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

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

Short title: Effects of living macrophytes on epiphyton

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

Abstract

1. 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.

2. 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.

3. 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.

4. 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.

5. 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 & Lodge, 1986), increase habitat complexity (McAbendroth *et al.*, 2005; O Hare & Murphy, 1999), provide a refuge against predation (Warfe & Barmuta, 2004; Warfe & Barmuta, 2006) and reduce water flow velocity in lotic ecosystems (Sand-Jensen & 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 (Bakker *et al.*, 2016; Wolters *et al.*, 2018a), 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 (Allan & Castillo, 2007; Cummins & Klug, 1979).

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 of light conditions, nutrient availability and herbivore grazing pressure than macrophytes with a simpler growth form (Ferreiro, Giorgi & Feijoo, 2013; Tessier *et al.*, 2008; Warfe & Barmuta, 2006). In doing so, they typically support more biofilm per unit area than simple macrophytes, despite similar total surface areas (Ferreiro *et al.*, 2013; Tessier *et al.*, 2008; Warfe & Barmuta, 2006). 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 (Burkholder & Wetzel, 1990; Carpenter & Lodge, 1986; Gross, 2003; Wigand *et al.*, 2000).

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, Chambers & Schindler, 2005), while DOC excretions can have a positive effect on bacterial biomass and productivity in that biofilm (Kirchman *et al.*, 1984; Theil-Nielsen & 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 (Gross, 2003; Wigand *et al.*, 2000). Epiphyton is however often less affected by these allelopathic compounds than phytoplankton (Hilt & 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 (Fink & Von Elert, 2006; Hart & Robinson, 1990), 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, Colmer & Sand-Jensen, 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 (Iwan Jones *et al.*, 2000; Zimba & Hopson, 1997). 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 Wolters et al. (2018b), 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 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 & 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 & Barko, 1985), which is essentially demineralised water with added minerals ($CaCl_2$ • 2 H_2O: 91.7 mg L^{-1}; $MgSO_4$ • 7 H2O: 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-NO_3^- L^{-1}, 11.4 µg N-NO_2^- L^{-1}, 22 µg N-NH_4^+ L^{-1}, 3.6 µg P-PO_4^{3-} L^{-1}, Alkanity: 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 S1).

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 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 section, 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

212 Huang and Schulte (1985). Sample P content was subsequently measured on ICP-OES (iCAP
213 6300 Duo view, Thermo Fisher, Waltham, Massachusetts, USA).
214 The number of biofilm bacteria was determined using epifluorescence microscopy
215 after staining with 4',6-diamidino-2-phenylindole (DAPI) following the general protocol of
216 Porter & Feig (1980). For this purpose, the macrophyte subsample that was collected per
217 experimental container during the harvest was stored in plastic 50 mL tubes containing 70%
218 ethanol at -18 °C until later microbial analyses. Biofilm bacteria were first detached from
219 these macrophyte fragments by vigorously shaking and by sonicating for 15 minutes in an
220 ultrasonic bath. Macrophyte fragments were then removed from the tubes and rinsed with
221 MilliQ water to remove potentially remaining biofilm bacteria. The 50 mL tubes containing
222 the bacterial suspension were centrifuged at 3000 rpm for 5 min and the supernatant was
223 discarded until 10 mL of sample remained. This was then resuspended by vigorously shaking
224 and sonicating for 15 minutes in an ultrasonic bath. Aliquots of 200 to 500 µL were
225 subsequently taken and filtered, together with 2 mL MilliQ to ensure a homogeneous
226 suspension of bacterial cells, over 0.2 µm polycarbonate Millipore GTTP filters (Sigma-
227 Aldrich, Poole, UK) supported by a 0.45 µm mixed cellulose ester backing filter (Sigma-
228 Aldrich, Poole, UK). Polycarbonate filters were hereafter cut in four quarters and one quarter
229 per filter was mounted on glass slides, to be mounted and stained with a Citifluor A1
230 (Citifluor Ltd., London, UK) and Vectashield (Vector laboratorios, Burlingame, California,
231 USA) buffer (4:1, v:v) to which DAPI was added to a concentration of 1 mg L^{-1}. This was
232 then allowed to incubate for at least 10 minutes in the dark, after which bacterial cells were
233 observed at 1000× magnification under a Zeiss Axioplan 2 epifluorescence microscope and
234 photographed with an EXi Blue Fluorescence Microscopy Camera (QImaging). A minimum
235 of 10 microscopic fields and 400 cells were counted for each sample (Kirchman, 1993).
236

Water quality measurements

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 & 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 collector-gatherer and *H. acuta* a scraper (Heino, 2005; Monakov, 2003). 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. Depending on the treatment (simple, complex, alive, artificial) 2 basal macrophyte fragments of 5 cm were added to the jars, as well as 5 individuals of one of the 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, Bretz & Westfall, 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, Harper & Ryan, 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 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 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 1).

Significant differences were also observed in the elemental composition of the epiphytic biofilm (Table 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.

No uniform distinction in bacterial density could be made between either simple and complex or artificial and living macrophytes (Figure 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 S2A, r = -0.846, P = 0.001).

Water quality measurements

All measured water quality parameters displayed significant differences over time during the experiment (Table 3), whereby significant differences among the different macrophyte treatments were observed for all N (i.e. $N\text{-}NH_4^+$, $N\text{-}NO_2^-$, $N\text{-}NO_3^-$ and total-N) and P parameters (Table 3). Interaction effects were observed for all parameters, except for EC, O_2, $N\text{-}NH_4^+$ and DOC (Table 3, Figure S3). Two different trends can be distinguished, regarding nutrient levels and dissolved inorganic carbon (DIC). N concentrations show a sharp decline and approach zero after the onset of the experiment (Figure S3I), 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 S3H). 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 S3A) and a lower alkalinity (Figure S3B), combined with lower concentrations of dissolved CO_2 (Figure S3C), were measured for the living macrophytes after this change. For the artificial macrophyte treatments, these changes showed a significant positive relationship with the abundance of epiphytic algae (Figure S2C&D, pH: r = 0.784, P = 0.007, CO_2: r = -0.742, P = 0.014), while they showed a significant positive correlation with final plant dry biomass in the living macrophyte

359 treatments (Figure S2E&F, pH: r = 0.949, P = 0.000, alkalinity: r = -0.879, P = 0.001, CO_2: r
360 = -0.950, P = 0.000). Additionally, biofilm Ca content showed a significant negative
361 correlation with dissolved CO_2 concentrations (Figure S2G, r = -0.801, P < 0.001).
362 Although DOC concentrations displayed large fluctuations over time, no clear
363 differences between the treatments were observed (Figure S3E), which was also true for the
364 DOC quality, expressed as SUVA (Figure S3F). No significant differences in EC were
365 observed before the water change, but *E. densa* treatments showed a significantly lower EC
366 after the water change, which also resulted in lower overall EC values (Figure S3G).
367
368 *Macroinvertebrate growth experiment*
369 Macroinvertebrates in all treatments increased in length during the experiment (Figure
370 3A&B), and this effect was significant for all *C. dipterum* treatments, except the simple
371 artificial one (Figure 3B, Table 4), and for all *H. acuta* treatments (Figure 3C, Table 4).
372 Additionally, *H. acuta* from the living *Egeria* treatment showed a significantly larger shell
373 length increase than snails from the other treatments (Figure 3C, Welch test: $F_{df=3,8.6} = 4.05$; P
374 = 0.047), whereas no significant differences in growth rate were observed for *C. dipterum*
375 (Figure 3C, one-way ANOVA; $F_{df=3,16} = 3.09$; P = 0.056).

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 & 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

401 ratio was clearly above 7, the ratio that indicates co-limitation (Hillebrand & Sommer, 1999),
402 suggesting that nutrient availability was a more important limiting factor for algal growth in
403 those situations.

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

413 Besides having a negative effect on the growth of epiphytic algae, $CaCO_3$
414 encrustations can also have a potential inhibiting effect on the development of heterotrophic
415 bacteria, as was demonstrated in this study by the strong negative relationship between
416 bacterial density and biofilm Ca content for all treatments except *E. densa*. This can be
417 explained by the strong adsorption of free DOC, amino acids and fatty acids to the $CaCO_3$ in
418 the biofilm, effectively immobilizing these substances and making them unavailable for
419 bacterial uptake (e.g. Wetzel & Rich, 1973). It is therefore all the more remarkable that the
420 highest bacterial density was observed in *E. densa* treatments, the macrophyte with the
421 highest biofilm Ca content. A possible explanation for this fact could be that *E. densa*
422 possesses polar leaves that take up HCO_3^- on the abaxial side of the leaf and excrete OH^--ions
423 on the adaxial side, so that $CaCO_3$ precipitation takes place only on the adaxial side, whereas
424 $CaCO_3$ is precipitated on both leaf sides for other macrophytes (Prins & Elzenga, 1989; Prins
425 *et al.*, 1980). 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 (Espinosa-Rodriguez *et al.*, 2016; Gao *et al.*, 2011; Gette-Bouvarot *et al.*, 2015). 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 & 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 (Bowman *et al.*, 2005; Burkholder & Wetzel, 1990), 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 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 & 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 (Allan & Castillo, 2007; Monakov, 2003). 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 (Heino, 2005; Monakov, 2003). 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 & Von Elert, 2006)), meaning that effects of biofilm quality would only be visible under conditions of low epiphyton quantity. However, no quantitative 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 & 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, Blindow & Hargeby, 2001). It might therefore be

475 expected that stoichiometric differences in epiphyton quality are better reflected in consumer
476 growth rate in these natural systems compared to our experiment.
477
478 *Conclusions*
479 This study observed significant differences in epiphyton cover between simple and complex
480 macrophytes and between artificial and living macrophytes. The influence of living
481 macrophytes on the epiphytic biofilm likely depends on plant-specific traits. A fast growth
482 rate, complex growth form, HCO_3^- usage, polar leaves and the exudation of strong
483 allelochemicals are hereby likely associated with a low epiphyton cover, while slow growing,
484 simple, CO_2 using plants with nonpolar leaves and without strong allelochemicals likely have
485 a higher epiphyton cover. Additionally, epiphytic biofilm C:N:P molar ratios were lower on
486 living macrophytes, probably due to the plant's role as nutrient pump, although this effect will
487 likely diminish under more eutrophic conditions. These changes in biofilm stoichiometry had
488 no effect on the growth of macroinvertebrate grazers at high biofilm quantities however,
489 although the bacterial stimulating effect of some macrophytes led to an increased growth of
490 one of the studied species. It can thus be concluded that, depending on their traits, living
491 macrophytes can have a positive effect on macroinvertebrate grazers by providing a large
492 surface area for colonisation by epiphytic algae and bacteria, by improving biofilm
493 stoichiometry and by stimulating bacterial growth.

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Tables

Table 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

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Table 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}

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Table 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	**0.000**	Time	4	28.223	**0.000**
Treatment × time	16	3.575	**0.000**	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	**0.000**	Time	4	7.929	**0.000**
Treatment × time	16	7.534	**0.000**	Treatment × time	16	2.713	**0.002**
CO_2				*Total N*			
Treatment	4	0.018	0.999	Treatment	4	15.074	**0.000**
Time	4	47.345	**0.000**	Time	4	66.801	**0.000**
Treatment × time	16	9.129	**0.000**	Treatment × time	16	3.237	**0.000**
O_2				*N-NH_4^+*			
Treatment	4	0.857	0.508	Treatment	4	3.866	**0.019**
Time	4	18.319	**0.000**	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	**0.000**
Time	4	28.365	**0.000**	Time	4	55.566	**0.000**
Treatment × time	16	1.265	0.244	Treatment × time	16	9.515	**0.000**
SUVA				*N-NO_3^-*			
Treatment	4	2.066	0.128	Treatment	4	14.164	**0.000**
Time	4	30.949	**0.000**	Time	4	74.975	**0.000**
Treatment × time	16	5.743	**0.000**	Treatment × time	16	2.843	**0.001**

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Table 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**

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695 # Figures

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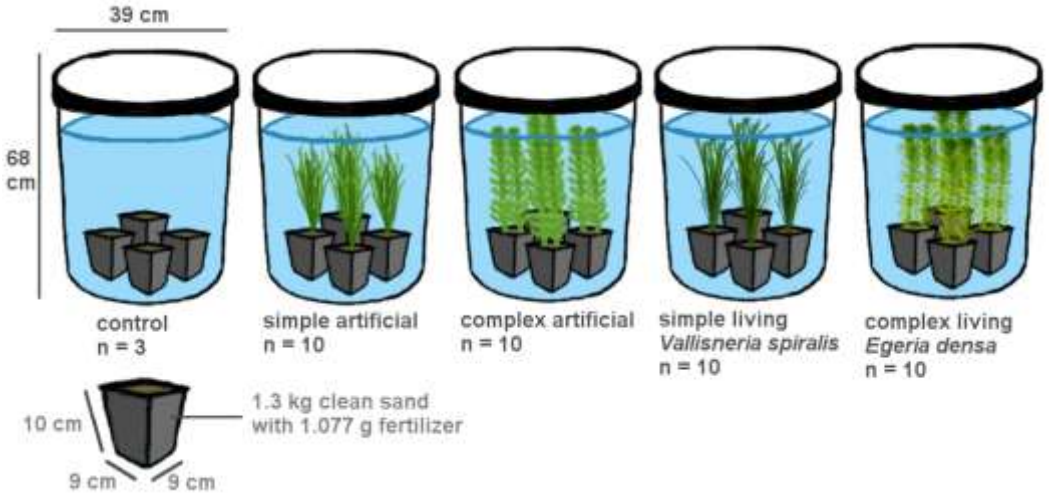

Figure 1. Schematic overview of the experimental setup at the start of the experiment.

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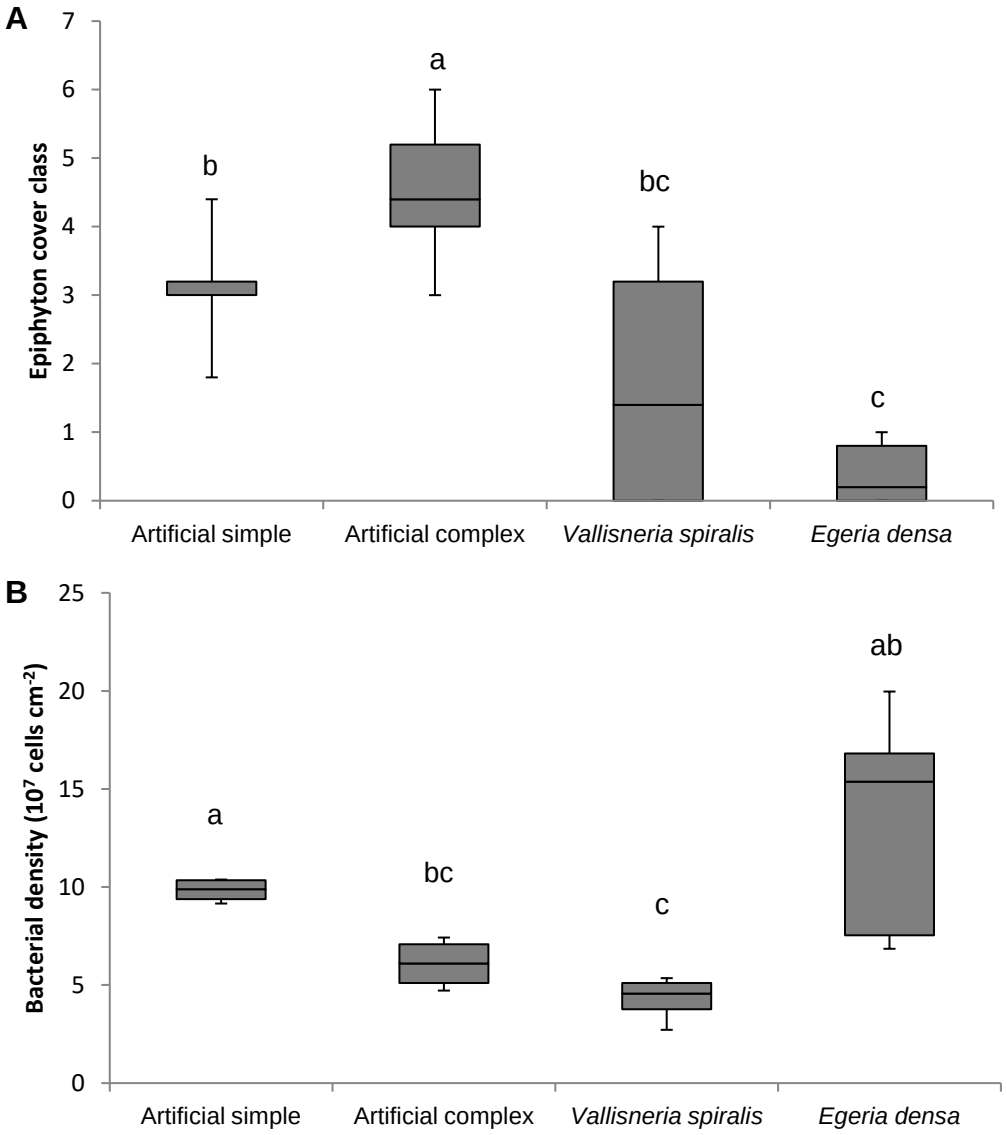

Figure 2. Epiphyton cover class (A) and bacterial density (B) for the different treatments. The boxes with the horizontal segment represent the first-third quartile range and the median of the data respectively, with the whiskers indicating minimum and maximum values. Different letters indicate significant ($P < 0.05$) differences among treatments.

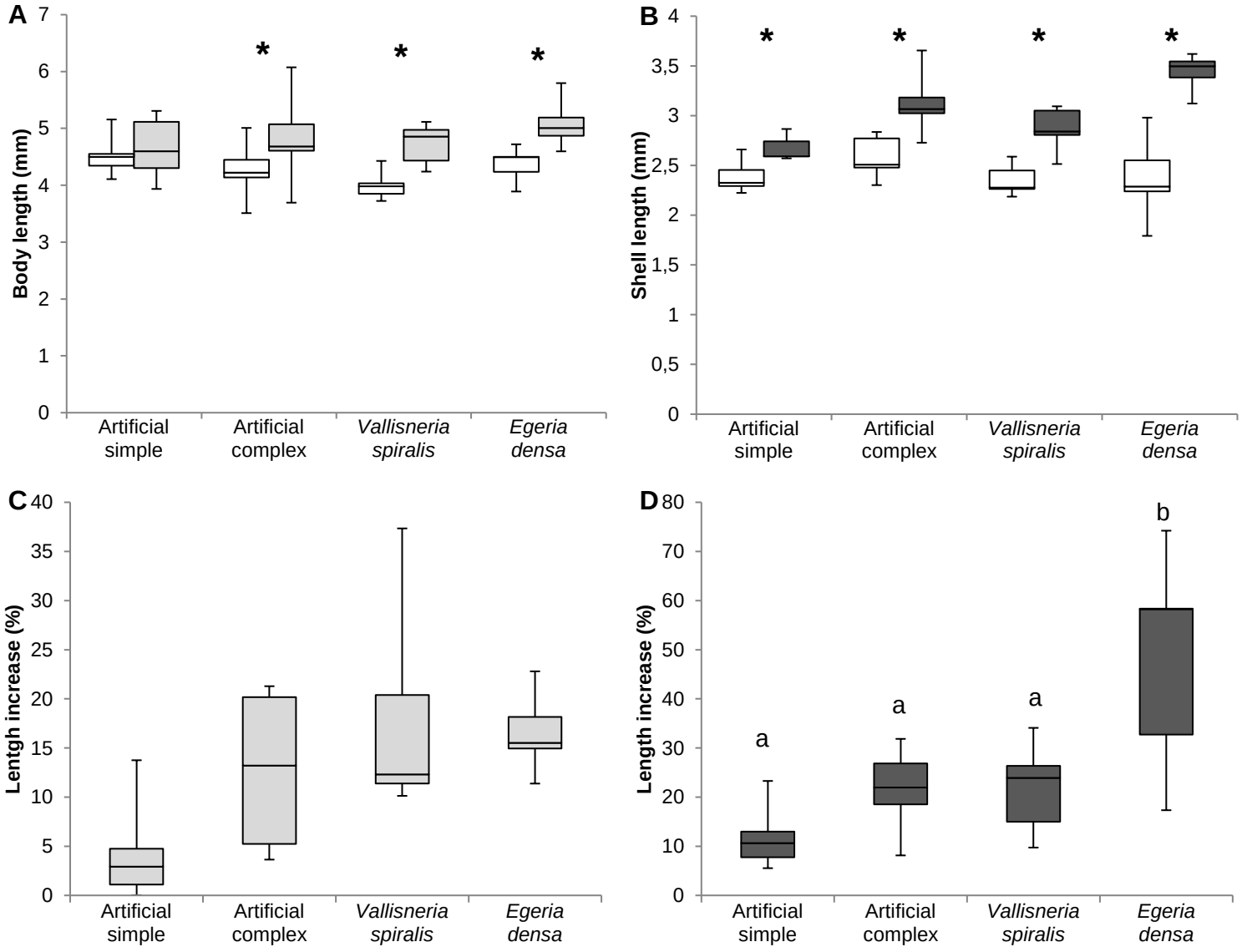

Figure 3. *C. dipterum* (A) and *H. acuta* (B) length before (white bars) and after (gray bars) the experimental period. Percent length increase of *C. dipterum* (C) and *H. acuta* (D) for the different treatments at the end of the experimental period is also shown. The boxes with the horizontal segment 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.

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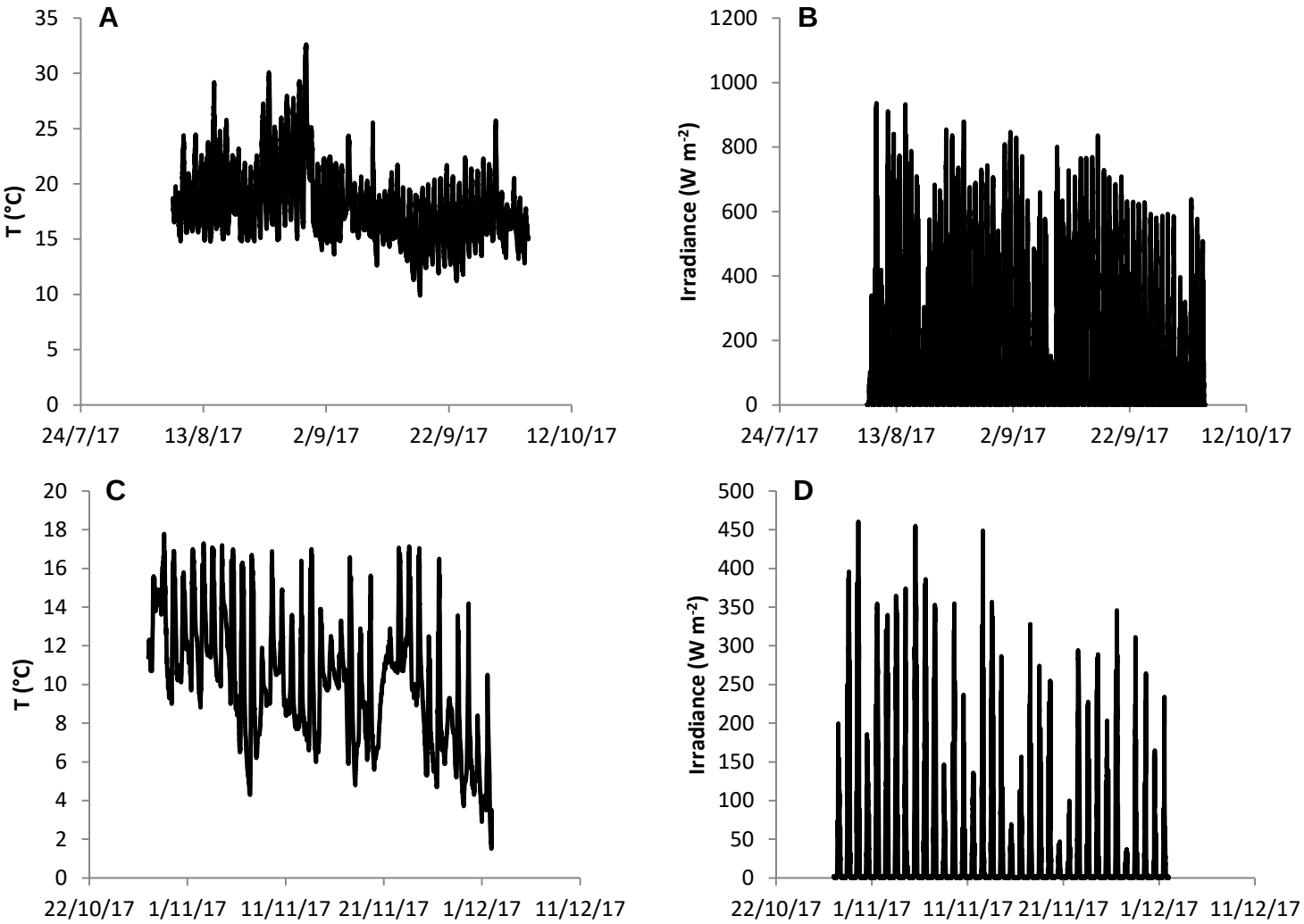

Figure S1. Temperature (A & C) and irradiance (B & D) inside the greenhouse facility during the epiphyton growth phase (A & B) and the macroinvertebrate growth experiment (C & D).

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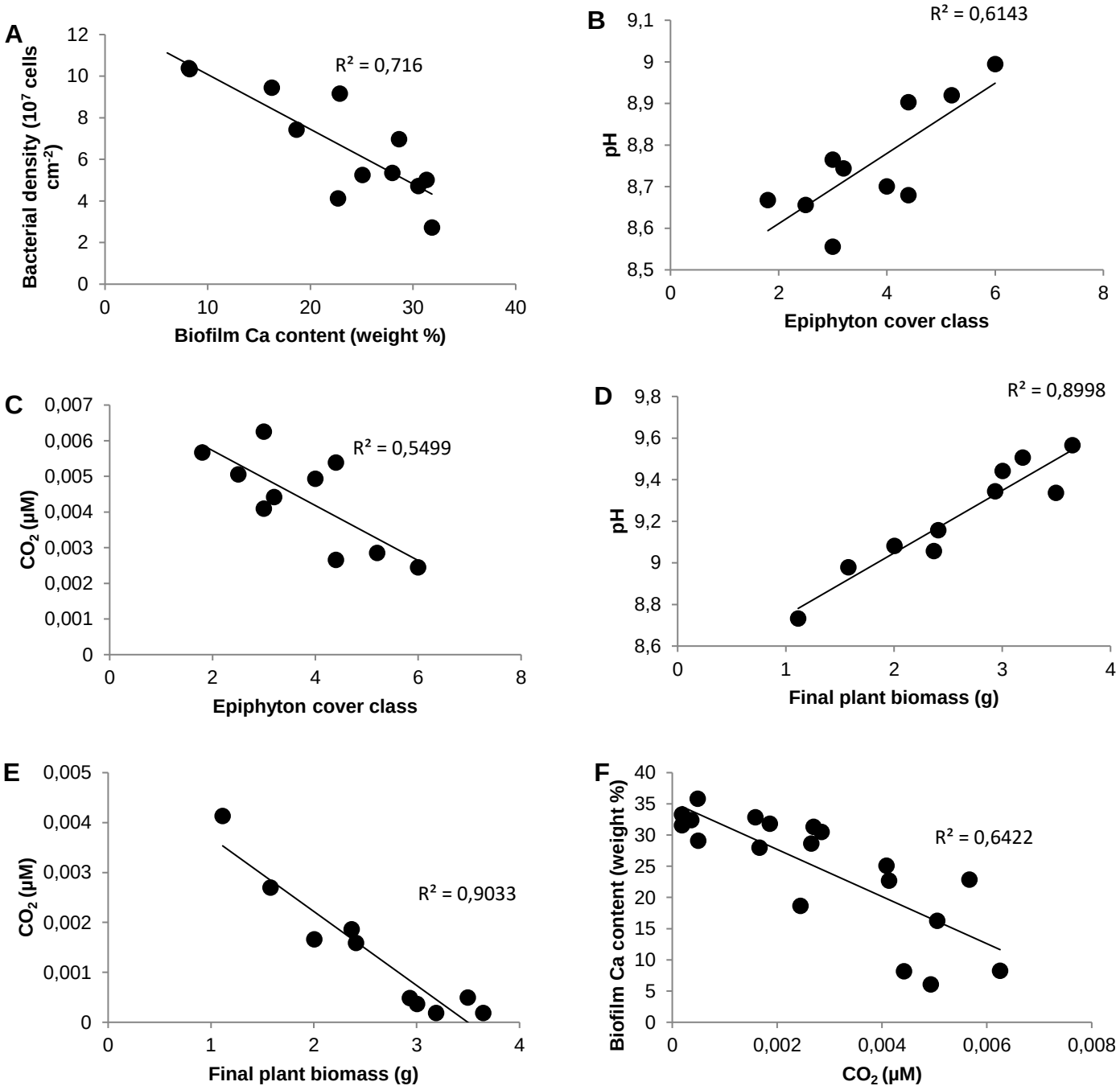

Figure S2. Relationship between biofilm Ca content and bacterial density (A) for all treatments except *E. densa*, relationship between epiphyton cover class and pH (B) and CO_2 concentration (C) for artificial treatments, relationship between final plant biomass and pH (D) and CO_2 concentration (E) for living plant treatments and the relationship between CO_2 concentration and biofilm Ca content (F) for all treatments.

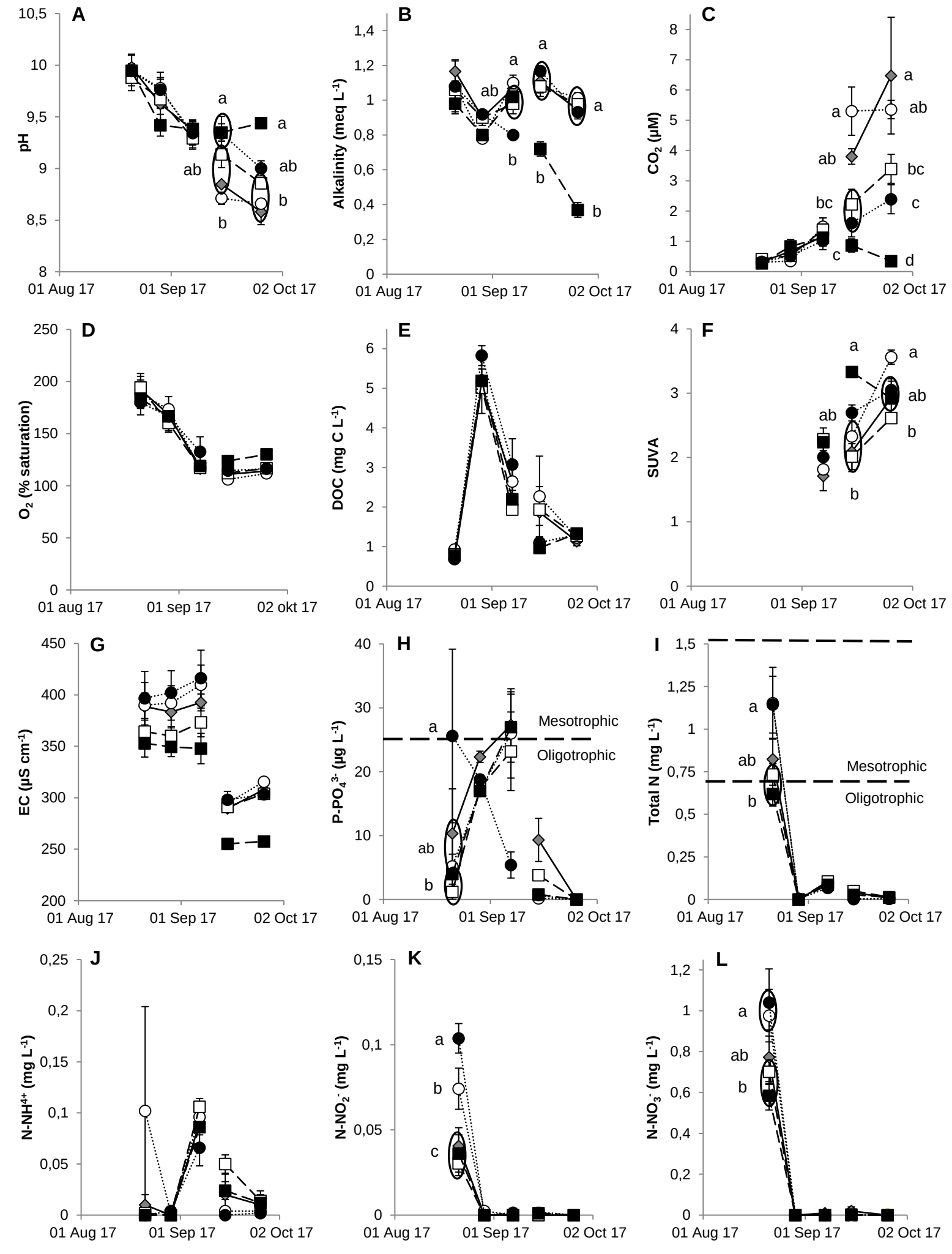
A
pH
10,5
10
9,5
9
8,5
8
a
ab
b
01 Aug 17
01 Sep 17
02 Oct 17
B
Alkalinity (meq L-1)
1,4
1,2
1
0,8
0,6
0,4
0,2
0
C
CO2 (µM)
8
7
6
5
4
3
2
1
0
bc
c
d
D
O2 (% saturation)
250
200
150
100
50
01 aug 17
01 sep 17
02 okt 17
E
DOC (mg C L-1)
F
SUVA
G
EC (µS cm-1)
450
400
350
300
250
200
H
P-PO4 3- (µg L-1)
40
30
20
10
Mesotrophic
Oligotrophic
I
Total N (mg L-1)
1,5
1,25
1
0,75
0,5
0,25
J
N-NH4+ (mg L-1)
0,25
0,2
0,15
0,1
0,05
K
N-NO2- (mg L-1)
0,15
0,1
0,05
L
N-NO3- (mg L-1)
1,2
1
0,8
0,6
0,4
0,2

Figure S3. 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. 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).

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