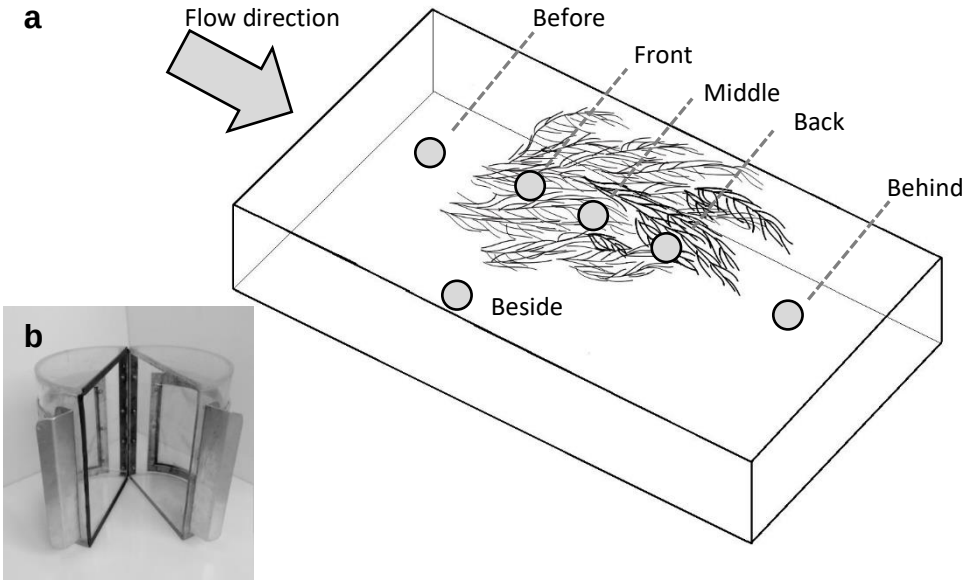

Figure 2.1 Schematic overview of the different sampling points (grey circles) in- and outside a macrophyte patch (a). Water flow velocity was measured in all 6 sections, while macroinvertebrates were only collected for the 3 sections within the macrophyte patch. The inset (b) shows a photograph of the box-sampler used in this study.

Water flow velocity

Water flow velocities in the stream were measured three days before both sampling events, to allow the macroinvertebrate community some time to recover from the slight disturbances, in the front, middle and back section of each patch. Measurements were performed 5 cm from the outer boundary of the patch. flow velocity was also measured 10 cm before the patch upstream boundary ('before'), 10 cm beside the patch outer boundary ('beside') and 10 cm behind the patch downstream boundary ('behind') to gain insight in the flow-attenuating effect of the vegetation (Figure 2.1). Measurements were performed at every 10 cm in the water column, starting at 5 cm above the stream bottom. All flow velocities were measured using a Valeport 801 ElectroMagnetic Flowmeter, programmed to return a 30 second average. For every location inside (front, middle and back) each vegetation patch, only velocity measurements taken within the vegetation patch itself were taken into account, while for locations outside (before, beside and behind) the patches the complete depth-averaged flow velocity was used.

Macroinvertebrate assemblages

Within each macrophyte stand, macroinvertebrate and plant material was collected using a cylindrical box-sampler (inner dimensions: 23.5 cm × 19 cm (length × diameter); total volume: 6663 cm^3) which was custom made for this purpose (Figure 2.1). During sampling, the sampler was gently lowered over the upper section of the vegetation stand, after which its two halves were gently closed and the vegetation within the sampler was cut off with a sharp knife. In this way, only the upper section of the patch was sampled to avoid any sediment in the samples. Samples were collected in the front, middle and back sections of each patch.

Immediately after the samples were taken, they were preserved with 4% formaldehyde. In the laboratory, these plant and macroinvertebrate samples were sieved (mesh size 500 µm) and the macroinvertebrates were separated from the plant material. Macroinvertebrates were then stored until identification (oligochaetes in 4% formaldehyde, other invertebrates in 70% ethanol), whereas macrophytes were stored at 4 °C until their structural complexity was measured. Macroinvertebrates were identified to the lowest taxonomic level (generally species) practical, with the exception of the Chironomidae which were identified to genus level.

After identification, a functional group was assigned to each macroinvertebrate taxon by combining information on their feeding modes and habit traits, according to Moog (1995), Moller Pillot (2009) and Verdonschot et al. (2012). The functional feeding groups provide information on the food sources and feeding modes of macroinvertebrates, while habit traits provide information on relative mobility and where food is obtained (Heino 2005).

For each macroinvertebrate sample, both taxonomic and functional richness and diversity were calculated. The taxonomic and functional richness in each sample was represented as the number of taxa and functional groups within the sample, whereas taxonomic and functional diversity were represented by calculating the Shannon diversity index by respectively using the different taxa and functional groups within each sample as individual species within the calculation (Hill 1973, Stevens et al. 2003).

Macrophyte structural complexity

As a measure of macrophyte structural complexity, fractal dimension based on perimeter (D_P or "boundary" fractal) was calculated for each macrophyte sample to get an indication of the degree of dissection of the plant (McAbendroth et al. 2005). After all macroinvertebrates were removed from the samples, a macrophyte subsample was taken and spread out over a white plastic plate of 1 m^2 that was covered in water to allow the natural separation of branches and leaves. Photographs were taken using a Nikon D300S with a Tokima 11-16 mm f/2.8 lens. Pictures were then converted into binary images (1 pixel = 0.13 mm), after which the fractal perimeter was calculated using ImageJ software (Rasband 1997-2012). ImageJ uses a box count algorithm to quantify the fractal dimension of the perimeter of the structures. A series of grid sizes ranging from 2 to 64 pixels (box sizes 0.26 - 8.32 mm) were used to estimate the perimeter covered by the structures at different measurement scales. Fractal dimension was estimated from the slope of the perimeter estimate plotted against the grid size (both $\log_{10}(x)$-transformed).

Statistical analyses

To test whether the measured parameters were normally distributed, both Shapiro-Wilk tests and visual inspection of Q-Q plots were used. Not normally

distributed data were tested for significant differences among groups using Kruskal-Wallis tests and Dunn's post hoc tests. Normally distributed data were checked for equality of error variances using Levene's tests. Significant differences among groups were assessed using one-way ANOVAs with Tukey post-hoc tests for equal variances or using Welch tests and Games-Howell post-hoc tests for non-equal variances. Relationships between environmental parameters, including macrophyte complexity and water flow velocity, and the occurrence of macroinvertebrate taxa and functional were defined using Pearson correlation coefficients and tested for significance using two-tailed t-tests. All tests were performed in SPSS version 23.0.

Multivariate analyses, performed in CANOCO for Windows version 5 (Ter Braak and Smilauer 2012), were used to describe the taxonomic and functional macroinvertebrate community composition among different years and different plant species (unconstrained analysis) and to identify relationships between the community composition and the environmental variables (constrained analysis). Based on exploratory multivariate analyses including the environmental variables plant identity (represented as dummy variables), plant structural complexity, flow velocity, sample biomass, water depth and patch surface area (Figure S2.1), it was decided to exclude the latter three variables from further analysis. Sample biomass did not have a significant effect on community composition, the effect of water depth was only caused by the inter-year difference in overall water height in the stream, rather than plant-specific properties, and patch surface area did not have a significant effect on community composition in the separate years, indicating that any explanatory power by this variable in the combined years could solely be attributed to inherent differences between years

For the unconstrained analyses, maximum gradient length was 3.3 for the taxonomic dataset and 2.0 for the functional group dataset, indicating that a Detrended Correspondence Analysis (DCA) and a Principal Component Analysis (PCA) would best fit the data (Ter Braak and Smilauer 2012). Community data were represented as the proportion of the total community consisting of a certain taxon or functional group, and these data were first $\log_{10}(x+1)$ transformed prior to the ordinations. Rare taxa were down-weighted for the DCA analysis of the taxonomic community composition, whereas the functional groups were centred for the PCA of the functional community composition.

Subsequently, constrained analyses were performed to estimate to what extent the environmental factors explained the observed variance in macroinvertebrate community composition. This was done by using the constrained variants of the earlier performed multivariate analyses, namely a Canonical Correspondence Analysis (CCA) for the taxonomic community composition and a Redundancy Analysis (RDA) for the functional one. Monte Carlo global permutation tests were then performed to determine if the variance explained by the canonical axes differed significantly from random. Finally, the total variation that was explained by the individual explanatory variables was calculated by variance partitioning, which tested for both the simple effects (i.e. variance the variable would explain if it were the only explanatory variable) and the conditional effects (i.e. variance the variable would explain if the other explanatory variables were covariates).

In addition to the CANOCO analyses, a TWINSPAN clustering was performed in WinTWINS (Hill and Smilauer 2005) to analyse the clustering of the different samples and to elucidate the importance of the different variables (e.g. year, plant species, patch or within-patch position) herein. Cut levels were set to 0, 0.02, 0.05, 0.1 and 0.2.

Results

Environmental variables

Highly significant (one-way ANOVA; $F_{df=2,40} = 129.1$; $p < 0.001$) differences in fractal dimension based on perimeter, used as a measure of plant complexity, were found among plant species (Table S2.2). *S. emersum* displayed the lowest edge complexity ($D_p = 1.065$), *C. obtusangula* displayed the highest complexity ($D_p = 1.215$), whereas *P. natans* occupied an intermediate position ($D_p = 1.135$). No differences in fractal dimension were observed between within-patch locations (one-way ANOVA; $F_{df=2,41} = 0.833$; $p = 0.442$) and between different years (one-way ANOVA; $F_{df=1,41} = 0.015$; $p = 0.902$).

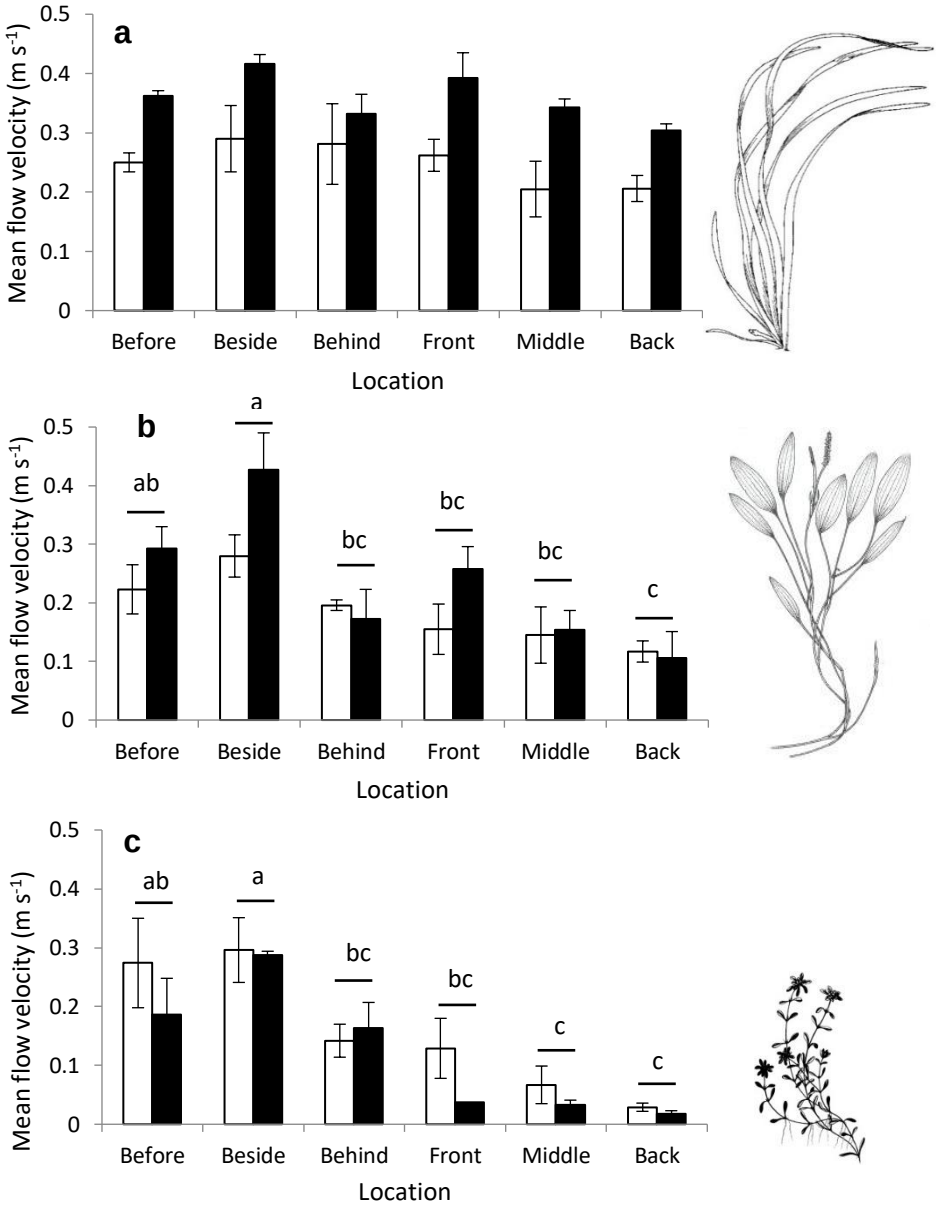

Figure 2.2 Water flow velocity measured on several locations in- and outside patches of *S. emersum* (a), *P. natans* (b) and *C. obtusangula* (c) in 2014 (white bars) and 2015 (black bars). Values are presented as means (n = 3) ± S.E. Locations indicated with different letters are significantly different at $p < 0.05$. Note that no significant differences were observed in *S. emersum* patches.

Measured water flow velocities differed significantly (one-way ANOVA; $F_{df=2,48}$ = 44.0; $p < 0.001$) among macrophyte patches of different growth forms, with the highest flow velocities in *S. emersum* patches and the lowest in *C. obtusangula* patches, with *P. natans* taking an intermediate position (Figure 2.2). Flow velocity differed significantly among the measured locations for *P. natans* and *C. obtusangula* (Table 2.1) and was also significantly higher in June 2015 compared to August 2014 in- and outside *S. emersum* and *P. natans* patches (Table 2.1). Within the macrophyte patches, a reduction of the incoming flow velocity was observed throughout the patch, although this was not significant for *S. emersum*. The flow attenuating effect was the most profound for *C. obtusangula*, with a 77.1% decrease in flow velocity within the patch, the least for *S. emersum*, with only a 6.45% decrease in flow velocity, and *P. natans* situated in between with a 42.1% decrease. Additionally, significant negative correlations were observed between the measured within-patch flow velocity and macrophyte complexity in the back of the patch in 2014 and for all within-patch locations in 2015 (Figure S2.2).

Macroinvertebrate community structure

Macroinvertebrates collected from the different macrophyte species included 114 different taxa, with 82 taxa being observed in both years, and 38,940 individuals, 12,224 for 2014 and 26,716 for 2015. The community in August 2014 was dominated by Simuliidae (28.6% of total individuals, 6 taxa) and Chironomidae (28.3%, 16 taxa), whereas the June 2015 community was dominated by Oligochaeta

Table 2.1. Two-factor analysis of variance (ANOVA) of the effects of location and year on the mean depth-averaged flow velocity. Significant values ($p < 0.05$) are indicated in bold.

		df	F	*p*
S. emersum	Location	5	1.851	0.141
	Year	1	26.404	**0.000**
	Location × year	5	0.379	0.858
P. natans	Location	5	8.88	**0.000**
	Year	1	4.355	**0.048**
	Location × year	5	1.396	0.261
C. obtusangula	Location	5	11.81	**0.000**
	Year	1	1.68	0.208
	Location × year	5	0.47	0.797

Table 2.2 Frequency of occurrence and number of individuals of the different macroinvertebrate functional groups from all samples taken in 2014 and 2015 (n = 27 for both years). Observed macroinvertebrate taxa are classified according to functional group.

Functional group	Frequency 2014 (%)	Abundance 2014 (#)	Frequency 2015 (%)	Abundance 2015 (#)	Example taxa
Burrower-Filterer	22	13	22	266	*Ephemera* sp., *Pisidium* sp.,
Burrower-Gatherer	22	23	56	832	Nematomorpha, *Prostoma sp*., Tubificinae, Lumbriculidae, *Chironomus* sp., *Cladopelma sp*., *Prodiamesa olivacea*, *Symplecta* sp., *Satchelliela* sp., *Paramormia* sp.,
Burrower-Predator	22	49	33	37	*Demicryptochironomus vulneratus*, *Cryptochironomus* sp., *Clinotanypus nervosus*, *Sialis lutaria*,
Burrower-Shredder	0	0	4	1	*Tipula* sp.,
Climber-Filterer	22	36	19	8	*Bithynia tentaculata*,
Climber-Gatherer	22	38	63	2171	*Ophidonais serpentina*, *Slavina appendiculata*, *Mystacides* sp.,
Climber-Parasite	33	14	15	4	*Theromyzon tesselatum*, *Hemiclepsis marginata*, *Piscicola geometra*,
Climber-Piercer	74	208	7	5	*Hydroptila* sp.,
Climber-Predator	78	125	59	88	*Helobdella stagnalis*, *Glossiphonia* sp., *Agabus* sp., *Porhydrus* sp., *Calopteryx splendens*, *Nepa cinerea*, *Lebertia* sp., *Sperchon* sp., *Atractides* sp., *Planaria* sp., *Polycelis* sp., *Girardia tigrina*, *Dugesia* sp.,
Climber-Scraper	22	12	4	2	*Haitia fontinalis*, *Radix balthica*, *Gyraulus albus*,
Climber-Shredder	81	77	7	2	*Hydrellia* sp., *Peltodytus* sp., *Haliplus* sp., *Cataclysta lemnata*, *Nymphula nitidulata*, *Paraponyx stratiota*, *Elophila nymphata*, *Halesus radiatus*
Clinger-Filterer	100	6804	100	4785	*Simulium* sp., *Rheotanytarsus* sp., *Dicrotendipus* sp., *Hydropsyche* sp., *Neureclipsis bimaculata*,
Clinger-Gatherer	41	32	96	1927	*Micropsectra* sp., *Polypedilum sp*, *Microtendipus* sp., *Cricotopus sylvestris*, *Synorthocladius semivirens*
Clinger-Predator	0	0	4	1	*Oecetis* sp.,

Table 2.2 (continued) Frequency of occurrence and number of individuals of the different macroinvertebrate functional groups from all samples taken in 2014 and 2015 (n = 27 for both years). Observed macroinvertebrate taxa are classified according to functional group.

Functional group	Frequency 2014 (%)	Abundance 2014 (#)	Frequency 2015 (%)	Abundance 2015 (#)	Example taxa
Sprawler-Gatherer	89	134	100	5073	*Parachironomus* sp., *Paracladopelma camptolabis* gr., *Phaenospectra* sp., *Psectrocladius* sp., *Corynoneura* sp., *Chaetocladius piger* gr., *Paratrichocladius rufiventris*, *Thienemaniella majuscula*, *Rheocricotopus fusciceps*, *Nanocladius dichromus* gr., *Tvetenia calvescens*, *Eukiefferiella claripennis*, *Orthocladius* sp., *Potthastia longimanna*,
Sprawler-Predator	48	61	48	247	*Apsectrotanypus trifascipennis*, *Conchapelopia* agg., *Macropelopia* sp., *Ablabesmyia sp*., *Procladius* sp., *Dicranota* sp.,
Sprawler-Shredder	85	2293	44	432	*Brillia longifurca*, *Notidobia ciliaris*, *Asellus aquaticus*, *Orconectus limosus*,
Swimmer-Gatherer	74	734	100	9750	*Stylaria lacustris*, *Nais* sp., *Sigara striata*,
Swimmer-Predator	63	75	33	84	*Erpobdella* sp., Ceratopogonini, *Orectochilus villosus*,
Swimmer-Scraper	96	1305	70	324	*Baetis* sp.,
Swimmer-Shredder	41	133	4	1	*Gammarus* sp., *Crangonyx pseudogracilis*,

(46.6%, 7 taxa) and Chironomidae (38.8%, 36 taxa). Regarding the functional groups (Table 2.2), the August 2014 community was dominated by clinger-filterers (56.1%) and sprawler-shredders (18.8%), while the June 2015 community was dominated by swimmer-gatherers (36.5%) and sprawler-gatherers (20.4%) (Table S2.5). Taxonomic and functional macroinvertebrate richness differed significantly among the three macrophyte growth forms (Figure 2.3a and b, Kruskal-Wallis test, taxa richness: $X^2_{df=2} = 37.6$; $p < 0.001$, functional richness: $X^2_{df=2} = 38.3$; $p < 0.001$) and showed a significant positive relationship with macrophyte complexity (Figure 2.4a and b). This difference was not significant between *S. emersum* and *P. natans* for the August 2014 samples, however. Although species diversity, expressed as

Shannon diversity, differed significantly between growth forms (one-way ANOVA; $F_{df=2,51} = 6.386$; $p = 0.003$), only the 2015 *P. natans* and *C. obtusangula* communities were significantly more diverse than the other communities (Figure 2.3c). Furthermore, no significant relationship with macrophyte complexity could be observed (Figure 2.4c). Functional diversity increased significantly with increasing plant complexity (Figure 2.3d, Welch test; $F_{df=2,32.7} = 43.7$; $p < 0.001$ and Figure 2.4d), with again no significant difference between *S. emersum* and *P. natans* in 2014. It should be noted that both taxonomic and functional diversity were higher in 2015

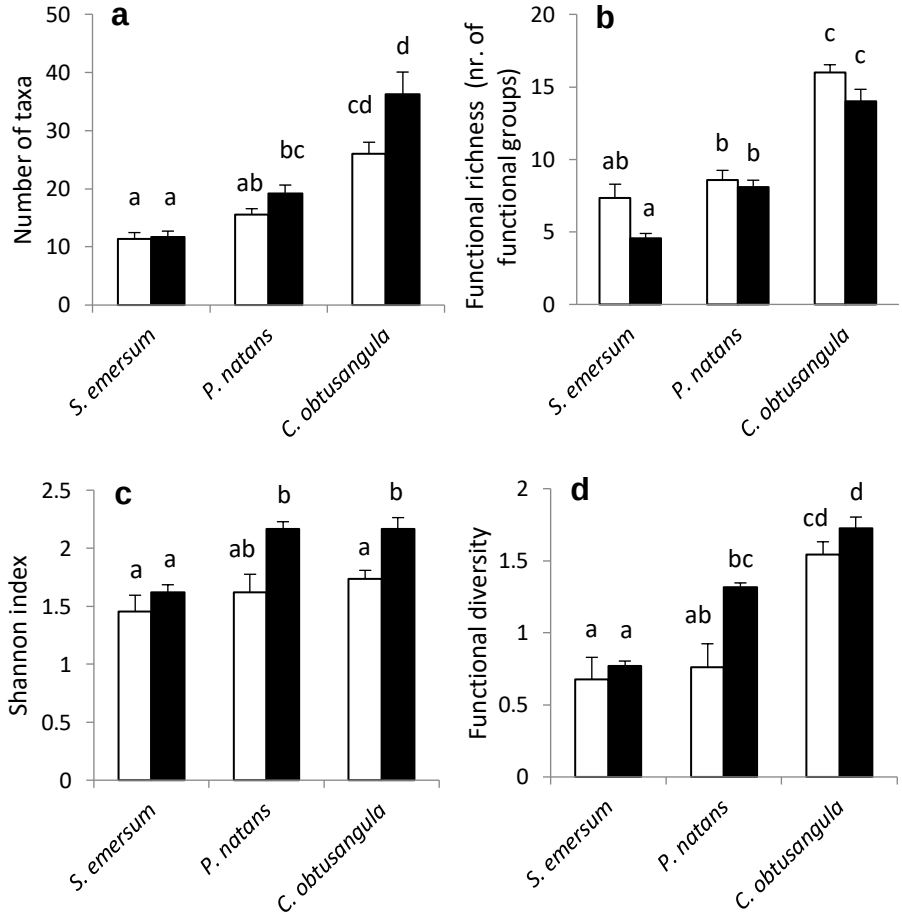

Figure 2.3 Macroinvertebrate community richness and diversity for 2014 (white bars) and 2015 (black bars). Species richness (a) and diversity (c) are shown, as well as functional richness (b) and diversity (d). Values are presented as means (n = 9) ± S.E. Different letters indicate significant differences at $p < 0.05$.

than in 2014 (Figure 2.3c, one-way ANOVA; $F_{df=1,52} = 14.5$; $p < 0.001$ and Figure 2.3d, Welch test; $F_{df=1,48.4} = 4.2$; $p = 0.047$).

Significant differences in the distribution of the different taxonomic and functional groups were observed between the different growth forms in the two years (Functional group distribution shown in Table 2.3, effect of sampling event shown in Table S2.4 and S2.5). The *C. obtusangula* community was functionally the richest (Figure 2.3b), with representatives of all functional groups observed and with the 2014 community being dominated by Clinger-Filterers (mostly *Simulium* sp. (Diptera: Simuliidae)), Sprawler-Shredders (almost exclusively *Asellus aquaticus* L. (Isopoda: Asellidae) and Swimmer-Scrapers (*Baetis* sp. (Ephemeroptera:

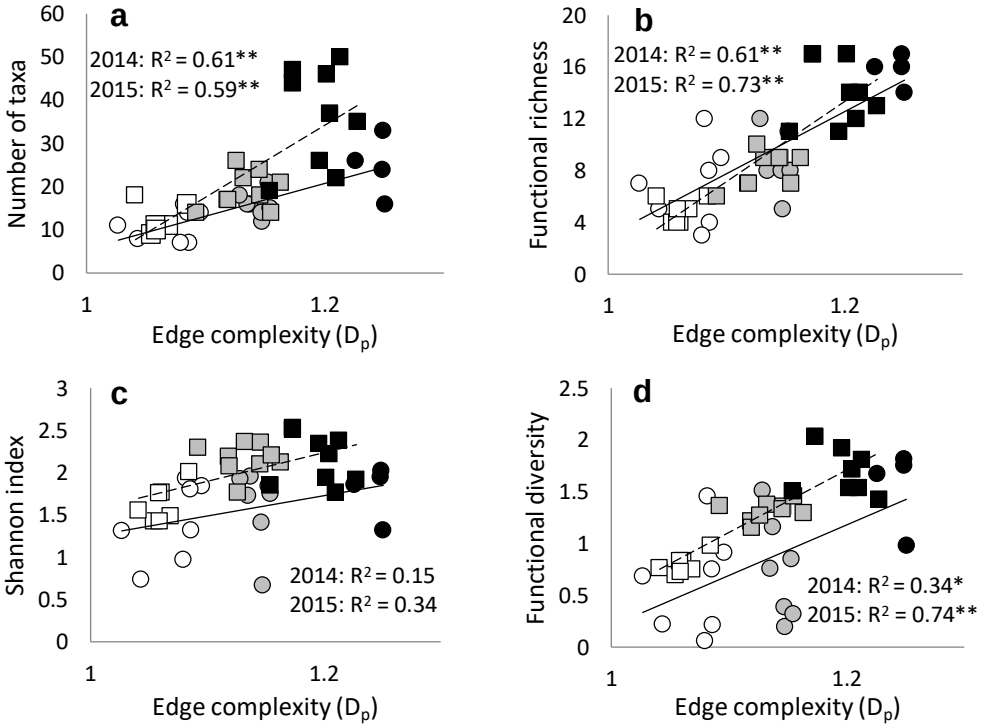

Figure 2.4 Relationship between macrophyte complexity and number of taxa (a), number of functional groups (b), Shannon index (c) and functional diversity (d). Circles and solid trendlines represent 2014 samples and squares and dashed trendlines 2015 samples, while *S. emersum*, *P. natans* and *C. obtusangula* samples are presented in white, grey and black respectively. Single and double asterisks indicate significance at the $p < 0.05$ and $p < 0.01$ level respectively.

Baetidae) and the 2015 community by Swimmer-Gatherers (almost exclusively *Stylaria lacustris* L. (Oligochaeta: Naididae)), Climber-Gatherers (mostly *Ophidonais serpentina* Müller (Oligochaeta: Naididae)) and Sprawler-Gatherers (mostly Orthocladiinae (Diptera: Chironomidae)). On the other hand, the *S. emersum* community can be seen as functionally the simplest (Figure 2.3b), with the fewest functional groups occurring there and with the community being dominated almost exclusively by Clinger-Filterers (mostly *Simulium* sp.) in 2014 and by Clinger-Filterers and Sprawler-Gatherers (mostly Orthocladiinae (Diptera: Chironomidae)) in 2015. Again, the *P. natans* communities took an intermediate position. The main differences between the communities from the different years can be summarised by the absence of Lepidoptera, Amphipoda and many of the Trichoptera taxa, an increased dominance of naidid Oligochaeta and a large increase in chironomid

Table 2.3 Mean percentage of the macroinvertebrate community in the macrophyte patches that consists of the different functional groups. Values are presented as means (n = 9) ± S.E. Different letters indicate differences among macrophyte species for both years simultaneously (values for 2015 on the next page) at $p < 0.05$.

	2014		
	Sparganium emersum	*Potamogeton natans*	*Callitriche obtusangula*
Burrower-Filterer	0^{a}	0^{a}	$0.36^{b} \pm 0.18$
Burrower-Gatherer	$0.06^{a} \pm 0.06$	0^{a}	$0.55^{ab} \pm 0.24$
Burrower-Predator	$0.06^{a} \pm 0.06$	0^{a}	$1.31^{ab} \pm 0.76$
Climber-Filterer	0^{a}	0^{a}	$0.69^{b} \pm 0.21$
Climber-Gatherer	0^{a}	0^{a}	$0.94^{ab} \pm 0.46$
Climber-Parasite	0.06 ± 0.06	0.18 ± 0.08	0.11 ± 0.06
Climber-Piercer	$2.49^{ab} \pm 0.87$	$0.46^{abc} \pm 0.23$	$1.99^{b} \pm 0.4$
Climber-Predator	$0.23^{ab} \pm 0.1$	$0.67^{ac} \pm 0.21$	$1.89^{c} \pm 0.57$
Climber-Scraper	0	0.23 ± 0.17	0.12 ± 0.07
Climber-Shredder	$1.3^{a} \pm 0.42$	$0.65^{a} \pm 0.22$	$0.45^{ac} \pm 0.15$
Clinger-Filterer	$82.94^{a} \pm 4.46$	$76.59^{a} \pm 6.94$	$32.58^{b} \pm 5.69$
Clinger-Gatherer	$0.18^{a} \pm 0.12$	$0.07^{a} \pm 0.07$	$0.5^{a} \pm 0.14$
Sprawler-Gatherer	$2.14^{ab} \pm 0.57$	$0.88^{a} \pm 0.19$	$0.86^{a} \pm 0.27$
Sprawler-Predator	$0.1^{a} \pm 0.1$	$0.11^{ab} \pm 0.07$	$0.86^{bc} \pm 0.22$
Sprawler-Shredder	$5.15^{ab} \pm 1.88$	$7.64^{ab} \pm 3.55$	$29.84^{a} \pm 7.04$
Swimmer-Gatherer	$1.02^{a} \pm 0.33$	$0.75^{a} \pm 0.27$	$9.11^{ab} \pm 1.94$
Swimmer-Predator	$0.22^{ab} \pm 0.15$	$1.67^{a} \pm 0.61$	$0.52^{a} \pm 0.18$
Swimmer-Scraper	$3.82^{ab} \pm 1.12$	$10.08^{a} \pm 2.89$	$15.38^{a} \pm 5.04$
Swimmer-Shredder	$0.24^{a} \pm 0.24$	$0.02^{a} \pm 0.02$	$1.95^{b} \pm 0.66$

diversity in 2015 compared to 2014. In contrast to the significant effects of both sampling event and macrophyte species on community composition, within-patch location had no observable effect on the occurrence of the different taxa (Table S2.5) and functional groups (Table S2.6) throughout the macrophyte patch.

Unconstrained multivariate analyses of the taxonomic (DCA) and functional (PCA) macroinvertebrate community composition for both years revealed clear differences between different years and growth forms (Figure 2.5a and b). The DCA biplot of the taxonomic community composition (Figure 2.5a) clearly distinguishes between samples from different years (with the August 2014 samples on the left side and the June 2015 samples on the right side of the plot) and between the different growth forms (with samples from *S. emersum* in the lowest section, *P. natans* slightly higher and *C. obtusangula* in the upper section). No clear separation

Table 2.3 (continued) Mean percentage of the macroinvertebrate community in the macrophyte patches that consists of the different functional groups. Values are presented as means (n = 9) ± S.E. Different letters indicate differences among macrophyte species for both years simultaneously (values for 2014 on the previous page) at $p < 0.05$.

	2015		
	Sparganium emersum	*Potamogeton natans*	*Callitriche obtusangula*
Burrower-Filterer	0^{a}	0^{a}	$1.28^{b} \pm 0.48$
Burrower-Gatherer	$0.34^{a} \pm 0.2$	$0.28^{ab} \pm 0.12$	$5.17^{b} \pm 2$
Burrower-Predator	0^{a}	$0.01^{a} \pm 0.01$	$0.2^{b} \pm 0.07$
Climber-Filterer	0^{a}	$0.03^{a} \pm 0.03$	$0.04^{ab} \pm 0.02$
Climber-Gatherer	0^{a}	$0.61^{bc} \pm 0.17$	$12.78^{c} \pm 2.47$
Climber-Parasite	0	0.01 ± 0.01	0.01 ± 0.01
Climber-Piercer	0^{c}	0^{c}	$0.02^{ac} \pm 0.02$
Climber-Predator	0^{b}	$0.31^{abc} \pm 0.08$	$0.35^{abc} \pm 0.09$
Climber-Scraper	0	0	0.01 ± 0.01
Climber-Shredder	0^{b}	0^{b}	$0.01^{bc} \pm 0.01$
Clinger-Filterer	$63.83^{a} \pm 4.14$	$32.37^{b} \pm 5.23$	$9.27^{c} \pm 1.07$
Clinger-Gatherer	$0.91^{ab} \pm 0.17$	$5.78^{b} \pm 1.14$	$8.35^{b} \pm 1.66$
Sprawler-Gatherer	$32.35^{c} \pm 3.97$	$28.68^{c} \pm 2.71$	$17.39^{bc} \pm 1.72$
Sprawler-Predator	0^{a}	$0.21^{a} \pm 0.11$	$1.3^{c} \pm 0.23$
Sprawler-Shredder	$0.07^{b} \pm 0.07$	$0.08^{b} \pm 0.07$	$2.85^{ab} \pm 1.02$
Swimmer-Gatherer	$2.4^{a} \pm 0.63$	$29^{b} \pm 5.69$	$39.38^{b} \pm 3.9$
Swimmer-Predator	0^{b}	0^{b}	$0.44^{a} \pm 0.17$
Swimmer-Scraper	$0.1^{c} \pm 0.07$	$2.62^{abc} \pm 0.75$	$1.13^{bc} \pm 0.31$
Swimmer-Shredder	0^{a}	0^{a}	$0.003^{a} \pm 0.003$

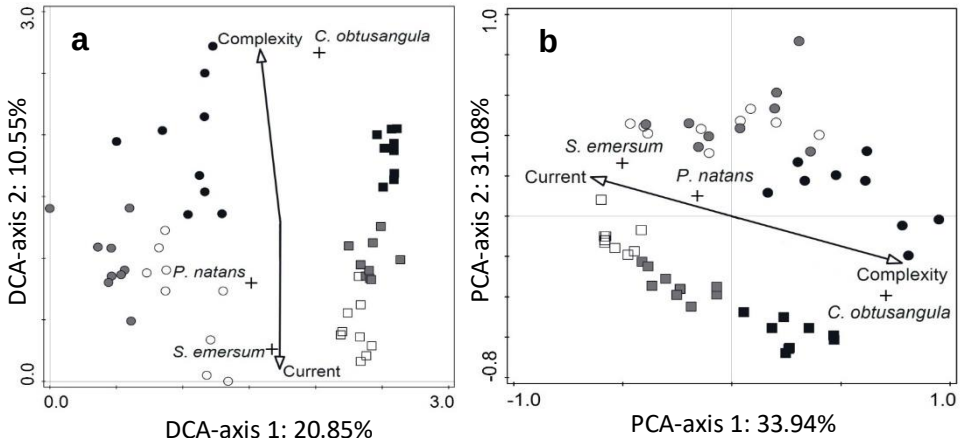

Figure 2.5 DCA biplot of the taxonomic macroinvertebrate community composition (a) and PCA biplot of the functional community composition (b) of 2014 and 2015. Circles represent 2014 samples and squares 2015 samples, while *S. emersum*, *P. natans* and *C. obtusangula* samples are presented in white, grey and black respectively. Continuous environmental variables are presented as black arrows, while the dummy variable plant identity is presented as + sign.

could be made between samples taken from different patches or different locations within the patch, as can be seen in the TWINSPAN clustering diagram (Figure S2.3). The first two DCA-axes in this plot explained 31.4% (20.85% and 10.55% respectively) of the variation in the species data.

The PCA biplot of the functional community composition (Figure 2.5b) shows comparable results with those from the taxonomic community composition, with a clear distinction between different years and different growth forms. However, this distinction between different growth forms was less pronounced for *S. emersum* and *P. natans* samples from 2014, with samples from both growth forms occupying the same area in the plot. Again, no clear separation could be made between samples taken from different patches or different locations within the patch. The first two PCA-axes in this plot explained 65.02% (33.94% and 31.08% respectively) of the variation in the species data.

Variables explaining community structure

As suggested by the multivariate ordination plots, significant linear correlations were found between several macroinvertebrate taxa and functional groups and the two continuous environmental variables, water flow velocity and plant fractal

complexity (Table S2.3 and S2.4). Because flow velocity and fractal complexity were negatively correlated to each other, the occurrence of many macroinvertebrate groups was correlated to both environmental parameters.

Variance partitioning for 2014, 2015 and both years together, including all three explanatory variables (plant identity, flow velocity and fractal complexity), yielded different results when either simple or conditional effects were tested.

Table 2.4 Percentage of the total variance in taxonomic and functional macroinvertebrate community structure that could be explained by the three environmental parameters, both individually as well as their combined effects overlapping with other parameters. Results are shown for the variance partitioning of both complete communities and communities consisting of animals occurring only in both years. Bold values indicate a significant amount of variation explained by individual environmental parameters (Monte Carlo permutation test, $p \leq 0.05$).

		Variance explained (%)							Total variance explained (%)
		Plant identity (P)	Fractal complexity (F)	Water flow velocity (W)	P+F	F+W	P+W	P+F+W	
Complete communities	**2014**								
	Taxonomic	**10**	-1.1	**2**	10.4	<0.1	-0.2	3.2	24.3
	Functional	**4.6**	-1.9	0.6	18.3	-0.2	0.3	6.3	28.0
	2015								
	Taxonomic	**10.8**	-0.4	-0.9	4.5	<0.1	0.8	30.7	45.5
	Functional	1.8	**2.7**	-0.8	12.0	-0.1	0.8	52.7	69.1
	2014+2015								
	Taxonomic	**11**	**6.9**	**0.9**	-2.8	-0.4	0.1	6	21.7
	Functional	**13.6**	**8.1**	**1.6**	-1.2	-0.1	-1.3	14.7	35.4
Macroinvertebrates occurring in both years	**2014**								
	Taxonomic	**18.4**	-1.1	1.7	7.5	<0.1	-0.6	6	31.9
	Functional	3.8	-2	-0.7	20.5	0	-0.3	26.7	48.0
	2015								
	Taxonomic	**4.2**	**2.5**	-0.4	10.2	-0.1	0.6	53.3	70.3
	Functional	**3.2**	**2.4**	-0.2	13.4	-0.1	0.8	56.7	76.2
	2014+2015								
	Taxonomic	**12.7**	**6.3**	0.2	-2.9	-0.1	1.7	10.4	28.3
	Functional	**12.6**	**5.8**	-0.7	1.9	-0.1	2.8	26.8	49.1

When an individual variable was used to explain all variance in the taxonomic or functional macroinvertebrate community structure (i.e. the simple effects), all explanatory variables maintained a highly significant effect on community composition. Contrastingly, when one variable was used to explain the variance in community composition and the other variables were used as covariates (i.e. the conditional effects were tested), more variation was observed in the relative importance of the individual parameters (Table 2.4). When looking at the community composition for both years together, all separate variables were significant in explaining the variance in both taxonomic and functional community composition when the other two variables were used as covariates. Of the three separate variables, plant identity explained most of the variation in community composition and was found to be significant in all cases except the 2015 functional community. For the separate years, both flow velocity and fractal complexity alone explained only a small percentage of the variation, with more variation explained by plant identity (all communities except the 2015 functional community), the combinations of plant identity and complexity (all communities) and the combinations of all three variables (both 2015 communities). Fractal complexity explained most variation after plant identity, except in the 2014 communities (although it should be noted that complexity combined with other factors explained far more variation than flow velocity combined with other factors in that year), and flow velocity explained the least. The total amount of variation explained was the highest for both 2015 communities, even though a large portion could not be directly attributed to a single variable. Environmental variables always explained more variation in the functional community than in the taxonomic community.

In June 2015, environmental variables explained roughly double the amount of variation in both taxonomic and functional community composition when compared to August 2014 (Table 2.4). This phenomenon also occurred when variance partitioning was performed only for taxa occurring in both years, to exclude the possibility that these differences were caused by seasonal taxa that would not inhabit the stream on both sampling events (Table 2.4).

Discussion

Results obtained in this study showed great variability in structural complexity and within-patch water flow velocity for the three plant species studied, which was reflected in the associated macroinvertebrate community. As expected, measurements of plant fractal complexity proved to be suitable quantitative estimates of structural complexity, confirming intuitive expectations of complexity, as was also observed in earlier studies (McAbendroth et al. 2005, Ferreiro et al. 2011). The observed increases in flow attenuation by complex plant species and a decreased flow velocity in down-stream patch sections were also expected based on literature and provided further distinction in environmental conditions within patches of different plant species (Sand-Jensen and Mebus 1996, Peralta et al. 2008). It should be noted that no differences in plant complexity were detected among different locations within the same patch, suggesting that within-patch flow velocity was influenced by plant architecture rather than vice versa.

Besides differences in macroinvertebrate community composition among different growth forms, considerable variation was observed between the two sampling events in the middle and end of the growing season. This variation in community composition is likely caused by seasonal emergence patterns of aquatic insects and by stochastic annual variation in macroinvertebrate assemblages, which are inherent to these systems (e.g. Armitage et al. 1995, Pardo and Armitage 1997).

Variables explaining community structure

The general trend observed in this study was that all three environmental variables were significant in explaining the variance in both taxonomic and functional macroinvertebrate community composition. Of these variables, plant identity appeared to be the most important, followed by fractal complexity and then flow velocity. However, it was also observed that these variables were highly inter-correlated, making it statistically impossible to distinguish between the separate effects of the individual environmental variables on the macroinvertebrate community. Future studies could tackle this limitation by focussing on mixed vegetation stands and longer gradients of macrophyte complexity, by including several complex plant species.

The true relevance of these separate environmental parameters for the individual taxonomic and functional macroinvertebrate groups likely depends on

the autecology and life history traits of the latter. The abundance of filter-feeding macroinvertebrates, for example is more likely to be directly influenced by flow velocity, a key factor determining the availability of their food resources, than by the identity or the complexity of their substrate (e.g. Tachet et al. 1992, Finelli et al. 2002). On the other hand, the abundance of flow-tolerating taxa that do not use the macrophytes or the attached epiphyton as a direct food source, such as Zygoptera larvae in this study, is probably stronger influenced by habitat structural complexity (e.g. Warfe and Barmuta 2006, Verdonschot and Peeters 2012). Finally, plant identity can be expected to be the dominant factor explaining the abundance of herbivorous taxa that depend on the macrophytes for their food supply, such as the several Lepidoptera larvae observed in this study (Gaevskaya 1969, Palm 1986).

Besides the differences in the importance of individual variables, the total amount of variation explained by all variables was also roughly two times higher in June 2015 than in August 2014 (Table 2.4). This increase in the amount of explained variation in community composition suggests that macrophytes were more important as a habitat for macroinvertebrates in the second sampling year. Because the sampling events took place in different months and different years, seasonal or yearly differences in macroinvertebrate presence might also be cited as a cause for this difference in explained variation (e.g. Armitage et al. 1995, Pardo and Armitage 1997). However, this option seems less likely when variance partitioning for animals that only occurred in both years produced roughly the same result as the earlier variance partitioning analysis using the complete macroinvertebrate community (Table 2.4), thus excluding stochastic yearly differences in the species pool or seasonal emergence patterns of aquatic insects as a possible cause for this difference. It might therefore be expected that internal stream factors, such as flow velocity or water chemistry, are causing the difference in the relative importance of macrophytes as a habitat for stream macroinvertebrates. Flow velocity was significantly higher in 2015 (Welch test; $F_{df=1,92.175} = 4.1$; $p = 0.047$), probably due to the lower water level and the general higher cross-sectional blockage later in the season (Verschoren et al. 2017). Furthermore, no differences in water chemistry were observed between the two occasions (monthly measurements, VMM - Flemish Environment Agency 2016), it therefore seems likely that flow is the main factor influencing the importance of aquatic macrophytes as a habitat for macroinvertebrates. In this sense, macrophyte patches can thus be seen as

instream flow refugia that become more important for macroinvertebrates with increasing flow velocity. Macroinvertebrates can occupy flow refugia to avoid adverse physical conditions during periods of increased discharge and flow velocity, after which they can use the refugia as a recolonisation source (e.g. Lancaster and Hildrew 1993, Winterbottom et al. 1997).

Additional variables explaining community structure

Although plant identity, fractal complexity and flow velocity had significant effects on community composition, it is likely that other variables might also have influenced the macroinvertebrate community. First of all, size of the macrophyte patches might have played a role, as larger habitats can typically support a larger number of taxa (Connor and McCoy 1979, Matias et al. 2010). However, our study did not observe any relationship between patch size and both richness and diversity of taxa and functional groups (data not shown), an observation in line with Taniguchi et al. (2003) who found that structural complexity affected macroinvertebrate community composition independently of patch size. In addition to habitat size, proximity to other vegetation patches that may function as a source of immigrating species, can also affect within-patch taxa richness (e.g. MacArthur and Wilson 1967). It should be noted though, that inter-patch distance in the studied stream was never more than a few meters, distances that generally form no migratory barrier for lotic macroinvertebrates (Elliott 2003).

Additional parameters that might have affected macroinvertebrate community composition, which are however related to macrophyte complexity and flow velocity, include the density of macrophyte structures and the availability of the food sources organic matter and epiphyton. An increased density of macrophyte structural elements leads to a lower within-patch flow velocity (e.g. Madsen et al. 2001) and might potentially also affect predator's foraging success (Crowder and Cooper 1982, Bartholomew et al. 2000, Bartholomew and Shine 2008), although there are studies suggesting this effect is less important than the effect of structural complexity (Warfe and Barmuta 2004). Additionally, an increased density of physical structures might limit the size or the diversity of the interstitial spaces between macrophyte structures, with associated negative effects on macroinvertebrate diversity (Thomaz and Cunha 2010, St Pierre and Kovalenko 2014). Density of structural elements was not measured in this study, limiting the

use of alternative measures of habitat complexity that incorporate structural density (e.g. McAbendroth et al. 2005, Kovalenko et al. 2009). Finally, the availability of the basal resources epiphyton and detritus in the aquatic food web has been shown to have a positive effect on food web complexity and thus on macroinvertebrate community structure (e.g. Townsend et al. 1998). Complex macrophytes have been shown to contain more epiphyton, irrespective of surface area, than simpler macrophytes (Chapter 6, Taniguchi et al. 2003, Warfe and Barmuta 2006), whereas the strong flow attenuating effect of complex macrophytes also causes more detritus to settle within these patches (Sand-Jensen and Mebus 1996, Schoelynck et al. 2013). Epiphyton and detritus availability was not measured however, so these parameters could not be linked to the macroinvertebrate community structure in this study.

Intra-patch variability

Although large differences in macroinvertebrate community composition were observed among the different macrophyte species, no such differences were observed for the macroinvertebrate community within these patches, despite the observed gradient in within-patch flow velocity. This lack of intra-patch community variability might be attributed to the large volume of the box-sampler (6663 cm^3) that was used in this study, which was three times that of the box-sampler used in an earlier study by Bell et al. (2013). Given the fact that this previous study only observed significant within-patch variability in invertebrate density for a third of the studied functional feeding groups (predators and filter-feeders in *Sparganium emersum*, shredders in *Callitriche platycarpa*), it stands to reason that increasing the sample volume will further obscure this small-scale spatial variability. Alternatively, the reductions in flow velocity within complex macrophyte patches, irrespective of location within the patch, could be sufficient to provide a suitable refuge for most macroinvertebrate taxa.

Conclusions

This study observed significant differences in the complexity of three studied macrophyte species and the associated within-patch flow velocity, which was found to be most attenuated by complex plant species. The macroinvertebrate community associated with these plants showed a clear distinction for the different

growth forms, reflecting the differences in environmental conditions. Variation in both taxonomic and functional community structure could be significantly explained by the environmental variables plant identity, plant complexity and flow velocity, although the relative importance of these parameters could not be determined due to their high degree of intercorrelation. Additionally, it was found that the explanatory power of these variables was higher under conditions of high flow velocity, suggesting a role of macrophyte patches as instream flow refugia for macroinvertebrates.

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Supplementary tables

Table S2.1 Macrophyte patch characteristics for both 2014 and 2015. Water depth values are presented as means (n = 3) ± S.E., while the remaining parameters are based on single measurements.

		Replicate	Length (m)	Width (m)	Surface (m^2)	Water depth (m)
2014	*S. emersum*	1	2.3	0.8	1.45	0.61 ± 0.03
		2	2.4	1.6	3.02	0.44 ± 0.02
		3	2.1	0.6	0.99	0.6 ± 0.01
	P. natans	1	3.6	1.1	3.11	0.71 ± 0.03
		2	7.5	0.5	2.95	0.59 ± 0.05
		3	6.8	1	5.34	0.54 ± 0.01
	C. obtusangula	1	2.1	0.4	0.66	0.41 ± 0.02
		2	1.8	0.9	1.27	0.4 ± 0.04
		3	3.5	1	2.75	0.42 ± 0.01
2015	*S. emersum*	4	2.4	0.9	1.7	0.32 ± 0.02
		5	5.9	0.6	2.78	0.28 ± 0.01
		6	6.8	1.1	5.87	0.26 ± 0.03
	P. natans	4	4.7	2.1	7.75	0.34 ± 0.07
		5	13.5	2.9	30.75	0.26 ± 0.03
		6	6.1	2.6	12.46	0.27 ± 0.06
	C. obtusangula	4	1.2	0.6	0.57	0.36 ± 0.01
		5	1.7	0.9	1.2	0.26 ± 0.02
		6	1.4	0.7	0.77	0.22 ± 0.03

Table S2.2 Mean edge complexity measured in different locations within macrophyte patches. Values are presented as means ± S.E. Different letters indicate differences among patch locations and plant species for each separate year at $p \leq 0.05$.

	n		Mean edge complexity (D_p)	
	2014	2015	2014	2015
Within plants				
Sparganium emersum				
- Front	2	2	1.05 ± 0.03	1.05 ± 0.007
- Middle	2	3	1.07 ± 0.03	1.06 ± 0.003
- Back	3	2	1.08 ± 0.002	1.07 ± 0.01
Potamogeton natans				
- Front	3	3	1.15 ± 0.002	1.15 ± 0.006
- Middle	2	3	1.13 ± 0.003	1.12 ± 0.01
- Back	2	3	1.14 ± 0.005	1.13 ± 0.01
Callitriche obtusangula				
- Front	2	3	1.24 ± 0.01	1.20 ± 0.02
- Middle	1	3	1.25	1.18 ± 0.01
- Back	1	3	1.25	1.20 ± 0.01
Among plants species				
S. emersum	7	7	1.07^{a} ± 0.010	1.06^{a} ± 0.005
P. natans	7	9	1.14^{b} ± 0.004	1.13^{b} ± 0.007
C. obtusangula	4	9	1.24^{c} ± 0.006	1.19^{c} ± 0.008

Table S2.3 Total macroinvertebrate abundance ordered by taxon for both 2014 and 2015.

Phylum	Class	Order	Family	Subfamily	Genus & species	2014	2015
Plathyhelmintes	Turbellaria	Tricladia	Dugesiidae		*Dugesia* sp.	10	4
					Girardia tigrina	11	0
			Planariidae		*Planaria torva*	5	1
					Polycelis sp.	12	1
					Turbellaria sp.	6	1
Nemathel-minthes					Nemathelminthes sp.	1	40
Nemertea	Enopla	Hoplo-nemertea			*Prostoma* sp.	1	0
Annelida	Clitelatta	Haplotaxida	Tubificidae	Naidinae	*Nais* sp.	0	1340
					Ophidonais serpentina	31	2086
					Slavina appendiculata	0	85
					Stylaria lacustris	733	8405
				Tubificinae	*Aulodrilus sp.*	0	49
					Potamothrix moldaviensis	17	469
		Lumbri-culida	Lumbriculidae		*Lumbriculus variegatus*	2	27
		Rhynchobdellida	Erpobdellidae		*Erpobdella octoculata*	41	8
					E. testacea	14	0
					E. sp.	6	1
			Piscicolidae		*Piscicola geometra*	1	3

Table S2.3 (continued) Total macroinvertebrate abundance ordered by taxon for both 2014 and 2015.

Phylum	Class	Order	Family	Subfamily	Genus & species	2014	2015
Annelida	Clitelatta	Rhynchobdellida	Glossiphoniidae		*Helobdella stagnalis*	8	1
					Hemiclepsis marginata	1	0
					Glossiphonia complanata	0	1
					G. verrucata	2	0
					G. sp.	1	0
Mollusca	Bivalvia	Veneroida	Sphaeriidae		*Pisidium amnicum*	13	265
	Gastropoda		Bithyniidae		*Bithynia tentaculata*	37	8
			Lymnaeidae	Amphipepleinae	*Radix balthica*	4	2
			Physidae	Physinae	*Haitia fontinalis*	5	0
			Planorbidae	Planorbinae	*Gyraulis albus*	2	0
Arthropoda	Malacostraca	Amphipoda	Crangonyctidae		*Crangonyx pseudogracilis*	67	0
			Gammaridae		*Gammarus pulex*	46	0
					G. tigrinis	17	0
					G. roeseli	2	0
					Amphipoda sp.	2	1
		Decapoda	Astacidae		*Orconectus limosus*	2	0
		Isopoda	Asellidae		*Asellus aquaticus*	2290	427

Table S2.3 (continued) Total macroinvertebrate abundance ordered by taxon for both 2014 and 2015.

Phylum	Class	Order	Family	Subfamily	Genus & species	2014	2015
Arthropoda	Arachnida	Prostigmata	Hygrobatidae		*Atractides sp.*	15	0
			Lebertiidae	Lebertiinae	*Lebertia sp.*	8	4
			Sperchontidae	Sperchontinae	*Sperchon sp.*	19	20
	Insecta	Ephemeroptera	Baetidae		*Baetis fuscatus*	0	207
					B. vernus	1305	117
			Ephemeridae		*Ephemera danica*	0	1
		Odonata	Calopterygidae		*Calopteryx splendens*	26	5
					Zygoptera sp.	2	2
		Heteroptera	Corixidae	Corixinae	*Sigara striata*	1	5
			Nepidae		*Nepa cinerea*	0	1
		Diptera	Chironomidae	Chironominae Tr. Tanytarsini	*Rheotanytarsus* sp.	3185	2498
					Micropsectra sp.	0	279
				Chironominae Tr. Chironomini	*Demicrypto-chironomus vulneratus*	0	2
					Cryptochirono-mus sp.	0	5
					Polypedilum scalaenum	7	128
					P. cf. *tritum*	0	27
					Microtendipus sp.	0	15
					Dicrotendipes sp.	0	8
					Chironomus sp.	0	4
					Cladopelma sp.	0	1
					Parachirono-mus acruatus	0	24
					P. biannulatus	0	3

Table S2.3 (continued) Total macroinvertebrate abundance ordered by taxon for both 2014 and 2015.

Phylum	Class	Order	Family	Subfamily	Genus & species	2014	2015
Arthropoda	Insecta	Diptera	Chironomidae	Chironominae Tr. Chironomini	*Paracladopelma camptolabis gr.*	0	1
					Phaenospectra sp.	0	6
				Orthocladiinae	*Psectrocladius* sp.	33	119
					Corynoneura lobata agg	9	0
					C. scutelata	0	11
					Chaetocladius gr. *piger*	0	1
					Paratrichocladius rufiventris	0	3
					Thienemanniella majuscula	73	402
					Rheocricotopus fusciceps	4	16
					Nanocladius gr. *dichromus*	18	84
					Cricotopus sylvestris	25	1596
					Tvetenia calvescens	0	158
					Eukiefferiella claripennis	0	1369
					Orthocladius sp.	0	3216
					Synorthocladius semivirens	0	2
					Brillia longifurca	0	5
				Tanypodinae	*Clinotanypus nervosus*	44	6
					Apsectrotanypus trifascipensis	11	4
					Conchapelopia agg	36	200
					Macropelopia sp.	0	47
					Ablabesmyia sp.	0	1
					Procladius sp.	14	8
				Diamesinae	*Potthastia longimana*	0	40
				Prodiamesinae	*Prodiamesa olivacea*	2	85

Table S2.3 (continued) Total macroinvertebrate abundance ordered by taxon for both 2014 and 2015.

Phylum	Class	Order	Family	Subfamily	Genus & species	2014	2015
Arthropoda	Insecta	Diptera	Ceratopogonidae		Ceratopogonini sp.	0	74
			Ephydridae		*Hydrellia* sp.	14	1
			Limoniidae	Limnophilinae	*Symplecta* sp.	0	2
			Pediciidae	Pediciinae	*Dicranota* sp.	1	0
			Psychodidae	Psychodinae	*Satcheliella* sp.	0	88
					Paramormia sp.	0	68
			Simuliidae	Simuliinae	*Simulium erythrocephalum*	1784	102
					S. vernum	175	21
					S. angustipes	234	1
					S. ornatum	1018	1723
					S. morsitans	101	19
					S. equinum	193	574
			Tipulidae	Tipulinae	*Tipula* sp.	0	1
		Trichoptera	Hydropsychidae	Hydropsychinae	*Hydropsyche angustipennis*	70	1
					H. pellucidula	82	1
			Hydroptilidae		*Hydroptila* sp.	208	5
			Leptoceridae		*Mystacides azurea*	7	0
					M. longicornis	1	0
					Oecetis sp.	0	1
			Limnephilidae		*Halesus radiatus*	0	1
			Polycentropodidae		*Neureclipsis bimaculata*	12	0
			Sericostomatidae		*Notidobia ciliaris*	1	0
		Megaloptera	Sialidae		*Sialis lutaria*	6	24

Table S2.3 (continued) Total macroinvertebrate abundance ordered by taxon for both 2014 and 2015.

Phylum	Class	Order	Family	Subfamily	Genus & species	2014	2015
Arthropoda	Insecta	Coleoptera	Dysticidae	Agabinae	*Agabus* sp.	0	44
				Hydropor-inae	*Porhydrus* sp.	0	1
			Gyrinidae		*Orectochilus villosus*	14	1
			Haliplidae		*Peltodytus* sp.	5	0
					Haliplus sp.	22	0
			Hydro-philioidea		Hydro-philioidea sp.	0	1
		Lepidoptera	Pyralidae	Nymphu-linae	*Cataclysta lemnata*	1	0
					Elophila nymphaeta	1	0
					Nymphula nitidulata	33	0
					Paraponyx stratiota	1	0

Table S2.4 Three-factor analysis of variance (ANOVA) of the effects of year, plant species and within-patch location on the proportion of macroinvertebrate taxa in the community. Significant values ($p < 0.05$) are indicated in bold. Only taxa of which at least 20 individuals were collected are included in the analysis.

	Taxon	df	F	*p*
Year	Nemathelminthes sp.	1	10.636	**0.002**
	Nais sp.	1	24.833	**0.000**
	Ophidonais serpentina	1	34.639	**0.000**
	Slavina appendiculata	1	2.482	0.124
	Stylaria lacustris	1	17.119	**0.000**
	Aulodrilus sp.	1	7.527	**0.009**
	Potamothrix moldaviensis	1	4.682	**0.037**
	Lumbriculus variegatus	1	2.201	0.147
	Erpobdella octoculata	1	4.634	**0.038**
	Pisidium amnicum	1	5.971	**0.020**
	Bithynia tentaculata	1	4.047	0.052
	Crangonyx pseudogracilis	1	2.055	0.160
	Gammarus pulex	1	6.878	**0.013**
	Asellus aquaticus	1	5.164	**0.029**
	Sperchon sp.	1	0.007	0.933
	Baetis vernus	1	14.897	**0.000**
	Baetis fuscatus	1	20.629	**0.000**
	Callopteryx splendens	1	2.239	0.143
	Rheotanytarsus sp.	1	1.110	0.299
	Microspectra sp.	1	10.349	**0.003**
	Polypedilum scalaenum	1	4.754	**0.036**
	Polypedilum cf. *tritum*	1	2.416	0.129
	Parachironomus acruatus	1	3.529	0.068
	Psectrocladius sp.	1	1.155	0.290
	Thienemanniella majuscula	1	37.228	**0.000**
	Nanocladius gr. *dichromus*	1	2.672	0.111
	Cricotopus sylvestris	1	14.186	**0.001**
	Tvetenia calvescens	1	21.390	**0.000**
	Eukiefferiella claripennis	1	56.708	**0.000**
	Orthocladius sp.	1	31.107	**0.000**

Table S2.4 (continued) Three-factor analysis of variance (ANOVA) of the effects of year, plant species and within-patch location on the proportion of macroinvertebrate taxa in the community. Significant values ($p < 0.05$) are indicated in bold. Only taxa of which at least 20 individuals were collected are included in the analysis.

	Taxon	df	F	*p*
Year	*Clinotanypus nervosus*	1	2.454	0.126
	Conchapelopia agg	1	13.945	**0.001**
	Macropelopia sp.	1	3.962	0.054
	Procladius sp.	1	0.695	0.410
	Potthastia longimana	1	8.929	**0.005**
	Prodiamesa olivacea	1	8.953	**0.005**
	Ceratopogonini sp.	1	3.508	0.069
	Satcheliella sp.	1	1.368	0.250
	Paramormia sp.	1	1.342	0.254
	Simulium erythrocephalum	1	14.012	**0.001**
	Simulium vernum	1	6.866	**0.013**
	Simulium angustipes	1	13.107	**0.001**
	Simulium ornatum	1	3.414	0.073
	Simulium morsitans	1	2.029	0.163
	Simulium equinum	1	6.282	**0.017**
	Hydropsyche angustipennis	1	17.375	**0.000**
	Hydropsyche pellucidula	1	11.325	**0.002**
	Hydroptila sp.	1	18.146	**0.000**
	Sialis lutaria	1	2.793	0.103
	Agabus sp.	1	7.251	**0.011**
	Haliplus sp.	1	7.806	**0.008**
	Nymphula nitidulata	1	21.780	**0.000**
Plant	Nemathelminthes sp.	2	4.203	**0.023**
	Nais sp.	2	5.428	**0.009**
	Ophidonais serpentina	2	35.018	**0.000**
	Slavina appendiculata	2	2.311	0.114
	Stylaria lacustris	2	13.971	**0.000**
	Aulodrilus sp.	2	7.075	**0.003**
	Potamothrix moldaviensis	2	5.214	**0.010**
	Lumbriculus variegatus	2	2.961	0.064
	Erpobdella octoculata	2	2.455	0.100

Table S2.4 (continued) Three-factor analysis of variance (ANOVA) of the effects of year, plant species and within-patch location on the proportion of macroinvertebrate taxa in the community. Significant values ($p < 0.05$) are indicated in bold. Only taxa of which at least 20 individuals were collected are included in the analysis.

	Taxon	df	F	*p*
Plant	*Pisidium amnicum*	2	7.266	**0.002**
	Bithynia tentaculata	2	9.136	**0.001**
	Crangonyx pseudogracilis	2	2.055	0.143
	Gammarus pulex	2	5.224	**0.010**
	Asellus aquaticus	2	7.834	**0.001**
	Sperchon sp.	2	4.277	**0.022**
	Baetis vernus	2	7.209	**0.002**
	Baetis fuscatus	2	8.644	**0.001**
	Callopteryx splendens	2	4.878	**0.013**
	Rheotanytarsus sp.	2	17.365	**0.000**
	Microspectra sp.	2	7.115	**0.002**
	Polypedilum scalaenum	2	2.144	0.132
	Polypedilum cf. *tritum*	2	0.655	0.525
	Parachironomus acruatus	2	3.529	**0.040**
	Psectrocladius sp.	2	1.729	0.192
	Thienemanniella majuscula	2	7.087	**0.003**
	Nanocladius gr. *dichromus*	2	6.269	**0.005**
	Cricotopus sylvestris	2	7.954	**0.001**
	Tvetenia calvescens	2	6.055	**0.005**
	Eukiefferiella claripennis	2	3.164	0.054
	Orthocladius sp.	2	8.287	**0.001**
	Clinotanypus nervosus	2	4.136	**0.024**
	Conchapelopia agg	2	21.485	**0.000**
	Macropelopia sp.	2	3.962	**0.028**
	Procladius sp.	2	10.505	**0.000**
	Potthastia longimana	2	7.531	**0.002**
	Prodiamesa olivacea	2	9.505	**0.000**
	Ceratopogonini sp.	2	3.508	**0.041**
	Satcheliella sp.	2	1.368	0.267
	Paramormia sp.	2	1.284	0.289
	Simulium erythrocephalum	2	13.748	**0.000**

Table S2.4 (continued) Three-factor analysis of variance (ANOVA) of the effects of year, plant species and within-patch location on the proportion of macroinvertebrate taxa in the community. Significant values ($p < 0.05$) are indicated in bold. Only taxa of which at least 20 individuals were collected are included in the analysis.

	Taxon	df	F	*p*
Location	Nemathelminthes sp.	2	0.112	0.894
	Nais sp.	2	0.622	0.543
	Ophidonais serpentina	2	2.092	0.138
	Slavina appendiculata	2	0.681	0.513
	Stylaria lacustris	2	1.007	0.375
	Aulodrilus sp.	2	1.245	0.300
	Potamothrix moldaviensis	2	0.325	0.724
	Lumbriculus variegatus	2	0.468	0.630
	Erpobdella octoculata	2	0.221	0.803
	Pisidium amnicum	2	0.045	0.956
	Bithynia tentaculata	2	0.963	0.391
	Crangonyx pseudogracilis	2	1.422	0.255
	Gammarus pulex	2	0.268	0.766
	Asellus aquaticus	2	0.449	0.642
	Sperchon sp.	2	2.362	0.109
	Baetis vernus	2	0.157	0.855
	Baetis fuscatus	2	0.236	0.791
	Callopteryx splendens	2	0.675	0.515
	Rheotanytarsus sp.	2	1.228	0.305
	Microspectra sp.	2	0.059	0.943
	Polypedilum scalaenum	2	0.053	0.948
	Polypedilum cf. *tritum*	2	0.388	0.681
	Parachironomus acruatus	2	0.251	0.780
	Psectrocladius sp.	2	0.295	0.746
	Thienemanniella majuscula	2	1.753	0.188
	Nanocladius gr. *dichromus*	2	0.390	0.680
	Cricotopus sylvestris	2	0.185	0.832
	Tvetenia calvescens	2	0.285	0.753
	Eukiefferiella claripennis	2	0.121	0.887
	Orthocladius sp.	2	0.018	0.982
	Clinotanypus nervosus	2	0.191	0.827

Table S2.4 (continued) Three-factor analysis of variance (ANOVA) of the effects of year, plant species and within-patch location on the proportion of macroinvertebrate taxa in the community. Significant values ($p < 0.05$) are indicated in bold. Only taxa of which at least 20 individuals were collected are included in the analysis.

	Taxon	df	F	*p*
Location	*Conchapelopia* agg	2	0.314	0.732
	Macropelopia sp.	2	0.460	0.635
	Procladius sp.	2	1.988	0.152
	Potthastia longimana	2	1.614	0.213
	Prodiamesa olivacea	2	1.436	0.251
	Ceratopogonini sp.	2	0.625	0.541
	Satcheliella sp.	2	1.065	0.355
	Paramormia sp.	2	0.914	0.410
	Simulium erythrocephalum	2	1.293	0.287
	Simulium vernum	2	0.241	0.787
	Simulium angustipes	2	1.300	0.285
	Simulium ornatum	2	0.950	0.396
	Simulium morsitans	2	0.745	0.482
	Simulium equinum	2	1.925	0.161
	Hydropsyche angustipennis	2	2.121	0.135
	Hydropsyche pellucidula	2	2.877	0.069
	Hydroptila sp.	2	1.153	0.327
	Sialis lutaria	2	1.578	0.220
	Agabus sp.	2	0.015	0.985
	Haliplus sp.	2	0.790	0.461
	Nymphula nitidulata	2	1.620	0.212
Year × Plant	Nemathelminthes sp.	2	3.545	**0.039**
	Nais sp.	2	5.428	**0.009**
	Ophidonais serpentina	2	32.948	**0.000**
	Slavina appendiculata	2	2.311	0.114
	Stylaria lacustris	2	9.216	**0.001**
	Aulodrilus sp.	2	7.075	**0.003**
	Potamothrix moldaviensis	2	4.497	**0.018**
	Lumbriculus variegatus	2	2.201	0.125
	Erpobdella octoculata	2	1.162	0.324
	Pisidium amnicum	2	5.971	**0.006**

Table S2.4 (continued) Three-factor analysis of variance (ANOVA) of the effects of year, plant species and within-patch location on the proportion of macroinvertebrate taxa in the community. Significant values ($p < 0.05$) are indicated in bold. Only taxa of which at least 20 individuals were collected are included in the analysis.

	Taxon	df	F	*p*
Year × Plant	*Bithynia tentaculata*	2	4.482	**0.018**
	Crangonyx pseudogracilis	2	2.055	0.143
	Gammarus pulex	2	5.224	**0.010**
	Asellus aquaticus	2	3.167	0.054
	Sperchon sp.	2	0.986	0.383
	Baetis vernus	2	5.825	**0.006**
	Baetis fuscatus	2	8.644	**0.001**
	Callopteryx splendens	2	2.239	0.121
	Rheotanytarsus sp.	2	0.627	0.540
	Microspectra sp.	2	7.115	**0.002**
	Polypedilum scalaenum	2	1.780	0.183
	Polypedilum cf. *tritum*	2	0.655	0.525
	Parachironomus acruatus	2	3.529	**0.040**
	Psectrocladius sp.	2	1.862	0.170
	Thienemanniella majuscula	2	11.815	**0.000**
	Nanocladius gr. *dichromus*	2	2.540	0.093
	Cricotopus sylvestris	2	7.381	**0.002**
	Tvetenia calvescens	2	6.055	**0.005**
	Eukiefferiella claripennis	2	3.164	0.054
	Orthocladius sp.	2	8.287	**0.001**
	Clinotanypus nervosus	2	2.261	0.119
	Conchapelopia agg	2	11.846	**0.000**
	Macropelopia sp.	2	3.962	**0.028**
	Procladius sp.	2	0.695	0.506
	Potthastia longimana	2	7.531	**0.002**
	Prodiamesa olivacea	2	8.622	**0.001**
	Ceratopogonini sp.	2	3.508	**0.041**
	Satcheliella sp.	2	1.368	0.267
	Paramormia sp.	2	1.284	0.289
	Simulium erythrocephalum	2	12.436	**0.000**
	Simulium vernum	2	4.263	**0.022**

Table S2.4 (continued) Three-factor analysis of variance (ANOVA) of the effects of year, plant species and within-patch location on the proportion of macroinvertebrate taxa in the community. Significant values ($p < 0.05$) are indicated in bold. Only taxa of which at least 20 individuals were collected are included in the analysis.

	Taxon	df	F	*p*
Year × Plant	*Simulium angustipes*	2	4.354	**0.020**
	Simulium ornatum	2	1.942	0.158
	Simulium morsitans	2	1.097	0.345
	Simulium equinum	2	2.092	0.138
	Hydropsyche angustipennis	2	17.750	**0.000**
	Hydropsyche pellucidula	2	0.103	0.902
	Hydroptila sp.	2	5.681	**0.007**
	Sialis lutaria	2	2.793	0.075
	Agabus sp.	2	5.869	**0.006**
	Haliplus sp.	2	2.726	0.079
	Nymphula nitidulata	2	9.780	**0.000**
Year × Location	Nemathelminthes sp.	2	0.252	0.779
	Nais sp.	2	0.622	0.543
	Ophidonais serpentina	2	2.183	0.127
	Slavina appendiculata	2	0.681	0.513
	Stylaria lacustris	2	0.772	0.470
	Aulodrilus sp.	2	1.245	0.300
	Potamothrix moldaviensis	2	0.375	0.690
	Lumbriculus variegatus	2	0.363	0.698
	Erpobdella octoculata	2	0.357	0.702
	Pisidium amnicum	2	0.027	0.973
	Bithynia tentaculata	2	1.238	0.302
	Crangonyx pseudogracilis	2	1.422	0.255
	Gammarus pulex	2	0.268	0.766
	Asellus aquaticus	2	0.444	0.645
	Sperchon sp.	2	0.007	0.993
	Baetis vernus	2	0.549	0.582
	Baetis fuscatus	2	0.236	0.791
	Calloperyx splendens	2	0.320	0.728
	Rheotanytarsus sp.	2	0.859	0.432
	Microspectra sp.	2	0.059	0.943

Table S2.4 (continued) Three-factor analysis of variance (ANOVA) of the effects of year, plant species and within-patch location on the proportion of macroinvertebrate taxa in the community. Significant values ($p < 0.05$) are indicated in bold. Only taxa of which at least 20 individuals were collected are included in the analysis.

	Taxon	df	F	*p*
Year × Location	*Polypedilum scalaenum*	2	0.099	0.906
	Polypedilum cf. *tritum*	2	0.388	0.681
	Parachironomus acruatus	2	0.251	0.780
	Psectrocladius sp.	2	0.592	0.559
	Thienemanniella majuscula	2	1.968	0.154
	Nanocladius gr. *dichromus*	2	0.122	0.886
	Cricotopus sylvestris	2	0.143	0.867
	Tvetenia calvescens	2	0.285	0.753
	Eukiefferiella claripennis	2	0.121	0.887
	Orthocladius sp.	2	0.018	0.982
	Clinotanypus nervosus	2	0.082	0.922
	Conchapelopia agg	2	0.030	0.971
	Macropelopia sp.	2	0.460	0.635
	Procladius sp.	2	3.721	**0.034**
	Potthastia longimana	2	1.614	0.213
	Prodiamesa olivacea	2	1.221	0.307
	Ceratopogonini sp.	2	0.625	0.541
	Satcheliella sp.	2	1.065	0.355
	Paramormia sp.	2	0.914	0.410
	Simulium erythrocephalum	2	1.117	0.338
	Simulium vernum	2	0.224	0.800
	Simulium angustipes	2	1.258	0.296
	Simulium ornatum	2	0.853	0.435
	Simulium morsitans	2	0.795	0.459
	Simulium equinum	2	0.957	0.394
	Hydropsyche angustipennis	2	1.973	0.154
	Hydropsyche pellucidula	2	2.741	0.078
	Hydroptila sp.	2	0.952	0.396
	Sialis lutaria	2	0.181	0.835
	Agabus sp.	2	0.015	0.985
	Haliplus sp.	2	0.790	0.461

Table S2.4 (continued) Three-factor analysis of variance (ANOVA) of the effects of year, plant species and within-patch location on the proportion of macroinvertebrate taxa in the community. Significant values ($p < 0.05$) are indicated in bold. Only taxa of which at least 20 individuals were collected are included in the analysis.

	Taxon	df	F	*p*
Year × Location	*Nymphula nitidulata*	2	1.620	0.212
Plant × Location	Nemathelminthes sp.	4	0.490	0.743
	Nais sp.	4	0.456	0.768
	Ophidonais serpentina	4	2.047	0.108
	Slavina appendiculata	4	0.727	0.579
	Stylaria lacustris	4	0.340	0.849
	Aulodrilus sp.	4	1.414	0.249
	Potamothrix moldaviensis	4	0.374	0.825
	Lumbriculus variegatus	4	0.468	0.759
	Erpobdella octoculata	4	0.540	0.707
	Pisidium amnicum	4	0.045	0.996
	Bithynia tentaculata	4	0.933	0.456
	Crangonyx pseudogracilis	4	1.422	0.247
	Gammarus pulex	4	0.147	0.963
	Asellus aquaticus	4	0.415	0.797
	Sperchon sp.	4	1.787	0.153
	Baetis vernus	4	0.461	0.764
	Baetis fuscatus	4	0.905	0.471
	Callopteryx splendens	4	0.675	0.614
	Rheotanytarsus sp.	4	0.131	0.970
	Microspectra sp.	4	0.146	0.964
	Polypedilum scalaenum	4	0.803	0.531
	Polypedilum cf. *tritum*	4	1.124	0.360
	Parachironomus acruatus	4	0.251	0.907
	Psectrocladius sp.	4	0.639	0.638
	Thienemanniella majuscula	4	1.149	0.349
	Nanocladius gr. *dichromus*	4	0.342	0.848
	Cricotopus sylvestris	4	0.157	0.959
	Tvetenia calvescens	4	1.227	0.317
	Eukiefferiella claripennis	4	0.106	0.980

Table S2.4 (continued) Three-factor analysis of variance (ANOVA) of the effects of year, plant species and within-patch location on the proportion of macroinvertebrate taxa in the community. Significant values ($p < 0.05$) are indicated in bold. Only taxa of which at least 20 individuals were collected are included in the analysis.

	Taxon	df	F	*p*
Plant × Location	*Orthocladius* sp.	4	0.162	0.956
	Clinotanypus nervosus	4	0.152	0.961
	Conchapelopia agg	4	0.208	0.933
	Macropelopia sp.	4	0.460	0.765
	Procladius sp.	4	1.988	0.117
	Potthastia longimana	4	1.402	0.253
	Prodiamesa olivacea	4	1.572	0.203
	Ceratopogonini sp.	4	0.625	0.648
	Satcheliella sp.	4	1.065	0.388
	Paramormia sp.	4	0.941	0.451
	Simulium erythrocephalum	4	1.337	0.275
	Simulium vernum	4	0.332	0.855
	Simulium angustipes	4	0.481	0.749
	Simulium ornatum	4	0.137	0.968
	Simulium morsitans	4	0.735	0.574
	Simulium equinum	4	0.542	0.706
	Hydropsyche angustipennis	4	2.010	0.114
	Hydropsyche pellucidula	4	0.453	0.770
	Hydroptila sp.	4	0.431	0.785
	Sialis lutaria	4	1.578	0.201
	Agabus sp.	4	0.049	0.995
	Haliplus sp.	4	1.589	0.198
	Nymphula nitidulata	4	1.770	0.156
Year × Plant × Location	Nemathelminthes sp.	4	0.587	0.674
	Nais sp.	4	0.456	0.768
	Ophidonais serpentina	4	2.134	0.097
	Slavina appendiculata	4	0.727	0.579
	Stylaria lacustris	4	0.224	0.923
	Aulodrilus sp.	4	1.414	0.249
	Potamothrix moldaviensis	4	0.428	0.788
	Lumbriculus variegatus	4	0.363	0.834

Table S2.4 (continued) Three-factor analysis of variance (ANOVA) of the effects of year, plant species and within-patch location on the proportion of macroinvertebrate taxa in the community. Significant values ($p < 0.05$) are indicated in bold. Only taxa of which at least 20 individuals were collected are included in the analysis.

	Taxon	df	F	*p*
Year × Plant × Location	*Erpobdella octoculata*	4	1.366	0.265
	Pisidium amnicum	4	0.027	0.998
	Bithynia tentaculata	4	1.196	0.329
	Crangonyx pseudogracilis	4	1.422	0.247
	Gammarus pulex	4	0.147	0.963
	Asellus aquaticus	4	0.429	0.786
	Sperchon sp.	4	0.028	0.998
	Baetis vernus	4	0.704	0.595
	Baetis fuscatus	4	0.905	0.471
	Callopteryx splendens	4	0.320	0.863
	Rheotanytarsus sp.	4	0.303	0.874
	Microspectra sp.	4	0.146	0.964
	Polypedilum scalaenum	4	0.690	0.604
	Polypedilum cf. *tritum*	4	1.124	0.360
	Parachironomus acruatus	4	0.251	0.907
	Psectrocladius sp.	4	0.534	0.711
	Thienemanniella majuscula	4	0.645	0.634
	Nanocladius gr. *dichromus*	4	0.098	0.982
	Cricotopus sylvestris	4	0.145	0.964
	Tvetenia calvescens	4	1.227	0.317
	Eukiefferiella claripennis	4	0.106	0.980
	Orthocladius sp.	4	0.162	0.956
	Clinotanypus nervosus	4	0.059	0.993
	Conchapelopia agg	4	0.164	0.955
	Macropelopia sp.	4	0.460	0.765
	Procladius sp.	4	3.721	**0.012**
	Potthastia longimana	4	1.402	0.253
	Prodiamesa olivacea	4	1.348	0.271
	Ceratopogonini sp.	4	0.625	0.648
	Satcheliella sp.	4	1.065	0.388
	Paramormia sp.	4	0.941	0.451

Table S2.4 (continued) Three-factor analysis of variance (ANOVA) of the effects of year, plant species and within-patch location on the proportion of macroinvertebrate taxa in the community. Significant values ($p < 0.05$) are indicated in bold. Only taxa of which at least 20 individuals were collected are included in the analysis.

	Taxon	df	F	*p*
Year × Plant × Location	*Simulium erythrocephalum*	4	1.245	0.309
	Simulium vernum	4	0.165	0.955
	Simulium angustipes	4	0.430	0.786
	Simulium ornatum	4	3.032	**0.030**
	Simulium morsitans	4	1.187	0.333
	Simulium equinum	4	1.979	0.119
	Hydropsyche angustipennis	4	2.084	0.103
	Hydropsyche pellucidula	4	0.402	0.806
	Hydroptila sp.	4	0.366	0.831
	Sialis lutaria	4	0.181	0.947
	Agabus sp.	4	0.049	0.995
	Haliplus sp.	4	1.589	0.198
	Nymphula nitidulata	4	1.770	0.156

Table S2.5 Three-factor analysis of variance (ANOVA) of the effects of year, plant species and within-patch location on the proportion of functional groups in the community. Significant values ($p < 0.05$) are indicated in bold. Burrower-Shredders and Clinger-Predators were only represented by a single individual and are thus excluded.

	Functional group	df	F	*p*
Year	Burrower-Filterer	1	2.501	0.123
	Burrower-Gatherer	1	5.502	**0.025**
	Burrower-Predator	1	1.886	0.178
	Climber-Filterer	1	7.645	**0.009**
	Climber-Gatherer	1	21.740	**0.000**
	Climber-Parasite	1	7.356	**0.010**
	Climber-Piercer	1	29.655	**0.000**
	Climber-Predator	1	9.171	**0.005**
	Climber-Scraper	1	3.142	0.085
	Climber-Shredder	1	28.586	**0.000**
	Clinger-Filterer	1	52.624	**0.000**
	Clinger-Gatherer	1	41.166	**0.000**
	Sprawler-Gatherer	1	199.593	**0.000**
	Sprawler-Predator	1	1.583	0.216
	Sprawler-Shredder	1	21.008	**0.000**
	Swimmer-Gatherer	1	79.394	**0.000**
	Swimmer-Predator	1	8.311	**0.007**
	Swimmer-Scraper	1	18.525	**0.000**
	Swimmer-Shredder	1	10.613	**0.002**
Plant	Burrower-Filterer	2	7.861	**0.001**
	Burrower-Gatherer	2	5.923	**0.006**
	Burrower-Predator	2	3.116	0.057
	Climber-Filterer	2	9.924	**0.000**
	Climber-Gatherer	2	25.331	**0.000**
	Climber-Parasite	2	1.023	0.370
	Climber-Piercer	2	4.160	**0.024**
	Climber-Predator	2	6.262	**0.005**
	Climber-Scraper	2	1.092	0.346
	Climber-Shredder	2	2.903	0.068
	Clinger-Filterer	2	59.395	**0.000**
	Clinger-Gatherer	2	9.240	**0.001**
	Sprawler-Gatherer	2	7.471	**0.002**

Table S2.5 (continued) Three-factor analysis of variance (ANOVA) of the effects of year, plant species and within-patch location on the proportion of functional groups in the community. Significant values ($p < 0.05$) are indicated in bold. Burrower-Shredders and Clinger-Predators were both only represented by a single individual and are therefore excluded.

	Functional group	df	F	*p*
Plant	Sprawler-Predator	2	31.007	**0.000**
	Sprawler-Shredder	2	9.263	**0.001**
	Swimmer-Gatherer	2	34.027	**0.000**
	Swimmer-Predator	2	3.393	**0.045**
	Swimmer-Scraper	2	3.584	**0.038**
	Swimmer-Shredder	2	7.306	**0.002**
Location	Burrower-Filterer	2	0.027	0.973
	Burrower-Gatherer	2	0.221	0.803
	Burrower-Predator	2	0.456	0.638
	Climber-Filterer	2	0.559	0.577
	Climber-Gatherer	2	0.613	0.547
	Climber-Parasite	2	0.185	0.832
	Climber-Piercer	2	1.703	0.196
	Climber-Predator	2	0.114	0.893
	Climber-Scraper	2	0.718	0.495
	Climber-Shredder	2	1.296	0.286
	Clinger-Filterer	2	0.365	0.697
	Clinger-Gatherer	2	0.745	0.482
	Sprawler-Gatherer	2	1.213	0.309
	Sprawler-Predator	2	0.149	0.862
	Sprawler-Shredder	2	0.431	0.653
	Swimmer-Gatherer	2	3.218	0.052
	Swimmer-Predator	2	0.035	0.966
	Swimmer-Scraper	2	1.069	0.354
	Swimmer-Shredder	2	1.598	0.216
Year × Plant	Burrower-Filterer	2	2.501	0.096
	Burrower-Gatherer	2	3.852	**0.030**
	Burrower-Predator	2	1.664	0.204
	Climber-Filterer	2	8.739	**0.001**
	Climber-Gatherer	2	18.718	**0.000**
	Climber-Parasite	2	0.692	0.507
	Climber-Piercer	2	4.098	**0.025**

Table S2.5 (continued) Three-factor analysis of variance (ANOVA) of the effects of year, plant species and within-patch location on the proportion of functional groups in the community. Significant values ($p < 0.05$) are indicated in bold. Burrower-Shredders and Clinger-Predators were both only represented by a single individual and are therefore excluded.

	Functional group	df	F	*p*
Year × Plant	Climber-Predator	2	3.148	0.055
	Climber-Scraper	2	1.087	0.348
	Climber-Shredder	2	2.989	0.063
	Clinger-Filterer	2	3.805	**0.032**
	Clinger-Gatherer	2	8.042	**0.001**
	Sprawler-Gatherer	2	5.750	**0.007**
	Sprawler-Predator	2	1.881	0.167
	Sprawler-Shredder	2	5.777	**0.007**
	Swimmer-Gatherer	2	17.275	**0.000**
	Swimmer-Predator	2	4.994	**0.012**
	Swimmer-Scraper	2	2.452	0.100
	Swimmer-Shredder	2	7.258	**0.002**
Year × Location	Burrower-Filterer	2	0.058	0.944
	Burrower-Gatherer	2	0.387	0.682
	Burrower-Predator	2	0.350	0.707
	Climber-Filterer	2	0.625	0.541
	Climber-Gatherer	2	0.465	0.632
	Climber-Parasite	2	0.144	0.867
	Climber-Piercer	2	1.649	0.206
	Climber-Predator	2	0.236	0.791
	Climber-Scraper	2	0.615	0.546
	Climber-Shredder	2	1.358	0.270
	Clinger-Filterer	2	0.017	0.983
	Clinger-Gatherer	2	0.674	0.516
	Sprawler-Gatherer	2	1.406	0.258
	Sprawler-Predator	2	1.211	0.310
	Sprawler-Shredder	2	0.618	0.545
	Swimmer-Gatherer	2	1.477	0.242
	Swimmer-Predator	2	0.201	0.819
	Swimmer-Scraper	2	1.091	0.347
	Swimmer-Shredder	2	1.587	0.219

Table S2.5 (continued) Three-factor analysis of variance (ANOVA) of the effects of year, plant species and within-patch location on the proportion of functional groups in the community. Significant values ($p < 0.05$) are indicated in bold. Burrower-Shredders and Clinger-Predators were both only represented by a single individual and are therefore excluded.

	Functional group	df	F	*p*
Plant × Location	Burrower-Filterer	4	0.027	0.998
	Burrower-Gatherer	4	0.341	0.848
	Burrower-Predator	4	0.322	0.861
	Climber-Filterer	4	0.549	0.701
	Climber-Gatherer	4	0.645	0.634
	Climber-Parasite	4	0.855	0.500
	Climber-Piercer	4	1.789	0.153
	Climber-Predator	4	0.333	0.854
	Climber-Scraper	4	0.820	0.521
	Climber-Shredder	4	2.145	0.095
	Clinger-Filterer	4	0.195	0.939
	Clinger-Gatherer	4	0.116	0.976
	Sprawler-Gatherer	4	0.417	0.795
	Sprawler-Predator	4	0.786	0.542
	Sprawler-Shredder	4	0.506	0.732
	Swimmer-Gatherer	4	1.108	0.368
	Swimmer-Predator	4	1.432	0.243
	Swimmer-Scraper	4	1.058	0.391
	Swimmer-Shredder	4	1.237	0.313
Year × Plant × Location	Burrower-Filterer	4	0.058	0.993
	Burrower-Gatherer	4	0.460	0.765
	Burrower-Predator	4	0.264	0.899
	Climber-Filterer	4	0.602	0.663
	Climber-Gatherer	4	0.510	0.728
	Climber-Parasite	4	0.870	0.491
	Climber-Piercer	4	1.800	0.150
	Climber-Predator	4	0.171	0.952
	Climber-Scraper	4	0.953	0.445
	Climber-Shredder	4	2.109	0.100
	Clinger-Filterer	4	2.894	**0.036**
	Clinger-Gatherer	4	0.068	0.991
	Sprawler-Gatherer	4	0.701	0.597

Table S2.5 (continued) Three-factor analysis of variance (ANOVA) of the effects of year, plant species and within-patch location on the proportion of functional groups in the community. Significant values ($p < 0.05$) are indicated in bold. Burrower-Shredders and Clinger-Predators were both only represented by a single individual and are therefore excluded.

	Functional group	df	F	*p*
Year × Plant × Location	Sprawler-Predator	4	1.895	0.133
	Sprawler-Shredder	4	0.678	0.612
	Swimmer-Gatherer	4	1.352	0.270
	Swimmer-Predator	4	1.161	0.344
	Swimmer-Scraper	4	1.156	0.346
	Swimmer-Shredder	4	1.221	0.319

Table S2.5 Macroinvertebrate taxa whose relative abundance is significantly ($p < 0.05$) linearly correlated to either water flow velocity or macrophyte structural complexity. Taxa written in bold were found to be significantly correlated at the $p < 0.01$ level, whereas taxa with asterisks were only recorded in a single year and are therefore not shown for the overall 2014 & 2015 dataset.

	Taxa positively correlated with flow velocity	Taxa negatively correlated with flow velocity	Taxa positively correlated with structural complexity	Taxa negatively correlated with structural complexity
2014	*Potamothrix moldaviensis*, ***Simulium ornatum***, *Hydropsyche pellucidula*, *Nymphula nitidulata**	*Stylaria lacustris*, ***Baetis vernus***	*Stylaria lacustris*, *Baetis vernus*, *Simulium erythrocephalum*, ***Asellus aquaticus***	*Theromyzon tesselatum*, ***Simulium ornatum***, *Thienemaniella majuscula* *Hydropsyche pellucidula*, *Peltodytes* sp.*, *Nymphula nitidulata**
2015	Nematomorpha, ***Simulium ornatum***, *S. equinum*, ***Eukiefferiella claripennis****, *Sperchon* sp.	***Stylaria lacustris***, *Ophidonais serpentina*, *Rheotanytarsus* sp., ***Cricotopus sylvestris***	***Stylaria lacustris***, ***Rheotanytarsus* sp.**, ***Cricotopus sylvestris***	Nematomorpha, ***Simulium ornatum***, *S. morsitans*, *S. equinum*, ***Eukiefferiella claripennis****, Ceratopogonini*
2014 & 2015	Nematomorpha, *Theromyzon tesselatum*, ***Simulium ornatum***, *Psectrocladius* sp., *Rheocricotopus* sp., *Hydropsyche pellucidula*, *H. angustipennis*, *Sialis lutaria*	***Stylaria lacustris***, *Ophidonais serpentina*, *Baetis vernus*, ***Cricotopus sylvestris***	***Stylaria lacustris***, ***Baetis vernus***, ***Asellus aquaticus***, ***Bithynia tentaculata***	**Nematomorpha**, ***Simulium ornatum***, ***S. equinum***, *Rheocricotopus* sp., *Hydropsyche pellucidula*, *Pisidium amnicum*

Table S2.6 Macroinvertebrate functional groups whose relative abundance is significantly ($p < 0.05$) linearly correlated to either water flow velocity or macrophyte structural complexity. Functional groups written in bold were found to be significantly correlated at the $p < 0.01$ level.

	Functional groups positively correlated with flow velocity	Functional groups negatively correlated with flow velocity	Functional groups positively correlated with structural complexity	Functional groups negatively correlated with structural complexity
2014	**Clinger-Filterer**	Swimmer-Gatherer, **Swimmer-Scraper**	Climber-Predator, **Sprawler-Shredder**, **Swimmer-Gatherer**, Swimmer-Scraper	Climber-Shredder, **Clinger-Filterer**, **Sprawler-Gatherer**
2015	**Clinger-Filterer**, **Sprawler-Gatherer**	Climber-Gatherer, **Clinger-Gatherer**, **Swimmer-Gatherer**	Climber-Gatherer, **Clinger-Gatherer**, **Swimmer-Gatherer**	**Clinger-Filterer**, **Sprawler-Gatherer**
2014 & 2015	**Clinger-Filterer**, Climber-Shredder, **Sprawler-Gatherer**	Climber-Gatherer, **Clinger-Gatherer**, **Swimmer-Gatherer**	**Climber-Filterer**, Climber-Predator, **Sprawler-Shredder**, **Swimmer-Gatherer**, Swimmer-Scraper	**Burrower-Filterer**, Climber-Shredder, **Clinger-Filterer**, Sprawler-Gatherer, Swimmer-Predator

Supplementary figures

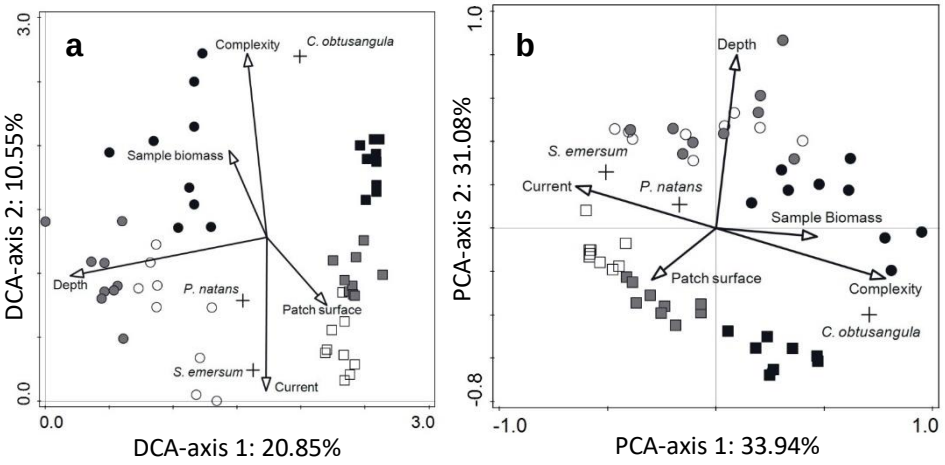


Figure S2.1 DCA biplot of the taxonomic macroinvertebrate community composition (a) and PCA biplot of the functional community composition (b) of 2014 and 2015, including all measured environmental parameters. Circles represent 2014 samples and squares 2015 samples, while *S. emersum*, *P. natans* and *C. obtusangula* samples are presented in white, grey and black respectively. Continuous environmental variables are presented as black arrows, while the dummy variable plant identity is presented as + sign.

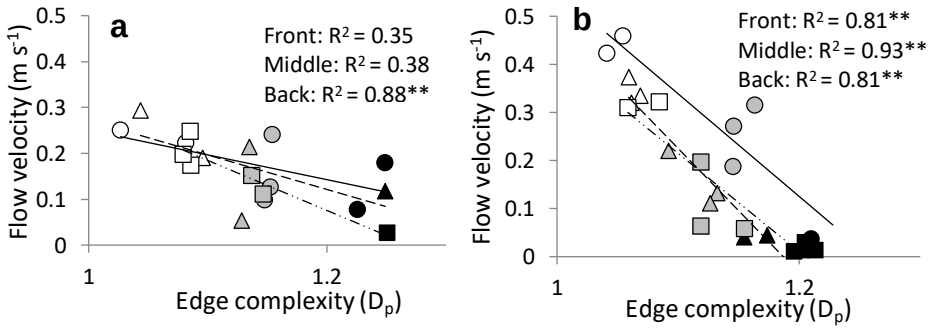
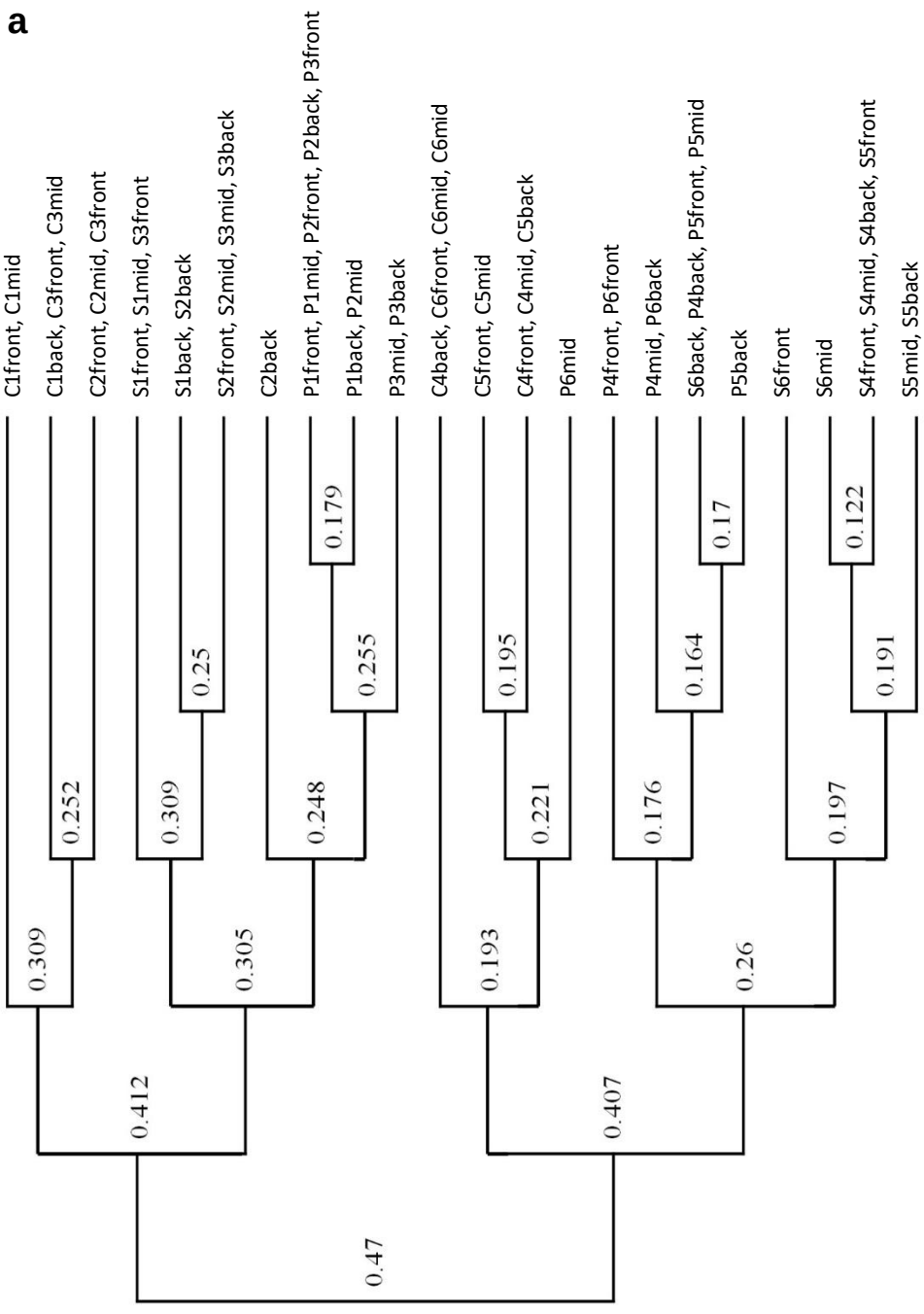


Figure S2.2 Relationship between within-patch water flow velocity and plant edge complexity for both 2014 (a) and 2015 (b). *S. emersum*, *P. natans* and *C. obtusangula* samples are presented in white, grey and black respectively, while front, middle and back locations are represented by circles and solid trendlines, triangles and dashed trendlines and squares and alternating dashed and dotted trendlines respectively. Double asterisks indicate significance at the $p < 0.01$ level.


a
C1front, C1mid
C1back, C3front, C3mid
C2front, C2mid, C3front
S1front, S1mid, S3front
S1back, S2back
S2front, S2mid, S3mid, S3back
C2back
P1front, P1mid, P2front, P2back, P3front
P1back, P2mid
P3mid, P3back
C4back, C6front, C6mid, C6mid
C5front, C5mid
C4front, C4mid, C5back
P6mid
P4front, P6front
P4mid, P6back
S6back, P4back, P5front, P5mid
P5back
S6front
S6mid
S4front, S4mid, S4back, S5front
S5mid, S5back
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0.252
0.309
0.25
0.305
0.248
0.179
0.255
0.412
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0.26
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0.47

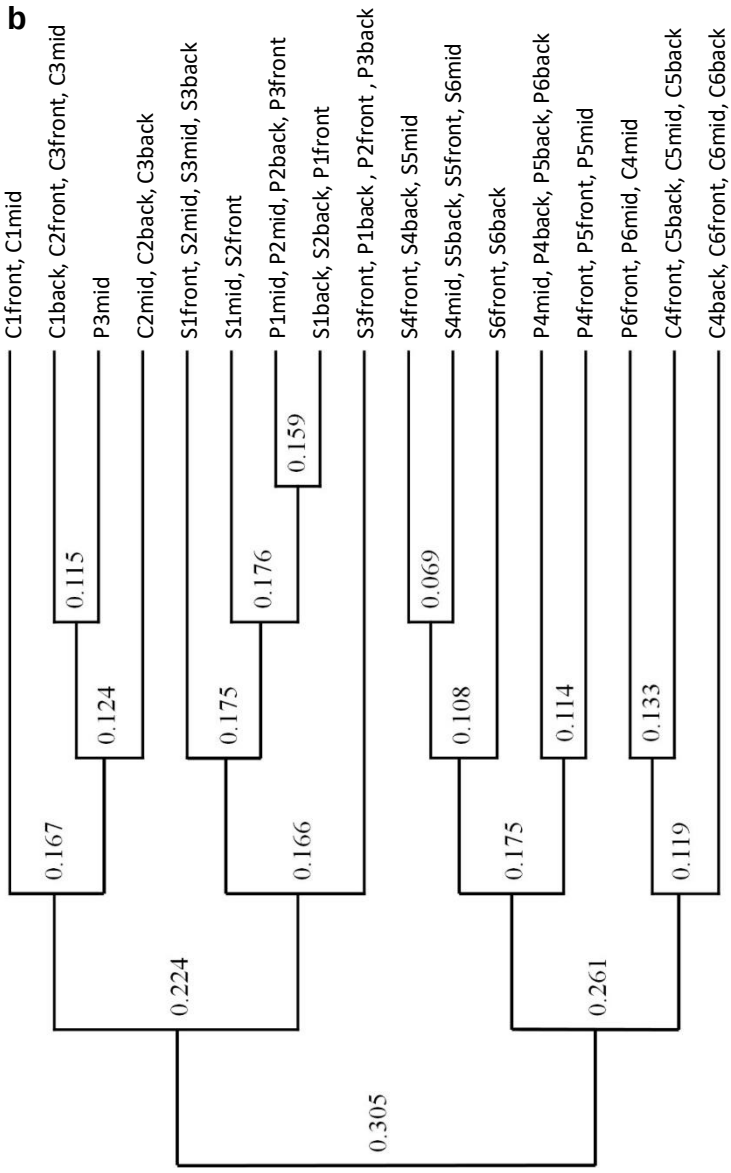
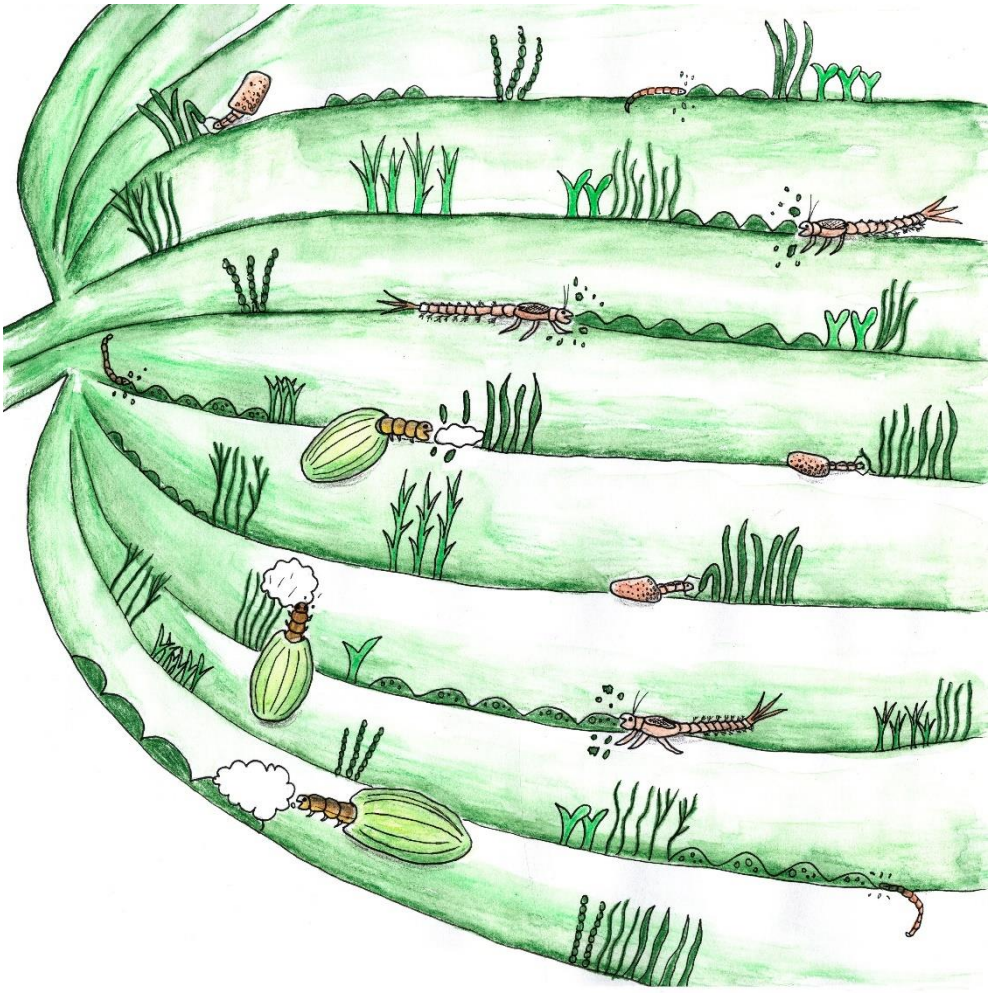


Figure S2.3 TWINSPAN clustering of the taxonomic (a) and functional (b) macroinvertebrate community composition for 2014 and 2015. Eigenvalues are indicated at every junction in the diagram. For the different samples, the first letter S, P or C indicates the plant species *S. emersum*, *P. natans* and *C. obtusangula* respectively, the number indicates the patch (where 1, 2 and 3 are sampled in 2014 and 4, 5 and 6 in 2015) and the last part indicates the within-patch location of the sample.



Chapter 3.

Stable isotope measurements confirm consumption of submerged macrophytes by macroinvertebrate and fish taxa

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Abstract

Many macrophyte species in lowland streams exhibit signs of grazing and herbivore damage, even though herbivory by aquatic macroinvertebrates and fish is generally considered to be of little importance. In this chapter we collected evidence for the hypothesis that herbivory on macrophytes by macroinvertebrates and fish is more widespread than assumed. We measured the dual stable isotope signatures ($\delta^{13}C$ and $\delta^{15}N$) of organic matter, epiphyton, submerged macrophytes, macroinvertebrates and fish in a Belgian lowland stream. There was a clear distinction in isotopic signatures of the different basal resources, allowing the use of the SIAR mixing model. These calculations revealed the consumption of macrophyte tissue not only by the phytophagous larvae of *Nymphula nitidulata* Hufnagel (Lepidoptera: Crambidae), but also by Baetidae nymphs (Ephemeroptera), Orthocladiinae larvae (Diptera: Chironomidae), the crayfish *Orconectus limosus* Rafinesque (Decapoda: Cambaridae) and the fish *Gobio gobio* L. (Cypriniformes: Cyprinidae) which are classified as feeding on other resources. Although the potential share of macrophyte biomass in the diet of macroinvertebrates and fish was demonstrated to be up to 49%, this amount is only a small percentage of the total standing macrophyte biomass in a lowland stream. However, the impact of this herbivory may still be substantial because consumption may comprise a significant fraction of the daily primary production. Additionally, small-scale herbivory may still have a negative impact on macrophyte growth and survival, for example through consumption of apical meristems and the increased susceptibility to diseases and toxins if the macrophyte's epidermis is damaged.

Keywords: Aquatic food web, stable isotope mixing model, epiphytic algae, generalist feeding strategy, temperate lowland stream

Introduction

Even though aquatic macrophytes can develop substantial biomass within lowland streams and rivers (Champion and Tanner 2000), it is commonly thought that living plant parts are rarely consumed by macroinvertebrates and fish (Mann 1988, Lampert and Sommer 2007, Moore and De Ruiter 2012). Instead, the majority of macrophyte primary production is assumed to enter the aquatic food web as detritus (Polunin 1984), which may serve as food source for resident macroinvertebrates or for animals living further downstream (cf. Vannote et al. 1980). The scarcity of direct macrophyte consumption by invertebrates and fish is probably not driven by its nutritive quality, as freshwater macrophytes generally have lower C:N ratios and contain lower amounts of hard to digest carbon-rich structural compounds, such as lignin and cellulose, in comparison to many terrestrial plant species (e.g. Lodge 1991, Bakker et al. 2016). However, many macrophyte species possess inhibitory secondary metabolites, such as alkaloids, glucosinolates and polyphenolics, which can act as a chemical defence against herbivory (Sotka et al. 2009, Gross and Bakker 2012). Feeding trials with omnivorous macroinvertebrates and fish confirm a preference for macrophytes with low concentrations of deterring chemicals (e.g. Li et al. 2004, Dorenbosch and Bakker 2011).

Despite this general consensus, there are numerous cases where significant amounts of invertebrate and fish-induced herbivore damage on aquatic vascular plants have been observed under natural conditions, both in older and recent literature (Jacobsen and Sand-Jensen 1992, Cronin et al. 1998, Körner and Dugdale 2003, Bakker et al. 2016, Wood et al. 2017). However, only a few macroinvertebrate and fish taxa are actually known to directly consume living macrophyte parts, including specialist Lepidoptera, Coleoptera and Diptera taxa, generalist omnivorous crabs and crayfish and generalist herbivorous fish (Newman 1991, Olsen et al. 1991, Cronin et al. 1998, Dorenbosch and Bakker 2011).

Although aquatic food webs have been reconstructed before, using both consumer stomach content and stable isotope analyses, aquatic macrophytes have often been excluded as possible food sources for macroinvertebrates and fish (e.g. Hamilton et al. 1992, Finlay 2001). However, studies that did include macrophytes as a food source reported varying results regarding macrophyte consumption; they either included only emergent and generally unpalatable species (Reid et al. 2008),

observed little evidence for direct macrophyte consumption (e.g. Jaschinski et al. 2011), or did indeed observe incorporation of macrophyte derived carbon in the aquatic food web, including consumption of living macrophytes by macroinvertebrate shredders (Watson and Barmuta 2011, Syväranta et al. 2016).

This study hypothesises that herbivory on aquatic macrophytes by macroinvertebrates and fish occurs in more generalist consumer taxa than normally considered, and will consequently also have a larger impact on the standing macrophyte biomass. This hypothesis is based both on personal observations of grazing damage on aquatic macrophytes in field conditions and on the expectation that from an evolutionary point of view, large feeding niches such as macrophytes can hardly be expected to remain unoccupied (e.g. Lodge 1991). In order to confirm or reject our hypothesis, we reconstruct the instream food web of a slow-flowing Belgian lowland stream using stable isotope measurements of typical producers and generalist consumers and by analysing the consumers' diet composition using a stable isotope mixing model.

Material and methods

Study site

Fieldwork was performed in the 18th and 19th of May 2015 in the Desselse Nete, a slow-flowing sand bottom lowland stream in the north of Belgium (51°14'53" N, 5°4'53" E) with a stream width varying between 3.5 and 5.5 m and an average depth of 60 cm. Summer nutrient concentrations were 95.0 ± 35.7 µg N-NH_4^+ l^{-1}, 672.5 ± 60.5 µg N-NO_3^- l^{-1} and 17.3 ± 4.0 µg P-PO_4^{3-} l^{-1}, with an average pH of 7.45 (Vlaamse Milieu Maatschappij 2017a). Catchment land use is mainly agricultural and anthropogenic impact on the stream is substantial, with stressors including hydromorphological degradation and water quality issues (i.e. pollution and agricultural nutrient inputs).

Sample collection, processing and isotopic analysis

In order to accurately reconstruct the aquatic food web, samples of all trophic levels were collected, including coarse particulate organic matter (CPOM) and fine particulate organic matter (FPOM), aquatic macrophytes, epiphyton, macroinvertebrates and fish. Sestonic FPOM was filtered from collected river water over 55 µm Whatmann glass-fibre filters (GF/C), while CPOM was collected in both

sestonic and benthic form, by sieving it from the river water and the upper 5 cm of the sediment, respectively. Aquatic macrophytes with their associated epiphyton and macroinvertebrates were collected using a cylindrical box-sampler (inner dimensions: 23.5 cm × 19 cm (length × diameter); mesh size: 500 µm; total volume: 6663 cm^3 (method after Chapter 2)), which was gently lowered over the vegetation stand, after which its two halves were gently closed and the aquatic vegetation within the sampler was cut off by hand with a sharp knife. Plant biomass was collected from three separate locations in the stream in order to obtain sufficient biomass and to account for small-scale spatial variability. Macroinvertebrates associated with sediment were additionally collected by taking five sediment core samples spread over bare and vegetated sections of the stream, using a plastic core sampler (diameter 5.4 cm). Although the invasive Chinese mitten crab (*Eriocheir sinensis* H. Milne-Edwards (Decapoda: Varunidae)), which was reported to feed on macrophyte tissue (Chapter 5), was also expected for this stream, no individuals from this species were found. Immediately after collection, samples were stored in 5 l plastic buckets and transported back to the laboratory where macroinvertebrates were separated from vegetation and stored at -20°C until further identification and processing. Macroinvertebrate guts were not removed, nor was gut clearance time provided, because these procedures were shown to not significantly affect stable isotope signatures of herbivorous and detritivorous macroinvertebrates (Jardine et al. 2005). To be able to reconstruct the food web, macroinvertebrates were identified to the lowest taxonomic level practical. Care was taken to remove carbonate shells of molluscs by dissection, to prevent it biasing the ^{13}C measurements (Jacob et al. 2005). Epiphyton was manually scraped from the macrophytes, using tweezers for large fragments and by carefully brushing the macrophytes with gloved fingers and rinsing them with distilled water for more tightly attached fragments. This was then stored at -20°C until further processing. Macrophytes that were cleaned of epiphyton in this way were sorted by species and oven-dried at 70 °C to a constant weight (at least 48 h). Fish were captured by electrofishing. After identification three individuals were collected per species and dissected to obtain muscle tissue, which was subsequently stored at -20 °C until further processing. As is common in isotope food web studies, only muscle tissue was extracted for isotope analysis in fish and the crayfish *Orconectus limosus* Rafinesque (Decapoda: Cambaridae), due to its intermediate turnover rate and low

variability compared to other tissues (Tieszen et al. 1983, Pinnegar and Polunin 1999). All other macroinvertebrates were stored and analysed as complete animals. Organic matter, epiphyton, macroinvertebrates and (cray-)fish tissue samples were subsequently freeze-dried, using a Heto PowerDry LL3000 (Thermo Scientific) and ground using a Retsch mixer mill (MM301). Dried macrophytes were all ground with a Retsch ZM200 ultra-centrifugal mill.

Subsamples of the powdered material were weighed in silver cups and acidified with one drop of 5% hydrochloric acid, to remove any carbonates (Jacob et al. 2005), and oven-dried at 60°C for 4 hours after which the cups were folded and analysed. 5 mg of sample was used for organic matter, macrophytes and epiphyton, and 1 mg was used for macroinvertebrates and fish. Whenever possible, macroinvertebrates were measured per separate vegetation or sediment sample in which they occurred, although it was sometimes necessary to pool animals from different samples in order to obtain enough biomass for stable isotope analyses. Fish muscle tissue was analysed for three separate individuals, although some species were caught in fewer numbers. Sample carbon and nitrogen content was measured using a Flash EA 1112 Elemental Analyser (Thermo Finnigan) or a EuroEA3000 (Eurovector) Elemental Analyser. The ^{13}C and ^{15}N stable isotope signatures were measured using a Delta V Advantage isotope ratio mass spectrometer (Thermo Finnigan) that was coupled, via a ConFlo III interface (Thermo Finnigan), to the Elemental Analysers.

Data analysis

To assess the relative importance of the food sources in the consumer's diet the stable isotope mixing model 'Stable Isotope Analysis in R' (SIAR, Parnell and Jackson 2013) package (version 4.2) was used under R 3.3.2 (R Development Core Team 2016). This Bayesian mixing model incorporates variation in the stable isotope (i.e. $\delta^{13}C$ and $\delta^{15}N$) signatures of the different food sources and consumers and, based on this information, calculates density plots of credible intervals for the estimated dietary proportion of each food source (Parnell et al. 2010, Parnell and Jackson 2013). Additionally, this mixing model has the ability to incorporate carbon and nitrogen content of food sources, allowing for a better resolution when analysing food sources with vastly different C and N concentrations (Phillips and Koch 2002),

for example for omnivores that may consume both nitrogen-poor detritus and nitrogen-rich animal material.

The model was applied to the following macroinvertebrate and fish taxa that, based on a literature survey and our own experience, were expected to potentially include macrophytes in their diets, either as living macrophyte parts or macrophyte-derived detritus (i.e. groups for which the mixing model analysis was useful cf. Phillips et al. 2014): *Pisidium* sp. (Bivalvia: Sphaeriidae), *Baetis* sp. (Ephemeroptera: Baetidae), Chironomini (Diptera: Chironomidae), Orthocladiinae (Diptera: Chironomidae), *Simulium* sp. (Diptera: Simuliidae), *Hydroptila* sp. (Trichoptera: Hydroptilidae), *Asellus aquaticus* L. (Isopoda: Asellidae), *Orconectus limosus* Rafinesque (Decapoda: Cambaridae), *Gobio gobio* L. (Cypriniformes: Cyprinidae), and *Perca fluviatilis* L. (Perciformes: Percidae). The phytophagous larvae of *Nymphula nitidulata* Hufnagel (Lepidoptera: Crambidae) were also included in the model as a reference group that is known to purposefully consume macrophytes (Gaevskaya 1969, Palm 1986).

Organic matter, epiphyton and macrophytes, being the three basal resources measured in this study, were used as the three possible food sources for the herbivorous/detritivorous invertebrate taxa in the SIAR model calculations. For the two omnivorous fish species and the crayfish *O. limosus*, macroinvertebrates were added as an additional possible food source, of which the mean stable isotope signature was calculated as the weighted average of all different measured invertebrate taxa (Phillips et al. 2005). Additionally, fish were included as a possible food source in the diet of *P. fluviatilis*, as our collected individuals measured over 10 cm and were thus large enough to have possibly undergone the ontogenetic shift to a piscivorous lifestyle (e.g. Mittelbach and Persson 1998). Again, the mean stable isotope signature of this food source was calculated as the weighted average of the relevant fish taxa (Phillips et al. 2005).

Before incorporation in the model, the food source carbon and nitrogen stable isotope signatures were corrected for trophic fractionation by adding 0.8‰ and 2.6‰, respectively, for animals of which only muscle tissue was analysed (i.e. *O. limosus* and fish) and by adding 0.4‰ and 2.3‰, respectively, for the remaining macroinvertebrates that were analysed in one piece, according to McCutchan et al. (2003). No $\delta^{15}N$ differentiation was used for *Hydroptila* sp. because these animals

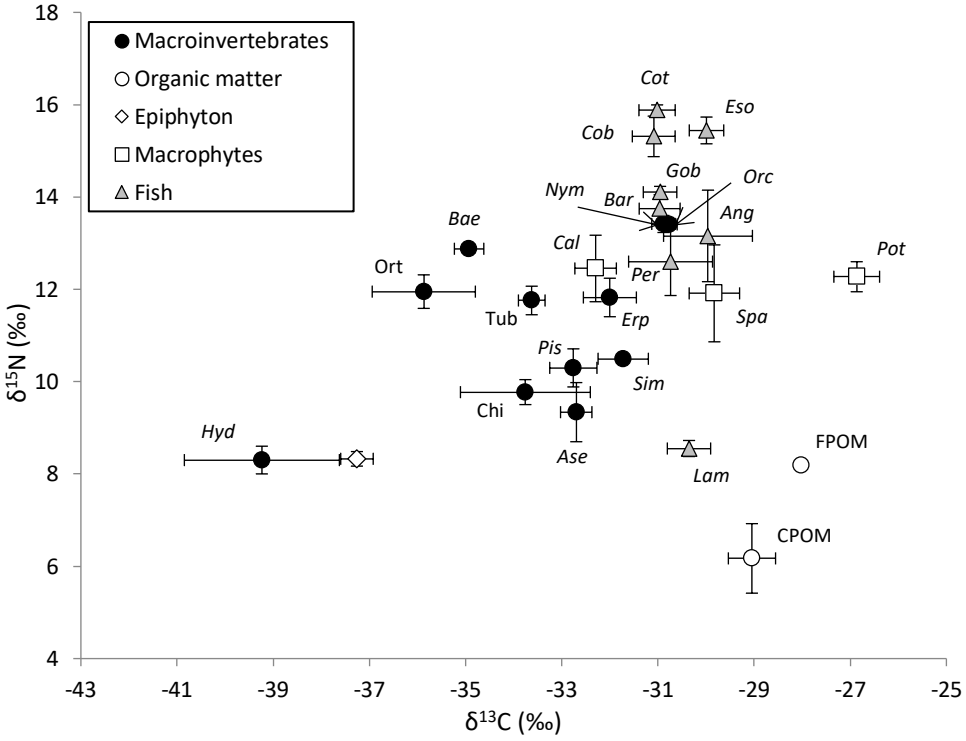
are considered fluid feeders and should therefore not be corrected for nitrogen (Keiper 1998, McCutchan et al. 2003).

After the calculation of the macrophyte proportion in the diet of invertebrate and fish consumers, the quantitative impact of macrophyte consumption by Orthocladiinae, *Baetis* sp. and *N. nitidulata* on the macrophyte standing biomass within the stream was calculated, based on these dietary composition data and literature data on macroinvertebrate feeding rates and distribution in the Desselse Nete. This was done by multiplying the calculated potential macrophyte proportion in the consumer's diet with its individual daily feeding rate, which was obtained from various literature sources (Zelinka 1984, Winterbourn et al. 1985, Nolte 1990, Cattaneo and Mousseau 1995, Monakov 2003). Furthermore, the animal's distribution data from the Desselse Nete were used from the literature. These specimens were collected on macrophytes (i.e. *Sparganium emersum* Rehmann (Sparganiaceae), *Potamogeton natans* L. (Potamogetonaceae) and *Callitriche obtusangula* Le Gall (Plantaginaceae), the same plant species as in this study) with a known plant biomass in the Desselse Nete on August 2014 and June 2015 (Chapter 2). Data from both years were hereby used in the calculation, in order to incorporate a degree of the seasonal differences in abundance that naturally occur in macroinvertebrate populations (Jacobsen and Sand-Jensen 1992, Gross et al. 2002). This calculation should be seen as an indicator of the scale of the impact of macroinvertebrate herbivory, rather than a definite account, as the time scale of the study cannot fully capture the natural fluctuations in herbivore population size.

Results

Natural ^{13}C and ^{15}N abundance

Clear differences in $\delta^{13}C$ and $\delta^{15}N$ signatures of different food web components were observed within the Desselse Nete (Figure 3.1). A clear distinction is hereby visible in the isotopic signatures of the basal resources in the food web: organic matter, epiphyton and macrophytes. With a relatively high $\delta^{13}C$ and a low $\delta^{15}N$ value, compared to other food web components, FPOM and CPOM were positioned at the lower right corner of the $\delta^{13}C$-$\delta^{15}N$ isotope biplot. Epiphyton on the other hand, with the majority of its biomass consisting of the green alga *Cladophora* sp. (Cladophoraceae), displayed the greatest depletion in ^{13}C values. This, combined



Macrophytes:	Macroinvertebrates (2):	Fish:
Cal: *Callitriche obtusangula*	*Erp*: *Erpobdella* sp.	*Ang*: *Anguilla anguilla*
Pot: *Potamogeton natans*	*Hyd*: *Hydroptila* sp.	*Bar*: *Barbatula barbatula*
Spa: *Sparganium emersum*	*Nym*: *Nymphula nitidulata*	*Cob*: *Cobitus taenia*
	Orc: *Orconectus limosus*	*Cot*: *Cottus perifretum*
Macroinvertebrates:	Ort: Orthocladiinae	*Eso*: *Esox lucius*
Ase: *Asellus aquaticus*	*Pis*: *Pisidium* sp.	*Gob*: *Gobio gobio*
Bae: *Baetis* sp.	*Sim*: *Simulium* sp.	*Lam*: *Lampetra planeri*
Chi: Chironomini		*Per*: *Perca fluviatilis*

Figure 3.1 Stable isotope signatures (mean ± SE) of $\delta^{13}C$ and $\delta^{15}N$ of different groups of primary producers, organic matter and different macroinvertebrate and fish taxa collected in the Desselse Nete. Abbreviations within the graph are explained in the textbox below the graph.

Table 3.1 Potential contributions of the different basal resources to the consumers' diets. Values are presented as proportions of the total diet (between 0 and 1). Empty cells indicate that a food source was not incorporated in the mixing model. Ranges represent 90% credible intervals (5–95 percentile ranges) with median contribution in parentheses, calculated using the SIAR mixing model. Food web resources with high potential contribution are indicated in bold (95 percentile ≥ 50%).

	Macrophytes	Epiphyton	Organic matter	Macroinvertebrates	Fish
Asellus aquaticus	0.00-0.28 (0.07)	**0.39-0.61 (0.50)**	**0.18-0.57 (0.42)**		
Chironomini	0.00-0.33 (0.07)	**0.18-0.79 (0.50)**	**0.07-0.69 (0.41)**		
Pisidium sp.	0.00-0.26 (0.08)	**0.35-0.64 (0.50)**	**0.20-0.57 (0.41)**		
Hydroptila sp.	0.00-0.41 (0.11)	**0.17-0.97 (0.59)**	**0.00-0.57 (0.24)**		
Simulium sp.	0.00-0.44 (0.18)	**0.13-0.65 (0.40)**	**0.09-0.65 (0.41)**		
Perca fluviatilis	0.00-0.37 (0.19)	0.02-0.37 (0.21)	0.01-0.41 (0.24)	0.00-0.38 (0.19)	0.00-0.34 (0.16)
Orthocladiinae	**0.09-0.50 (0.31)**	**0.23-0.86 (0.57)**	0.00-0.38 (0.11)		
Orconectus limosus	**0.15-0.60 (0.37)**	0.05-0.35 (0.20)	0.00-0.27 (0.10)	**0.00-0.57 (0.31)**	
Gobio gobio	**0.08-0.67 (0.37)**	0.01-0.36 (0.18)	0.00-0.35 (0.12)	**0.00-0.58 (0.30)**	
Baetis sp.	**0.07-0.66 (0.49)**	**0.18-0.83 (0.45)**	0.00-0.27 (0.07)		
Nymphula nitidulata	**0.11-0.80 (0.49)**	0.01-0.47 (0.27)	**0.00-0.55 (0.26)**		

with relatively low $\delta^{15}N$ values, resulted in its bottom left position within the isotope biplot. The macrophytes *S. emersum*, *P. natans* and *C. obtusangula* showed mean $\delta^{13}C$ values that ranged from -32.2‰ (*C. obtusangula*) to -26.9‰ (*P. natans*), in addition to mean $\delta^{15}N$ values that were higher than those of most invertebrate consumers, with the exception of *N. nitidulata* and *O. limosus*, and ranged from 11.9‰ (*S. emersum*) to 12.5‰ (*C. obtusangula*). After correcting for trophic fractionation, macroinvertebrate isotopic signatures were generally positioned within the mixing triangle of the basal resources, except for the ^{15}N enriched *N. nitidulata* and *O. limosus* and the ^{13}C depleted *Hydroptila* sp., Orthocladiinae and *Baetis* sp. taxa. The group with the highest $\delta^{13}C$ and $\delta^{15}N$ signatures were the fishes, with the exception of the FPOM filtering *Lampetra planeri* Bloch (Petromyzontiformes: Petromyzontidae), and the aforementioned macroinvertebrates *N. nitidulata* and *O. limosus* which also possessed high $\delta^{15}N$ signatures.

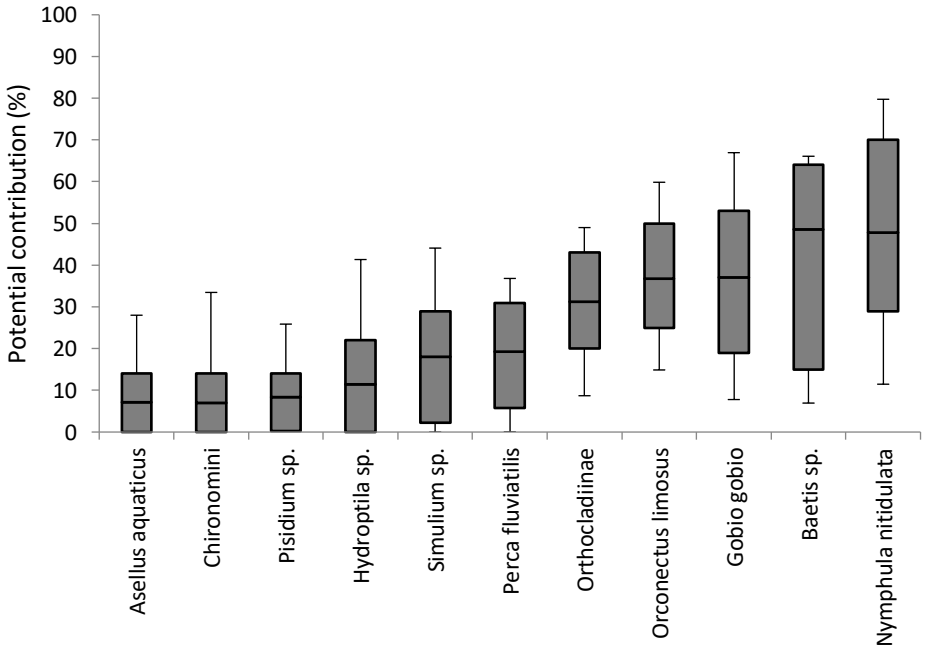


Figure 3.2 Potential contribution ranges of macrophytes to the consumers' diets. Boxplots show median (line), 25–75 percentile range (box) and 5–95 percentile range (whiskers).

Table 3.2 Daily consumption of macrophyte standing biomass by Orthocladiinae larvae, *Baetis* sp. nymphs and *N. nitidulata* larvae in the Desselse Nete as calculated from dietary composition data from this study and quantitative distribution data and consumption rates from the literature. Macrophyte biomass and consumer distribution data are means ± SE, while calculated potential macrophyte consumption rates are presented as 90% credible intervals (5–95 percentile ranges) with median contribution in parentheses. Data from [a]Chapter 2, [b]Nolte (1990), [c]Cattaneo & Mousseau (1995), [d]Zelinka (1984), [e]Winterbourn et al. (1985), [f]Monakov (2003), [g]Nielsen et al. (1985) and [h]Madsen et al. (2001).

	2014			2014
	S. emersum	*P. natans*	*C. obtusangula*	Total
Macrophyte dry weight (g sample^{-1})[a]	0.79 ± 0.17	4.27 ± 0.9	3.89 ± 1.14	3.00 ± 0.56
Orthocladiinae larvae sample^{-1} [a]	5.59 ± 1.56	3.23 ± 0.56	9.24 ± 2.53	6.02 ± 1.08
Baetis sp. nymphs sample^{-1} [a]	9.00 ± 2.52	31.00 ± 6.96	105.00 ± 30.89	48.33 ± 12.97
N. nitidulata larvae sample^{-1} [a]	2.78 ± 0.81	0	0.89 ± 0.26	1.22 ± 0.36
Orthocladiinae feeding rate (mg dry matter ind^{-1} day^{-1})[b,c]	0.23	0.23	0.23	0.23
Baetis sp. feeding rate (mg dry matter ind^{-1} day^{-1})[d,e]	1.75	1.75	1.75	1.75
N. nitidulata feeding rate (mg dry matter ind^{-1} day^{-1})[f]	11.54	11.54	11.54	11.54
Biomass consumption Orthocladiinae (% macrophyte standing stock consumed day^{-1})	0.01-0.06 (0.04)	0.002-0.01 (0.008)	0.003-0.02 (0.01)	0.006-0.03 (0.02)
Biomass consumption *Baetis* sp. (% macrophyte standing stock consumed day^{-1})	0.13-1.19 (0.89)	0.10-0.96 (0.71)	0.27-2.54 (1.89)	0.17-1.57 (1.16)
Biomass consumption *N. nitidulata* (% macrophyte standing stock consumed day^{-1})	0.47-3.42 (2.05)	0	0.05-0.38 (0.23)	0.17-1.27 (0.76)
% Ingestion total	0.69-5.26 (3.35)	0.10-0.97 (0.72)	0.42-3.78 (2.74)	0.39-3.22 (2.19)
% Ingestion of daily vegetation growth rate [g,h]	13.70-105.29 (67.00)	2.60-24.31 (18.00)	6.43-58.17 (42.15)	

Mixing model analysis

Besides differences in stable isotope signatures of organic matter, epiphyton, macrophytes and macroinvertebrates, there were also marked differences in the C:N ratios of these food sources. Organic matter, with a C:N ratio of 21.3 ± 4.8 (mean ± SE), was hereby relatively the poorest in nitrogen, and macroinvertebrates were relatively rich in nitrogen with a C:N ratio of 5.7 ± 0.3. Epiphyton and macrophytes were situated in between with C:N ratios of 10.8 ± 0.3 and 13.2 ± 0.7, respectively. The mixing model analysis revealed varying potential contributions of macrophytes in the diet of the analysed macroinvertebrate and fish taxa, with median values ranging from 7% for *A. aquaticus* and Chironomini to 49% for *Baetis*

Table 3.2 continued.

	2015			2015
	S. emersum	*P. natans*	*C. obtusangula*	Total
Macrophyte dry weight (g sample^{-1})[a]	0.63 ± 0.07	2.96 ± 0.52	3.78 ± 0.55	2.60 ± 0.36
Orthocladiinae larvae sample^{-1} [a]	85.48 ± 12.37	193.72 ± 40.51	496.56 ± 94.34	258.59 ± 47.56
Baetis sp. nymphs sample^{-1} [a]	0.33 ± 0.24	11.89 ± 1.98	23.78 ± 7.69	12.00 ± 3.16
N. nitidulata larvae sample^{-1} [a]	0	0	0	0
Orthocladiinae feeding rate (mg dry matter ind^{-1} day^{-1})[b,c]	0.23	0.23	0.23	0.23
Baetis sp. feeding rate (mg dry matter ind^{-1} day^{-1})[d,e]	1.75	1.75	1.75	1.75
N. nitidulata feeding rate (mg dry matter ind^{-1} day^{-1})[f]	11.54	11.54	11.54	11.54
Biomass consumption Orthocladiinae (% macrophyte standing stock consumed day^{-1})	0.24-1.29 (0.84)	0.14-0.75 (0.48)	0.3-1.62 (1.04)	0.23-1.22 (0.79)
Biomass consumption *Baetis* sp. (% macrophyte standing stock consumed day^{-1})	0.006-0.06 (0.05)	0.06-0.56 (0.41)	0.08-0.77 (0.57)	0.05-0.46 (0.34)
Biomass consumption *N. nitidulata* (% macrophyte standing stock consumed day^{-1})	0	0	0	0
% Ingestion total	0.32-1.74 (1.13)	0.20-1.31 (0.9)	0.38-2.38 (1.61)	0.30-1.81 (1.22)
% Ingestion of daily vegetation growth rate [g,h]	6.40-34.82 (22.60)	4.99-32.66 (22.50)	5.91-36.62 (24.77)	

sp. and *N. nitidulata* (Table 3.1, Figure 3.2). Macrophyte consumption was significantly ($p < 0.05$) above 0 (i.e. the 5th percentile was higher than 0) for Orthocladiinae, *O. limosus*, *G. gobio*, *Baetis* sp. and *N. nitidulata*, indicating macrophyte consumption by these taxa (Figure 3.2). On the other hand, *A. aquaticus*, Chironomini, *Pisidium* sp., *Hydroptila* sp., *Simulium* sp. and *P. fluviatilis* did not consume significant portions of macrophyte tissue (Figure 3.2).

From the calculated diet composition of Orthocladiinae, *Baetis* sp. and *N. nitidulata*, which were coupled to quantitative literature data of macrophyte biomass, macroinvertebrate distribution and the feeding rates of these animals, the relative daily consumption of macrophyte standing biomass was calculated (Table 3.2). Although considerable variation was observed between the different years, invertebrate taxa and plant species, the general calculated consumption of macrophyte standing biomass was low with median values ranging from 0.72% (*P. natans* in 2014) to 3.35% macrophyte biomass consumed day^{-1} (*S. emersum* in 2014) (Table 3.2).

Discussion

Reconstruction of consumers' diets

With a potential median contribution of 49%, macrophytes formed the most important food source for the aquatic larvae of the lepidopteran *N. nitidulata* and the ephemeropteran nymph *Baetis* sp. The fact that the rest of the diet of *N. nitidulata* consisted of epiphytic algae was unexpected since these larvae are typically described as oligophagous macrophyte specialists that only feed on a number of host plants (Gaevskaya 1969, Palm 1986). A most likely explanation for this observation could be the ingestion of attached epiphyton during macrophyte consumption. Similarly, yet the other way around, the median potential inclusion of 49% and 31% macrophyte biomass in the diets of *Baetis* sp. and Orthocladiinae, respectively, could very well be caused by accidental ingestion of macrophyte tissue during the grazing of epiphyton. These two taxa are both known to feed on epiphytic algae, whereby *Baetis* nymphs are generally classified in the scraper functional feeding group (cf. Cummins 1973) and Orthocladiinae larvae as gatherers. *Baetis* nymphs hereby mostly feed on algal species that are tightly attached to the macrophytes, whereas larvae of many Orthocladiinae species prefer to feed on the more loosely attached algal species in the outer epiphyton

layer (Tall et al. 2006, Maasri et al. 2010). The fact that *Baetis* nymphs consume epiphyton closer to the macrophyte surface than Orthocladiinae larvae might result in a higher accidental scraping of macrophyte tissue during grazing, which is also reflected in the animals' diet calculated in this study (Figure 3.2). The accidental consumption and incorporation of aquatic macrophyte tissue by gathering and scraping macroinvertebrates is rarely mentioned in studies (but see Yule 1986), yet might be more common than based on the records in literature alone. Another possible route for the consumption of macrophyte-derived material might be the direct or accidental consumption of senescing macrophyte parts, which are generally colonised by many epiphytic algae and bacteria, are softer and are poorer in inhibitory secondary metabolites (Suren 1989, Suren and Lake 1989, Newman 1991).

Although macrophyte leaf damage was not assessed in this study, other researchers also observed leaf erosion through accidental scraping by Ephemeroptera nymphs, whereby Ephemeroptera taxa that consumed more adnate algal taxa caused more erosion than taxa that consumed algae that were farther removed from the leaf surface (Karouna and Fuller 1992). Furthermore, the larvae of the *Hydroptila* genus of microcaddisflies, another algivorous taxon, are known to feed on green filamentous algae (e.g. *Cladophora* sp.) which are far removed from their macrophyte substrates (e.g. Keiper 1998). It is therefore no surprise that macrophytes did not seem to be part of the diet of *Hydroptila* sp. larvae at all (Figure 3.2). This theory of accidental leaf erosion could be tested in future studies by assessing the damage done to macrophyte leaves by different groups of grazing macroinvertebrates, using electron microscopy (cf. Karouna and Fuller 1992).

Other macroinvertebrates that did not include significant potential fractions of macrophytes in their diet were Chironomini larvae and *A. aquaticus* (Figure 3.2). These animals are generally considered to be predominantly detritivorous, though macrophyte miners like some representatives of the genus *Endochironomus* exist, and are classified in the gatherer and shredder functional feeding group, respectively (e.g. Usseglio-Polatera et al. 2000, Moller Pillot 2009). It is hereby interesting to note that, while the calculated diet of the Chironomini larvae is in accordance with literature (e.g. Moller Pillot 2009), the calculated diet of *A. aquaticus* diverts from the assumption that this animal lives from

allochthonous and autochthonous detritus colonised by a bacterial/fungal biofilm (e.g. Graça et al. 1994). A possible explanation for this deviation could be that *A. aquaticus* supplements its relatively nitrogen-poor diet with small amounts of alternative food sources which are richer in nitrogen such as epiphytic algae and dead animals, which has previously been shown for a variety of detritivorous invertebrates that mostly feed on nutritionally poor resources such as leaf litter (Anderson 1976, Crenier et al. 2017).

Furthermore, no significant potential contributions of macrophyte tissue were observed in the diet of the filter-feeding invertebrates *Pisidium* sp. and *Simulium* sp. (Figure 3.2). This is in accordance with the literature that names bacteria, algae and FPOM as major food sources for these taxa (Wallace and Merritt 1980, Monakov 2003). However, it can be expected that macrophyte-derived detritus might be consumed by these animals, particularly at the end of the growing season when most macrophytes decay and are broken down, as this is also a more nutritious resource than living macrophyte tissue (Newman 1991).

Finally, a significant dietary fraction of macrophyte biomass was calculated in the diet of the omnivorous crayfish *O. limosus* and the fish *G. gobio* (Figure 3.2), confirming earlier observations of macrophyte consumption by these taxa (Lodge et al. 1994, Michel and Oberdorff 1995). Although it can be expected that omnivorous taxa might only consume macrophytes when more nutritious food sources such as animal prey are in short supply (e.g. Dorenbosch and Bakker 2011), some studies have instead demonstrated distinct preferences for either macrophytes or detritus in certain aquatic omnivores (Gherardi et al. 2004, Gherardi and Barbaresi 2007). It is hereby expected that other factors, such as a higher carbon assimilation efficiency of macrophytes, together with their higher availability and easier handling, contribute to this selectivity (e.g. Gherardi et al. 2004). More research, including behavioural studies, is needed in order to clarify the driving forces that determine feeding preferences in freshwater omnivores.

Conclusions and ecological relevance

Based on stable isotope measurements and mixing model calculations, this study observed the consumption of macrophyte tissue in a number of herbivorous and omnivorous macroinvertebrate and fish taxa in a Belgian lowland stream. Besides the taxa that purposefully consume living macrophytes (e.g. *N. nitidulata* and *O.*

limosus), evidence was found for the incorporation of macrophyte-derived carbon and nitrogen in the macroinvertebrate taxa Orthocladiinae and *Baetis* sp., which are normally described as algivores feeding on periphyton (Elliott and Humpesch 2010, Moller Pillot 2013). The magnitude and timing of this behaviour is unknown, as this study only reconstructed the food web of a single stream at a single moment. Yet we argue that this study may be the first explicitly demonstrating the assimilation of macrophyte tissues in algivorous macroinvertebrates.

While the destructive effects of (invasive) crayfish and crabs, that actually consume very little of what they destroy, are well known (Chapter 5, Lodge et al. 1994, Jin et al. 2003), the ecological effects of smaller-scale consumption are less well known and can potentially be underestimated. In this study we demonstrate the potential relevance of macrophytes in the diet of macroinvertebrates, although the scale of this herbivory on the standing macrophyte biomass remains relatively small, with only a few percent consumed by the dominant macroinvertebrate taxa each day (Table 3.2). In comparison, the daily consumption of epiphyton standing biomass has been estimated at 50-70% by some authors (Kesler 1981, Armitage et al. 1995). However, when compared to the macrophytes' growth rates, the percentage of macrophytes consumed each day by the macroinvertebrates accounts for 18 to 105% of the daily primary production (Table 3.2), meaning that these animals can potentially hamper or even restrict the growth of macrophytes. However, it should be noted that herbivore density in these calculations is based on only two sampling events. This may only provide a limited image of the actual seasonal fluctuations in herbivore population size, leading to a potential over- or underestimation of macrophyte consumption (Jacobsen and Sand-Jensen 1992, Gross et al. 2002).

Although complete consumption of macrophyte biomass was not observed in this study, the consequences of herbivory by macroinvertebrates and fish on the instream aquatic vegetation can vary in magnitude depending on the prevailing environmental conditions and the nature of the macrophyte consumption. Small scale consumption of plant tissue can possibly induce vigorous regrowth combined with additional branching and investment in new undamaged shoots (e.g. Belsky et al. 1993, Pieczyńska 2003). On the other hand, damaging of the macrophyte cuticle and epidermis might expose the plant to bacterial and fungal infections or toxic compounds, which can lower the overall fitness of the vegetation (Suren 1989). The

negative effects of macrophyte consumption on standing plant biomass are likely to be more pronounced under prevailing ambient conditions that already have a negative influence on macrophyte growth, such as a turbid water layer and high epiphyton cover. Under these conditions, even small amounts of herbivory can lead to a significant decline in underwater vegetation (Chapter 5, Hidding et al. 2016). More research may be needed to quantitatively assess the role of small scale herbivory on the development and functioning of the aquatic macrophyte community.

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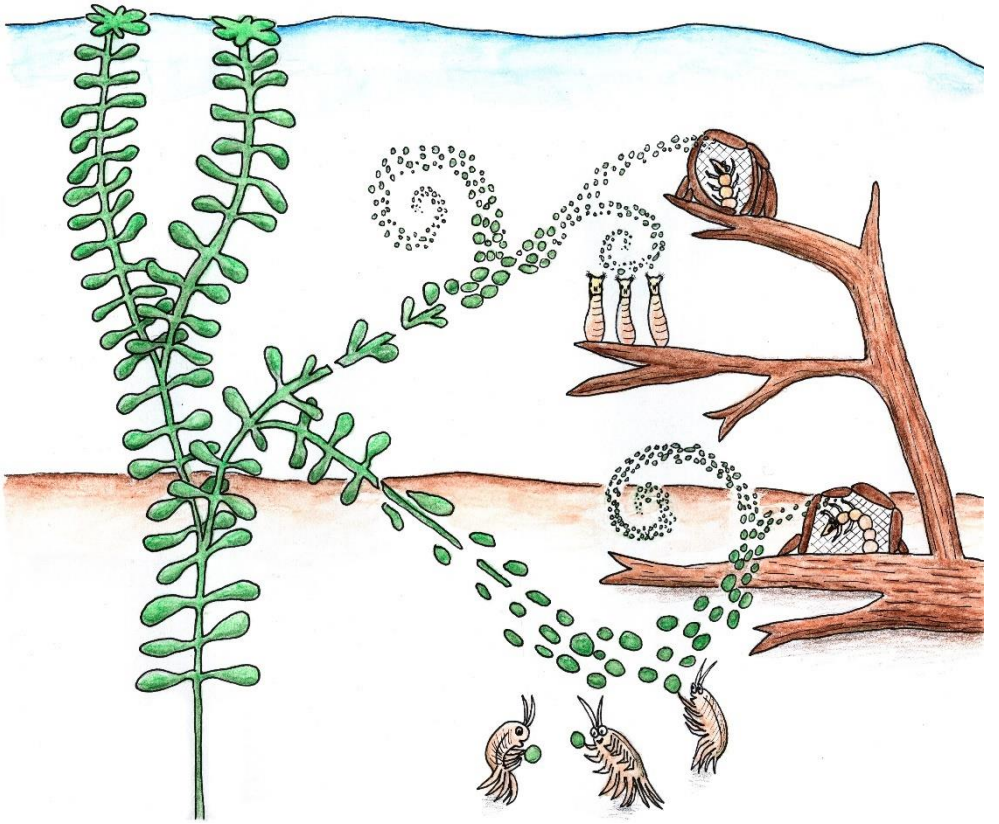

Chapter 4.

Seasonal changes in fatty acid composition indicate consumption of submerged macrophyte-derived organic matter by macroinvertebrates in a Belgian lowland stream

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Abstract

Living macrophytes are generally not considered to be a dominant food source for generalist macroinvertebrate consumers in temperate lowland streams, but instead enter the detrital food web at the end of the growing season. This study uses fatty acids (FAs) in order to investigate whether this macrophyte-derived organic material is subsequently consumed by detritivorous macroinvertebrates and thus contributes to the aquatic food web in this way. Over the course of the 2016 growing season, we measured the FA composition of macrophytes, epiphytic algae, particulate organic matter and the macroinvertebrate species *Gammarus roeseli* Gervais (Amphipoda: Gammaridae), *Simulium* sp. (Diptera: Simuliidae) and *Hydropsyche* sp. (Trichoptera: Hydropsychidae) in a Belgian lowland stream. Significant differences in FA content and FA composition were observed among the different basal resources and macroinvertebrate consumers, in addition to a significant temporal variation in FA composition. The polyunsaturated fatty acids (PUFAs) 18:2ω6 and 18:3ω3 were dominant in living macrophytes and fine particulate organic matter (FPOM) was enriched in these PUFAs in autumn. This coincided with a strong enrichment of long chain saturated fatty acids (LSFAs; C20:0-C28:0), characteristic of terrestrial detritus, in the coarse particulate organic matter (CPOM). These seasonal changes were also reflected in *G. roeseli* and *Hydropsyche* larvae at the end of the macrophyte growing season in autumn. Based on the consumer Σω3:Σω6 ratio, indicative of autochthonous vs. allochthonous consumption, we conclude that for the autumn period, allochthonous leaf litter likely forms the majority of the diet of *G. roeseli*, but that the diet of *Hydropsyche* and *Simulium* larvae also includes macrophyte-derived organic matter.

Keywords: Macroinvertebrates, macrophytes, fatty acids, aquatic food web, seasonal changes

Introduction

In lowland streams in temperate regions, many macroinvertebrate species directly or indirectly depend on aquatic macrophytes as a habitat, refuge or food source (Cummins and Klug 1979, Heck and Crowder 1991, O'Hare and Murphy 1999, Warfe and Barmuta 2004, 2006, Allan and Castillo 2007, Bakker et al. 2016). The development of aquatic macrophyte biomass generally follows a seasonal pattern, with macrophyte growth in spring, maximum biomass in summer and macrophyte senescence and die-off in autumn, although some species reach their peak biomass in spring or autumn (e.g. Sand-Jensen et al. 1989). This seasonal variation in the macrophyte community has potentially large consequences for aquatic macroinvertebrates and the instream food web, through changes in both macrophyte quantity and quality.

During the growing season, the surface area and habitat complexity provided by the regrowth of macrophytes creates a complex habitat for macroinvertebrates and increases food web complexity, also by providing a surface for the attachment of epiphyton and by the retention of particulate organic matter (McAbendroth et al. 2005, Warfe and Barmuta 2006, Kefi et al. 2012, Borst et al. 2018). Macrophyte regrowth also presents a new food source in the form of macrophyte tissue to many omnivorous and herbivorous invertebrates, which is most palatable at the start of the growing season (Elger and Willby 2003). However, living macrophytes are not considered to be the main food source for many invertebrate taxa, due to the presence of inhibitory secondary metabolites, such as alkaloids, glucosinolates and polyphenolics, which can act as a defence against herbivory (Sotka et al. 2009, Gross and Bakker 2012). At the end of the growing season, the growth of macrophytes comes to a halt and certain macrophyte species start to senescence and plant parts die-off. Some macrophytes survive winter as belowground tubers and turions, from which they germinate again in the following year (e.g. *Sparganium emersum* Rehmann (Sparganiaceae)), while others always remain wintergreen (e.g. *Myriophyllum spicatum* L. (Haloragaceae)) or remain wintergreen depending on the prevalent stream conditions during the winter months (e.g. *Callitriche obtusangula* Le Gall (Plantaginaceae)) (Hynes 1970, Stanley et al. 1976, Wiegleb et al. 2014). During the process of senescing, macrophytes leach many inhibitory compounds and may thus become more palatable (Brönmark 1989, Newman 1991). The subsequent decay of the macrophytes causes the plants

to enter the instream detritus pool, where they become colonised by microorganisms, further increasing their nutritious value to detritivorous macroinvertebrates (Arsuffi and Suberkropp 1984, Newman 1991, France 2011). Remaining macrophyte tissue is then broken down to fine particulate organic matter (FPOM) and converted to bacterial and fungal biomass through the microbial loop, from where it re-enters the aquatic food web (Polunin 1984, Pusch et al. 1998, Hieber and Gessner 2002). After the growing season, the disappearance of many macrophyte species in winter drastically reduces the available habitat surface for many invertebrate species, contributing to a decrease in macroinvertebrate biomass during this period without macrophytes (e.g. Hargeby et al. 1994, Shupryt and Stelzer 2009).

Although consumption of macrophyte-derived coarse particulate organic matter (CPOM) by macroinvertebrate shredders in freshwater systems has been experimentally demonstrated before (e.g. Smock and Harlowe 1983, Suren and Lake 1989), the subsequent consumption of macrophyte-derived FPOM by gatherers or filter-feeders, as has been shown for estuaries (Machas et al. 2003, Wang et al. 2015), has not been demonstrated in detail. Therefore, the aim of this study is: i) to qualitatively demonstrate the consumption of macrophyte-derived FPOM by macroinvertebrates, and ii) to look for possible dietary shifts in invertebrate populations during the year, as the availability of living macrophytes and macrophyte-derived FPOM drastically changes in this period. In this way, we aim to follow the flow of macrophyte-derived CPOM and FPOM through the detritivorous food web.

For this purpose, we measured the fatty acid (FA) composition of a number of different basal food sources in the stream food web (i.e. CPOM, FPOM, macrophytes and epiphyton) and macroinvertebrate consumers over the course of one year. FAs are present in all organisms and are frequently used as conservative biomarkers to trace food sources of consumers in marine and freshwater ecosystems (Desvilettes et al. 1997, Arts and Wainman 1999). The fact that FAs remain unaltered during their transfer from primary producers to primary consumers and higher trophic levels makes them particularly suitable as biomarkers (Desvilettes et al. 1997). Furthermore, some polyunsaturated fatty acids (PUFA) are essential for the functioning of animals cells, in the sense that they cannot be synthesized by the animals themselves but have to be acquired from

their diets (Arts et al. 2001). A number of these FAs can only be synthesised by specific groups of organisms and can therefore be used as indicators for these specific groups, whereby linoleic and linolenic acid have for example been established as diagnostic for vascular plant and aquatic plant material (Rozentsvet et al. 2002, Nesterov et al. 2009, Mortillaro et al. 2011). Finally, the ratio of Σω3 FAs : Σω6 FAs is used as a marker for terrestrial (<1.0) vs aquatic (>1.0) matter (Desvilettes et al. 1994, Torres-Ruiz et al. 2007). By measuring the FA composition of different food web components throughout the year, we aim to observe evidence for the consumption of macrophyte derived material by macroinvertebrates.

We expect to observe a reflection of macrophyte development throughout the growing season in the consumers' diets, whereby we specifically expect an increase in FAs diagnostic for macrophytes (i.e. linoleic and linolenic acid) in the tissues of macroinvertebrate filter-feeders at the end of the growing season, when the majority of the macrophyte biomass dies off and is degraded to FPOM.

Material and methods

Study site

Fieldwork was performed in the Zwarte Nete, a slow-flowing sand bottom lowland stream in the north of Belgium (51°15'3" N, 5°4'53" E) on five occasions; the 30th of March, the 20th of June, the 3rd of August and the 12th of October 2016 and on the 10th of January 2017. This stream is characterised by extensive instream plant growth in summer whilst overhanging and riparian vegetation is limited by the intensive agricultural use of the grasslands adjacent to the stream. In the studied reach, average stream width is 4.5 m with an average depth of 55 cm over the studied period and a discharge varying between 0.2 and 0.5 $m^3 s^{-1}$. Summer nutrient concentrations are 187.5 ± 69.1 µg $N\text{-}NH^{4+}$ l^{-1}, 1022.5 ± 103.0 µg $N\text{-}NO^{3-}$ l^{-1} and 56.25 ± 11.7 µg $P\text{-}PO4^{3-}$ l^{-1}, with an average pH of 7.2 (Vlaamse Milieu Maatschappij 2017a). Dominant macrophytes in the studied stream section included *Sparganium emersum* Rehmann (Sparganiaceae), *Callitriche obtusangula* Le Gall (Plantaginaceae) and *Myriophyllum spicatum* L. (Haloragaceae). The macrophyte growing season occurs from March to August, with vegetation remaining present until October.

Sample collection and preparation

On the five sampling events, samples were collected of aquatic macrophytes, epiphyton, suspended fine particulate organic matter (FPOM, grain size < 1 mm), benthic coarse particulate organic matter (CPOM, grain size > 1 mm) and macroinvertebrates.

Aquatic macrophytes included *Sparganium emersum*, which was only present during the June, August and October sampling events, and *Callitriche obtusangula*, which was additionally present in March. Macrophytes were hand-picked from three locations in the stream, in order to obtain sufficient biomass for later FA analyses and to account for small-scale spatial variability, after which they were transported back to the laboratory where any associated macroinvertebrates and attached epiphyton were removed. Epiphyton was removed by manually scraping it off the plants, followed by brief sonication of the macrophytes (e.g. Leff et al. 1994). Cleaned macrophytes were then oven-dried at 70 °C for at least 48 h, after which they were ground with a Retsch ZM200 ultra-centrifugal mill, whereas the collected epiphyton was freeze-dried, using a Heto PowerDry LL3000 (Thermo Scientific), and ground using a Retsch mixer mill (MM301).

Suspended FPOM was collected by taking 20 l samples of stream water from the middle of the water column and filtering this back in the lab over 55 µm Whatmann glass-fibre filters (GF/C) until these filters were clogged. Benthic CPOM was collected by sieving four sediment core samples (diameter 5.4 cm), two from vegetated locations and two from non-vegetated locations, of the upper 5 cm of the sediment over a 1 mm sieve. Both the FPOM filters and the sorted CPOM were then freeze-dried, after which the FPOM filters were weighed and the CPOM was ground with a Retsch mixer mill (MM301).

Because the aim of the study was to measure the importance of macrophyte-derived organic matter in the diet of shredding and filter-feeding macroinvertebrates, we selected the shredder *Gammarus roeseli* Gervais (Amphipoda: Gammaridae), the filter-feeder *Simulium* sp. (Diptera: Simuliidae) and the filter-feeder *Hydropsyche* sp. (Trichoptera: Hydropsychidae (both *H. angustipennis* Curtis and *H. pellucidula* Curtis were observed in the samples)) as study organisms, because these taxa were expected to consume macrophyte-derived organic matter at the end of the growing and were found to be well represented in the studied stream. Macroinvertebrates were collected by kick-

netting a small section of the stream, which included both macrophyte stands as well as bare sediment, using a 0.5 mm mesh kick-net. This was done until enough of the target organisms for later FA analyses were collected, or until it was decided that none of them could be found in the stream on that sampling event (in case of *Hydropsyche* sp. in June 2016). Immediately after collection, these mixed samples were stored in 5 L plastic buckets and transported back to the laboratory were the animals were sorted and identified to the lowest taxonomic level practical. Animals that were removed from the collected macrophytes were also included in the analysis. All target organisms were sorted and stored overnight at 4 °C to enable gut clearance. Afterwards, the animals were frozen, freeze-dried and ground with a Retsch mixer mill (MM301).

Lipid analysis

Whenever sufficient biomass was collected, lipid analyses were performed on duplicate samples of different types of organic matter, macrophytes and macroinvertebrates. Samples were processed following a slightly modified version of Meziane et al. (2007) and Mortillaro et al. (2011), who used a modified method from Bligh and Dyer (1959). First of all, 10 µg tricasanoic acid was added to the sample as internal standard. Lipids were extracted by sonication for 20 min with a H_2O:$CHCl_3$:MeOH (1:1:2, v:v:v) mixture (20 ml). The addition of a 10 ml H_2O:$CHCl_3$ mixture (1:1, v:v) formed a two layer system enhanced by centrifugation (3000 rpm, 5 min). The lower $CHCl_3$ phase containing the lipids was retained, concentrated under a N_2 flow, and the residue saponified under reflux (90 min) with a 2 ml 2 mol NaOH:MeOH (1:2, v:v) mixture. Saponification and methylation were performed using a slightly modified version of Meziane and Tsuchiya (2002) in order to obtain the total lipids as methyl esters. First, 0.25 ml HCl solution (25%) was added, after which 2 × 2 ml $CHCl_3$ were added to recover the lipids. This fraction was dried under N_2 and the fatty acids of the total lipids were converted to methyl esters under reflux with 2 ml of 14% BF3-methanol for 10 min. Total lipids were re-extracted with chloroform (2ml) and washed with 2 ml H_2O. After evaporation under N_2, the extracts were weighed and redissolved in 0.25 ml $CHCl_3$:MeOH (2:1; v:v).

Lipid groups were separated by the Thin-Layer Chromatography (TLC) technique using Merck plates coated with Kieselgel 60 silica (Darmstadt, Germany) and the mobile phase hexane: diethyl ether: acetic acid (70:30:1). Bands containing

fatty acids (as methyl esters; FAMEs) were scraped and dissolved in a mixture of 10 ml HCl_3:MeOH (2:1; v:v) at 40 °C for 60 min. The sample was filtered, dried under N_2 and dissolved in 100µl HCl_3:MeOH (2:1; v:v) for analysis by gas chromatography.

The FAs were separated and quantified with gas chromatography (GC; Hewlett Packard: HP6891 series equipped with flame ionization detector). Separation was performed using a Supelco OMEGAWAX 320 column (30 m × 0.32 mm internal diameter, 0.25 µm film thickness) with H_2 as carrier gas. After injection of 1 µl of sample at 60 °C, the temperature was raised to 150 °C at 40 °C min^{-1}, then to 240 °C at 3 °C min^{-1} and kept 14 min at 240°C. Most FA peaks were identified by comparing their retention times with those of authentic standards (Supelco™ 37, PUFA-1 Marine Source, and Bacterial Mix; Supelco Inc., Bellefonte, PA, USA). FAs are designated as X:YωZ, where X is the number of carbons, Y the number of double bonds and *Z* is the position of the ultimate double bond from the terminal methyl group.

Statistical analyses

For the presentation and the statistical analysis of the FA data, the observed FAs were classified according to their potential role as biomarker. Potential biomarkers that were observed included markers for diatoms (i.e. 14:0, 16:1ω7 and 20:5ω3 (Dunstan et al. 1994, Napolitano et al. 1996)), bacteria (15:0, 17:0 and 18:1ω7 (Kaneda 1991, Desvilettes et al. 1997)), aquatic plants (18:2ω6 and 18:3ω3 (Rozentsvet et al. 2002, Nesterov et al. 2009, Mortillaro et al. 2011)) and terrestrial detritus (C20:0-C28:0 (Napolitano 1999, Mills et al. 2003)). Other FAs that were not identified as biomarkers were classified as ubiquitous. Additionally, the Σω3 FAs : Σω6 FAs was calculated as a marker for the relative amount of autochthonous aquatic (Σω3:Σω6 > 1) and allochthonous terrestrial (Σω3:Σω6 < 1) matter (Pollero et al. 1981, Desvilettes et al. 1994, Torres-Ruiz et al. 2007).

To test whether the measured FAs were normally distributed, both Shapiro-Wilk tests and visual inspection of Q-Q plots were used. Not normally distributed data were tested for significant differences among groups using Kruskal-Wallis tests and Dunn's post hoc tests. Normally distributed data were checked for equality of error variances using Levene's tests. Significant differences among groups were assessed using one-way ANOVAs with Tukey post-hoc tests for equal variances or using Welch tests and Games-Howell post-hoc tests for non-equal

variances. Relationships between FAs and time were defined using Pearson correlation coefficients and tested for significance using two-tailed t-tests. All tests were performed in SPSS version 24.0.

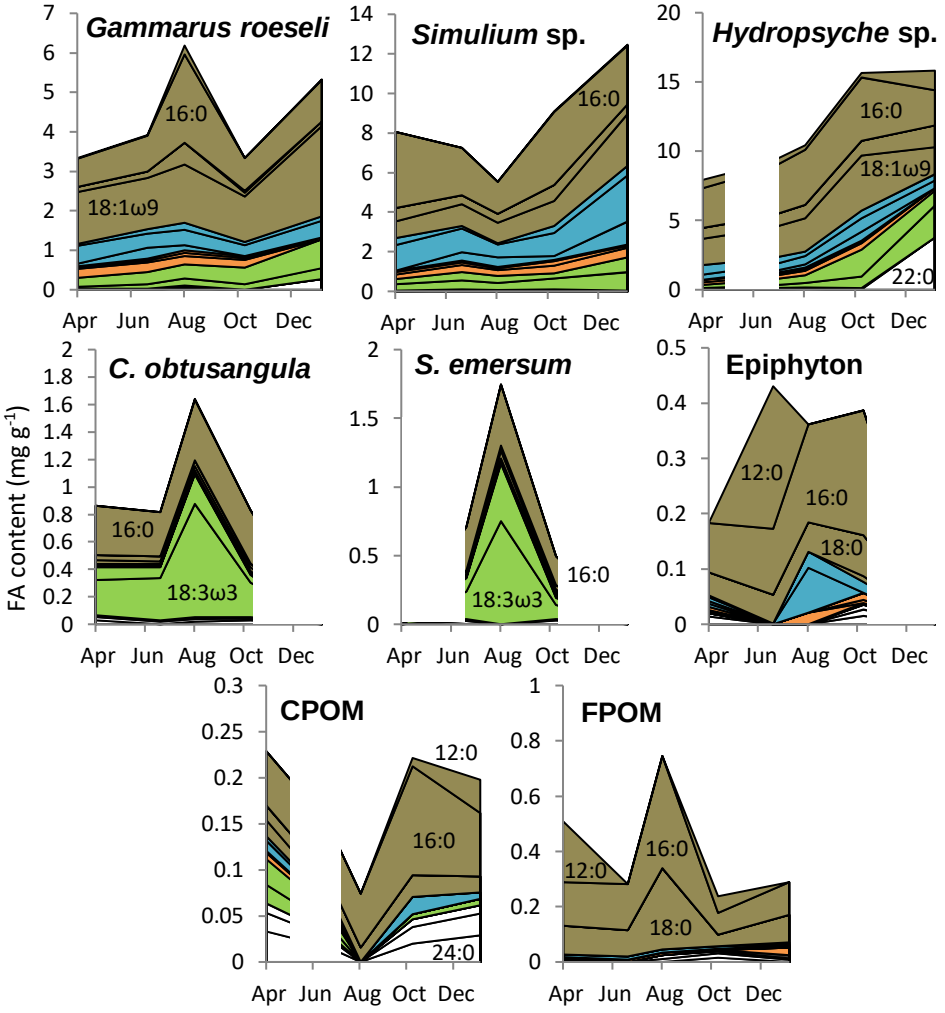
Proportional differences in FA profiles among the different food web components were visualised using nonmetric multidimensional scaling (NMDS) on Bray-Curtis distances in CANOCO for Windows version 5 (Ter Braak and Smilauer 2012). These differences were subsequently tested for significance among the different food web components using one-way analysis of similarity (ANOSIM) (Clarke 1993), whereby the statistic test was computed after 9999 permutations. ANOSIM tests were performed in PAST 3.17 (Hammer et al. 2001).

Results

FA composition of basal resources

A total of 16 FAs were identified in the basal resources FPOM, CPOM, epiphyton and the macrophytes *S. emersum* and *C. obtusangula*, whereby the total amount of FAs differed significantly among the different basal resources, with macrophytes having a significantly higher FA content than CPOM (Figure 4.1, Kruskal-Wallis test; $X^2_{df=4} = 14.4$; $p < 0.01$). Significant differences in the proportions of the different FAs were detected among the different basal resources (ANOSIM; R = 0.35, $p < 0.01$), except between the macrophytes *S. emersum* and *C. obtusangula* (ANOSIM; R = 0.02, $p = 0.31$), between *S. emersum* and CPOM (ANOSIM; R = 0.39, $p = 0.09$) and between epiphyton, CPOM and FPOM (ANOSIM; R = -0.01, $p = 0.48$). These differences are also clearly visible in the NMDS plot (Figure 4.2), which shows a distinct difference between macroinvertebrate consumers, macrophytes, OM and epiphyton. The stress value (Kruskal and Wish 1978) of this plot was 0.06.

For all basal resources, except the macrophytes, ubiquitous FAs constituted the majority of the total FAs in epiphyton (between 64 and 99%) and organic matter (76-94% for FPOM and 40-100% for CPOM), with 16:0 being the most common. Within these three basal resources, the terrestrial marker 24:0 was detected in significantly higher proportions in CPOM than in epiphyton and FPOM (one-way ANOVA; $F_{df=4,15} = 3.6$; $p = 0.03$). For FPOM, the proportion of the aquatic plant marker 18:2ω6 also showed a significant increase over time (r = 0.89, $p = 0.04$). The FA composition of the aquatic macrophytes *S. emersum* and *C. obtusangula* was dominated by the aquatic plant markers 18:2ω6 and 18:3ω3, which respectively



Ubiquitous FA	Diatom markers	Bacterial markers	Vascular plant markers	Allochthonous detritus markers
12:0	14:0	15:0	18:2ω6	20:0
16:0	16:1ω7	17:0	18:3ω3	22:0
17:1ω7	20:5ω3	18:1ω7		24:0
18:0				
18:1ω9				

Figure 4.1 FA content of macroinvertebrate consumers, macrophytes, epiphyton and organic matter measured in the Zwarte Nete over the course of 12 months. Individual FAs are classified in groups as defined in the material and methods section, with most abundant FAs specified in the graphs. Note the differences in scale for the Y-axes.

made up 31-67% and 37-63% of the total FA profile and were significantly higher than observed in epiphyton and organic matter (one-way ANOVA; 18:2ω6: $F_{df=4,15}$ = 11.9; $p < 0.01$, 18:3ω3: $F_{df=4,15}$ = 33.8; $p < 0.01$, Σaquatic plant markers: $F_{df=4,15}$ = 30.4; $p < 0.01$).

FA composition of macroinvertebrate consumers

A total of 16 FAs were identified in the macroinvertebrate consumers *G. roeseli*, *Simulium* sp. and *Hydropsyche* sp., with the total amount of FAs differing significantly among the different consumers, whereby *G. roeseli* had a significantly lower FA content than the other two consumers (Figure 4.1, Welch test; $F_{df=2,5.63}$ = 10.1; $p = 0.01$). All three consumers differed significantly among one another in FA profile (Figure 4.2, ANOSIM; R = 0.58, $p < 0.01$). This difference was primarily caused by the significantly higher content of ubiquitous FAs in *Hydropsyche* sp. compared to the other consumers (one-way ANOVA; $F_{df=2,11}$ = 16.0; $p < 0.01$) and the

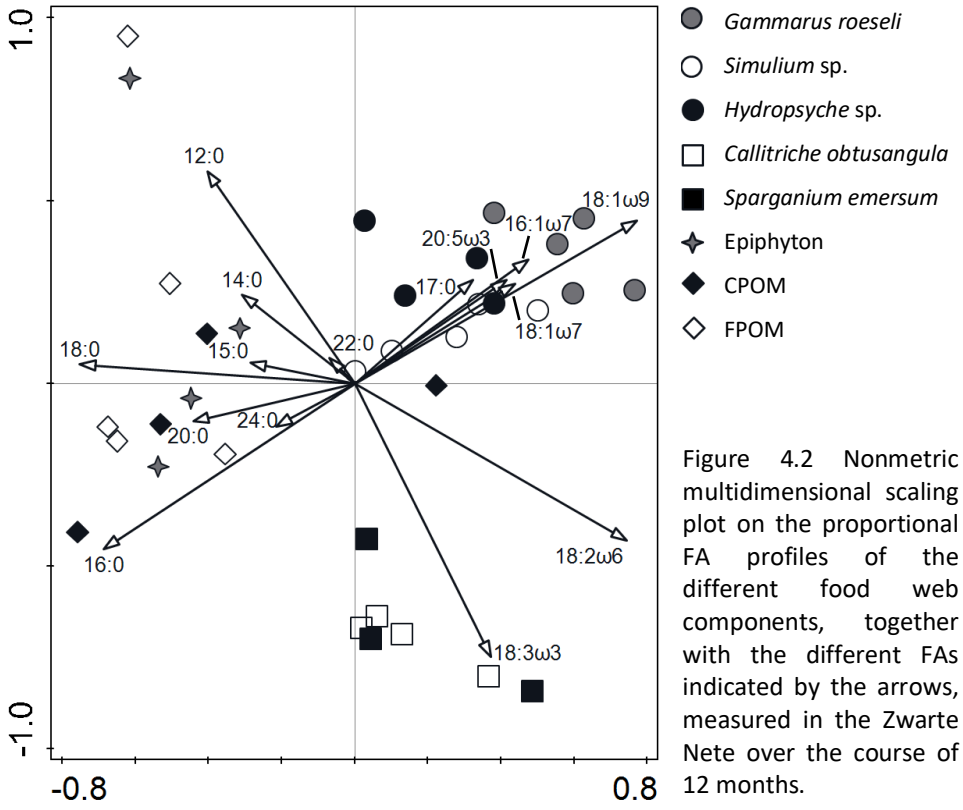


Figure 4.2 Nonmetric multidimensional scaling plot on the proportional FA profiles of the different food web components, together with the different FAs indicated by the arrows, measured in the Zwarte Nete over the course of 12 months.

significantly higher content of diatom and bacterial FAs in *Simulium* sp. compared to *G. roeseli* (one-way ANOVA, diatom markers; $F_{df=2,11}$ = 5.1; p = 0.03, bacterial markers; $F_{df=2,11}$ = 4.9; p = 0.03).

As was observed in the basal resources, ubiquitous FAs constituted the majority of FAs in the macroinvertebrate consumers (between 47 and 78%), whereby 16:0 was again the dominant form (16-47%). Ubiquitous FAs were followed in proportional dominance by diatom marker FAs (between 6 and 32%), of which 16:1ω7 was the dominant form (6-18%). Both the proportion of 16:1ω7 and the total diatom markers were present in significant higher proportions in *Simulium* sp. compared to *G. roeseli* and the other consumers together respectively (one-way ANOVA, 16:1ω7; $F_{df=2,11}$ = 19.0; p < 0.01, Σdiatom markers; $F_{df=2,11}$ = 9.9; p < 0.01). *Simulium* sp. also had a significantly higher Σω3:Σω6 ratio than the other consumers (one-way ANOVA; $F_{df=2,11}$ = 7.5; p = 0.01), which was always higher than 1.0 and showed a significant positive correlation with time (Figure 4.2, r = 0.89, p = 0.04). The Σω3:Σω6 ratio of the other invertebrates was lower and generally below 1.0, with the exception of the second sampling event for *G. roeseli* and the final event for *Hydropsyche* sp. (Figure 4.3).

Although no significant change in the total FA content was observed over time, significant increases in aquatic plant FAs were observed for *G. roeseli* (18:2ω6; r = 0.95, p = 0.01, 18:3ω3; r = 0.89, p = 0.04, Σaquatic plant markers; r = 0.95, p = 0.01), together with significant increases in the ubiquitous FA 12:0 (r = 0.90, p = 0.04), the bacterial marker 18:1ω7 (r = 0.92, p = 0.03) and the aquatic plant marker 18:3ω3 (r = 0.91, p = 0.03) in *Simulium* sp. No significant changes in total FA content were observed over time for *Hydropsyche* sp., although a significant proportional decrease in the bacterial marker 17:0 (r = -0.98, p = 0.02) and a significant proportional increase in total aquatic plant markers (r = 0.97, p = 0.03) was observed in this consumer.

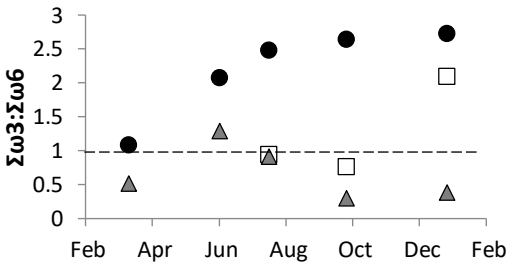


Figure 4.3 Macroinvertebrate consumer FA Σω3:Σω6 ratio for *G. roeseli* (grey triangles), *Hydropsyche* sp. (white squares) and *Simulium* sp. (black circles). Data are presented as means ± SE, although the latter are too small to be visible on the graph. The dashed line represents the threshold indicating terrestrial (<1) and aquatic (>1) diets.

Discussion

The aim of this study was to investigate the consumption of macrophyte-derived CPOM and FPOM by macroinvertebrates during the year and to look for possible dietary shifts in invertebrate populations during this period. Over the course of the year, great variation was observed in the proportion of different FAs for individual macroinvertebrate taxa, as well as in total macroinvertebrate FA content (Figure 4.1).

Consumption of macrophytes by macroinvertebrates

A significant increase in the proportion of vascular plant markers (18:2ω6 and 18:3ω3) over the course of the year was observed in *G. roeseli* and especially in *Hydropsyche* larvae, while 18:3ω3 increased significantly in *Simulium* larvae. The increase of these FAs in the consumers at the end of the growing season coincides with the simultaneous increase of 18:2ω6 in the FPOM, which can be attributable to the expected autumnal breakdown of macrophyte material into smaller particles (e.g. Naiman et al. 2010). As filter-feeding invertebrates that includes FPOM in their diet (e.g. Wallace and Merritt 1980, Monakov 2003), it is likely that *Hydropsyche* and *Simulium* larvae include degraded macrophyte tissue in their diet in this way.

It should however be noted that 18:2ω6 and 18:3ω3 are also dominant in terrestrial vascular plants (Napolitano 1999, Mills et al. 2003), and consequentially in terrestrial leaf litter, so that the increase in these FAs could also be interpreted as an increased consumption of OM derived from terrestrial plants, caused by autumnal leaf-shedding and the associated pulse in allochthonous OM in the stream. This is also in accordance with the sudden higher contribution of 22:0 in *Hydropsyche* larvae during winter (Figure 4.1). Together with other long chain saturated fatty acids (LSFAs; C20:0-C28:0), 22:0 is an indicator of allochthonous leaf litter, commonly being found in the cuticular waxes of higher plants (Napolitano 1999, Mills et al. 2003). However, this alternative hypothesis is not supported by the *Hydropsyche* and *Simulium* larvae Σω3:Σω6 ratio, which was higher than 1 during winter (Figure 4.3), indicating a reliance on autotrophic production instead of allochthonous leaf litter (Desvilettes et al. 1994, Torres-Ruiz et al. 2007). It seems likely that the shredder *G. roeseli* depends for a greater portion of its diet on allochthonous production in autumn and winter though, as indicated by its low Σω3:Σω6 ratio and other literature data (Gessner et al. 1999, Graça 2001).

Regarding the OM profile, it is furthermore interesting to note the great similarities among CPOM, FPOM and epiphyton (Figure 4.1 & 4.2). This is surprising, as epiphyton is generally considered to be an important food source for herbivorous macroinvertebrates and to have a higher FA, especially PUFA, content than (terrestrially derived) organic matter (Torres-Ruiz et al. 2007, Lau et al. 2009, Descroix et al. 2010, Guo et al. 2016, Crenier et al. 2017). A possible explanation for this deviation from literature could be a very low algal and bacterial density, with a high FA content, in which way the biofilm is dominated by intercepted FPOM.

Macroinvertebrate total FA content

Differences in total macroinvertebrate FA content are probably caused by species-specific differences in dietary habits and physiology. Larvae of aquatic insects are generally richer in total FAs and PUFAs than non-insects, such as *G. roeseli*, because of their more intensive accumulation of lipids during their juvenile life stages (Bychek and Gushchina 1999, Cripps et al. 1999). Besides differences in average total consumers FA content, there are also clear differences in the temporal evolution of this FA content, which are likely caused by differences in life-histories of the different species, such as the number of yearly generations and the timing of metamorphosis. In accordance with literature, *G. roeseli* has, as the only studied consumer that inhabits the stream as an adult, the highest FA content during the species' reproductive peak in late spring/early summer, due to the development of eggs in females (Arts et al. 1995, Pöckl et al. 2003). On the other hand, the population of *Simulium* larvae in the Zwarte Nete consists of several generations per year, with multiple fast developing summer generations and one slow developing overwintering generation (Smart 1934, Lock and Van Maanen 2014). These summer generations were observed to have a lower FA content than the winter generation, which might be caused by an inherent lower FA content of the summer generations but also by the large size-range of this generation and the sample's consequential larger proportion of earlier instar larvae, with a lower FA content than later instars (e.g. Arts and Wainman 1999). Similarly, the population of *Hydropsyche* larvae in the Zwarte Nete consists of two generations per year, with one fast summer generation and one slower overwintering generation (Higler 2008). Unfortunately, insufficient biomass was collected for the summer generation on the only moment this generation was dominantly present in June, resulting

in FA content data of the slow growing winter generation only. It is hereby clearly visible that the FA content of the *Hydropsyche* larvae increases over the year, which can be explained by the higher FA content of later instar larvae (Arts and Wainman 1999). Another pattern, observable in all macroinvertebrate consumers, is the increased FA content at the end of the year and the lower FA content at the start of the year (Figure 4.1). It might be expected that this decrease in FA content is caused by the depletion of fat reserves during winter (Downer and Matthews 1976).

Conclusions

This study observed significant differences in FA composition among the different basal resources and macroinvertebrate consumers in a lowland stream in addition to a significant temporal variation in FA composition of these different food web components. In the basal resources, this was expressed by the dominance of 18:2ω6 and 18:3ω3 in the macrophytes, by the increase of these two marker FAs in the transported FPOM in autumn and by the strong autumnal increase of C20:0-C28:0 FAs, characteristic of terrestrial detritus, in the CPOM. The latter two shifts in FA profile are hereby expected to be indicative for the autumnal breakdown of macrophyte material in the stream and the increase of terrestrial leaf litter through leaf fall respectively. In the macroinvertebrate consumers, these seasonal changes are also reflected as a significant increase in vascular plant FA markers in *G. roeseli* and *Hydropsyche* larvae at the end of the growing season, together with the appearance of LSFAs in these same organisms. Based on the consumer Σω3:Σω6 ratio, it is expected that *G. roeseli* mainly consumes allochthonous leaf litter in autumn, but that the diet of *Hydropsyche* larvae also includes macrophyte-derived organic matter. This might be further confirmed by future studies that focus on the temporal evolution of the ^{13}C and ^{15}N stable isotope signatures of basal resources and consumers, to further distinguish aquatic and terrestrial carbon sources.

Acknowledgements

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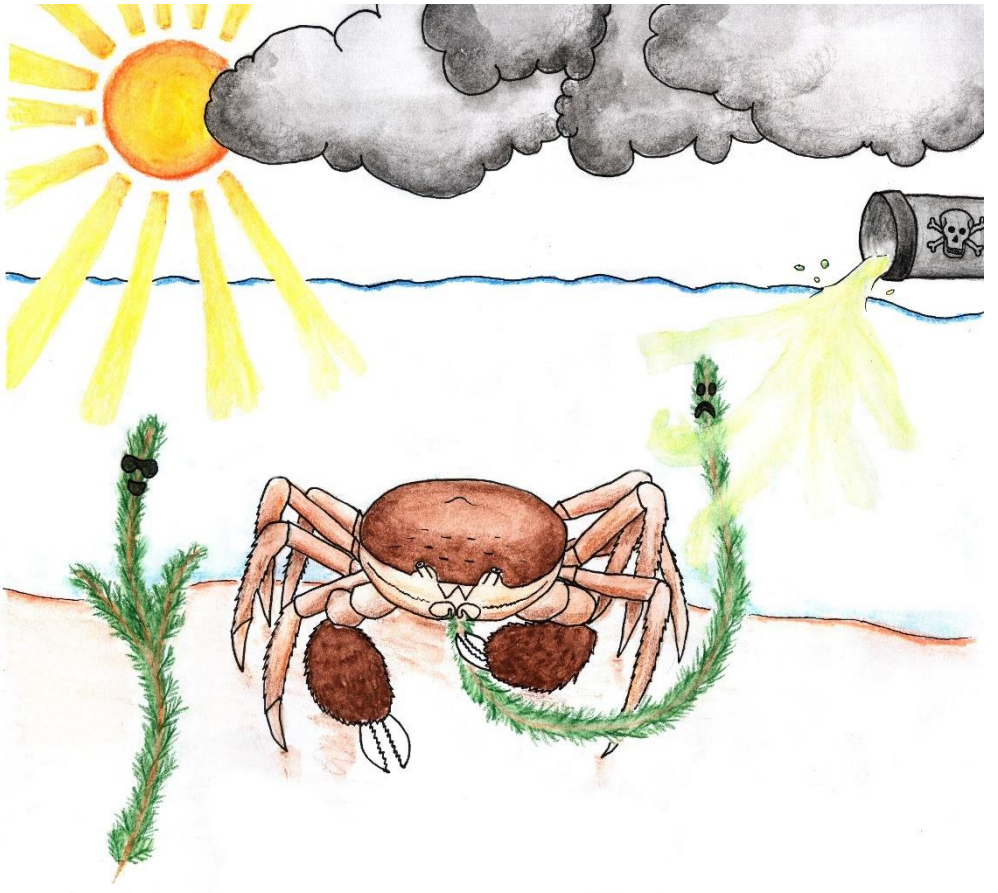

Chapter 5.

Experimental evidence for the decline of submerged vegetation in freshwater ecosystems by the invasive Chinese mitten crab (Eriocheir sinensis)

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Abstract

The Chinese mitten crab (*Eriocheir sinensis*) is a damaging invader which is designated as a species of Union Concern within the European Union. A negative impact of the crabs on macrophyte vegetation in lowland streams is suspected but not yet proven or quantified. We have performed a mesocosm study that combines a density gradient of Chinese mitten crabs (0, 0.3, 1.0 and 2.5 ind. m^{-2}) with chemical stress (2350 µg EDTA l^{-1} + 258 µg glyphosate l^{-1}) or light limitation stress (-70% irradiance compared to control) on water plants (*Myriophyllum spicatum*). The results clearly demonstrate that the crabs are capable of removing plant shoots effectively which can lead to a complete elimination of the vegetation. Generally, the higher the crab density, the sooner the plants started to disappear and the sooner the vegetation was completely removed. Additional light and chemical stress accelerated this process, resulting in more rapid plant disappearance. The additional stressors also led to plant disappearance at a crab density of 0.3 ind. m^{-2} compared to 1.0 ind. m^{-2} in the control treatments. Video recording, plant strength and crab pinch strength measurements and stable isotope signatures of $\delta^{13}C$ and $\delta^{15}N$ in the Chinese mitten crabs and their possible food sources showed that directly eating the plants is causing only minor damage to the plants. Most damage comes from the movement of the crabs and crab-crab interactions during which they use their chelae to grasp the shoots.

Keywords: *Myriophyllum spicatum*, mesocosm experiment, invasive species, glyphosate, EDTA, light reduction, plant stress

Introduction

Invasive alien species (IAS) are major drivers for biodiversity loss. The economic costs of these invasions, in Europe alone, are estimated to be 12 billion euro each year, with a strong likeliness to increase in the coming years (European Environmental Agency (EEA) 2012). Not surprisingly, applied ecological research on IAS populations is mentioned as one of the most urgent current nature conservation issues by the EEA. Member states are obliged to take measures to eradicate these species. The Chinese mitten crab (*Eriocheir sinensis* H. Milne-Edwards (Decapoda: Varunidae)) is one of the 49 species listed in EU regulations (European Union 2014). Since the first observation of the crab in the German River Aller in 1912, this IAS has spread rapidly throughout many parts of Europe.

In vegetated lowland streams and rivers, aquatic plants fulfil an important role in the aquatic ecosystem (e.g. O'Hare et al. 2018). They play a major role in the evaluation criteria of the European Water Framework Directive (WFD) and/or nature conservation programs (e.g. Natura 2000 goals). Since the summer of 2013, a nearly total absence of macrophytes is observed in a large part of the Grote Nete stream in Belgium, where previously plant growth was abundant. Chinese mitten crabs were observed in the Nete catchment, but not exclusively in the section where the macrophytes were lost (Vlaamse Milieu Maatschappij 2015, 2017b). These studies however, do not mention crab densities, which may have varied in different parts of the catchment. Chinese mitten crabs are omnivorous and opportunistic and feed on almost any organic food source they can find, including living macrophytes (Jin et al. 2001, 2003, Mao et al. 2016). Crayfish in general can threaten the development of submerged macrophytes (Van der Wal et al. 2013). This may pose a threat to the resident flora and fauna and may lead to loss of biodiversity (Rogers 2000, Wójcik et al. 2015). Burrowing activities also often make stream beds and banks more susceptible to erosion which can induce resuspension of sediment, increase in turbidity and decrease in light availability (Jin et al. 2003, Bouma and Soes 2010, Wang et al. 2017). These effects increase the vulnerability of plants to uprooting, which may subsequently influence the release of nutrients and pollutants from the sediment (Wang et al. 2017). It has also been shown that macrophytes can be more susceptible to herbivory when they are already under stress (Hidding et al. 2016, Wang et al. 2017). The combination of increased environmental stress and an increased crab population in lowland streams may

thus pose a threat to aquatic vegetation (which could be the case in the aforementioned section of the Grote Nete stream).

Increased concentrations of ethylenediaminetetraacetic acid (EDTA) and glyphosate were also often found throughout the Nete catchment, especially near the effluent of waste water treatment plants (Vlaamse Milieu Maatschappij 2015, 2017b). EDTA is a chelating agent that forms very stable complexes with essential metal ions (e.g. Fe) and major cations such as Ca^{2+} and Mg^{2+}, which can result in limited uptake and ensuing deficiency of these elements in macrophytes (Hangarter and Stasinopoulos 1991). Glyphosate (N-(phosphonomethyl)glycine) is the active compound of various commercially available herbicides. It inhibits an enzyme in plants (5-Enylpyruvylshikimate-3-phosphate synthase, EPSPS) which is a key step in making aromatic amino acids, thereby preventing the synthesis of metabolites, including flavonoids, lignins and other phenolic compounds (Dill 2005). Insufficient phytotoxic evidence, however, was found to point to the right cause of macrophyte loss in the Grote Nete stream (Vlaamse Milieu Maatschappij 2015).

The objective of this study is to investigate whether the activities of invasive Chinese mitten crabs (herbivory and cutting) can cause a decline of native mature aquatic vegetation. To achieve this objective, a mesocosm experiment was conducted in which the decline of vegetation patches was followed under different crab densities, and in combination with different types of abiotic stress factors on the vegetation. The abiotic stress factors (chemical stress and light limitation stress) were inspired by the case study described above (Grote Nete stream), and the following hypotheses are put forward: (i) beyond a certain crab density threshold, macrophyte shoots are negatively affected by crab activity such as consuming or cutting plants; (ii) this can result in a decimation of the entire vegetation patch and a hampered regrowth from its root system; and (iii) abiotic stressors on plants (e.g. chemical and light limitation stress) can influence the level of damage caused by crab activity.

Material and methods

Experimental setup

The experiment took place in 12 circular mesocosm tanks (2 m diameter). These tanks (ponds) are located in a greenhouse of the Mesodrome research facility at the University of Antwerp. The greenhouse is a semi-controlled environment in a sense that daylight (length and intensity) and temperature are natural, but other influences such as precipitation, wind etc. are controlled. The experiment was executed between April and June 2017, which is during the growth period of the vegetation and during the anadromous migration of the juvenile crabs used in the experiment.

A layer of 5 cm coarse (0-2 mm) commercially bought river sand was added to each tank. Tap water was added to create a water column depth of 0.5 m (sand bulk density = 1.97 g DM cm^3; sand:water volume ratio = 0.1). Water quality parameters P-PO_4^{3-}, TDIN, pH, electrical conductivity (EC) and dissolved oxygen were measured at the beginning of the experiment and subsequently monitored once per month (Table 5.1). A colorimetric segmented flow analyser was used for nutrient analysis (SAN++, Skalar, Breda, The Netherlands) and a WTW Multi 3430 SET F multimeter (Weilheim, Germany) for pH, EC and O_2 measurements. Water temperature was logged continuously with an iButton Hygrochron Temperature/Humidity Data Logger (Maxim Integrated Products, Sunnyvale, CA, USA). Light irradiance above the water surface was logged continuously with Hobo data loggers (Onset, S-LIA-M003, PAR Sensor). In the middle of each tank, a plastic tray was placed (33×41×10 cm). This tray was filled with the same sand as in the rest of the tank. The top was sealed with a mesh wire with a mesh size of 25 mm so that crabs could not fully dig into the sand to avoid uprooting, though their legs and chelae could still access the root system to a certain extent. In each tray 150 shoots of commercially bought *Myriophyllum spicatum* L. (Haloragaceae) were planted, with a combined weight of 90.0 ± 15.9 g fresh weight (FW) (7.5 ± 1.3 g dry weight - DW). Furthermore, ceramic flower pots were added as a shelter for the crabs (one pot for each crab to avoid competition), and an air stone was installed to aerate the tanks. Finally, leaf litter consisting of 5 common tree species, *Quercus robur* L. (Fagaceae), *Q*. *rubra* L., *Fagus sylvatica* L. (Fagaceae), *Castanea sativa* Mill (Fagaceae) and *Populus×canadensis* Moench (Salicaceae), was added to provide an

Table 5.1 Abiotic conditions of the tanks per month and per treatment (chemical stress, light limitation stress and control). Data are mean values ± SE (n = 4) for $P\text{-}PO_4^{3-}$, total dissolved nitrogen (TDIN), pH, electrical conductivity (EC) and O_2. Temperature was logged continuously and averaged for the whole month period (± standard deviation). Irradiance was logged continuously and the median of all day-time values was calculated for the whole month period.

	$P\text{-}PO_4^{3-}$	TDIN	pH	EC	O_2	O_2	Temp	Irradiance
Control	**(mg P l^{-1})**	**(mg N l^{-1})**	**(-)**	**(µS cm^{-1})**	**(%)**	**(mg l^{-1})**	**(°C)**	**(W m^{-2})**
Start	<0.02	3.7 ± 0.0	8.2 ± 0.0	607 ± 0	105 ± 0	10.5 ± 0.1	-	-
April	0.02 ± 0.00	3.7 ± 0.0	8.6 ± 0.0	-	106 ± 1	10.3 ± 0.0	15.0 ± 0.5	163
May	<0.02	2.6 ± 0.1	8.9 ± 0.1	712 ± 13	126 ± 3	10.4 ± 0.2	23.3 ± 0.5	157
June	<0.02	0.2 ± 0.0	9.0 ± 0.1	664 ± 19	-	-	22.4 ± 0.5	230
Chemical								
Start	<0.02	3.7 ± 0.0	8.2 ± 0.0	607 ± 0	105 ± 0	10.5 ± 0.1	-	-
April	0.02 ± 0.00	3.7 ± 0.0	8.6 ± 0.0	-	106 ± 0	10.3 ± 0.0	15.0 ± 0.5	163
May	<0.02	2.1 ± 0.1	9.0 ± 0.0	712 ± 14	131 ± 2	10.8 ± 0.2	22.8 ± 0.5	157
June	<0.02	0.0 ± 0.0	8.3 ±0.1	714 ± 12	-	-	21.6 ± 0.5	230
Light								
Start	<0.02	3.7 ± 0.0	8.2 ± 0.0	607 ± 0	105 ± 0	10.5 ± 0.1	-	-
April	0.02 ± 0.00	3.7 ± 0.0	8.6 ± 0.0	-	108 ± 0	10.4 ± 0.1	15.5 ± 0.5	164
May	<0.02	2.7 ± 0.1	8.6 ± 0.0	752 ± 20	114 ± 0	9.4 ± 0.1	21.9 ± 0.5	56
June	<0.02	1.2 ± 0.1	8.4 ± 0.1	720 ± 19	-	-	20.9 ± 0.5	59

alternative food source for the crabs. Tree leaves were oven-dried for 72h at 70 °C and then preconditioned for 1 week in pond water. The leaves were divided over 12 plastic trays (18 × 33 × 10 cm) and brought into the 12 tanks (650 g FW (163 g DW) per mesocosm). The trays were covered with mesh (mesh size 25 mm) to keep the leaves in the trays and avoid floatation. Two holes of 5x5 cm allowed the crabs to easily access the trays. Plants were allowed to acclimatise, root and grow under optimal conditions for 3 weeks before chemical and light limitation stress were imposed (Figure 5.1).

Chemical stress was introduced by adding EDTA and glyphosate together to the first four tanks. EDTA was added as $Na_2H_2EDTA.2H_2O$ at a concentration of 2350 ± 58 µg l^{-1} measured five weeks after introduction (not filtered; Gas Chromatography, ISO standard 16588). Glyphosate was added as Roundup® which had a glyphosate concentration of 258 ± 27 µg l^{-1} measured five weeks after introduction (not filtered; Gas Chromatography, ISO standard 16588). Note that the added concentration of both chemicals at the beginning was higher (5000 µg EDTA l^{-1} and 500 µg glyphosate l^{-1}) since a certain fraction will degrade or can adsorb to surfaces (plastic of the containers, sand grains, organic matter etc.) and become inactive. The resulting measured concentrations are high, but are in the same order of magnitude of values found in the Grote Nete catchment, i.e. 2150 µg EDTA l^{-1} found in effluent of the wastewater treatment plant discharging into the Grote Nete stream and up to 140 µg glyphosate l^{-1} found in waterways in Flanders (Vlaamse Milieu Maatschappij 2015).

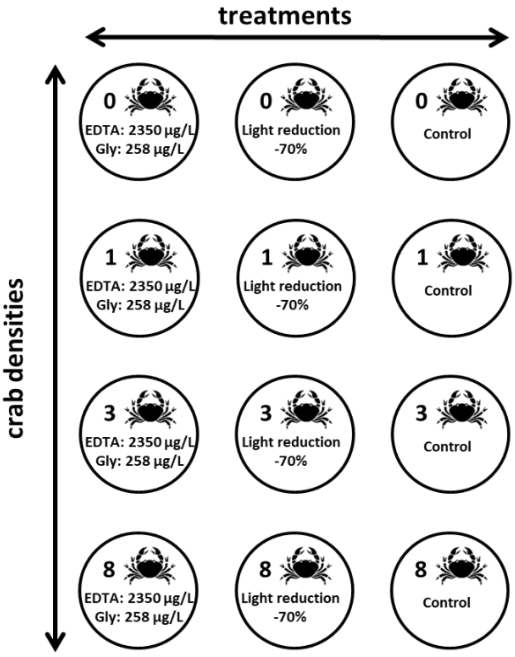

Figure 5.1 Experimental setup. Twelve mesocosm tanks were installed combining 4 crab densities with 3 treatments.

Light limitation stress was imposed as a ~70% light reduction

to a second series of four tanks by covering the tanks with layers of a white, plastic shade cloth. The imposed light reduction was based on experiments by Barko and Smart (1981) and Zefferman (2014) in which the effect of different shading intensities on *M. spicatum* were tested. They demonstrated that a 70% light reduction resulted in similar shoot numbers and shoot lengths as in the control treatment (Barko and Smart 1981), and a limited reduced biomass and relative growth rate compared to the full light level (Zefferman 2014). Therefore, a 70% reduction can be considered to be stressful for the plants, without impeding their growth. A small test prior to the experiment in which a comparable range of light reduction was used, showed similar results as the Barko and Smart (1981) and Zefferman (2014) experiments (unpublished data).

The remaining four tanks are the control treatments. One week after the addition of the light limitation and chemical stress, most of the shoots had reached the water surface and started to produce flowers which indicated they had become mature plants. At this moment, crabs were added to the tanks (Figure 5.1). Crabs were preselected based on their condition (visual appearance of good health based on mobility, colour, and presence of all limbs) and similarity in size and weight (21.9 ± 2.1 g FW). No gender selection was made. For each stress treatment and control, 1 tank received no crabs (reference within each treatment), 1 tank received 1 crab, 1 tank received 3 crabs and 1 tank received 8 crabs. This corresponds to crab densities of 0 ind. m^{-2}, 0.3 ind. m^{-2}, 1.0 ind. m^{-2} and 2.5 ind. m^{-2}, respectively. Because related omnivorous sesarmid (Sesarmidae) crabs are known to supplement their diet with animal tissue such as fish carcasses, to provide a major part of their dietary nitrogen requirements, crabs were offered fish meat weekly (± 1 g anchovies per crab) to ensure a balanced diet and to avoid cannibalism (Thongtham and Kristensen 2005, Kristensen et al. 2010). Each two to five days, plant shoots that were cut by the crabs and floated in the tanks were retrieved, oven-dried at 70°C for at least 48 hours and weighed. The experiment lasted for 25 days because at this time most of the vegetation in the stressed treatments with the highest crab density was gone.

After the experiment, the crabs were retrieved and weighed again, then stored frozen at -20 °C until further analysis. The remaining plant biomass in the trays was cut by hand just above the mesh wire, oven-dried at 70 °C for at least 48 hours and weighed. The sum of the remaining plant biomass and the floating plant

biomass collected during the experiment gives the total plant biomass. All 12 trays were then together transferred to a tank with fresh tap water and optimal conditions. The water quality was the same as at the start of the previous experiment (Table 5.1) and was not monitored further. Regrowth of the shoots was visually evaluated weekly during 7 weeks (short term period) after which the biomass was determined again by cutting all shoots just above the mesh wire, drying and weighing. The presence of flowers was noted. The trays were then placed back into the tank for a new, long term period of regrowth. After 1 year, all biomass was determined again by cutting all shoots just above the mesh wire, drying and weighing. The presence of flowers was again noted.

Plant nutrient concentrations

To compare the nutrient condition of the plants between the treatments, total phosphorus content of plants from all 12 tanks was determined near the end of the experiment according to Walinga et al. (1989): samples were digested with H_2SO_4, salicylic acid and H_2O_2 and analysed on a colorimetric segmented flow analyzer (SAN++, Skalar, Breda, The Netherlands). Total carbon and nitrogen content of plants from all 12 tanks was determined during the procedure for stable isotope analysis (see below).

Crab pinch test

To compare the force of the crabs with the resisting strength of the plants, crab pinch strength of 40 individuals of each sex and of different body mass was measured using a Charge Amplifier (Kistler Instrumente AG, Type 5995, Winterthur, Switserland). The crabs were preselected based on their condition (visual appearance of good health). Five pinches were measured for each crab, using 1 chela at the time, and randomly alternating between chelae. Maximum pinch strength (out of 5) and average pinch strength (n = 5) were then plotted against crab biomass (g FW).

Plant shear test

Shearing tests were used to measure the stem's resistance to fracture. For each treatment, the test was undertaken on 5 stems. Tests were conducted with a leaf-cutting device following Ang et al. (2008), mounted on a universal testing machine

(Instron 5942, Canton, MA, USA). A single stainless-steel blade of a straight razor (Dovo, Solinge, Germany) was mounted on the moving head of the testing machine with an approach angle of 20°. The stem was positioned on 2 supports (with a 15 mm span), with the blade being equidistant from the 2 supports. The blade was moved downward at a constant speed of 10 mm s^{-1} shearing the stem into 2 parts. The maximum load applied to the leaf (N) was recorded with a frequency of 10 Hz and used to calculate the maximum force to shear the stem (N) and the shear strength (maximum force divided by the cross-sectional area, MPa). To take into account the high proportion of lacunae in stems of aquatic plants, the cross-sectional area used to calculate shear strength was the effective cross sectional area, calculated as the difference between total cross-sectional area and total lacuna area. This correction is used to quantify the effective cross-sectional area supporting forces in the shearing tests. To measure cross-sectional area of the stem sheared, thin cuts adjacent to the shearing plane were made. Images of the cuts were taken using a binocular and a digital camera and analysed with Leica Application Suite (v4.3, Leica Microsystems, Switzerland) to calculate total stem cross-sectional area (mm^2) and total lacuna area (mm^2).

Stable isotope analysis

To calculate the relative importance of the food sources, stable isotope signatures ($\delta^{13}C$ and $\delta^{15}N$) were measured for all different 'food web' components in the mesocosms; the crabs as consumers and *M. spicatum*, leaf litter and supplementary fish as food sources. Crabs were dissected to collect their gill tissue, which was subsequently freeze-dried using a Heto PowerDry LL3000 (Thermo Scientific) and ground using a Retsch mixer mill (MM301). Only gill tissue was extracted for isotope analysis, due to its low turnover rate compared to other tissues (Lorrain et al. 2002). Dried *M. spicatum* shoots and leaf litter were ground with a Retsch ZM200 ultra-centrifugal mill.

Powdered samples were weighed in silver cups and acidified with one drop 5% hydrochloric acid, to remove any carbonates (Jacob et al. 2005), and oven-dried at 80°C for 4 hours after which the cups were folded and analysed. Sample weight were 5 mg for leaf litter and macrophytes, and 1 mg for crab tissue. Sample carbon and nitrogen content were measured using a Flash EA 1112 Elemental Analyzer (Thermo Finnigan). The ^{13}C and ^{15}N stable isotope signatures were measured using

a Delta V Advantage isotope ratio mass spectrometer (Thermo Finnigan) that was coupled, via a ConFlo III interface (Thermo Finnigan), to the Elemental Analyzer.

Video recording

Two crabs similar to ones used in the experiment, and a patch of 15 mature *M. spicatum* shoots were placed in a 100 l aquarium. Crab movements were observed with a camera (Sony CX550) during 3 consecutive 12 hours day and night cycles (using the infrared mode of the camera). The aim was to record the interaction of the crabs with the plants in a qualitative way to observe whether crabs climb into the patch, if they use there chelae to grasp the shoots and whether they cut the shoots directly.

Statistical analyses

First, the impacts of the different treatments and crab densities on plant biomass is compared. An empirical model for the fraction of biomass remaining (FBR) as a function of time (*t*) is proposed:

$$Fraction\ of\ Biomass\ Remaining = \frac{1}{1+e^{-\delta[\tau-t]}} \qquad (1)$$

The FBR-model (Equation 1) computes the fraction of biomass remaining after a time *t* of running the experiment. At time *t* = 0 s, the FBR equals one. When the experiment duration t approaches τ [units s], the FBR starts to decay at an exponential decay rate δ [units s^{-1}]. For t significantly larger than τ the FBR converges to zero. The larger is τ, the longer it takes for the system to start declining. Every dataset for FBR as a function of time from each mesocosm tank will have its own set of parameters τ and δ. The parameters are obtained by fitting the dataset to the model. By doing so the different stress impacts and crab densities can be compared using the corresponding parameters τ and δ. More detailed information, and every fit output is given in the supplementary material. These tests were performed in R 3.3.2 (R Development Core Team 2016).

Secondly, differences in crab fresh weight before and after the experiment and between stress treatments were tested. Mesocosm tanks with different crab densities but the same stress treatment were used as 4 replicas. Data were first checked for normality via Shapiro-Wilk tests and visual inspection of Q-Q plots. Not

normally distributed data were tested for significant differences among groups using Kruskal-Wallis tests and Dunn's post hoc tests. Normally distributed data were checked for equality of error variances using Levene's tests. Significant differences among groups were assessed using one-way ANOVAs with Tukey post-hoc tests for equal variances or Welch tests and Games-Howell post-hoc tests for non-equal variances. Interaction could not be tested because of the experimental design, though this is not expected to happen and if it occurs it is included in the residual variation of the applied test. Additionally, differences in crab pinch strength between sexes were tested following the same statistical procedure as described above. Relationships between crab pinch strength and crab biomass were defined using Pearson correlation coefficients and tested for significance using two-tailed t-tests. All tests were performed in SPSS version 24.0.

Thirdly, mesocosm tanks with different stress treatments but with the same crab density were used as 3 replicates to test the effect of crab density on plant biomass. Mesocosm tanks with different crab densities but the same stress treatment were used as 4 replicas to test for differences in plant nutrient concentrations, plant shear force, and total plant biomass between different stress treatments. The same statistical procedure was used as described above for testing differences in crab characteristics.

Finally, stable isotope data were used to calculate the relative importance of the food sources in the crab's diet. The stable isotope mixing model 'Stable Isotope Analysis in R' (SIAR, Parnell and Jackson 2013) was used in R 3.3.2 (R Development Core Team 2016). This Bayesian mixing model incorporates variation in the stable isotope ($\delta^{13}C$ and $\delta^{15}N$) signatures of the different food sources and the consumer and subsequently calculates density plots of credible intervals for the estimated dietary proportion of each food source (Parnell et al. 2010, Parnell and Jackson 2013). Furthermore, this mixing model allows the incorporation of food source C and N content, thereby enabling better resolution when analysing food sources with vastly different C and N concentrations (Phillips and Koch 2002), e.g. for omnivores that may consume both nitrogen-poor detritus and nitrogen-rich animal material. Before incorporation in the model, the food source carbon and nitrogen stable isotope signatures were corrected for trophic fractionation by adding 0.8‰ and 2.6‰ respectively, for animals of which only muscle tissue was analysed (McCutchan et al. 2003).

Results

The results clearly demonstrate that the crabs were able to remove plant shoots effectively, leading to a complete elimination of the plant patch (Figure 5.2). If no crabs were present (reference tanks), no shoots were cut. When crabs were present, many shoots were cut and the amount and timing was influenced by crab density and additional stress treatment. The fraction of biomass remaining as a function of time follows a sigmoid pattern (Figure S5.1-S5.3). For all treatments (light limitation and chemical stress, and the control treatment), the threshold value (τ) exponentially decreased as a function of crab density with an exponential coefficient α_{light} and $\alpha_{chemical}$ and $\alpha_{control}$ respectively (Figure S5.5, S5.7, S5.9). Essentially, a higher crab density resulted in an earlier decline of the system: e.g., with 0.3 ind. m^{-2} in the control treatment (Figure 5.2a), most biomass was still present by the end of the experiment (reduction of only 5%), whereas an increasing crab density in the control treatment led to a removal of all biomass by the end of the experiment (reduction of 100%), which occurred earlier at the highest crab density (at day 25 for 1.0 ind. m^{-2} and at day 12 for 2.5 ind. m^{-2}). No significant correlation between the rate of collapse (δ) and crab density was observed once the threshold was reached.

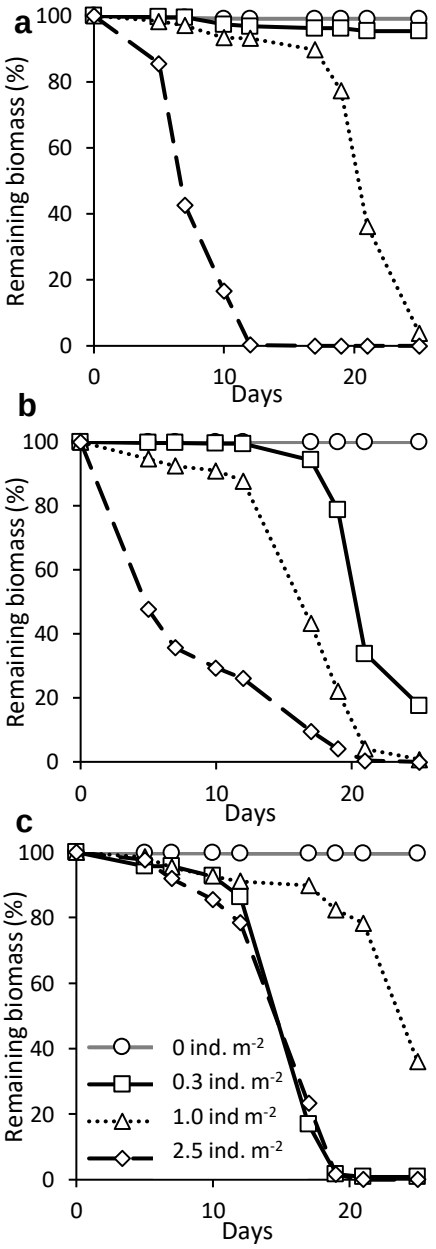

Figure 5.2 Reduction of plant biomass in the control (a), chemical stress (b) and light reduction stress (c) ponds with different crab densities.

Application of a nonlinear least-squares data fit showed that the exponential decay rate of (τ) as function of crab density is larger for the chemical or light treatment. The decay rates are [1.70 ± 0.55], [2.30 ± 1.29] and [0.82 ± 0.11] respectively. No significant difference was observed between the impact of light and chemical stress on macrophyte biomass development.

Crab characteristics

Only 3 crabs were found dead in the first few days of the experiment and were immediately replaced by a similar individual. Crab fresh weight at the end of the experiment had increased significantly from 21.9 ± 6.5 g to 33.6 ± 10.7 g FW (Kruskal-Wallis test; $X^2_{df=1} = 29.2$; $p < 0.001$). Male crabs had a significantly higher average and maximum pinch strength than females, proportional to their body mass (Welch test, maximum strength; $F_{df=1,59} = 19.5$; $p < 0.001$, Average strength;

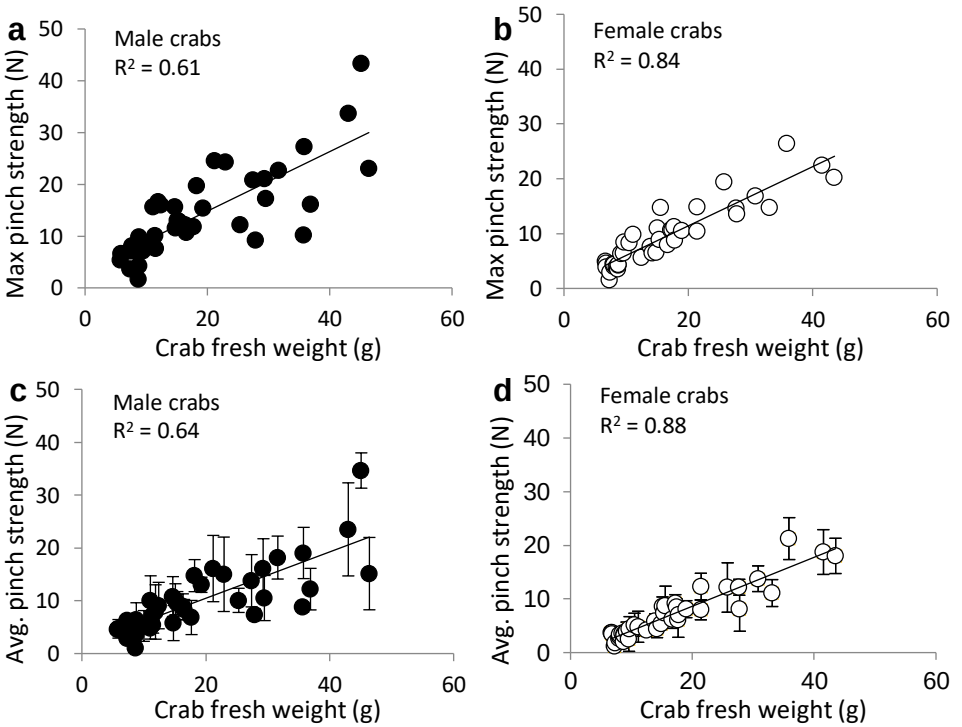

Figure 5.3 Relationship between maximum (a&b) and average (c&d) crab pinch strength and crab fresh weight (g). Black circles represent male crabs (a&c), while white circles represent female crabs (b&d). The error bars for the average pinch strength represent the standard deviation. All relationships were significant at the $p < 0.01$ level.

$F_{df=1,61}$ = 18.6; p < 0.001). Additionally, both pinch strengths showed a significant positive relationship with body mass for both male and female crabs (Figure 5.3).

Plant characteristics

Visually, the plants appeared healthy at the end of the initial growth period. They filled the entire water column, produced flowers and had no signs of necrosis or other symptoms of aberrant growth. Physiologically, plants grown under light limitation stress had significantly higher concentrations of N (29.8 ± 2.4 mg N g DW^{-1} (mean ± SE), one-way ANOVA; $F_{df=2,9}$ = 21.8; p < 0.01) and P (2.4 ± 0.4 mg P g DW^{-1}, one-way ANOVA; $F_{df=2,9}$ = 15.2; p = 0.01) than the plants from the control (15.7 ± 3.5 mg N g DW^{-1} and 1.1 ± 0.1 mg P g DW^{-1}) and chemical treatment (17.4 ± 5.2 mg N g DW^{-1} and 1.4 ± 0.3 mg P g DW^{-1}). Because carbon content did not vary significantly among the treatments, the C:N and C:P ratios of plants grown under light limitation stress (C:N = 12.7 ± 0.2, C:P = 136.2 ± 9.3) were consequently lower than in the control (C:N = 19.3 ± 1.5, C:P = 287.5 ± 27.3) and chemical treatment (C:N = 16.6 ± 2.2, C:P = 210.2 ± 17.0), although these differences were not significant. Plants grown under light limitation stress also had a significantly lower shear strength (0.17 ± 0.03 MPa) than the ones from the chemical treatment (0.26 ± 0.02 MPa, one-way ANOVA; $F_{df=2,12}$ = 4.3; p = 0.039). The maximum force to shear the stems of the control plants (0.66 ± 0.14 N) was significantly higher than from plants from the light treatment (0.40 ± 0.1 N, Kruskal-Wallis test; $X^2_{df=2}$ = 5.9; p = 0.049).

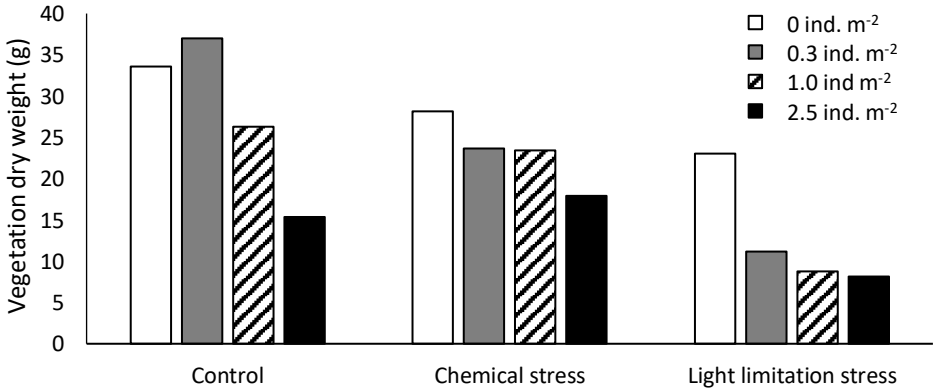

Figure 5.4 Total plant biomass in each of the treatments and per crab density. Total plant biomass is the sum of the floating plant biomass collected during the experiment and the remaining plant biomass at the end of the experiment.

Total plant biomass recovered from the control tanks at the end of the experiment varied between 23.0 - 33.6 g DW, depending on the abiotic stressor: control > chemical stress > light limitation stress (Figure 5.4). Treatment type did have a significant effect on total plant biomass (one-way ANOVA; $F_{df=2,9} = 4.62$; $p = 0.042$), with light limited plants having a significantly lower biomass than plants from the control treatment ($p = 0.038$). Within each treatment, total plant biomass was not significantly affected by crab density (one-way ANOVA; $F_{df=3,8} = 1.46$; $p = 0.30$). Nevertheless, there were large variations in total plant biomass between crab density treatments, e.g. more than 50% less biomass if 2.5 ind. m^{-2} are present compared to the control situation (Figure 5.4).

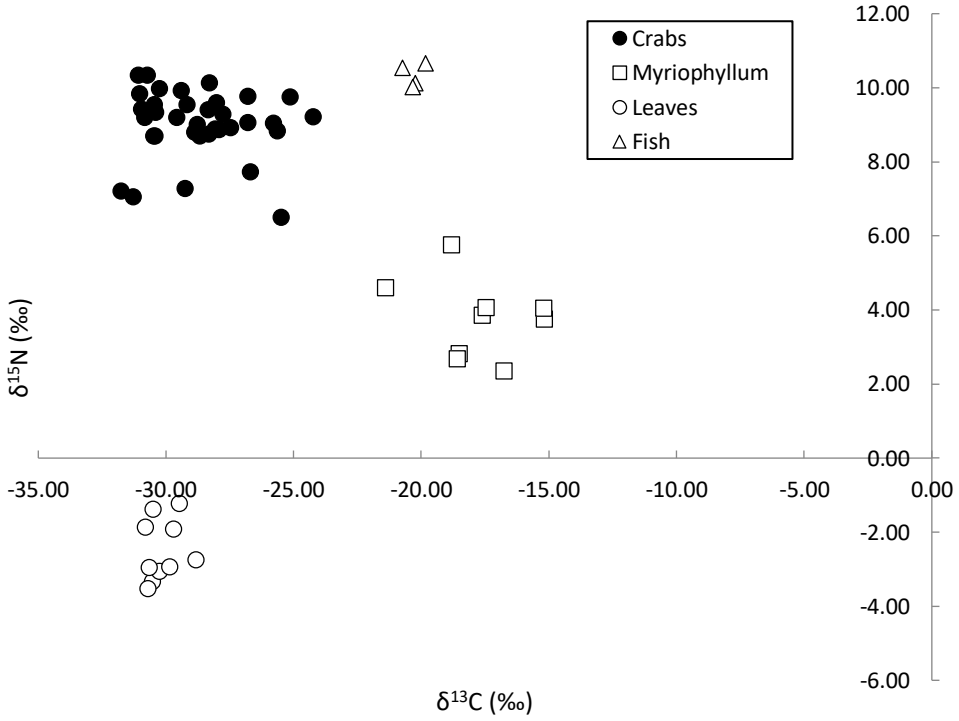

Figure 5.5 Stable isotope signatures of $\delta^{13}C$ and $\delta^{15}N$ of the Chinese mitten crabs and their possible food sources *M. spicatum*, terrestrial leaves and supplemented fish meat.

Crab diet reconstruction

Clear differences in $\delta^{13}C$ and $\delta^{15}N$ signatures could be observed between the crabs and the three measured possible food sources (Figure 5.5). In this biplot, the crabs are positioned at the upper left part, being relatively depleted in ^{13}C, but with high $\delta^{15}N$ signatures. Terrestrial leaves were the most depleted in ^{15}N, followed by *M. spicatum* with slightly higher values and by supplemented fish which was the most enriched in ^{15}N. Terrestrial leaves were the most depleted in ^{13}C in comparison to *M. spicatum* and fish, which both had comparable $\delta^{13}C$ signatures.

Mixing model calculations showed that the majority of the crabs' diet consisted of terrestrial leaves and fish meat, with a variable but smaller proportion consisting of *M. spicatum* (Table 5.2). The 5th percentile value of *M. spicatum* was always 0, indicating that the chance of *M. spicatum* not being consumed was at least 5% (Table 5.2). The only treatments in which *M. spicatum* constitutes a potential important food source (i.e. have a potential maximum contribution higher than 50%), also had the highest uncertainty due to the low number of crabs being measured, inherent to the crab density imposed. Differences in potential food sources between treatments and crab densities were not consistent.

Table 5.2 Potential contributions of the different possible food sources to the crabs' diets. Ranges represent 90% credible intervals (5–95 percentile ranges) with median contribution in parentheses, calculated using the SIAR mixing model. The analysis of the single crab in the control treatment had analytical errors and no data could be generated.

Treatment	**Crab densities (ind. m^{-2})**	***M. spicatum***	**Tree leaves**	**Fish**
Control	0.3	-	-	-
Control	1.0	0 - 0.45 (0.13)	0.35 - 0.81 (0.66)	0.13 - 0.29 (0.21)
Control	2.5	0 - 0.29 (0.05)	0.50 - 0.81 (0.73)	0.17 - 0.26 (0.21)
Light limitation	0.3	0 - 0.62 (0.27)	0.14 - 0.86 (0.52)	0.01 - 0.48 (0.16)
Light limitation	1.0	0 - 0.62 (0.31)	0.10 - 0.78 (0.45)	0.06 - 0.49 (0.22)
Light limitation	2.5	0 - 0.22 (0.05)	0.57 - 0.80 (0.73)	0.18 - 0.25 (0.22)
Chemical	0.3	0 - 0.64 (0.32)	0.06 - 0.78 (0.44)	0.01 - 0.54 (0.21)
Chemical	1.0	0 - 0.47 (0.16)	0.31 - 0.72 (0.63)	0.08 - 0.37 (0.19)
Chemical	2.5	0 - 0.29 (0.06)	0.53 - 0.86 (0.75)	0.13 - 0.24 (0.18)

Table 5.3 Regrowth of plant biomass after (a)biotic stresses were relieved. Data are plant dry mass (g DM) after a short and long term period. The symbol * indicate the presence of flowers at the time of evaluation.

Treatment	Crab densities (ind. m^{-2})	Regrowth after 47 days (g DM)	Regrowth after 345 days (g DM)
Control	0	6.9*	21.5*
Control	0.3	0	2.3*
Control	1.0	0	0.2
Control	2.5	0	0.4*
Light limitation	0	3.2*	14.6*
Light limitation	0.3	0	0.1
Light limitation	1.0	0	0.6
Light limitation	2.5	0	0
Chemical	0	6.7*	22.0*
Chemical	0.3	0	1.0*
Chemical	1.0	0	0.2
Chemical	2.5	0.1	4.2*

Regrowth experiment

On day 16 of the regrowth experiment, the first shoots reappeared in the patches from the control treatment tanks (i.e. without crabs). On day 47 (short term period), flowers appeared again in the new-grown patches. Biomass mainly returned in patches from the control treatment (Table 5.3), and one shoot grew back in the tray from the chemical treatment with the highest crab density. No plant regrowth was observed in the other trays (Table 5.3). After one year of regrowth (long term period), the biomass of the patches from the control treatment all had flowers and macrophyte biomass was similar to the biomass in the previous year (Table 5.3, Figure 5.4). All other trays only had a few shoots and only some of them managed to produce flowers (Table 5.3).

Discussion

This study has shown that the Chinese mitten crabs can have a negative impact on freshwater flora. One crab (0.3 ind. m^{-2}), in combination with light limitation or chemical stress, resulted in complete eradication of the experimental vegetation patch in less than 25 days. Within the constraints of this experiment, this crab density could be considered a threshold value above which vegetation is likely to

be impacted by crabs in presence of other stress factors. This value is similar to the density threshold of 0.25 – 0.5 ind. m^{-2} established by Jin et al. (2001) for macrophytes in the crab's home range in China. In all experiments with 1.0 and 2.5 ind. m^{-2}, vegetation was severely diminished or completely gone within 25 days. However, since this is no field study, the parameters presented here do not account for the many *in situ* variables which could mitigate or exacerbate the impact, including the daily movements and seasonal migration of the crabs, presence or absence of other food sources, combination and intensity of additional biotic and abiotic plant (and crab) stress factors, and the ratio of plant coverage to crab density.

Locally and during certain periods of time (e.g. during migrations), it cannot be excluded that crab densities in streams and rivers exceed this threshold, resulting in the complete disappearance of aquatic vegetation. This is especially likely when the plants are already stressed by other factors (Hidding et al. 2016, Wang et al. 2017) and other food sources are restricted. In a natural stream, contrary to our experimental conditions, several vegetation patches are present over a much larger area and crabs are free to move around. Such conditions probably gives patches time to recover rather than being continuously exposed to the same crab density (which was the case in our experiment). Exposure time is also dependent on vegetation coverage which may be higher in low-order streams (hence a higher threshold value for a given crab density) and lower in high-order streams (hence a lower threshold value for a similar crab density). In the case of our experiment, the above-mentioned threshold value of 0.3 ind. m^{-2} could be recalculated to the density of crabs per vegetated surface area, instead of per total surface area of the tank, which increases it to 7.4 ind. m^{-2} vegetation. In order to estimate the risk for the vegetation it is advised to report crab density values in relation to the vegetation cover. Note that our experiment was executed with only 1 size class of crabs, and the threshold values observed will probably no longer hold when considering smaller or larger crabs.

The regrowth experiment demonstrated that following removal by the crabs, plants were hindered in their ability to grow back in the short and long term, even after their optimal (a)biotic conditions were restored. This may point to a hysteresis effect in the critical crab density that removes vegetation and the critical crab density that allows recovery. The mesh wire prevented the crabs from burrowing in the sediment, but it is likely that their chelae and/or legs were able to

reach and damage the root system, inhibiting regrowth. Long-term experiments such as those presented herein are needed to simulate stream restoration projects in which crab densities are reduced. A (semi-)permanent reduction or even total loss of vegetation can have dramatic consequences for the aquatic ecosystem since macrophytes are ecological engineers (Schoelynck et al. 2012) fulfilling many important roles in the aquatic ecosystem (e.g. Chapter 2, 3, 4 and 6). A sudden loss of vegetation may induce sediment erosion, which can be intensified by the burrowing activities of the crabs and which may further inhibit vegetation recovery in the long term.

Isotope analyses showed that only a minority of the plant biomass is actually assimilated and that leaf litter was the main food source for the crabs. This corroborates the findings of Czerniejewski et al. (2010) and Roswarne et al. (2016), who showed that macrophytes contribute approximately 10 - 16% of the crabs' diet, respectively (based on gut-content analyses), and with a mesocosm experiment by Rudnick et al. (2005) showing that the main food source for crabs are tree leaves. The crabs' cutting behaviour, and not consumption, was found to be the main cause of macrophyte shoot removal, which corroborates previous studies on the destructive effects of (invasive) crayfish and crabs (e.g. Lodge et al. 1994, Jin et al. 2003, Van der Wal et al. 2013). Crab pinch strength proved to be an order of magnitude higher than what is needed to cut the plant stems. Though video recording did not observe crabs pinching through and removing macrophyte stems, crab movement through the vegetation and crab-crab interactions resulted in breaks and snaps in the macrophyte shoots (Figure 5.6). Therefore, we propose the hypothesis that crabs mostly do not pinch the shoots at full force, but rather just grasp them. Yet this may be enough to damage plant cells (trauma), causing the shoot to die locally (necrosis), which results in plant fragments being repelled after which they start floating. The process of necrosis takes some time, which may also explain the time lag between the introduction of the crabs and observation of the first floating shoots. Variation in total macrophyte biomass was found among setups with different crab densities under the same treatment. This variation can only be explained by a reduced plant growth in tanks where crabs were present, because similar initial macrophyte biomass was planted, abiotic circumstances were the same within the same stress treatment and crabs consumed only little biomass. Growing and branching potential of the macrophytes decreases with

every trauma and repelled shoot. This results in a smaller shoot density, in lower biomass production and a higher risk for further crab damage.

The additional influence of abiotic stress had a significant effect on the speed of vegetation decline. In the presence of chemical stress, the start of vegetation loss appeared sooner in time and led to more severe losses after 25 days even at the lowest crab density, compared to the control treatment. Macrophytes exposed to chemical contaminants such as EDTA, glyphosate and its metabolite aminomethyl-phosphonic acid (AMPA) are expected to experience stress or injury which could accelerate the necrosis in the shoots that were damaged by the crabs (Reddy et al. 2004). Note that the concentrations used in this experiment were at

Figure 5.6 Images of video recording (a-c) and pictures (d-f) of the interaction of 2 Chinese mitten crabs with a macrophyte patch. (a) A crab grasping the macrophyte with front chelae. (b) The crabs disturbing the macrophyte by clambering on top of it and substantially bending its stems. (c) A crab presumably eating the macrophyte. This goes on for a few minutes in this instance. (d-f) Breaks and snaps (trauma) in some of the macrophytes stems due to crab activities.

The upper end of what can be found in streams in, for example, the Nete catchment, but are realistic near the mixing zone of effluent discharge or during periods of drought with the corresponding reduced dilution of contaminants. Also note that we have used Roundup® to add glyphosate, but we did not analyse the adjuvants in the herbicide, which can also be stressful, nor are any interaction or additive toxicological effects between EDTA and glyphosate considered. Plants under the reduced light treatment reacted ambiguously. At the lowest crab density, plants were cut loose much sooner than in the control treatment, with higher crab densities resulting in even earlier removal of macrophyte biomass. The higher nutrient content (i.e. higher N and P content and lower C:N and C:P ratios) of plants grown under the reduced light treatment would indicate that they are more nutritious and more likely to be consumed, if other factors regarding plant palatability such as structural defences or secondary compounds are constant between the treatments (Elser et al. 2000, Gross and Bakker 2012). Additionally, the lower shear strength of these plants would make them easier to cut and more vulnerable to crab pinches than plants in the other treatments. However, this effect was only clear in case of 0.3 ind. m^{-2} crab density (notably where crab-crab interactions are not present). Additionally, we hypothesise that lower light intensity may have itself had an ambiguous effect on the crab's behaviour, as it is a mainly nocturnal animal (Gilbey et al. 2008). Darker conditions might decrease the time that crabs hide in the vegetation, resulting in a lower plant trauma prevalence during crab-crab interactions, but it might also have increased crab mobility which may result in higher plant trauma prevalence. Specific behaviour experiments are needed to sort this out.

The impact of the Chinese mitten crab on the aquatic ecosystems in Europe remains largely unknown. However, our study suggests that the crabs can potentially have a large impact on macrophyte communities under high crab densities. Actual density data are not often published, which is a shortcoming to estimate the risk for aquatic vegetation. Yet absolute values of crab numbers caught show an increase across Europe in the last decades, e.g. Baltic Sea region (Ojaveer et al. 2007), Spain (Garcia-de-Lomas et al. 2010), Poland (Normant et al. 2000, Wójcik-Fudalewska and Normant-Saremba 2016), The Netherlands, Belgium, France, Germany and the UK (Herborg et al. 2003, Herborg et al. 2005). Ecological niche modelling demonstrated that most of Europe is vulnerable to invasion by

Chinese mitten crabs and especially rivers flowing into the Mediterranean Sea appear to be a highly suitable habitat (Herborg et al. 2007). With climate change, river water temperatures are projected to increase on average by 0.8–1.6 °C by 2100 (some European river systems like Rhine, Danube and Rhone even up to 2.1 °C) (Van Vliet et al. 2013), which may favour the spread of the crabs (Herborg et al. 2007). At the same time, abiotic stress to macrophytes caused by climate change is also likely to increase in the near future (Short et al. 2016, Reitsema et al. 2018), which can make plants more vulnerable to crabs (Hidding et al. 2016). Many streams and rivers may become devoid of macrophytes, which is currently the case for parts of the Grote Nete stream in Belgium (Vlaamse Milieu Maatschappij 2017b). This may result in negative consequences for the aquatic system as a whole and makes it hard to reach the Water Framework Directive goals aiming at good water quality by 2027.

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Supplementary material

1 Material and Methods

1.1Datasets

The datasets for the fraction of biomass remaining (FBR) as a function of time for the control, light reduction stress and chemical stress experiment are presented in Figure S5.1a, b and c respectively.

1.2 Model

To compare the different impacts an idealized model for the fraction of biomass remaining (FBR) as a function of time t is proposed. The model is shown in expression S1.

$$\text{Fraction of Biomass Remaining} = \frac{1}{1 + e^{d\,[t\,t]}} \qquad \text{(S1)}$$

The model from expression 1 is a function of two parameters d and t which have a clear intuitive interpretation. t can be interpreted as a measure for the time threshold when the system starts to collapse. The bigger t, the longer it takes for the system to collapse. d is a measure for consumption speed of plant material once the threshold t is reached. The interpretation of the parameters t and d is illustrated in Figure S5.2a and b respectively.

Every dataset for FBR as a function of time has its own set of parameters t and d . The parameters are obtained by fitting the dataset to the model. Once the parameters t and d are estimated for different stress impact, they can be compared. By doing so the different stress impacts itself can be compared using the corresponding parameters t and d.

2 Results

2.1 Model fit

Control The data fit and the output of the R nls() function are presented in Figure S5.3 and S5.4 respectively.

Light Reduction Stress The data fit and the output of the R nls() function are presented in Figure S5.5 and S5.6 respectively.

Chemical Stress The data fit and the output of the R nls() function are presented in Figure S5.7 and S5.8 respectively.

2.2 Parameter Comparison

The parameters t and d as a function of crab density for the different experiments are presented in Figure S5.9a and b respectively. The data fits are presented as well. The chosen model for the data fits are an exponential decaying function for t and a linear function for d. The corresponding nls() function output is presented in listings 12 and 13 for the t and d data fit respectively.

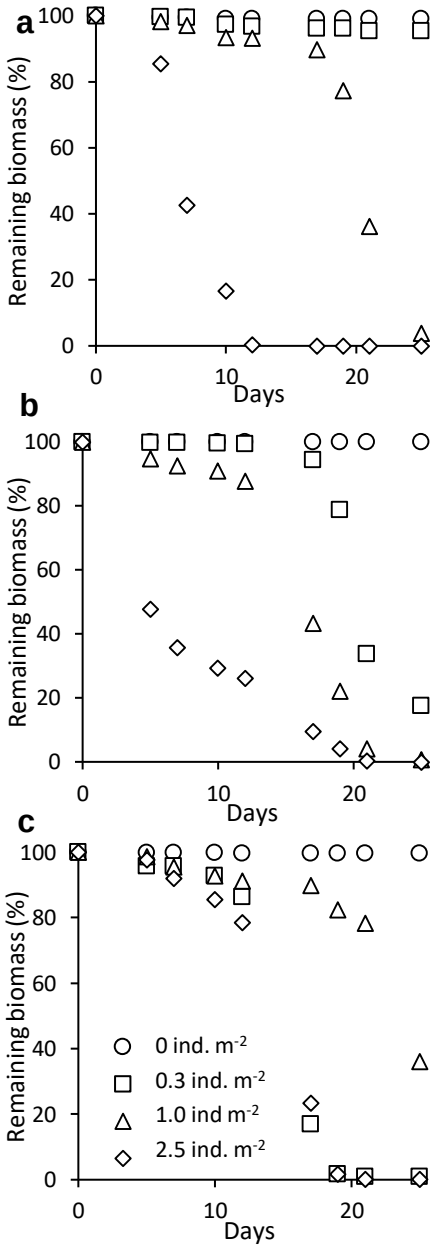


Figure S5.1 Reduction of plant biomass in the control (a), chemical stress (b) and light reduction stress (c) ponds with different crab densities.

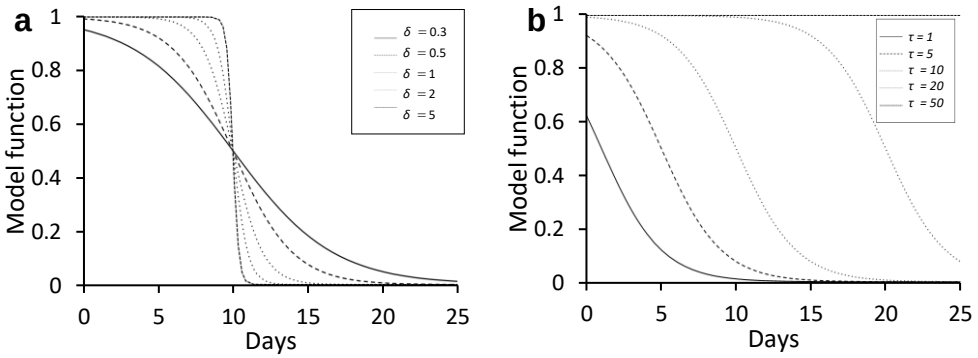


Figure S5.2 Illustration of the interpretation of the model parameters τ (in days) and δ (in days^{-1}). Model for varying δ and fixed τ = 10 days (a). Model for varying τ and fixed δ = 0.5 days^{-1} (b).

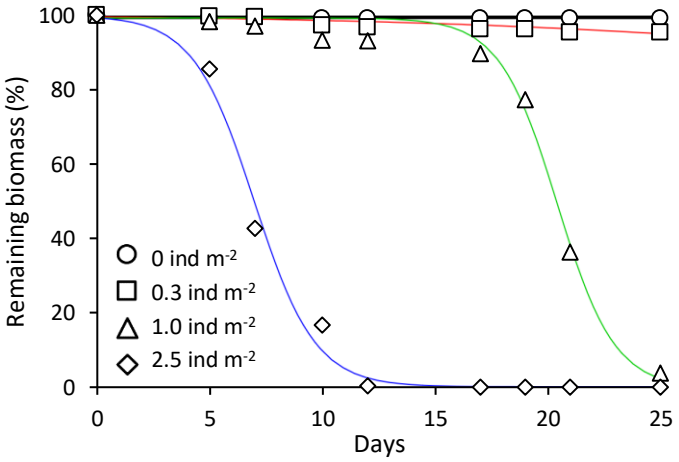


Figure S5.3 Datafit of model for fraction of biomass remaining for different crab densities for the control treatment.

Listing 1 R – nls() fit output for 0 ind m^{-2}

```
Formula: fu ~ 1 / (1 + exp(-a * (- days + b)))
Parameters:
Estimate Std.ErrortvaluePR( >|t| )
δ 0.03475     0.01389     2.502     0.408*
τ 155.89278   55.68485    2.800     0.0265*
---
Signif. Codes: 0 ***  0.001 **  0.01 *  0.05
0.1  1

Residual standard error: 0.002155 on 7
degrees of freedom

Number of iterationsconverge: 23
Achieved convergence tolerance: 5.907e-06
```

Listing 2 R – nls() fit output for 0.3 ind m^{-2}

```
Formula: fu ~ 1 / (1 + exp(-a * (- days + b)))
Parameters:
Estimate Std.ErrortvaluePR( >|t| )
δ 0.07293     0.01819     4.01     0.005121**
τ 64.12518    11.20399    5.723    0.000718***
---
Signif. Codes: 0 ***  0.001 **  0.01 *  0.05
0.1  1

Residual standard error: 0.009085 on 7
degrees of freedom

Number of iterationsconverge: 15
Achieved convergence tolerance: 4.581e-06
```

Listing 3 R – nls() fit output for 1.0 ind m^{-2}

```
Formula: fu ~ 1 / (1 + exp(-a * (- days + b)))
Parameters:
Estimate Std.ErrortvaluePR( >|t| )
δ 0.7821      0.1116      7.011     0.0002***
τ 20.3632     0.1771      144.995   9.92e-13***
---
Signif. Codes: 0 ***  0.001 **  0.01 *  0.05
0.1  1

Residual standard error: 0.04203 on 7 degrees
of freedom

Number of iterationsconverge: 13
Achieved convergence tolerance: 9.419e-06
```

Listing 4 R – nls() fit output for 2.5 ind m^{-2}

```
Formula: fu ~ 1 / (1 + exp(-a * (- days + b)))
Parameters:
Estimate Std.ErrortvaluePR( >|t| )
δ 0.7389      0.1001      7.379     0.000152***
τ 6.9091      0.1830      37.747    2.38e-09***
---
Signif. Codes: 0 ***  0.001 **  0.01 *  0.05
0.1  1

Residual standard error: 0.04124 on 7
degrees of freedom

Number of iterationsconverge: 12
Achieved convergence tolerance: 2.878e-06
```

Figure S5.4 The listings of the R nls() function output for the control treatment.

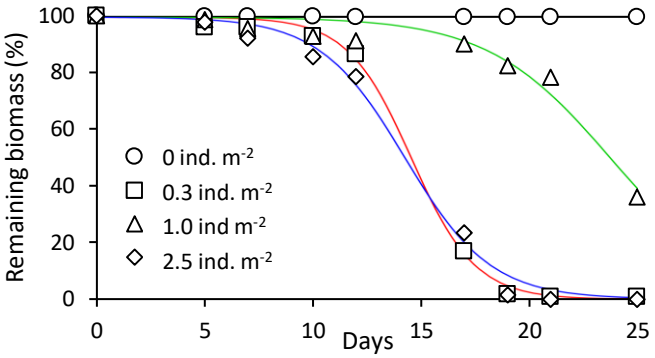


Figure S5.5 Datafit of model for fraction of biomass remaining for different crab densities for the light reduction treatment.

Listing 5 R – nls() fit output for 0.0 ind m^{-2}

Formula: fu ~ 1 / (1 + exp(-a * (- days + b)))
Parameters:
Estimate Std.ErrortvaluePR(>|t|)
δ 0.04086 0.01357 3.012 0.0190*
τ 152.26191 44.8525 3.395 0.0115*

Signif. Codes: 0 *** 0.001 ** 0.01 * 0.05 0.1 1
Residual standard error: 0.001022 on 7 degrees of freedom
Number of iterationsconverge: 38
Achieved convergence tolerance: 7.8e-06

Listing 6 R – nls() fit output for 0.3 ind m^{-2}

Formula: fu ~ 1 / (1 + exp(-a * (- days + b)))
Parameters:
Estimate Std.ErrortvaluePR(>|t|)
δ 0.6770 0.0496 13.64 2.68e-06***
τ 14.5545 0.2048 71.08 2.87e-11***

Signif. Codes: 0 *** 0.001 ** 0.01 * 0.05 0.1 1
Residual standard error: 0.02716 on 7 degrees of freedom
Number of iterationsconverge: 9
Achieved convergence tolerance: 1.732e-06

Listing 7 R – nls() fit output for 1.0 ind m^{-2}

Formula: fu ~ 1 / (1 + exp(-a * (- days + b)))
Parameters:
Estimate Std.ErrortvaluePR(>|t|)
δ 0.35259 0.0521 6.771 0.0002***
τ 23.7101 0.4478 52.951 2.25e-10***

Signif. Codes: 0 *** 0.001 ** 0.01 * 0.05 0.1 1
Residual standard error: 0.049 on 7 degrees of freedom
Number of iterationsconverge: 15
Achieved convergence tolerance: 9.7e-06

Listing 8 R – nls() fit output for 2.5 ind m^{-2}

Formula: fu ~ 1 / (1 + exp(-a * (- days + b)))
Parameters:
Estimate Std.ErrortvaluePR(>|t|)
δ 0.5057 0.0492 10.28 1.78e-05***
τ 14.2183 0.3033 46.88 5.26e-10***

Signif. Codes: 0 *** 0.001 ** 0.01 * 0.05 0.1 1
Residual standard error: 0.04206 on 7 degrees of freedom
Number of iterationsconverge: 9
Achieved convergence tolerance: 1.912e-06

Figure S5.6 The listings of the R nls() function output for the light reduction treatment.

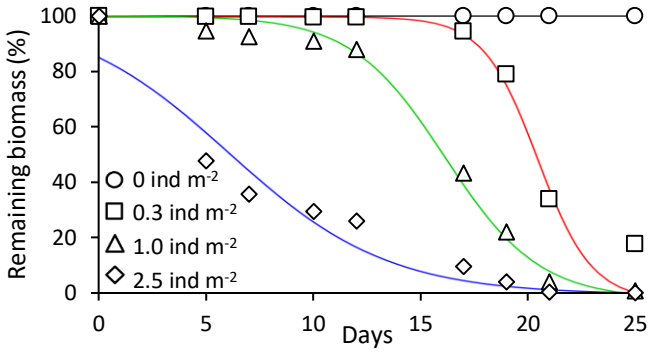


Figure S5.7 Datafit of model for fraction of biomass remaining for different crab densities for the chemical treatment.

Listing 9 R – nls() fit output for 0.0 ind m^{-2}

```
Formula: fu ~ 1 / (1 + exp(-a * (- days + b)))
Parameters:
Estimate Std.ErrortvaluePR( >|t| )
δ 0.0786     0.0322     2.435     0.045*
τ 120.5157   41.2669    2.920     0.0223*
---
Signif. Codes: 0 ***  0.001 **  0.01 *  0.05
0.1  1
Residual standard error: 0.0001528 on 7
degrees of freedom
Number of iterationsconverge: 34
Achieved convergence tolerance: 6.512e-06
```

Listing 10 R – nls() fit output for 0.3 ind m^{-2}

```
Formula: fu ~ 1 / (1 + exp(-a * (- days + b)))
Parameters:
Estimate Std.ErrortvaluePR( >|t| )
δ 0.7900     0.1650     4.788     0.0020**
τ 20.4011    0.2564     79.573    1.3e-11***
---
Signif. Codes: 0 ***  0.001 **  0.01 *  0.05
0.1  1
Residual standard error: 0.0610 on 7
degrees of freedom
Number of iterationsconverge: 27
Achieved convergence tolerance: 6.528e-06
```

Listing 11 R – nls() fit output for 1.0 ind m^{-2}

```
Formula: fu ~ 1 / (1 + exp(-a * (- days + b)))
Parameters:
Estimate Std.ErrortvaluePR( >|t| )
δ 0.4597     0.0465     9.826     2.40e-09***
τ 16.1669    0.2787     58.014    1.19e-10***
---
Signif. Codes: 0 ***  0.001 **  0.01 *  0.05
0.1  1
Residual standard error: 0.04012 on 7 degrees
of freedom
Number of iterationsconverge: 11
Achieved convergence tolerance: 5.513e-06
```

Listing 12 R – nls() fit output for 2.5 ind m^{-2}

```
Formula: fu ~ 1 / (1 + exp(-a * (- days + b)))
Parameters:
Estimate Std.ErrortvaluePR( >|t| )
δ 0.2703     0.0572     4.727     0.0021**
τ 6.0924     0.7553     8.066     8.65e-05***
---
Signif. Codes: 0 ***  0.001 **  0.01 *  0.05
0.1  1
Residual standard error: 0.08806 on 7
degrees of freedom
Number of iterationsconverge: 12
Achieved convergence tolerance: 8.004e-06
```

Figure S5.8 The listings of the R nls() function output for the chemical treatment.

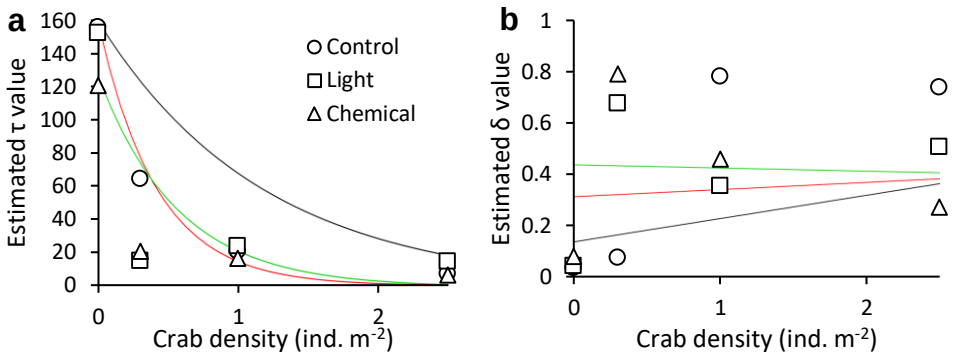


Figure S5.9 Model parameters τ (a) and δ (b) for the different experiments as a function of crab density and the corresponding datafit.

Listing 13 R – nls() fit output for control

```
Formula: fu ~ b * exp(-a * crabs)
Parameters:
Estimate Std.ErrortvaluePR( >|t| )
a 0.8156      0.1029      7.927      0.0154*
b 154.6160    7.4417      20.777     0.0023**
---
Signif. Codes: 0 ***  0.001 **  0.01 *  0.05
0.1  1
Residual standard error: 7.524 on 2 degrees of
freedom
Number of iterationsconverge: 5
Achieved convergence tolerance: 8.84e-06
```

Listing 14 R – nls() fit output for light stress

```
Formula: fu ~ b * exp(-a * crabs)
Parameters:
Estimate Std.ErrortvaluePR( >|t| )
a 2.300       1.281       1.796      0.214
b 152.214     19.462      7.821      0.016*
---
Signif. Codes: 0 ***  0.001 **  0.01 *  0.05
0.1  1
Residual standard error: 19.46 on 2 degrees
of freedom
Number of iterationsconverge: 8
Achieved convergence tolerance: 3.538e-06
```

Listing 15 R–nls() fit output for chemical stress

```
Formula: fu ~ b * exp(-a * crabs)
Parameters:
Estimate Std.ErrortvaluePR( >|t| )
a 1.7018      0.5433      3.132      0.0886
b 120.3244    11.7854     10.210     0.0095**
---
Signif. Codes: 0 ***  0.001 **  0.01 *  0.05
0.1  1
Residual standard error: 11.79 on 2 degrees of
freedom
Number of iterationsconverge: 9
Achieved convergence tolerance: 2.023e-06
```

Figure S5.10 nls() exponential fit output for the different treatments (Fig S5.9a).

Listing 16 Pearson test for control treatment

```
Pearson's product-moment correlation

Data: control0_dat$crabs & control0_dat$fu
t = 1.8184, df = 2, p value = 0.2106
Alternative hypothesis: true correlation
Isnotequalto0
95percentconfidenceinterval:
-0.7114951   0.9953395
Sample estimates:
cor
0.7893703
```

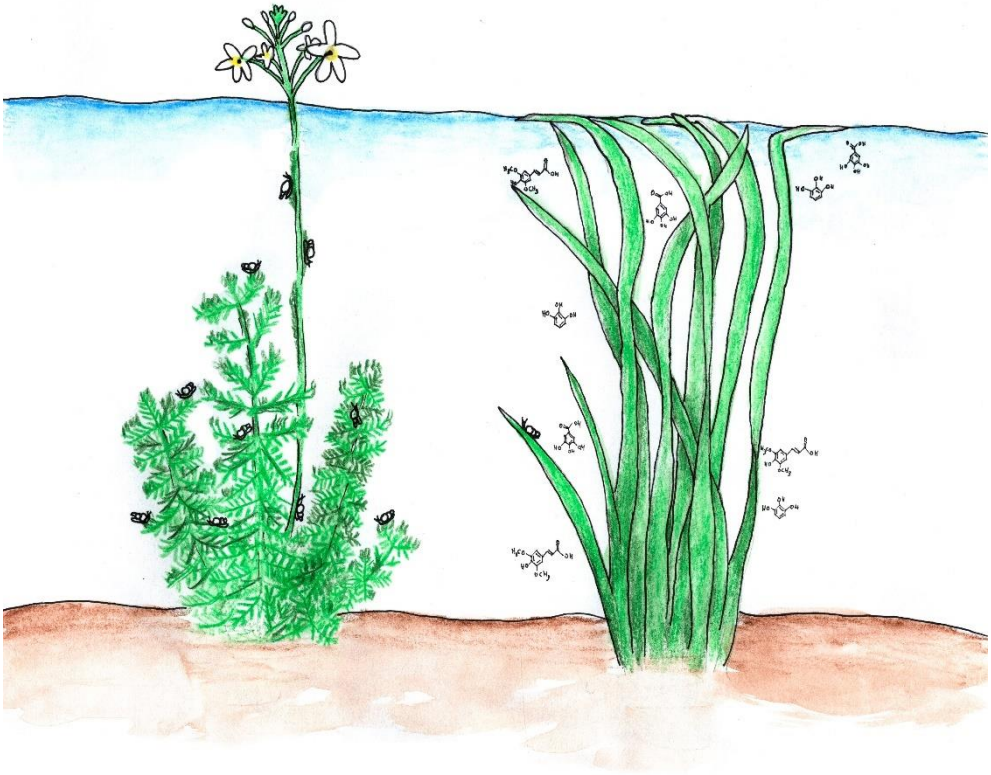
Listing 17 Pearson test for light treatment

```
Pearson's product-moment correlation

Data: control1_dat$crabs & control1_dat$fu
t = 0.55381, df = 2, p value = 0.6354
Alternative hypothesis: true correlation
Isnotequalto0
95percentconfidenceinterval:
-0.9182480   0.9816922
Sample estimates:
cor
0.3646383
```

Listing 18 Pearson test for light treatment

```
Pearson's product-moment correlation

Data: control3_dat$crabs & control3_dat$fu
t = -0.20507, df = 2, p value = 0.8565
Alternative hypothesis: true correlation
Isnotequalto0
95percentconfidenceinterval:
-0.9707107   0.9483842
Sample estimates:
cor
-0.1435028
```

Figure S5.11 Pearson's product-moment correlation test for the δ model parameter and crab density.



Chapter 6.

Macrophyte-specific effects on epiphyton quality and quantity and resulting effects on grazing macroinvertebrates

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Abstract

Aquatic macrophytes can have a significant impact on their associated community of epiphytic algae and bacteria through the provisioning of structural habitat complexity through different growth forms, the exudation of nutrients and the release of allelochemicals. In turn, this effect on epiphytic biofilm biomass and nutrient content has a potential effect on the macroinvertebrates that depend on epiphyton as a food source. In this study, we studied the effect of living macrophytes and their growth form on biofilm development in a semi-controlled replicated microcosm experiment. Conditions of a nutrient-poor water layer and nutrient-rich sediment were created to study the effects of nutrient exudation by living macrophytes. We compared biofilm quantity and quality on structurally simple (*Vallisneria spiralis*) versus complex (*Egeria densa*) living plants and artificial analogues. Subsequently, the biofilm that had developed on the plants was fed, in a laboratory growth experiment, to two species of macroinvertebrate grazers (the snail *Haitia acuta* and the mayfly nymph *Cloeon dipterum*). This enabled us to assess if and how the macrophyte-induced effects on the epiphyton can influence macroinvertebrate grazers.

Living macrophytes were found to have a significant effect on epiphytic algal cover, which was mostly expressed by a lower cover on living macrophytes compared to their artificial analogues. Additionally, epiphyton cover on artificial macrophytes was found to be higher on complex structures compared to simple ones, yet this was not observed on living macrophytes. Plant specific traits, such as the release of allelopathic substances, competition for nutrients and DIC, and the amount of $CaCO_3$ deposition on plant surfaces might explain these results. The density of epiphytic bacteria was found to be negatively correlated with biofilm Ca content from macrophytes in every treatment except living *E. densa*, which differed in leaf anatomy from the other plants by possessing polar leaves. Furthermore, biofilm on living macrophytes had lower C:N:P molar ratios compared to that on artificial plants, which is likely to be explained by nutrient exudation by the living plants. Although it was expected that a more nutritious biofilm would lead to increased grazer growth, this was observed only for *H. acuta* on *E. densa*. Because biofilm quantity was not a limiting factor, this lack of effect may be caused by compensatory feeding. It can be concluded that, depending on their traits, living macrophytes can have a positive effect on macroinvertebrate grazers by providing

a large surface area for colonisation by epiphytic algae and bacteria, by improving biofilm stoichiometry and by stimulating bacterial growth.

Keywords: Epiphytic algae; allelopathy; nutrient exudation; nutrient stoichiometry; phytomacrofauna

Introduction

The presence of aquatic macrophytes can have a large effect on the aquatic ecosystems in which they occur, including associated aquatic macroinvertebrate communities. By forming underwater structures, macrophytes provide a habitat for macroinvertebrates (Carpenter and Lodge 1986), increase habitat complexity (O'Hare and Murphy 1999, McAbendroth et al. 2005), provide a refuge against predation (Warfe and Barmuta 2004, 2006) and reduce water flow velocity in lotic ecosystems (Sand-Jensen and Mebus 1996, Schoelynck et al. 2013), thereby creating a habitat for more limnophilous macroinvertebrate species. Although living and decaying macrophytes may also serve as food source for herbivorous and omnivorous macroinvertebrates (Chapter 3, Chapter 4, Bakker et al. 2016), it is generally assumed that the epiphytic algae play a more important role in the diet of these animals than the macrophytes they are attached to (Cummins and Klug 1979, Allan and Castillo 2007).

By acting as a substrate for epiphytic algae and bacteria, macrophytes can have an indirect effect on the primary production-based green food web through their various influences on the attached epiphytic biofilm. First of all, macrophyte complexity, and thus growth form (e.g. McAbendroth et al. 2005), has been shown to significantly affect the amount of epiphytic biofilm on macrophyte surfaces, whereby more complex macrophytes create a greater heterogeneity in light conditions, nutrient availability and herbivore grazing pressure than macrophytes with a simpler growth form (Warfe and Barmuta 2006, Tessier et al. 2008, Ferreiro et al. 2013). In doing so, they typically support more biofilm per unit area than simple macrophytes, despite similar total surface areas (Warfe and Barmuta 2006, Tessier et al. 2008, Ferreiro et al. 2013). Furthermore, both living and decaying macrophytes have been shown to exude a wide variety of chemicals to the water layer, including allelochemicals, nitrogen (N), phosphorus (P) and dissolved organic

carbon (DOC), affecting its associated epiphytic biofilm (Carpenter and Lodge 1986, Burkholder and Wetzel 1990, Wigand et al. 2000, Gross 2003).

The excretion of N and P from macrophytes to the phyllosphere can have a positive effect on biofilm biomass and nutritious quality (i.e. lower C:N and C:P molar ratios) (Bowman et al. 2005), while DOC excretions can have a positive effect on bacterial biomass and productivity in that biofilm (Kirchman et al. 1984, Theil-Nielsen and Sondergaard 1999). Allelochemicals excreted by macrophytes can in turn limit epiphytic algal growth on macrophyte surfaces allowing more light to reach the plant surface by reducing shading (Wigand et al. 2000, Gross 2003). Epiphyton is however often less affected by these allelopathic compounds than phytoplankton (Hilt and Gross 2008).

Individual effects of macrophyte complexity, nutrient exudation and allelopathy on the epiphytic biofilm have been studied before, yet there is no consensus on the net effect of living macrophytes on algal and bacterial quantity and quality in the biofilm. Furthermore, the effects of the interactions between living macrophytes and the epiphytic biofilm on grazing macroinvertebrates have, to our knowledge, never been studied at the same time. Although previous experiments have shown that increased nutrient availability leads to a higher nutritive quality (i.e. lower C:N:P ratios) of periphytic algae (Bowman et al. 2005), which in turn leads to higher macroinvertebrate growth rates (Hart and Robinson 1990, Fink and Von Elert 2006), these results were all obtained from algae growing on non-living substrates.

This study had two objectives: i) to investigate the effects of macrophyte metabolism (artificial vs. living macrophytes) and growth form (simple vs. complex) on epiphytic algal quantity, algal community composition, bacterial content and biofilm elemental composition and ii) how these differences in biofilm quality affected the growth of macroinvertebrate grazers. For the first objective, we compared the epiphytic communities of two living macrophyte species and two types of artificial plant that differ in their growth form in a semi-controlled replicated greenhouse experiment (cf. Grutters et al. 2017). It was hypothesised that complex macrophytes would harbour more epiphytic algae and bacteria than simple macrophytes and that the influence of living macrophytes would include allelopathic effects, nutrient leaching and DOC leaching. Additionally, it was hypothesised that the underwater photosynthesis of living macrophytes and, to a

lesser degree, epiphytic algae, would lead to an increase in water pH and thus to the precipitation of $CaCO_3$ and Ca-P minerals on the macrophyte leaves, in turn resulting in higher concentrations of these elements in the biofilm (e.g. Hartley et al. 1997, Pedersen et al. 2013).

For the second research question, we studied the effects of these changes in biofilm quality on the growth of macroinvertebrate grazers by conducting a semi-controlled replicated macroinvertebrate growth experiment, wherein the different kinds of biofilm were offered in abundance to two species of invertebrate grazers. We hypothesised that macroinvertebrate growth would be higher on biofilm from living macrophytes because this biofilm was expected to contain more nutrients and to have a nutrient stoichiometry more suitable for macroinvertebrate growth.

Material and methods

Selected plant species

Two species of macrophytes and two artificial plant analogues were selected for the experiment. *Vallisneria spiralis* (Hydrocharitaceae) has a simple growth form and *Egeria densa* (Hydrocharitaceae) a more complex one. These plants were bought from a commercial plant nursery and, prior to the experiment, were incubated for one week in artificial ponds filled with tap water in the same greenhouse as where the main experiment would take place. Additionally, plastic *Vallisneria* and *Egeria* analogues were selected as artificial macrophytes (20 cm plastic plants, Hobby Aquaristik, Germany).

Before the start of the experiment, the epiphytic biofilm was removed from the living macrophytes by vigorously shaking the plants for 1 minute in water, followed by 10 minutes sonication in an ultrasonic bath. Although the effectiveness of this method was not microscopically confirmed in this study, other studies reported removal efficiencies of 90% for only vigorously shaking (Zimba and Hopson 1997, Jones et al. 2000). By combining this method with 10 minutes of sonication, very high removal efficiencies may be expected. Pilot experiments showed that this did not impair the plant's viability, although the sonication may have caused some damage to the plants through cell rupture and by heating the water.

Macrophyte fractal complexity, as an indication of the degree of dissection and complexity of the plant (McAbendroth et al. 2005), was measured at the start

and end of the experiment. Fractal complexity measurements were performed as described in Chapter 2, whereby macrophytes were spread out over a white plastic plate of 1 m^2 and photographed using a Nikon D300S with a Tokima 11-16 mm f/2.8 lens. These pictures were then converted into binary images (1 pixel = 0.13 mm), after which the fractal dimension based on perimeter (D_p or "boundary" fractal) was calculated with ImageJ software (Rasband 1997-2012), using a series of grid sizes ranging from 2 to 64 pixels (box sizes 0.26 - 8.32 mm) to estimate the perimeter covered by the structures at different measurement scales. Macrophyte surface area was also calculated at the end of the experiment by dissecting plant sections, with a known length, and spreading the parts out over a white plastic plate of 1 m^2. Pictures were then taken with the same camera and the total surface area was calculated with ImageJ (Rasband 1997-2012).

Experimental setup

Both living and artificial macrophytes were incubated as monocultures for 8 weeks, from the 8th of August to the 3rd or 4th of October 2017, in 40 plastic 80 l containers (39 cm diameter, 68 cm high) in a fully randomised experiment (n = 10). Each container held 4 plastic 0.81 l pots (9 × 9 × 10 cm (L × W × H)), with one plant of the same type in each (Figure 6.1). Additionally, we added 3 control containers without macrophytes. In order to adequately study the possible nutrient excreting role of living macrophytes, we aimed for conditions of high sediment nutrient availability and low water nutrient availability, conditions that are also found in many natural systems (Bloemendaal and Roelofs 1988). This was achieved by filling each 0.81 l pot with a mixture of 1.3 kg clean sand and 1.077 g (i.e. 1.33 g l^{-1}) Basacote slow-release fertiliser (Basacote 6M Plus, 16-8-12 NPK, COMPO, Münster, Germany). Containers were filled with 44 l of Smart and Barko medium (Smart and Barko 1985), which is essentially demineralised water with added minerals ($CaCl_2 \bullet 2\ H_2O$: 91.7 mg l^{-1}; $MgSO_4 \bullet 7\ H_2O$: 69.0 mg l^{-1}; $NaHCO_3$: 58.4 mg l^{-1}; $KHCO_3$: 15.4 mg l^{-1}). This medium did not contain any prior nutrients, although these likely leached to the water from the sand and fertiliser mixture or from the macrophytes immediately after setup. This has resulted in the following mean starting conditions (n = 5): pH: 7.49, 7.28 mg O_2 l^{-1}, electrical conductivity: 270 µS cm^{-1}, 4.6 µg $N\text{-}NO_3^-$ l^{-1}, 11.4 µg $N\text{-}NO_2^-$ l^{-1}, 22 µg $N\text{-}NH_4^+$ l^{-1}, 3.6 µg $P\text{-}PO_4^{3-}$ l^{-1}, alkalinity: 0.82 meq l^{-1}, 0.51 mg DOC l^{-1}. Because of increasing phytoplankton growth, the water in all containers

was replaced with Smart and Barko medium after 30 days. Because all plants survived the experiment, there was no need to remove dead plants or plant sections. The experimental containers were placed inside the greenhouse facility of the University of Antwerp, with natural light conditions and temperature that followed the outdoor conditions (Figure S6.1).

At the start of the experiment, 120 ml algal inoculum was added to the containers to allow the cleaned macrophytes to be colonised by epiphytic algae and bacteria. This inoculum consisted of a mix of the epiphytic biofilm that was removed from the macrophytes at the start of the experiment and biofilm collected from other experimental setups in the greenhouse.

Epiphytic biofilm methods

On the 3rd and 4th of October 2017, 56 and 57 days after the onset of the experiment respectively, the macrophytes from half of the experimental containers (n = 5) were harvested, in order to measure epiphytic algal quantity, community composition, bacterial content in the biofilm and biofilm elemental composition. The other half of the containers would later be used to assess the effects of the different treatments on biofilm nutritional quality and macroinvertebrate growth. From the harvested containers, only the lowest 5 cm of the macrophytes were used because these 'basal sections' were all present from the start of the experiment, so that no difference in colonisation time existed among the different treatments. From all

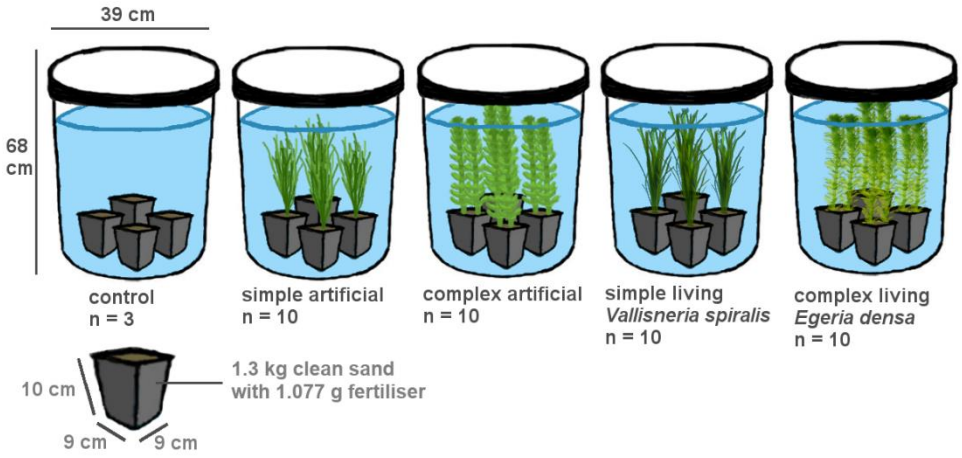

Figure 6.1 Schematic overview of the experimental setup at the start of the experiment.

harvested basal plant sections per experimental container, 1 or 2 sections (for complex and simple macrophytes respectively) were used for the biofilm quantity measurements, 1 or 2 sections (again for complex and simple macrophytes respectively) were used for the measurements of biofilm bacteria and the rest of the basal sections were used for the elemental analyses of the biofilm.

For the biofilm quantity measurements, this subsample was preserved in 4% formaldehyde until later taxonomic identifications. For each sample, 10 subsections of macrophyte tissue of approximately 1 cm^2, representing all different regions of the macrophyte, were selected after which any present epiphytic algae were identified up to order or genus under a Leica MZ12.5 stereomicroscope at 100× magnification. Epiphytic algal community composition was hereby defined as the estimated cover percentage of the total community that consisted of a certain order or genus. In addition, epiphytic algal cover on these 1 cm^2 subsections was estimated subjectively on a scale of 0 to 10, with 0 being no algal cover and 10 being a completely covered leaf. Although these subjective cover estimates are not the most accurate methods for determining the quantity of epiphytic algae, as biofilm thickness is not taken into account, they provided enough resolution to answer our research questions.

For the elemental analyses of the biofilm, the subsample of macrophyte basal sections were scoured of biofilm by vigorously shaking the plants in water for 1 minute, followed by 10 minutes sonication in an ultrasonic bath. This biofilm was then stored in plastic 1 l pots at 4 °C until later elemental analyses. To determine the C, N and P content of this biofilm, it was filtered over precombusted 1µm GF/C glass-fibre filters (Macherey-Nagel, Düren, Germany) and 0.45 µm nitrocellulose filters (Macherey-Nagel, Düren, Germany). Epiphytic algae and bacteria were not separated from the inorganic matrix of the biofilm in this way, and the measurements thus represent the elemental composition of the entire epiphytic biofilm. The glass-fibre and nitrocellulose filters were subsequently oven-dried to a constant weight at 70 °C (at least 48 h) and weighed. Glass-fibre filters were folded into tin cups and biofilm C and N content were measured using a Flash 2000 CN-analyser (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Biofilm P content was determined by acid digesting the complete nitrocellulose filters, with the precipitated biofilm, according to the method of Huang and Schulte (1985).

Sample P content was subsequently measured on ICP-OES (iCAP 6300 Duo view, Thermo Fisher, Waltham, Massachusetts, USA).

The number of biofilm bacteria was determined using epifluorescence microscopy after staining with 4',6-diamidino-2-phenylindole (DAPI) following the general protocol of Porter & Feig (1980). For this purpose, the macrophyte subsample that was collected per experimental container during the harvest was stored in plastic 50 ml tubes containing 70% ethanol at -18 °C until later microbial analyses. Biofilm bacteria were first detached from these macrophyte fragments by vigorously shaking and by sonicating for 15 minutes in an ultrasonic bath. Macrophyte fragments were then removed from the tubes and rinsed with MilliQ water to remove potentially remaining biofilm bacteria. The 50 ml tubes containing the bacterial suspension were centrifuged at 3000 rpm for 5 min and the supernatant was discarded until 10 ml of sample remained. This was then resuspended by vigorously shaking and sonicating for 15 minutes in an ultrasonic bath. Aliquots of 200 to 500 µl were subsequently taken and filtered, together with 2 ml MilliQ to ensure a homogeneous suspension of bacterial cells, over 0.2 µm polycarbonate Millipore GTTP filters (Sigma-Aldrich, Poole, UK) supported by a 0.45 µm mixed cellulose ester backing filter (Sigma-Aldrich, Poole, UK). Polycarbonate filters were hereafter cut in four quarters and one quarter per filter was mounted on glass slides, to be mounted and stained with a Citifluor A1 (Citifluor Ltd., London, UK) and Vectashield (Vector laboratorios, Burlingame, California, USA) buffer (4:1, v:v) to which DAPI was added to a concentration of 1 mg l^{-1}. This was then allowed to incubate for at least 10 minutes in the dark, after which bacterial cells were observed at 1000× magnification under a Zeiss Axioplan 2 epifluorescence microscope and photographed with an EXi Blue Fluorescence Microscopy Camera (QImaging). A minimum of 10 microscopic fields and 400 cells were counted for each sample (Kirchman 1993).

Water quality parameters

Water physicochemical parameters were measured on day 13, 21, 30, 38 and 49 of the experiment, in all containers in which the epiphytic biofilm would be harvested for taxonomic composition, total cover, elemental composition and bacterial analyses (n = 5 per treatment) and in the control containers (n = 3). In each container we measured temperature, pH, electrical conductivity and dissolved

oxygen (multiline F/set-3 multimeter), alkalinity (SAN^{++}, Skalar, Breda, The Netherlands), and the concentrations of $N\text{-}NO_3^-$, $N\text{-}NO_2^-$ $N\text{-}NH_4^+$ and $P\text{-}PO_4^{3-}$ in 0.45 µm filtered water (Chromafil® Xtra MV-45/25, Macherey-Nagel, Düren, Germany) (SAN^{++}, Skalar, Breda, The Netherlands). CO_2 concentrations were calculated from pH and alkalinity measurements (Stumm and Morgan 2012). Additionally, DOC quantity and quality, the latter expressed as the specific UV absorbance at 254 nm ($SUVA_{254}$) (Weishaar et al. 2003), was also recorded from 0.45 µm filtered water (Chromafil® PET -45/25, Macherey-Nagel, Düren, Germany) (SAN^{++}, Skalar, Breda, The Netherlands). Due to technical problems, SUVA was not measured during the first two measuring events.

Macroinvertebrate growth experiment

To assess the effects of the nutritional quality of epiphytic biofilm grown under the different treatments, a macroinvertebrate growth experiment was carried out with the remaining plants from the unharvested containers (n = 5) for 5 weeks, from the 28th of October to the 1st of December 2017. The macroinvertebrate consumers used in this experiment were nymphs of the mayfly *Cloeon dipterum* (Ephemeroptera: Baetidae) and the freshwater snail *Haitia acuta* (Gastropoda: Physidae). Both are classified as epiphytic biofilm grazers, whereby *C. dipterum* is considered a gatherer and *H. acuta* a scraper (Monakov 2003, Heino 2005). These animals were collected from another greenhouse mesocosm that was used to temporarily store macrophytes for another experiment. Before the experiment started individuals were measured on graph paper under a Zeiss SteREO Discovery V12 dissection microscope with an Axiocam ICc 1 camera (*C. dipterum*: head to abdomen, excluding tails; *H. acuta*: shell length (i.e. shell apex to basal lip), both to the nearest 0.01 mm) and starved for 24 hours. Per species, 20 2 l jars filled with water from the experimental containers were used as experimental units. Jars were placed in the greenhouse, where they were continuously aerated. A number of 5 cm basal macrophyte fragments (4 for simple treatments and 2 for complex treatments) of one of the treatments were then added to the jars, as well as 5 individuals of one of the macroinvertebrate species. Macrophyte fragments were replaced weekly by fresh fragments to provide the macroinvertebrates with sufficient food. Observations of *C. dipterum* nymphs during the experiment revealed that the animals always had full stomachs, indicating that it was unlikely

that food quantity was a limiting factor. At the end of the experiment, all invertebrates were collected and measured again under the dissection microscope in order to calculate their growth.

Statistical analyses

Throughout the experiment, the individual containers, rather than the 4 pots within each of them, were treated as the independent experimental units. Whenever samples from multiple plant sections were taken, this was done from pooled plant sections originating from different pots in the same container.

The effects of treatment and time, and their interaction effects, on the measured water quality parameters and macroinvertebrate size data were tested using linear mixed models, combined with a Tukey post hoc test in R 3.4.2 (R Development Core Team 2017) and using the packages '*multcomp*' (Hothorn et al. 2008) and '*nlme*' (Pinheiro et al. 2017). Treatment and time were hereby treated as fixed factors and the individual experimental containers and jars as random factor.

Differences in algal community composition among the different treatments were tested for significance using one-way analysis of similarity (ANOSIM) (Clarke 1993), whereby the statistic test was computed after 9999 permutations. This test was performed in PAST 3.17 (Hammer et al. 2001). Remaining data were tested for normality using both Shapiro-Wilk tests and visual inspection of Q-Q plots. Not normally distributed data were tested for significant differences among groups using Kruskal-Wallis tests and Dunn's post hoc tests. This was also done for the ordinal data of the epiphyton cover classes. Normally distributed data were checked for equality of error variances using Levene's tests. Significant differences among groups were assessed using one-way ANOVAs with Tukey post-hoc tests for equal variances or using Welch tests and Games-Howell post-hoc tests for non-equal variances. Relationships between parameters were defined using Pearson correlation coefficients and tested for significance using two-tailed t-tests. These tests were performed in SPSS version 24.0.

Because it was expected that the underwater photosynthesis of the macrophytes and algae could result in significant $CaCO_3$ deposition on the macrophyte leaves (e.g. Pedersen et al. 2013), which was also observed in this study, we anticipated that this non-cellular C would confound the calculation and interpretation of epiphyton C:N and C:P ratios. To counteract this possibility, we

calculated the molar amount of C in these ratios by subtracting the molar amount of Ca from the raw value of C (assuming a 1:1 molar ratio in biofilm $CaCO_3$). Although this method does not take into account the intracellular amount of Ca, we expect that this amount is so low compared to the extracellular $CaCO_3$ deposition as to fall within the normal error range of the ratios.

Results

Epiphyton

Significant differences in epiphytic algal cover were observed among the different treatments (Figure 6.2a, Kruskal-Wallis test; $\chi^2(3) = 10.53$; $p = 0.015$), whereby living macrophytes had a significant negative effect on epiphyton cover (two-way ANOVA; $F_{df=1,1} = 17.90$; $p = 0.001$). This effect was mostly caused by the significantly higher epiphyton cover on complex artificial macrophytes compared to the low cover on complex living macrophytes (with a D_p of 1.497 and 1.317 for artificial and real *Egeria* respectively), whereas epiphyton cover was comparable between simple artificial and simple living macrophytes (with a

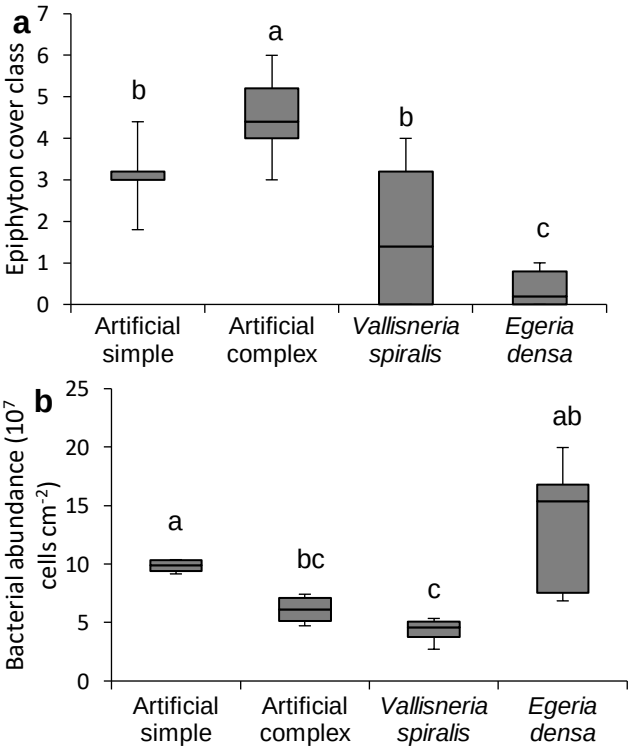

Figure 6.2 Epiphyton cover class (a) and bacterial density (b) for the different treatments. The boxes with the horizontal segment represent the first-third quartile range and the median of the data respectively, with the whiskers indicating minimum and maximum values. Different letters indicate significant ($p < 0.05$) differences among treatments.

D_p of 1.141 for both artificial and living *Vallisneria*). No significant effect of macrophyte growth form on epiphyton cover was observed (two-way ANOVA; $F_{df=1,1}$ = 0.26; p = 0.619), although a significant interaction effect between living macrophytes and growth form (two-way ANOVA; $F_{df=1,1}$ = 7.52; p = 0.014) indicated that the effect of macrophyte growth form on epiphyton cover differed between living and artificial macrophytes. Epiphyton community structure did not differ significantly among the different treatments (ANOSIM; R = -0.10, p = 0.903), with the community being dominated for 63-81% by cyanobacteria, and the remaining part consisting of Chlorophyta and diatoms (Bacillariophyceae), as well as a small percentage Desmidiaceae on the simple artificial plants (Table 6.1).

Significant differences were also observed in the elemental composition of the epiphytic biofilm (Table 6.2). The general pattern was that the biofilm on complex and living plants had a higher Ca content, a lower C and N content and a lower C:N molar ratio compared to the biofilm on simple and artificial plants. In addition, more $CaCO_3$ precipitation was visually observed on complex and living plants. Biofilm P content was lower on complex artificial macrophytes compared to the other treatments, which was only significantly expressed as a higher C:P and N:P molar ratio for that treatment.

Table 6.1 Average composition of the epiphytic algal community for the different treatments. Data indicate the percentage cover of each algal group (represented as order or genus) of the total epiphytic community and are presented as means ± S.E.

	Artificial simple	Artificial complex	*Vallisneria spiralis*	*Egeria densa*
Cyanobacteria				
Oscillatoriales	43.7 ± 11.4	51.2 ± 6.5	40.4 ± 12.2	60.0 ± 15.3
Chroococcales	19.0 ± 7.8	12.6 ± 6.6	30.8 ± 14.6	20.8 ± 7.0
Chlorophyta				
Coleochaetales (*Coleochaete*)	14.6 ± 7.3	10.8 ± 6.4	4.4 ± 2.6	6.0 ± 4.0
Oedogoniales (*Oedogonium*)	2.2 ± 2.2	0	2.6 ± 1.9	0
Zygnematales (*Mougeotia*)	0	6.0 ± 6.0	0.8 ± 0.8	0
Zygnematales (*Cosmarium*)	3.6 ± 3.4	0	0	0
Bacillariophyceae				
Achnanthales	16.9 ± 7.5	19.4 ± 12.1	21.0 ± 9.8	13.2 ± 9.5

No uniform distinction in bacterial density could be made between either simple and complex or artificial and living macrophytes (Figure 6.2b). Bacterial density was significantly higher on simple artificial macrophytes than on complex artificial macrophytes and on simple living macrophytes, whereby bacterial density on the latter was also significantly lower than on complex living macrophytes (Welch test; $F_{df=3,6.8}$ = 19.3; p = 0.001). In addition, the amount of heterotrophic bacteria in all treatments except on *E. densa*, showed a significant negative correlation with biofilm Ca content (Figure S6.2a, r = -0.85, p = 0.001).

Water quality measurements

All measured water quality parameters displayed significant differences over time during the experiment (Table 6.3), whereby significant differences among the different macrophyte treatments were observed for all N (i.e. N-NH_4^+, N-NO_2^-, N-NO_3^- and total-N) and P parameters (Table 6.3). Interaction effects were observed for all parameters, except for EC, O_2, N-NH_4^+ and DOC (Table 6.3, Figure S6.3). Two different trends can be distinguished, regarding nutrient levels and dissolved inorganic carbon (DIC). N concentrations show a sharp decline and approach zero

Table 6.2 Elemental composition of the epiphytic biofilm in the different treatments, expressed as weight percentages for the separate elements and molar ratios for the C, N and P ratios. C:N and C:P ratios are corrected for biofilm Ca content (see material and methods). Values are presented as means ± S.E. Different letters indicate significant (p < 0.05) differences among treatments.

	Artificial simple	Artificial complex	*Vallisneria spiralis*	*Egeria densa*
%C	24.99 ± 3.01^{a}	20.39 ± 2.93^{ab}	17.03 ± 1.61^{ab}	13.63 ± 0.93^{b}
%N	2.55 ± 0.55^{a}	1.15 ± 0.19^{ab}	1.09 ± 0.27^{ab}	0.68 ± 0.2^{b}
%P	0.15 ± 0.04	0.02 ± 0.01	0.13 ± 0.03	0.18 ± 0.09
%Ca	13.88 ± 3.55^{a}	21.79 ± 4.42^{ab}	29.34 ± 1.85^{ab}	32.43 ± 1.09^{b}
%Mg	0.15 ± 0.07^{ab}	0.50 ± 0.09^{ab}	0.16 ± 0.03^{a}	0.56 ± 0.04^{b}
%K	0.33 ± 0.08	0.40 ± 0.16	0.16 ± 0.06	0.10 ± 0.06
C:N	10.37 ± 0.63^{a}	10.10 ± 0.41^{ab}	8.67 ± 0.47^{ab}	6.83 ± 1.46^{b}
C:P	129.58 ± 13.88^{ab}	384.58 ± 140.16^{a}	59.18 ± 14.25^{b}	59.70 ± 22.06^{b}
N:P	27.45 ± 1.69^{ab}	87.46 ± 30.90^{a}	15.05 ± 3.60^{b}	20.60 ± 6.17^{ab}

after the onset of the experiment (Figure S6.3i), while P-concentrations in all treatments, except *V. spiralis*, first show a stable increase and only decline to non-detectable levels after the water change (Figure S6.3h). Before the water change, no clear differences among the different macrophyte treatments are apparent in DIC-related parameters (i.e. pH, alkalinity and CO_2), but a higher pH (Figure S6.3a) and a lower alkalinity (Figure S6.3b), combined with lower concentrations of dissolved CO_2 (Figure S6.3c), were measured for the living macrophytes after this change. For the artificial macrophyte treatments, these changes showed a

Table 6.3 Summary statistics of linear mixed models for individual and interactive effects of treatment and time on the different water quality parameters. Significant factors ($p < 0.05$) are indicated in bold.

	df	F	*p*		df	F	*p*
pH				*EC*			
Treatment	4	0.109	0.978	Treatment	4	1.828	0.168
Time	4	32.597	< **0.001**	Time	4	28.223	< **0.001**
Treatment × time	16	3.575	< **0.001**	Treatment × time	16	0.792	0.690
Alkalinity				*P-PO_4^{3-}*			
Treatment	4	1.266	0.320	Treatment	4	5.705	**0.004**
Time	4	7.462	< **0.001**	Time	4	7.929	< **0.001**
Treatment × time	16	7.534	< **0.001**	Treatment × time	16	2.713	**0.002**
CO_2				*Total N*			
Treatment	4	0.018	0.999	Treatment	4	15.074	< **0.001**
Time	4	47.345	< **0.001**	Time	4	66.801	< **0.001**
Treatment × time	16	9.129	< **0.001**	Treatment × time	16	3.237	< **0.001**
O_2				*N-NH_4^+*			
Treatment	4	0.857	0.508	Treatment	4	3.866	**0.019**
Time	4	18.319	< **0.001**	Time	4	5.427	**0.001**
Treatment × time	16	0.963	0.505	Treatment × time	16	1.02	0.447
DOC				*N-NO_2^-*			
Treatment	4	0.084	0.986	Treatment	4	48.214	< **0.001**
Time	4	28.365	< **0.001**	Time	4	55.566	< **0.001**
Treatment × time	16	1.265	0.244	Treatment × time	16	9.515	< **0.001**
SUVA				*N-NO_3^-*			
Treatment	4	2.066	0.128	Treatment	4	14.164	< **0.001**
Time	4	30.949	< **0.001**	Time	4	74.975	< **0.001**
Treatment × time	16	5.743	< **0.001**	Treatment × time	16	2.843	**0.001**

significant positive relationship with the abundance of epiphytic algae (Figure S6.2c&d, pH: r = 0.784, p = 0.007, CO_2: r = -0.74, p = 0.014), while they showed a significant positive correlation with final plant dry biomass in the living macrophyte treatments (Figure S6.2e&f, pH: r = 0.95, p < 0.001, alkalinity: r = -0.88, p = 0.001, CO_2: r = -0.95, p < 0.001). Additionally, biofilm Ca content showed a significant negative correlation with dissolved CO_2 concentrations (Figure S6.2g, r = -0.80, p < 0.001).

Although DOC concentrations displayed large fluctuations over time, no clear differences between the treatments were observed (Figure S6.3e), which was also true for the DOC quality, expressed as SUVA (Figure S6.3f). No significant differences in EC were observed before the water change, but *E. densa* treatments showed a significantly lower EC after the water change, which also resulted in lower overall EC values (Figure S6.3g).

Macroinvertebrate growth experiment

Macroinvertebrates in all treatments increased in length during the experiment (Figure 6.3a&b), and this effect was significant for all *C. dipterum* treatments, except the simple artificial one (Figure 6.3b, Table 6.4), and for all *H. acuta* treatments (Figure 6.3c, Table 6.4). Additionally, *H. acuta* from the living *Egeria* treatment showed a significantly larger shell length increase than snails from the other treatments (Figure 6.3c, Welch test: $F_{df=3,8.6}$ = 4.05; p = 0.047), whereas no significant differences in growth rate were observed for *C. dipterum* (Figure 6.3c, one-way ANOVA; $F_{df=3,16}$ = 3.09; p = 0.056).

Table 6.4 Summary statistics of linear mixed models for individual and interactive effects of treatment and time on measured *C. dipterum* and *H. acuta* size. Significant factors (p < 0.05) are indicated in bold.

	df	F	p
C. dipterum			
Treatment	3	0.971	0.431
Time	1	0.575	0.459
Treatment × time	3	3.185	0.052
H. acuta			
Treatment	3	0.857	0.483
Time	1	5.899	**0.027**
Treatment × time	3	8.156	**0.002**

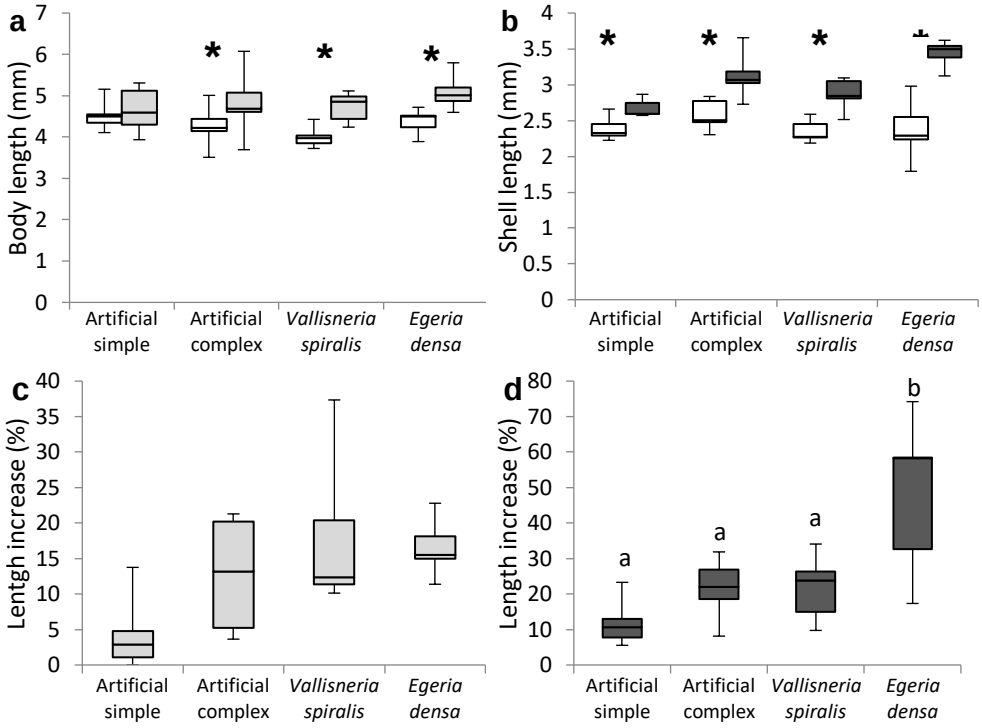

Figure 6.3 *C. dipterum* (a) and *H. acuta* (b) length before (white bars) and after (gray bars) the experimental period. Percent length increase of *C. dipterum* (c) and *H. acuta* (d) for the different treatments at the end of the experimental period is also shown. The boxes with the horizontal segment represent the first-third quartile range and the median of the data respectively, with the whiskers indicating minimum and maximum values. Significant length differences between the start and end of the experiment are indicated with an asterisk and different letters indicate significant ($p < 0.05$) differences in growth among treatments.

Discussion

Significant differences in epiphyton quantity and quality among the different macrophyte treatments have been observed in this study, suggesting that living macrophytes play a more active role than just a neutral substrate for epiphyton growth. Algal growth on simple artificial macrophytes was lower than on complex artificial macrophytes. As structural complexity was the only differentiating factor between the artificial treatments, it seems likely that the higher algal cover was caused by the increase in habitat heterogeneity and the amount of colonisable

microhabitats (Hooper et al. 2005, Warfe and Barmuta 2006). Similarly, the horizontal leaf orientation of complex artificial macrophytes in this study can cause more light to reach the epiphyton compared to a vertical leaf orientation, as in simple artificial macrophytes, resulting in more epiphyton on the former (Pettit et al. 2016).

This pattern of higher epiphyton cover on complex growth forms was not reflected in the living macrophytes. Possible explanations for these observations include the competition for DIC and nutrients by growing plants and the exudation of species-specific allelochemicals that inhibit the growth of epiphytic algae. These processes always occurred together and it was thus not possible to disentangle their separate effects on epiphyton cover. Given the strong negative relationship between dissolved CO_2 concentrations and final plant biomass in this study, it is possible that the growth and photosynthesis of living macrophytes caused DIC limitation for the epiphytic algae (e.g. Pedersen et al. 2013). Before the water change, it might be expected that phytoplankton growth and photosynthesis also caused DIC limitation. Despite the CO_2 produced in the biofilm by the respiration of heterotrophic bacteria (Wetzel 1993), it seems likely that this carbon limitation could in turn result in a lower algal cover on the living macrophytes, which is in line with other studies that found a lower epiphyton cover on fast growing plant species (e.g. Jones et al. 2002, Grutters et al. 2017). Carbon limitation did not seem to be an issue for the artificial macrophyte treatments, as the biofilm C:N molar ratio was clearly above 7, the ratio that indicates co-limitation (Hillebrand and Sommer 1999), suggesting that nutrient availability was a more important limiting factor for algal growth in those situations.

Some green algae and macrophytes, including *V. spiralis* and *E. densa* (Van Lookeren Campagne 1957, Pierini and Thomaz 2004), are able to utilise dissolved HCO_3^- as a carbon source, in addition to CO_2, in a process that produces OH^--ions, lowers the pH at the leaf surface and leads to the precipitation of $CaCO_3$ on the leaf (e.g. Pedersen et al. 2013). In our study, this was represented by the occurrence of visible $CaCO_3$ encrustations on the plants, in addition to the negative correlation between biofilm Ca content and dissolved CO_2 concentrations. These $CaCO_3$ encrustations are known to hinder the development of epiphytic algae, which can further explain the negative effect of living macrophytes on algal cover (Cattaneo and Kalff 1978, Sand-Jensen 1983).

Besides having a negative effect on the growth of epiphytic algae, $CaCO_3$ encrustations can also have a potential inhibiting effect on the development of heterotrophic bacteria, as was demonstrated in this study by the strong negative relationship between bacterial density and biofilm Ca content for all treatments except E. densa. This can be explained by the strong adsorption of free DOC, amino acids and fatty acids to the $CaCO_3$ in the biofilm, effectively immobilizing these substances and making them unavailable for bacterial uptake (e.g. Wetzel and Rich 1973). It is therefore all the more remarkable that the highest bacterial density was observed in *E. densa* treatments, the macrophyte with the highest biofilm Ca content. A possible explanation for this fact could be that *E. densa* possesses polar leaves that take up HCO_3^- on the abaxial side of the leaf and excrete OH^--ions on the adaxial side, so that $CaCO_3$ precipitation takes place only on the adaxial side, whereas $CaCO_3$ is precipitated on both leaf sides for other macrophytes (Prins et al. 1980, Prins and Elzenga 1989). Due to this absence of $CaCO_3$ encrustations and the limited competition by algae, which are light limited on the abaxial leaf side, half of the leaf would be suitable for bacterial colonisation. Additionally, macrophyte respiration can cause a nightly drop in water layer pH, potentially causing part of the $CaCO_3$ encrustation to dissolve, rereleasing the DOC, amino acids and fatty acids in the process.

The exudation of allelopathically active growth-inhibiting substances by *V. spiralis* and *E. densa* might be an additional reason for the lower amount of epiphytic algae on the living macrophytes, as both plant species have been shown to exude these substances (Gao et al. 2011, Gette-Bouvarot et al. 2015, Espinosa-Rodriguez et al. 2016). Based on the results obtained in this study, it might also be expected that there are species-specific differences in the potency of these allelochemicals, with *E. densa* having a stronger inhibiting effect on algal growth than *V. spiralis*.

Fast growing macrophyte species could also compete with epiphytic algae for nutrients in the water layer, which was generally oligotrophic, inhibiting algal growth in this way. However, biofilm C:N and C:P molar ratios were lower in living macrophyte treatments and indicated co-limitation of all three elements (Hillebrand and Sommer 1999). Nutrient excretion by living macrophytes could possibly also explain the biofilm's lower C:N and C:P molar ratios on these macrophytes (Burkholder and Wetzel 1990, Bowman et al. 2005). although its

relative importance compared to the macrophytes' competition for DIC could not be determined. Furthermore, it is expected that this potential positive effect of living macrophytes, through nutrient excretion, on algal cover is offset by the negative effects of DIC limitation and allelopathy in this study, leading to the observed lower epiphyton quantities on living macrophytes.

Macroinvertebrate growth

Because epiphytic biofilm quantity was not assumed to be a limiting factor for macroinvertebrate growth during the experiment (all macrophytes were still covered with biofilm after 1 week of grazing and *C. dipterum* nymphs always had full guts) and because no significant differences in epiphytic algal community composition were observed among the different treatments (Table 6.1), it seems likely that differences in biofilm quality were responsible for the observed differences in macroinvertebrate growth rate. It was expected that, based on the low biofilm C:N:P molar ratios on living macrophytes, macroinvertebrates on living macrophytes would have a higher growth rate because of the higher quality of their food (e.g. Sterner and Elser 2002). However, this was only represented by significantly higher growth rates for *H. acuta* on *E. densa*. A possible explanation for this could be the high bacterial density in *E. densa* biofilms, another potentially important and nutritious food source in the diet of gastropod scrapers (Monakov 2003, Allan and Castillo 2007). This would also explain the absence of this response in *C. dipterum*, as these animals are unable to consume the tightly attached bacterial biofilm and only collect the higher standing epiphytic algae (Monakov 2003, Heino 2005). The lack of an effect of biofilm C:N:P molar ratio on macroinvertebrate growth rate might be explained by ingestion of higher food quantities to compensate for the lower nutrient concentrations (i.e. compensatory feeding (Fink and Von Elert 2006)), meaning that effects of biofilm quality would only be visible under conditions of low epiphyton quantity. However, no quantitative algal consumption rates were measured.

Under natural conditions, macroinvertebrate growth has been shown to be consumer density-dependent, implying that macroinvertebrate grazers are frequently limited by the amount of epiphytic algae (Lamberti et al. 1995, Stelzer and Lamberti 2002). Indeed, in temperate lowland streams, the highest grazer densities are often found on the boundaries of macrophyte patches, where the

epiphyton density is highest, despite the greater risks of predation in those regions (e.g. Marklund et al. 2001). It might therefore be expected that stoichiometric differences in epiphyton quality are better reflected in consumer growth rate in these natural systems compared to our experiment.

Conclusions

This study observed significant differences in epiphyton cover between simple and complex macrophytes and between artificial and living macrophytes. The influence of living macrophytes on the epiphytic biofilm likely depends on plant-specific traits. A fast growth rate, complex growth form, HCO_3^- usage, polar leaves and the exudation of strong allelochemicals are hereby likely associated with a low epiphyton cover, while slow growing, simple, CO_2 using plants with nonpolar leaves and without strong allelochemicals likely have a higher epiphyton cover. Additionally, epiphytic biofilm C:N:P molar ratios were lower on living macrophytes, probably due to the plant's role as nutrient pump, although this effect will likely diminish under more eutrophic conditions. These changes in biofilm stoichiometry had no effect on the growth of macroinvertebrate grazers at high biofilm quantities however, although the bacterial stimulating effect of some macrophytes led to an increased growth of one of the studied species. It can thus be concluded that, depending on their traits, living macrophytes can have a positive effect on macroinvertebrate grazers by providing a large surface area for colonisation by epiphytic algae and bacteria, by improving biofilm stoichiometry and by stimulating bacterial growth.

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Supplementary figures

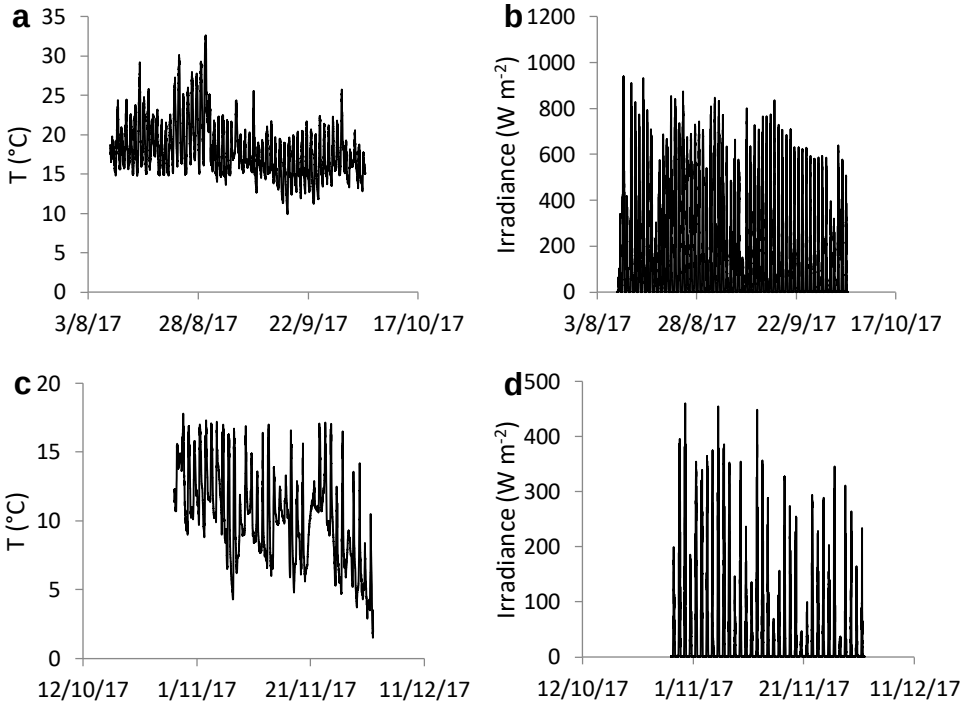


Figure S6.1 Temperature (a&c) and irradiance (b&d) inside the greenhouse facility during the epiphyton growth phase (a&b) and the macroinvertebrate growth experiment (c&d).

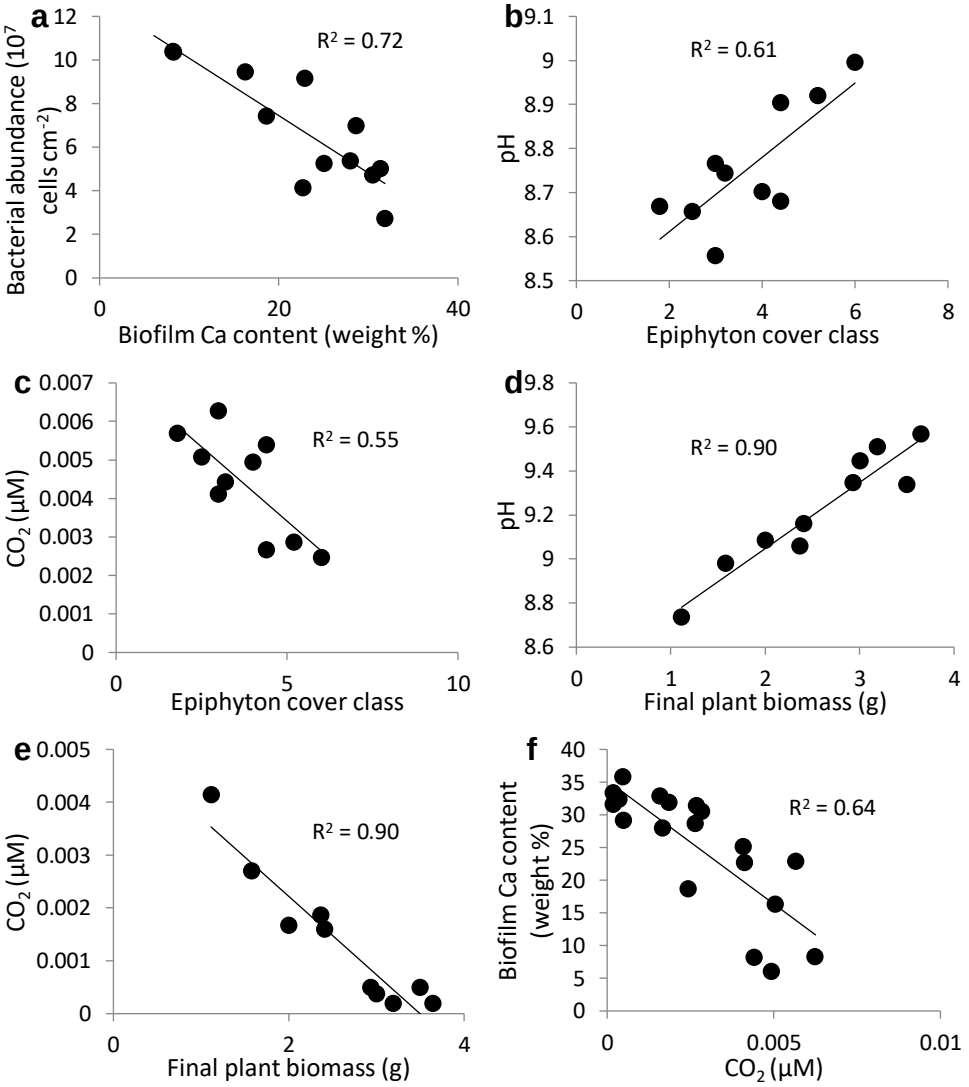


Figure S6.2 Relationship between biofilm Ca content and bacterial density (a) for all treatments except *E. densa*, relationship between epiphyton cover class and pH (b) and CO_2 concentration (c) for artificial treatments, relationship between final plant biomass and pH (d) and CO_2 concentration (e) for living plant treatments and the relationship between CO_2 concentration and biofilm Ca content (f) for all treatments.

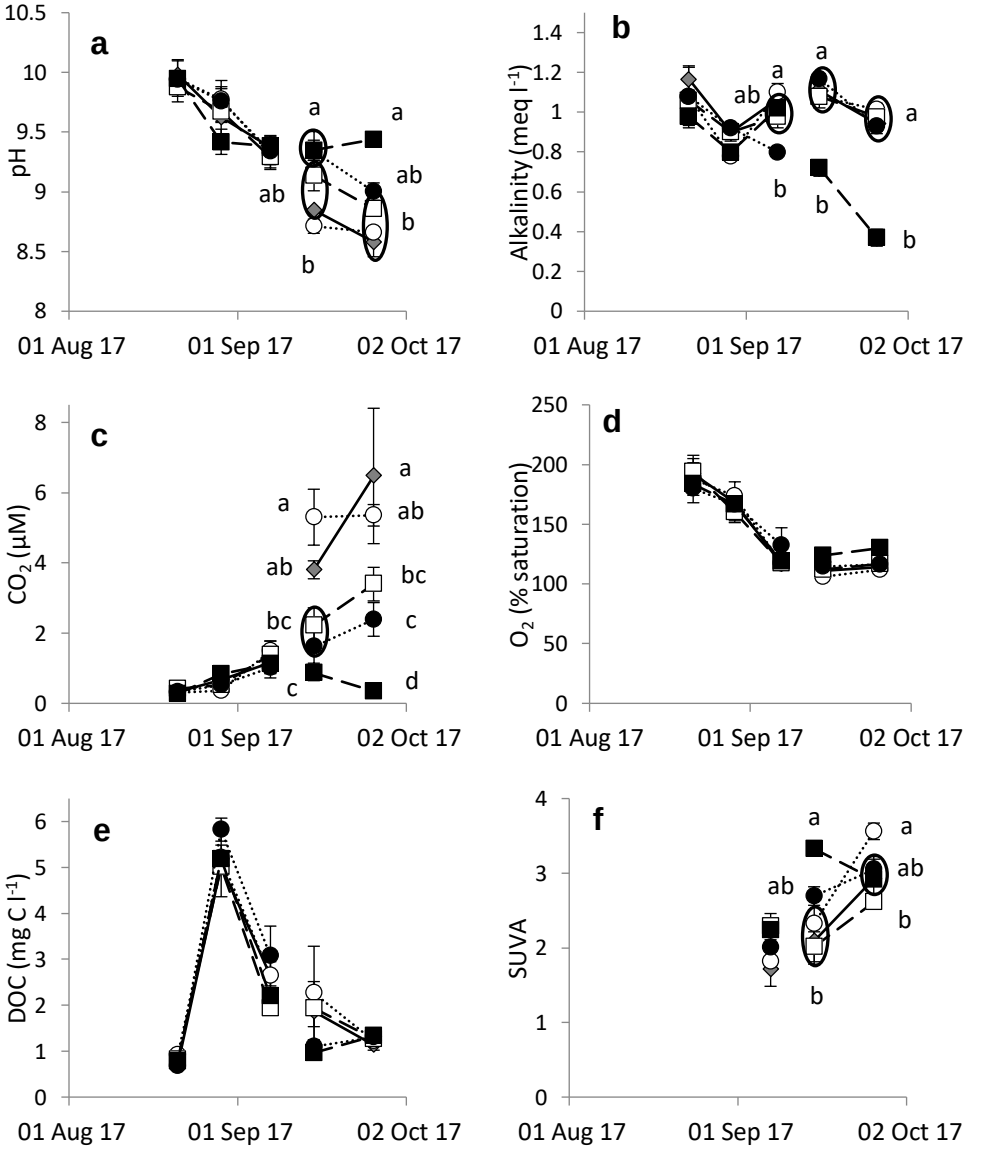


Figure S6.3 Development of water quality parameters over time, starting 13 days after the onset of the experiment (i.e. 21 August 2017) and ending with the harvest of the epiphyton (26 September 2017). Grey diamonds connected with a solid line represent the control treatment, white and black circles connected with a dotted line represent artificial and real *Vallisneria* (*spiralis*) respectively, while white and black squares connected with a dotted line represent artificial and real *Egeria* (*densa*) respectively. The water change on the 7th of September 2017 is represented by an interruption of the connecting lines.

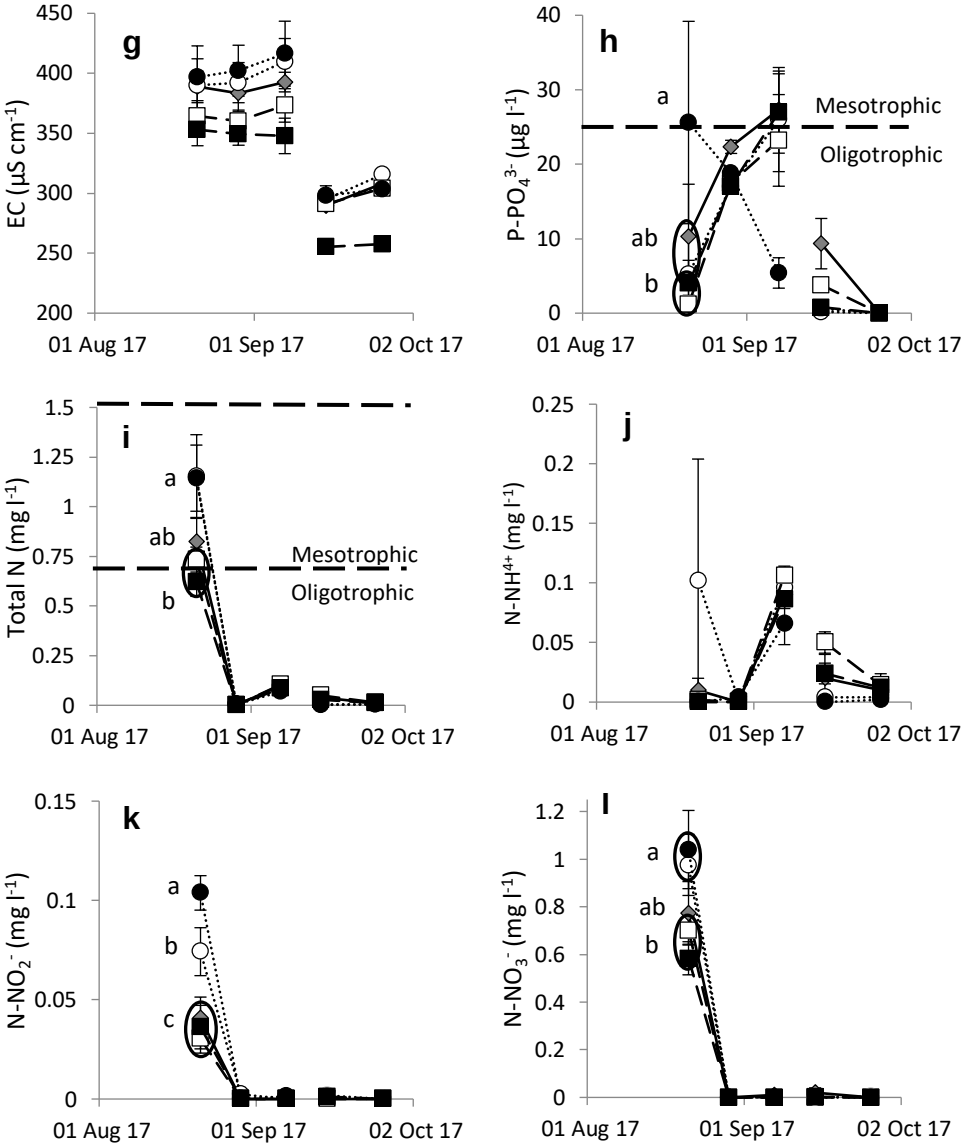


Figure S6.3 (continued) For each parameter, different letters indicate significant (p < 0.05) differences among treatments on a specific measuring event. The boundary values for oligotrophic-mesotrophic conditions in streams are shown for phosphorus (represented as phosphate) (h) and total nitrogen (i), whereby the mesotrophic-eutrophic boundary is also shown for total nitrogen in the upper part of the graph, according to Dodds et al. (1998).

Chapter 7.

Synthesis

Through their form and functioning, aquatic macrophytes can have a major impact on their aquatic environment as well as on the community of aquatic macroinvertebrates in the ecosystem. Although certain effects of macrophytes on their environment, including macroinvertebrates, have been well studied before, there is still much unknown about the role of macrophytes within the aquatic food web. The aims of this thesis were to (i) elucidate the role of macrophytes within the aquatic food web, including both their direct (e.g. direct consumption of living macrophytes and macrophyte-derived organic matter) and indirect (e.g. influence on other food sources, such as epiphytic algae and bacteria) role, and to (ii) study how the non-trophic interactions between macrophytes and macroinvertebrates would affect the macroinvertebrate community in lowland streams. This was integrated in the main research question 'To what extent are macroinvertebrate assemblages in temperate lowland streams influenced by the presence of living macrophytes?'. In this chapter, I will discuss how the research performed in this thesis contributes to answering this research questions and how the obtained results relate to the existing literature. Furthermore, I will discuss the strengths and limitations of the work performed in this thesis, together with practical applications for management and opportunities for future study.

Observations from this thesis

The research performed in this thesis studied the trophic- and non-trophic interactions between aquatic macrophytes and macroinvertebrates. As the only chapter to deal exclusively with non-trophic interactions, **Chapter 2** describes the highly significant effect of macrophyte growth form on habitat structural complexity and flow velocity inside vegetation patches, with complex macrophytes having a stronger hampering effect on flow velocity. In turn, macroinvertebrate communities on complex macrophytes showed a higher taxonomic and functional richness and diversity. However, it was not possible to separate the effects of plant identity, structural complexity and flow velocity on the macroinvertebrate community, due to their high degree of intercorrelation. Additionally, the explanatory power of these factors was higher under conditions of high flow velocity, suggesting a role of macrophyte patches as instream flow refugia for macroinvertebrates.

In **Chapter 3 and 4**, evidence was found for the direct consumption of macrophyte tissue by macroinvertebrates and fish, both as living plant tissue and as macrophyte-derived organic matter. Food web reconstruction with stable isotopes in **Chapter 3** showed the consumption of macrophyte tissue by the phytophagous larvae of *Nymphula nitidulata* Hufnagel (Lepidoptera: Crambidae), but also by *Baetis* sp. nymphs (Ephemeroptera: Baetidae), Orthocladiinae larvae (Diptera: Chironomidae), *Orconectus limosus* Rafinesque (Decpapoda: Cambaridae) and the fish *Gobio gobio* L. (Cypriniformes: Cyprinidae). It is expected that macrophyte consumption in *Baetis* sp. nymphs and Orthocladiinae larvae is the result of accidental ingestion during scraping activities associated with the feeding on epiphyton, contrary to the other species that are expected to purposefully consume macrophyte tissue.

In **Chapter 4**, fatty acid measurements, which were taken from a number of basal resources and consumers over the course of one year, suggest that filter-feeding *Simulium* sp. (Diptera: Simuliidae) larvae and *Hydropsyche* sp. (Trichoptera: Hydropsychidae) larvae consume increasing amounts of macrophyte-derived fine particulate organic matter (FPOM) in autumn, when many stream macrophytes senescence and the upper parts die-off.

The potential impact of herbivory, in combination with environmental stressors, is demonstrated in **Chapter 5.** In a case study featuring the invasive crab *Eriocheir sinensis* H. Milne-Edwards (Decapoda: Varunidae), the destructive potential of these crabs is shown in a series of mesocosm experiments in which two stressors (shade and chemical stress) make the vegetation more susceptible to destruction by herbivory.

The indirect role of macrophytes on the aquatic food web is demonstrated in **Chapter 6** in a series of microcosm experiments. In these experiments, living macrophytes are shown to harbour a lower amount of epiphytic algae and bacteria in their epiphytic biofilm compared to artificial macrophytes. However, the biofilm on living macrophytes was also shown to have lower C:N:P ratios compared to artificial macrophytes. This is probably caused by the interaction between the exudation of allelopathic substances and nutrients by living macrophytes. In a related macroinvertebrate growth experiment, these lower C:N:P ratios probably also resulted in the higher observed growth rate of the snail *Haitia acuta* Draparnaud (Gastropoda: Physidae) on complex living macrophytes.

Summarising, these data show that submerged macrophytes have a large effect on aquatic macroinvertebrates and exert influence on their community structure through a variety of non-trophic and trophic interactions. Especially the demonstrated direct and indirect role of macrophytes in the aquatic food web is a topic to which little attention was paid previously.

Strengths and limitations

This thesis offers a broad perspective on the subject of macrophyte-macroinvertebrate interactions. Through a combination of field studies (in the Desselse and Zwarte Nete lowland streams) and greenhouse experiments a variety of effects of macrophytes on macroinvertebrates (and vice versa) was demonstrated. Adopting such a broad scope has a number of advantages and disadvantages. Focussing on many different aspects of macrophyte-macroinvertebrate interactions has the advantage of obtaining a broad overview of the studied ecosystems and enables the incorporation of data from multiple independent studies, as was done for the quantitative consumption of macrophytes in Chapter 3. A disadvantage of this approach is that it is not possible to go into the same level of detail as a more narrow and focussed study on one specific subject.

A similar consideration applies to the usage of either field observations or (semi-)controlled greenhouse experiments. Although it would be ideal to perform all different studies in the same environment to make the results easily comparable, there are some studies that are better suited for either a field study or for an experiment. Field studies have the advantage of registering environmental variation and complex ecological interactions, which makes them very suitable for observing 'real world' patterns such as the distribution and diet of macroinvertebrates under natural circumstances. Many characteristic attributes of lowland streams, such as a the flow-mediated transport of FPOM or the colonisation of macrophyte patches with macroinvertebrates from the local species pool, are very difficult to create under controlled experimental conditions, making field studies a necessity when incorporating these phenomena in the study design. However, field studies can only provide correlative evidence for ecological processes, whereas experiments are required to infer causation from this correlation. Additionally, experiments offer the opportunity to study the isolated

effects of individual environmental factors that are masked by the number and complexity of simultaneously occurring processes under natural conditions. For this reason, greenhouse experiments were performed to test the interaction between different levels of herbivory by crabs and environmental stressors (Chapter 5) and the effect of living macrophytes, compared to artificial ones, on the epiphytic biofilm (Chapter 6). However, care should still be taken to extrapolate the results from these experiments, which were performed under conditions without water flow, to field situations in lowland streams. For example, high water flow velocities might form an additional stress factor for macrophytes (Riis et al. 2000, Riis and Biggs 2003), making them less resilient to other forms of stress (e.g. Chapter 5). Furthermore, water flow might decrease the level of DIC limitation for macrophytes and epiphytic algae, by transporting DIC to the macrophyte patch and by reducing the thickness of the diffusive boundary layer, resulting in faster diffusion rates of dissolved gasses such as CO_2 (Koch 1993, Madsen et al. 1993). This might in turn decrease the negative effect of living macrophytes on epiphytic algae through DIC limitation.

How does it all fit together?

The research performed in this thesis demonstrated a wide variety of trophic and non-trophic interactions between submerged macrophytes and aquatic macroinvertebrates. Together with existing literature, an overview of the interactions between macrophytes, macroinvertebrates and the lowland stream environment can be constructed that reflects the complexity of the ecosystem (Figure 7.1). It should be noted that, although the scheme looks complex, it is still a gross oversimplification of the true complexity of these ecosystems, as many factors are not taken into account for the sake of readability.

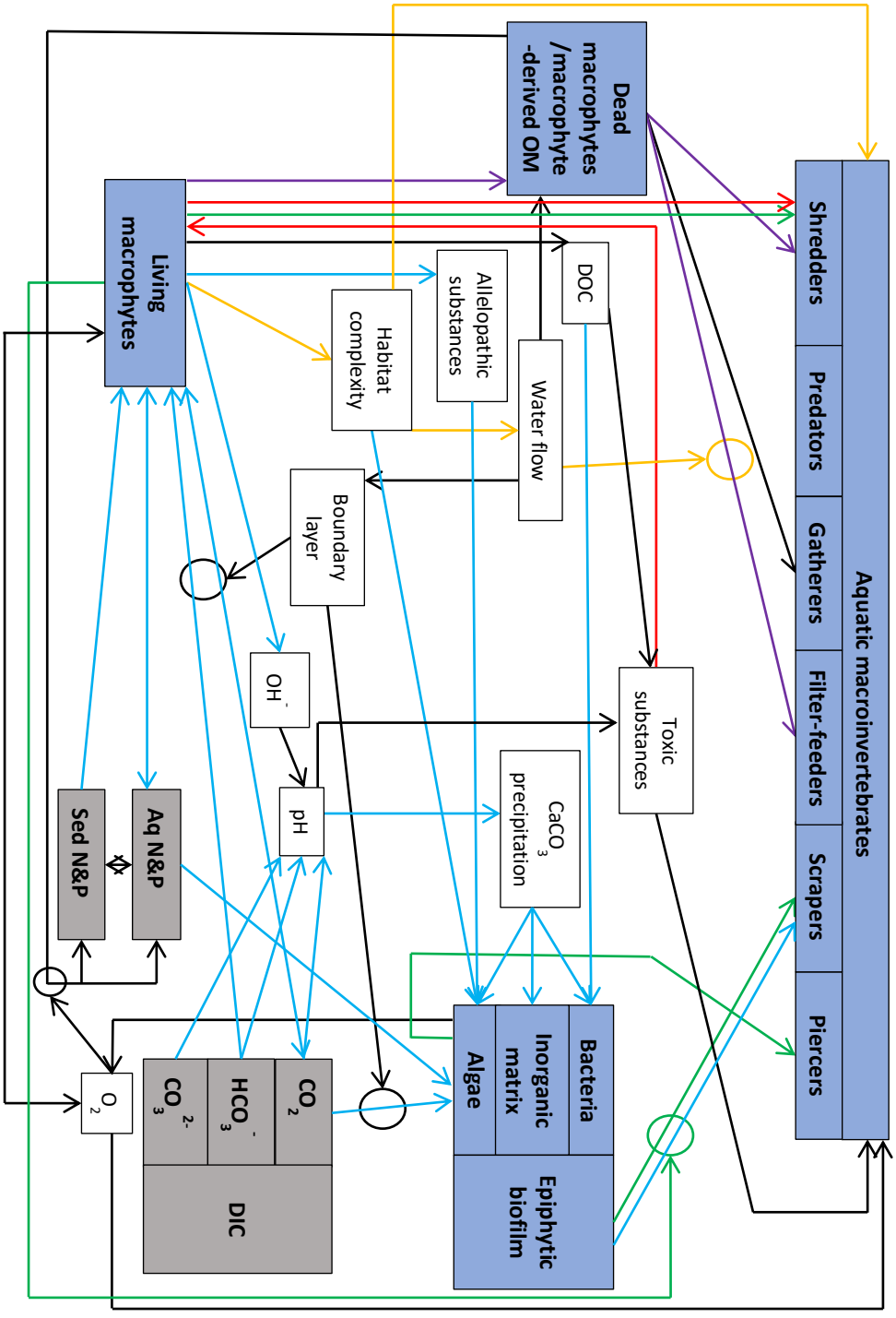
Effects of macrophyte structural complexity

Macrophytes, and actually any biological or non-biological physical structure, are known to increase the structural complexity of a habitat, leading to a higher diversity of animal body-size distributions due the increased physical habitat heterogeneity (Schmid et al. 2002, McAbendroth et al. 2005, Ferreiro et al. 2011), an increased amount of colonisable microhabitats (McNett and Rypstra 2000, McAbendroth et al. 2005) and a better refuge against predation (Warfe and

Barmuta 2004, 2006). This results in a higher macroinvertebrate richness and diversity, together with a higher food web complexity, as the complexity of the macrophyte increases (Chapter 2; Kefi et al. 2012, Borst et al. 2018). The presence of macrophyte structures in the water layer also provides resistance to water flow, reducing flow velocity in macrophyte stands and thereby creating a habitat for limnophilous macroinvertebrate species (Chapter 2; Sand-Jensen and Mebus 1996, Bell et al. 2013). This also causes the settlement and disposition of organic material within the macrophyte patch (Sand-Jensen and Mebus 1996, Schoelynck et al. 2013). The reduction in flow velocity is positively correlated to the structural complexity and density of the macrophytes that form the macrophyte stand (Chapter 2; Madsen et al. 2001, Bell et al. 2013, Schoelynck et al. 2013). Additionally, the flow-attenuating effect of macrophyte patches creates a gradient of high flow velocity at the upstream section of a macrophyte patch and a lower flow velocity at the downstream section, in this way further increasing environmental heterogeneity (Chapter 2; Peralta et al. 2008, Bell et al. 2013). Lastly, structurally complex macrophytes can support more epiphyton, an important food source for many macroinvertebrates, than plants with a simpler growth form, despite having a similar surface area (Chapter 6; Taniguchi et al. 2003, Warfe and Barmuta 2006). All these habitat-creating and habitat-modifying effects of macrophytes have a significant effect on the epiphytic macroinvertebrate community. Stands of simple macrophytes, with a high prevailing flow velocity, were observed to be inhabited by a relatively small number of rheophilous macroinvertebrates, predominantly filter-feeders (Chapter 2; Bell et al. 2013). On the other hand, a far richer and more diverse macroinvertebrate community was observed in vegetation patches consisting of more complex macrophytes, with a corresponding gradient in flow velocities (Chapter 2; Bell et al. 2013).

Macrophyte-epiphyton interactions

In addition to the positive effect of macrophyte complexity on the abundance of epiphyton described earlier, macrophytes interact with their epiphytic biofilm in a number of different ways (Figure 7.1). It should hereby be noted that macrophytes and epiphytic algae are both primary producers and compete for the same light conditions and nutrient sources. A high epiphyton cover can severely reduce the macrophyte's growth and survival due to excessive shading (e.g. Brönmark 1989).


Aquatic macroinvertebrates
Shredders
Predators
Gatherers
Filter-feeders
Scrapers
Piercers
Dead macrophytes /macrophyte -derived OM
Living macrophytes
DOC
Allelopathic substances
Habitat complexity
Water flow
Boundary layer
OH-
pH
Toxic substances
CaCO3 precipitation
Sed N&P
Aq N&P
O2
CO2
HCO3-
CO3 2-
DIC
Bacteria
Inorganic matrix
Algae
Epiphytic biofilm

Furthermore, macrophytes with a high epiphyton cover have been shown to be more susceptible to herbivory (Hidding et al. 2016). Living macrophyte therefore secrete a number of allelopathic compounds, such as phenolics, sulphuric compounds or alkaloids, that inhibit the growth of specific epiphytic algae (e.g. Wium-Andersen et al. 1983, Elakovich and Yang 1996, Gross 2003). Additionally, DIC, particularly CO_2, is depleted in the water layer as a result of the underwater photosynthesis, causing a rise in water pH and thus also contributing to the precipitation of $CaCO_3$ on the leaf surface (e.g. Chapter 6; Pedersen et al. 2013). These $CaCO_3$ encrustations are known to hinder the development of epiphytic algae and bacteria, for example through the competition for space or the adsorption of DOC, amino acids and fatty acids in the biofilm (Wetzel and Rich 1973, Cattaneo and Kalff 1978, Sand-Jensen 1983). On the other hand, macrophytes also excrete dissolved phosphorus and DOC to the water layer, that have been shown to be beneficial to the growth of epiphytic algae and bacteria respectively (e.g. Sondergaard 1981, Wetzel 1983, Burkholder and Wetzel 1990). DOC excreted by aquatic macrophytes can also limit the toxicity of heavy metals and certain other toxicants (e.g. Gensemer et al. 1999, Christl et al. 2001), whereas the increase in water pH, caused by their photosynthesis (e.g. Chapter 6; Pedersen et al. 2013), reduces the toxicity of certain toxicants and facilitates their hydrolysis (Chapman and Cole 1982, Cusimano et al. 1986, Brogan and Relyea 2014). Additionally, the oxygen produced during photosynthesis, which is excreted to the sediment through the macrophytes' roots, oxygenates the sediment so that the toxic sulphide is converted to non-toxic sulphate (Lamers et al. 2013). This oxidation may however also mobilise sulphide-bound metals, leading to an increase in their bioavailability (Teuchies et al. 2011, De Jonge et al. 2012). Furthermore, macrophytes may also accumulate heavy metals from the sediment, enabling their entry in the aquatic food web upon macrophyte consumption or senescence and decay (e.g. Jackson 1998).

Figure 7.1 (previous page). Schematic overview of the interactions between macrophytes, epiphytic biofilm, macroinvertebrates and the abiotic lowland stream environment. Interactions that were studied in Chapter 2 are indicated in orange arrows, interactions from Chapter 3 in green arrows, interactions from Chapter 4 in purple arrows, interactions from Chapter 5 in red arrows and interactions from Chapter 6 in blue arrows. Interactions not studied in this thesis are indicated by black arrows.

In the end, it seems likely that the net effect of living macrophytes on their associated epiphytic biofilm depends on a number of plant specific traits that determine the strength of the earlier mentioned effects of macrophytes on their epiphyton (Chapter 6; Jones et al. 2002, Grutters et al. 2017). It might for example be expected that slow growing, complex or mainly CO_2-using macrophytes will have a higher epiphyton cover than plants that have a higher growth rate, a simple growth form or are able to utilise HCO_3^- (Jones et al. 2002, Grutters et al. 2017). In addition to all these direct effects of macrophytes on their associated epiphytic biofilm, macrophytes also indirectly influence the epiphyton through the formation of discrete macrophyte patches and their influence on water flow velocity (cf. Chapter 2; Schoelynck et al. 2012, Schoelynck et al. 2018). Inside dense macrophyte stands, light availability decreases drastically, whereas the macrophytes' photosynthesis can lead to a depletion of dissolved CO_2 and a consequential rise in pH (Carpenter and Lodge 1986, Carter et al. 1991). At the top and borders of macrophyte patches, these conditions are more favourable for epiphyton growth, so that more epiphyton is generally found at these locations (Blindow 1987, Vis et al. 2006). Additionally, the higher water flow velocities at the borders of macrophyte patches in lotic systems can also reduce the thickness of the diffusive boundary layer, resulting in faster diffusion rates of dissolved gases such as CO_2 (Koch 1993, Madsen et al. 1993).

Herbivory

All these effects of living macrophytes on their associated epiphytic biofilm also have a significant effect on the herbivorous macroinvertebrate community that depends on the epiphyton as their main food source (Chapter 3; Chapter 6; Cummins and Klug 1979). Indeed, grazer densities have been observed to follow the pattern of epiphyton densities inside macrophyte patches, as the highest grazer densities are often found at the edges of macrophyte patches, where the epiphyton density is highest, despite the greater risks of predation in those regions (Marklund et al. 2001). Additionally, macrophytes can also lower the C:N:P stoichiometry, and thus improve the nutritive quality, of the epiphytic biofilm through the excretion of nutrients (Chapter 6; Burkholder and Wetzel 1990, Bowman et al. 2005). In turn, this can have a positive effect on the growth and survival of macroinvertebrate grazers (e.g. Sterner and Elser 2002). Grazing activities on epiphytic algae by

macroinvertebrates form an important part of the aquatic food web, with 50-70% of the standing epiphyton biomass being consumed each day (Kesler 1981, Armitage et al. 1995). On the other hand, the aquatic macrophytes themselves were generally not considered to form an important food source for many generalist herbivorous macroinvertebrates, due to the presence of inhibitory secondary metabolites, such as alkaloids, glucosinolates and polyphenolics, which can act as a chemical defence against herbivory (Sotka et al. 2009, Gross and Bakker 2012). Feeding trials with omnivorous macroinvertebrates and fish have indeed confirmed a preference for macrophytes with low concentrations of deterring chemicals (Li et al. 2004, Dorenbosch and Bakker 2011). Most of the herbivory on submerged macrophytes is attributed to a few specialist Lepidoptera, Coleoptera and Diptera taxa, generalist omnivorous crabs and crayfish and generalist herbivorous fish, whereas the majority of generalist macroinvertebrates are instead assumed to consume mostly epiphyton (Chapter 3; Chapter 5; Newman 1991, Olsen et al. 1991, Cronin et al. 1998, Dorenbosch and Bakker 2011). However, these previous studies did not take into account the accidental consumption of macrophyte tissues by macroinvertebrate grazers during their grazing activities on the epiphytic biofilm (Chapter 3; Yule 1986, Karouna and Fuller 1992). Grazing macroinvertebrates hereby scrape and ingest the upper layer of the macrophyte leaf during their grazing on the epiphytic biofilm, after which these macrophyte tissues are also assimilated by the animals (Chapter 3; Karouna and Fuller 1992). This ingestion of macrophyte tissue during grazing is strongest for animals that consume algae that are closely attached to the leaf surface, i.e. invertebrates from the scraper functional group such as *Baetis* sp. (Ephemeroptera: Baetidae) nymphs, whereas it is less pronounced for animals that graze farther from the leaf surface (Figure 7.2), for example gatherers like Orthocladiinae (Diptera: Chironomidae) larvae (Chapter 3). The impact of this consumption on standing macrophyte biomass seems small, with only a few percent consumed by the dominant macroinvertebrate taxa each day (Chapter 3), compared to the high turnover rates of epiphyton. However, when compared to the macrophytes' growth rates, the percentage of macrophytes consumed each day by the macroinvertebrates accounts for 18 to 105% of the daily primary production (Chapter 3), meaning that these animals can potentially hamper or even restrict the growth of macrophytes.

In addition to this accidental ingestion of macrophyte tissue by relatively small macroinvertebrates, there also exists herbivory on a larger scale by omnivorous crabs and crayfish, such as the invasive crab *Eriocheir sinensis* H. Milne-Edwards (Decapoda: Varunidae) and the crayfish *Orconectus limosus* Rafinesque (Decapoda: Cambaridae), that can potentially have a devastating effect on submerged macrophyte stands (Chapter 3; Chapter 5; Lodge et al. 1994, Jin et al. 2003, Soes et al. 2007). However, the majority of the destruction by these generalist omnivores is not caused by actual consumption, but by the cutting and uprooting of macrophytes during their foraging and feeding activities (Chapter 5; Lodge et al. 1994, Jin et al. 2003). The destructive impact of these animals on the submerged vegetation is of course dependent on their density (Chapter 5; Wang et al. 2017) but also on the vegetation's resilience and resistance to herbivory (e.g. Hidding et

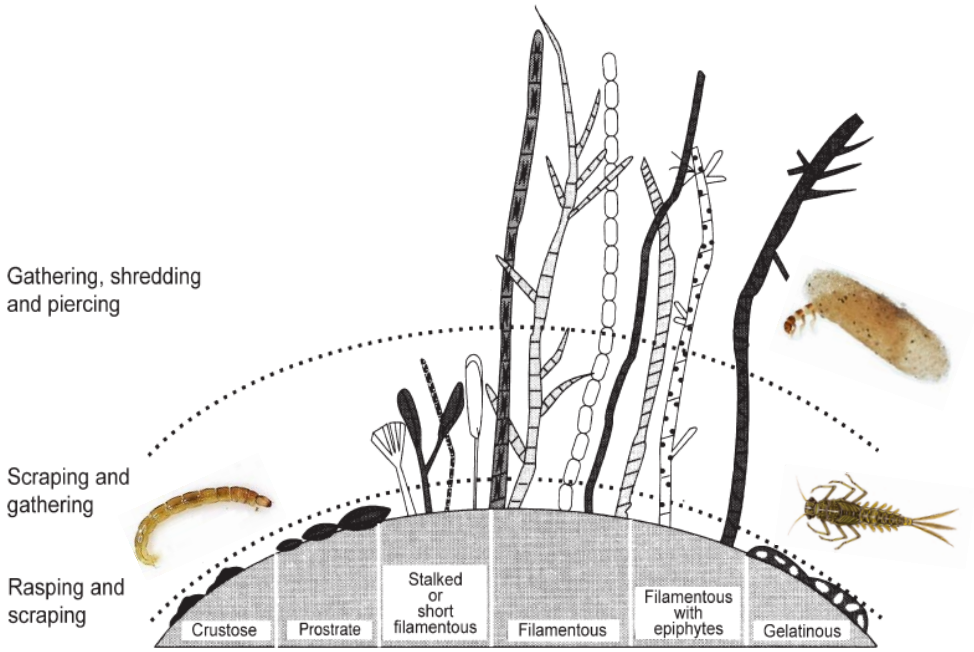


Figure 7.2 Overview of the major growth forms of epiphyton assemblages, together with the different modes of herbivory that are expected to be most effective with the particular growth forms. For each layer and feeding mode, a representative macroinvertebrate is shown that was also encountered in this thesis. From top to bottom: a piercer; *Hydroptila* sp. (Trichoptera: Hydroptilidae) larva, a gatherer; an Orthocladiinae larva and a scraper; a *Baetis* sp. nymph. Note that the animals and algae are not drawn to scale. (Adapted from Steinman 1996, Allan and Castillo 2007).

al. 2016). Under similar herbivore densities, additional stressors, such as light limitation or toxic substances, have been shown to lead to an increased decline in submerged macrophytes (Chapter 5), possibly explaining the sudden disappearance of macrophytes in some natural systems (Vlaamse Milieu Maatschappij 2015). Light limitation could for example be caused by an increased turbidity, for example due to bioturbation by crabs or crayfish, or by competition with phytoplankton or epiphytic algae, both caused by a high nutrient availability (e.g. Brönmark 1989, Soes et al. 2007).

The role of macrophyte-derived organic matter in the food web

Besides all these aforementioned interactions between macroinvertebrates and living macrophytes, dead and decaying macrophytes also play an important role in the lowland stream ecosystem. As macrophytes senescence and decay, they leach many of their inhibitory compounds and become colonised by microorganisms, becoming more palatable and nutritious to herbivorous and detritivorous macroinvertebrates (Brönmark 1989, Newman 1991, France 2011). This macrophyte-derived coarse particulate organic matter (CPOM) is then subsequently consumed by detritivorous macroinvertebrate shredders, such as Limnephilidae (Trichoptera) or Gammaridae (Amphipoda), and broken down to FPOM (Smock and Harlowe 1983, Suren and Lake 1989). It should however be noted that macrophyte-derived CPOM does not constitute the majority of the diet of these animals, that instead mostly consists of allochthonous CPOM and its associated biofilm (Chapter 3; Chapter 4; France 2011). Fatty acid measurements have shown that filter-feeding macroinvertebrates such as *Simulium* sp. (Diptera: Simuliidae) and *Hydropsyche* sp. (Trichoptera: Hydropsychidae), in turn consume the macrophyte-derived FPOM, whereby this diet source becomes especially important at the end of the growing season, when many macrophytes die off and are broken down (Chapter 4). The remaining macrophyte-derived organic matter which is not consumed as CPOM or FPOM by macroinvertebrates is converted to bacterial and fungal biomass through the microbial loop, from where it re-enters the aquatic food web (Polunin 1984, Pusch et al. 1998, Hieber and Gessner 2002).

Conclusion

The main research question of this thesis was to what extent macroinvertebrate assemblages in lowland streams are influenced by the presence of living macrophytes. Based on literature and the observations described in this thesis, it can be concluded that aquatic macrophytes play a very important key role in the temperate lowland stream ecosystem and that they thereby have a significant positive effect on the diversity of the macroinvertebrate community. By their physical structures and role as ecosystem engineers, macrophytes increase habitat complexity and act as foundation species though their non-trophic facilitation of food web complexity, which includes acting as a substrate for epiphytic algae and bacteria (Chapter 2, Chapter 6, Borst et al. 2018). Besides these non-trophic interactions, macrophytes also have a more direct effect on food web dynamics, by serving as a direct food source for herbivorous and detritivorous macroinvertebrates (Chapter 3, Chapter 4, Chapter 5).

Applications for management

The research described in this thesis is of a fundamental origin and is thus not directly driven by a specific question driven by practical need. However, observations from this thesis may still be useful for the management of temperate lowland streams.

In many of these streams, the development of aquatic vegetation increases hydraulic resistance, which leads to reduced flow velocities and increased stream water levels (e.g. De Doncker et al. 2009). To ensure the drainage of adjacent arable land and to lower the risk of flooding during high precipitation events, macrophytes are regularly mown and removed from the stream. This macrophyte removal is generally indiscriminate and results in a complete removal of all macrophytes from the mown stream section. Although macrophyte regrowth from stem fragments or roots is generally fast and can occur within three to five weeks (Crowell et al. 1994, Bal et al. 2006), the removal of the majority of macrophyte biomass is likely to have a very large effect on macroinvertebrate populations, for which the macrophytes constitute their primary habitat and who depend on the food web facilitated by the macrophytes (Chapter 2, Chapter 3, Chapter 6, Borst et al. 2018). In order to minimise damage to the stream ecosystem it is advised to leave patches of standing macrophyte biomass unmown, which can serve as (flow) refugia for remaining

macroinvertebrates, from which they can later recolonise the regrown vegetation patches. These alternative mowing patterns have been shown to only minimally increase hydraulic resistances compared to traditional mowing and in this way still ensure an appropriate amount of stream discharge (Bal et al. 2011). Mowing vegetation in this way might also create a mosaic of competitive and disturbance-tolerant pioneer macrophyte species, in this way increasing stream macrophyte diversity and thus ultimately macroinvertebrate diversity (Chapter 2, Schoelynck 2011). In turn, a high species diversity might increase the ecosystem's resilience to other disturbances (e.g. Covich et al. 2004).

Additionally, it is advised to prioritise actions to reduce the amount of environmental stress the macrophytes and the associated biota are subjected to in the lowland stream environment, especially targeting eutrophication, siltation and organic pollution. Besides situations where the amount of environmental stress is enough to directly eliminate aquatic vegetation, for example, as a result of massive algal blooms triggered by excess nutrients, even small amounts of stress can result in macrophytes becoming more susceptible and less resilient to other disturbances such as herbivory, which can in turn lead to a decline or even collapse of the aquatic vegetation (Chapter 5, Hidding et al. 2016). In order to prevent vegetation from collapsing and the shift to a far less biodiverse unvegetated state (e.g. Heck and Crowder 1991), investments should be made to decrease the runoff from agricultural fields, by for example reducing the amounts of applied fertiliser and by establishing riparian buffer zones, and to reduce the number of sewage overflows.

Future research

As shown in the previous sections, the interactions between living macrophytes and aquatic macroinvertebrates are numerous and complex, with plants and animals influencing each other through various direct or indirect means. The research performed in this thesis obtained new results, such as the previously underestimated direct and indirect role of macrophytes and macrophyte-derived material in the aquatic food web, which further improve our understanding of macrophyte-macroinvertebrate interactions. However, many aspects of these interactions are still unclear, and several new questions have arisen based on the results obtained during this thesis. An overview of these knowledge gaps, together with possible study directions is presented here:

- The research presented in **Chapter 2** showed that macrophyte taxonomic identity, together with growth form and the effect on water flow velocity, had a significant impact on macroinvertebrate community structure. However, due to the correlative nature of this study, it was not possible to disentangle the individual effect of these three different environmental factors. To make things more complicated, these three factors are also closely correlated with each other, as 1.) flow velocity determines whether a specific plant species can colonise and persist in lotic habitats (e.g. Bloemendaal and Roelofs 1988), 2.) macrophyte growth form is determined by both the plant species and the prevailing water flow conditions (Madsen et al. 2001), and 3.) water flow is influenced by macrophyte density and growth form (Sand-Jensen and Mebus 1996, Bell et al. 2013, Schoelynck et al. 2013). It was hereby hypothesised that some macroinvertebrate functional groups would be more heavily influenced by one or several of these factors than others. Specialist herbivorous macroinvertebrates would, for example, be most heavily influenced by the species of macrophyte present, whilst flow velocity would be more important for filter-feeders (Gaevskaya 1969, Tachet et al. 1992, Finelli et al. 2002). The individual importance of these three environmental variables could, for example, be studied in a controlled field or laboratory situation (e.g. artificial stream mesocosms), whereby the strength of different environmental factors can be controlled. By comparing macroinvertebrate communities on plastic plant patches with communities on living plant patches, with the same complexity and flow conditions, it would for example be possible to measure the importance of plant identity compared to the macrophyte's influence on water flow velocity. Similarly, flow velocity can be artificially manipulated to measure the strength of this environmental factor on the composition of the macroinvertebrate community. However, the effect of water flow-induced macrophyte reconfiguration on macrophyte complexity should also be taken into account (Schoelynck et al. 2013). It should furthermore be noted that this study approach is quite labour-intensive due to the large sample size when a full factorial design is implemented.

- Another interesting opportunity for further research manifested itself in **Chapter 3**, where evidence was presented for the accidental consumption of macrophyte tissue by macroinvertebrate grazers during their feeding activities on the epiphytic biofilm. This theory is based on literature data on the feeding mode

of different groups of epiphyton grazers, combined with stable isotope data and a study that showed varying degrees of leaf erosion from different macroinvertebrate grazers during epiphyton feeding (Karouna and Fuller 1992). It would be very interesting to test this theory and attempt to witness this accidental leaf consumption first-hand. This could for example be done by filming the behaviour of a selection of macroinvertebrate grazer species under a microscope during feeding and subsequently investigating the macrophyte leaf surface for traces of grazing damage using electron microscopy.

- Furthermore, it would be interesting to get a more complete overview of food web dynamics and the trophic role of macrophytes, as presented in **Chapter 3 & 4**, over a longer time period and for different systems. By measuring both stable isotope and fatty acid signatures of primary producers and consumers over the course of at least a full year, a more detailed overview of the fate of macrophyte-derived material in the aquatic food web could be derived. Furthermore, this provides an opportunity to detect shifts in consumer diets, as the availability of different food sources changes during the year. Possible difficulties with this approach, and the reason why this hasn't been attempted in this thesis in the first place, are the high financial and labour costs of the different analyses, and the large amount of biomass needed for the measurements.

- The studies described in this thesis were done in a limited number of environments; two adjacent lowland streams and a greenhouse in Belgium. An interesting study opportunity would therefore be to see how these observed processes and interactions occur in different aquatic systems. Although it is expected that many of these principles, such as the positive effect of habitat complexity on biodiversity, are universal and occur in the majority of lowland streams and freshwater systems (McAbendroth et al. 2005, St Pierre and Kovalenko 2014), some others may behave differently under different environmental conditions. Under eutrophic conditions, the interactions between living macrophytes and their biofilm might for example be expected to deviate sharply from the one observed in this thesis, as the epiphytic algae become less dependent on nutrients by the macrophytes.

- Finally, many of the field studies or experiments in this thesis have been performed for only a small number of macrophyte species at a time. While this has certainly shed light on many aspects of macrophyte-macroinvertebrate

relationships, it also brought forth a number of new questions on how the nature of these interactions varies across a range of different macrophyte species, which vary in their functional traits. For example, significant differences in responses of the epiphytic biofilm and macroinvertebrate grazers were detected between *Vallisneria spiralis* L. (Hydrocharitaceae) and *Egeria densa* Planch (Hydrocharitaceae) (**Chapter 6**). To better study the effects that individual macrophyte traits, such as growth form, method of DIC use, growth rate, allelochemical potential, growth strategy or structural defences against herbivory, have on their direct and indirect interactions with macroinvertebrates, these interactions can be tested across a wide range of macrophyte species, all with different traits (cf. Grutters 2017).

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Curriculum vitae

Jan-Willem Wolters was born on 16 June 1990 in Groningen, the Netherlands. Already at a young age, he was fascinated by nature, with a special love for cold-blooded and aquatic creatures. Because of this special interest, it was no surprise that he decided to study biology at the Radboud University Nijmegen in 2008. During his study, Jan-Willem specialised in aquatic ecology and biogeochemistry. He performed his Bachelor internship at the Bargerveen Foundation, where he studied the effects of the mineral composition of invertebrate prey on *Lacerta agilis* consumers. In his first Master internship at the NIOZ Yerseke, he travelled to Thailand to investigate the effects of catchment land use on the nutrient status of the adjacent mangrove forests. For his second Master internship at B-Ware Research Centre, he studied the ecohydrology of a groundwater-fed fen and the origin of the high groundwater nitrate concentrations. Jan-Willem graduated Cum Laude in 2013.

Driven by his interest in scientific research and his love for nature, Jan-Willem started a PhD in 2014 at the Ecosystem Management Research Group (ECOBE) at the University of Antwerp, in cooperation with Wageningen Environmental Research. In this PhD study, he investigated the various trophic and non-trophic interactions between macrophytes and macroinvertebrates in lowland streams. Currently, he is employed as an ecologist at the province of Overijssel.

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