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Polishing wastewater effluent using plants: floating plants perform better than submerged plants in both nutrient removal and reduction of greenhouse gas emission

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ABSTRACT

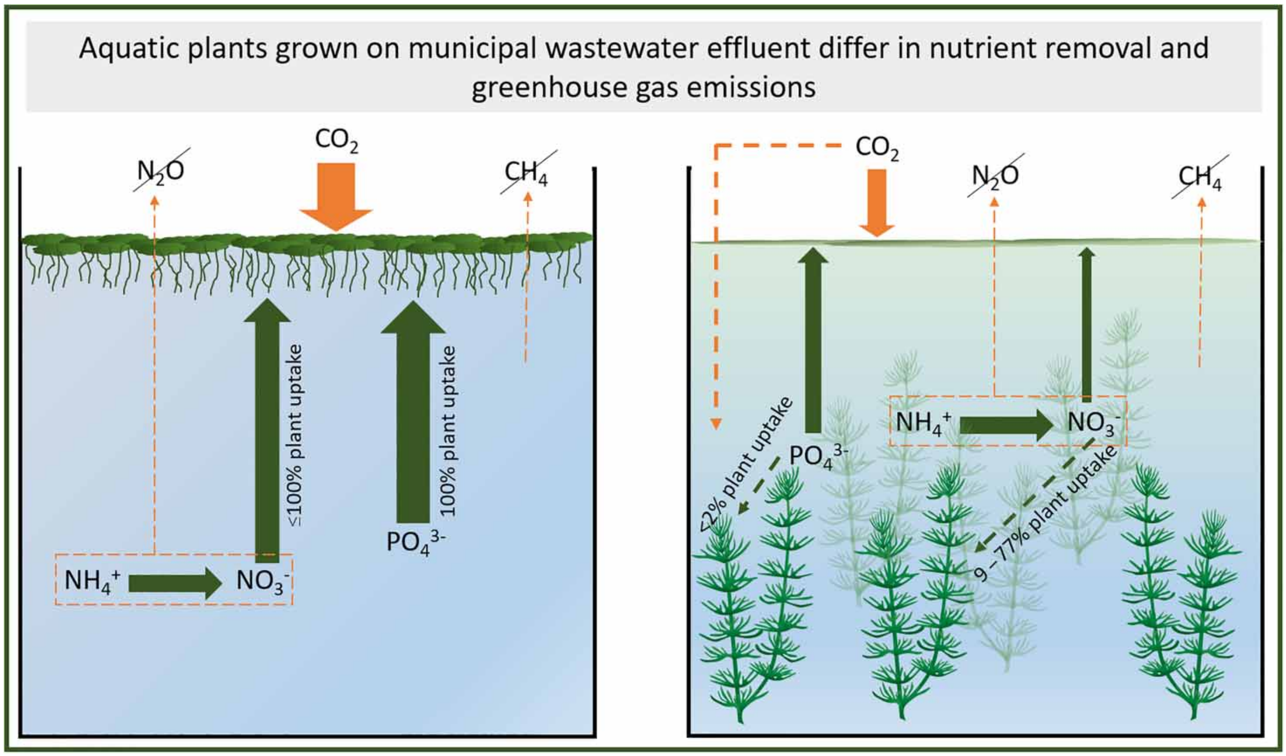
While research on aquatic plants used in treatment wetlands is abundant, little is known about the use of plants in hydroponic ecological wastewater treatment, and its simultaneous effect on greenhouse gas (GHG) emissions. Here, we assess the effectiveness of floating and submerged plants in removing nutrients and preventing GHG emissions from wastewater effluent. We grew two species of floating plants, *Azolla filiculoides* and *Lemna minor*, and two species of submerged plants, *Ceratophyllum demersum* and *Callitriche platycarpa*, on a batch of domestic wastewater effluent without any solid substrate. In these systems, we monitored nitrogen and phosphorus removal and fluxes of CO_2, CH_4 and N_2O, for 2 weeks. In general, floating plants produced the most biomass, whereas submerged plants were rapidly overgrown by filamentous algae. Floating plants removed nutrients most efficiently; both floating species removed 100% of the phosphate while *Lemna* also removed 97–100% of the inorganic nitrogen, as opposed to a removal of 81–88% in submerged plants with algae treatments. Moreover, aquaria covered by floating plants had roughly three times higher GHG uptake than the treatments with submerged plants or controls without plants. Thus, effluent polishing by floating plants can be a promising avenue for climate-smart wastewater polishing.

Key words: carbon dioxide, hydroponic, methane, nitrogen, nitrous oxide, phosphorus

HIGHLIGHTS

- Floating plants rapidly reached high biomass, while submerged plants were overgrown by algae.
- Nutrient uptake by floating plants was responsible for most N and P removal, while algae removed N and P in the submerged plant treatment.
- *Lemna* was most efficient in removing N and P (up to 100% removal).
- All treatments resulted in net greenhouse gas uptake. Small peaks in N_2O and CH_4 emissions were fully compensated by CO_2 uptake.

GRAPHICAL ABSTRACT



INTRODUCTION

Wastewater treatment plants (WWTPs) can account for up to 45% of the total nutrient loading in surface waters (e.g. Groenendijk *et al.* 2016). Because nutrient levels in domestic wastewater treatment effluents are relatively high (330–700 μmol/l N; 18–50 μmol/l P; Carey & Migliaccio 2009; CBS 2021), WWTPs contribute substantially to eutrophication of natural waterbodies (Carey & Migliaccio 2009), and furthermore contribute to high greenhouse gas (GHG) emissions from these waterbodies (Beaulieu *et al.* 2019). Reducing WWTP-derived nutrient loads can therefore reduce GHG emissions in receiving waterbodies. GHG emissions are set to be reduced by 55% in 2030 (European Commission 2021) and regional water authorities and wastewater managers can have a substantial role in this reduction.

Aquatic plants in hydroponic effluent polishing

Since the 1990s, the concept of ecological water treatment using aquatic plants (macrophytes) has gained interest (Wang 1987), but despite its many benefits, it is not yet widely used. Moreover, the focus has been on effluent polishing through nutrient uptake, but not yet on low GHG emission polishing. Next to constructed wetlands, a relatively new treatment of effluent has now started to gain interest, in which a closed system without a sediment layer is used for macrophyte growth, and effluent treatment takes place in a hydroponic way (e.g. Magwaza *et al.* 2020). By using the self-purification principle of natural waterbodies – the uptake and transformation of nutrients mediated by aquatic plants – effluent can be treated to reach nutrient concentrations below the critical values set by the European Water Framework Directive (WFD) (Norström *et al.* 2004).

Trait-specific effects on nutrient removal and GHG fluxes

Aquatic plants mediate nutrient removal both directly and indirectly. Directly, plants can extract inorganic phosphorus (P) and nitrogen (N) from wastewater, incorporating them into their biomass, and thus enabling N, P and carbon (C) harvesting and reuse (e.g. Norström *et al.* 2004). Indirectly, they alter conditions in water and sediment. For example, they alter oxygen concentrations and provide a surface for biofilm formation, thereby favouring coupled nitrification-denitrification and altering the production and emission of CH_4 (Danhorn & Fuqua 2007; Veraart *et al.* 2011; Law *et al.* 2012). During nitrification and denitrification, N_2O can be formed (Law *et al.* 2012), and aquatic plants have multiple ways in which they directly and indirectly affect this emission. At the same time, their photosynthesis removes CO_2 from the atmosphere or water layer while fixing C in their biomass.

Different aquatic plant growth forms have their own characteristics in removing nutrients and altering GHG emissions (Attermeyer *et al.* 2016; Christiansen *et al.* 2016). Submerged plant species can provide a large surface for epiphytic biofilm formation, altering microbial processes in these biogeochemically heterogeneous sites (Eriksson & Weisner 1999). At the same time, submerged macrophytes may inhibit denitrification by their oxygen leakage and by competing for nitrate with denitrifying bacteria (Toet *et al.* 2003). Floating plants, on the other hand, can form a dense mat on top of the water column, creating a reaeration barrier. Local conditions determine whether this barrier favours oxygen depletion or oxygen trapping. Although lower oxygen concentrations under such mats induce higher denitrification rates and CH_4 production (Veraart *et al.* 2011), the O_2 trapped under the macrophytes through radial oxygen loss (ROL) may enhance nitrification and CH_4 oxidation, making the outcome in terms of nutrient removal and GHG emission system specific (Kosten *et al.* 2016). Since floating plants can only cover the top layer of the water column, their growth easily becomes space-limited. Consequently, N and P uptake may stall when floating plants achieve full coverage (Si *et al.* 2019). Additionally, reduced surface for epiphytic biofilm formation potentially lowers the potential for microbial nutrient conversions, especially in systems without a sediment layer. Lastly, the floating fern *Azolla filiculoides* has a symbiosis with N-fixing microorganisms, which makes them less efficient in removing N, but highly efficient in removing P, because their growth does not stall once N is depleted in the water column (Brouwer *et al.* 2018).

The goal of this study was to explore the nutrient removal efficiency of two different macrophyte growth forms, floating vs. submerged, and their potential to lower GHG emissions when grown on WWTP effluent. We compared floating plants covering only the water column surface with submerged plants filling the entire water column. We expected that floating plants would stimulate denitrification, because they lower O_2 concentrations in the water column, and that submerged plants can stimulate nitrification because of their O_2 release. We expected the highest nutrient removal in systems with submerged plants because of their high uptake combined with a large surface area for biofilm formation. In addition, we expected that CO_2 uptake by photosynthesis would fully compensate for CO_2 production from respiration of organic carbon present in the wastewater effluent, leading to net CO_2 uptake. N_2O emission was expected during both nitrification and denitrification, where we expected highest emissions from systems covered by floating plants due to higher denitrification rates. Lastly, CH_4 emission was expected to be low in all cases, because of the lack of strictly anoxic habitats.

METHODS

Experimental setup

We quantified nutrient removal and GHG emissions of two floating plant species (*Azolla filiculoides* (hereafter: *Azolla*) and *Lemna minor* (*Lemna*)) and two submerged species (*Ceratophyllum demersum* (*Ceratophyllum*) and *Callitriche platycarpa* (*Callitriche*)). Additionally, control of effluent without plants was included, resulting in a total of five experimental treatments, each consisting of four replicates. The experiment was performed at the Radboud University greenhouse facility, in glass aquaria of $24 \times 24 \times 30$ cm, distributed in a randomised block design to avoid confounding microclimatic effects in the greenhouse. We maintained a light/dark cycle of 16 h/8 h, by using 400 W high-pressure sodium lamps (Hortilux-Schréder, Monster, The Netherlands), which turned on when the natural daylight intensity fell below 250 W/m^2.

Wastewater effluent originated from the municipal wastewater treatment plant in Remmerden, the Netherlands, which has a 2,100 m^3/h hydraulic capacity and serves 46,000 households. It is a UCT (University of Cape Town) carrousel (Østgaard *et al.* 1997) which had the following effluent concentrations in 2021, ranging between 80 and 500 μmol/l NH_4^+-N; 20 and 220 μmol/l NO_3^--N; 1 and 30 μmol/l PO_4^{3-}-P; 17 and 56 mg/l chemical oxygen demand (COD) and 1.9 and 8.9 mg/l biological oxygen demand (BOD_5) (Hoogheemraadschap de Stichtse Rijnlanden, WWTP Rhenen).

At the start of the experiment, we added 15 l of domestic wastewater effluent to each aquarium and introduced the assigned plant species to this effluent. Because of their different growth strategies and different morphological traits, floating plants were introduced to a surface area coverage of 25%, whereas submerged plants started at 25% volume in the water column. For each of the treatments, the wet weight of this 25% cover was determined, and an extra batch of plants was used to obtain the wet to dry ratio, to estimate initial dry biomass.

In each aquarium, we monitored nutrient concentrations and GHG emissions for 14 days, as well as physical–chemical properties of the water (temperature, pH, dissolved O_2). We measured three times on the first day, once a day during days

2–5, and every other day in the remaining period. On the last day, all plants were harvested to determine wet and dry biomass and plant nutrient content. We additionally harvested the filamentous green algae that started to grow in some of the treatments.

Water quality measurements

Concentrations of NH_4^+-N, NO_3^--N and PO_4^{3-}-P were measured colorimetrically in rhizon-filtered samples (membrane pore size 0.12/0.18 μm, Rhizon SMS 10 cm, Rhizosphere Research, Wageningen, The Netherlands) on an auto analyser III (Bran and Luebbe GmbH, Norderstedt, Germany) after being stored at −20 °C. Total phosphorus was measured in acidified water (0.1 ml 10% nitric-acid) on an ICP-OES (IRIS Interpid II, Thermo Fisher Scientific, Franklin, MA, USA) after being stored at 4 °C. Total inorganic carbon (TIC) was measured in unfiltered samples (ABB Advance optima Infrared Gas Analyzer (IRGA), Frankfurt, Germany) immediately after sample collection. The pH, temperature (°C) and dissolved O_2 (mg/l) concentrations in the water column of each aquarium were measured using a Portable Multi Meter (HQ2200, HACH, Loveland, CO, USA).

Elemental concentrations in plant tissue

The plants that were harvested at the end of the experiment as well as the extra batch of each plant species at the beginning of the experiment were dried at 70 °C for 4 days, after which they were ground manually. The same was done for the filamentous algae that were collected on the last day. N and C contents were determined in plant material (3 mg) using an elemental CNS analyser (Vario Micro Cube, Elementar, Langenselbold, Germany). P content was determined on the ICP-OES after microwave digestion, adding 4 ml HNO_3 (65%) and 1 ml H_2O_2 (35%) to 200 mg dried plant material in Teflon vessels, followed by heating in an EthosD microwave (Milestone, Sorisole Lombardy, Italy).

GHG measurements

GHG fluxes (CO_2, CH_4, N_2O) were measured using a Greenhouse Gas Analyser (G2508, Picarro, Santa Clara, CA, USA) connected to a transparent acrylic glass floating chamber (7.1 dm^3 headspace). In each aquarium, we measured diffusive fluxes of CO_2, CH_4 and N_2O over a period of 4 min, counted from when concentrations started to change. In between the measurements, the chamber was aerated until gas concentrations returned to atmospheric levels.

Data analysis

Total dissolved inorganic N (TDIN) was obtained by summing NH_4^+-N and NO_3^--N. Total dissolved P (TDP) concentrations were obtained from elemental ICP analysis of the filtered water samples (μmol/l).

GHG fluxes (mg/m^2/day) were calculated according to Almeida *et al.* (2016). A global warming potential of 29.8 for CH_4 and 273 for N_2O was used (100-year time frame; IPCC 2021) to convert fluxes to CO_2 equivalents (g CO_2-eq/m^3/day).

For element stocks (C, N and P), the plant content was multiplied by the dry weight of the plants. The total uptake of C, N and P (in μmol) was then obtained by subtracting the total mass of each element at the end of the experiment from the total mass at the start.

Plant growth was calculated by the difference in dry weight between the start and end of the experiment. The dry weight of filamentous algae harvested on the last experimental day was added to the plant growth data. Differences in plant growth and C, N and P plant uptake between treatments were analysed using ANOVA with a Tukey post hoc test (R 4.1.1 (R Core Team 2021), stats::*aov*; *multcompView*::*TukeyHSD* (Graves *et al.* 2019)).

The efficiency of N and P removal by plant uptake for the different plant species was calculated from the change in plant N and P content compared to dissolved inorganic N and P uptake from the water column. Efficiency was shown as a percentage, in which 100% indicated a complete removal due to plant uptake. A negative percentage showed a net release of N or P to the water column.

RESULTS

Effluent conditions

Dissolved O_2 concentrations and pH were stable (4–6 mg/l and 7.3, respectively) until day 4 when filamentous algae started to appear (Fig. S1). After this, pH rose to 8.5 for the floating plants, 8.7 for *Callitriche* and 9.5 for the *Ceratophyllum* and the control treatment. Dissolved O_2 concentrations increased as well and ended at concentrations of 8–9 mg/l for the floating plants, 10 mg/l for *Callitriche*, and 13–15 mg/l for *Ceratophyllum* and the control treatment.

Nutrient removal, GHG emission and biomass production over time

In less than 8 days, all NH_4^+-N was removed from the water column in all treatments (Figure 1(a)). NO_3^--N concentrations increased during the first few days and decreased during the remainder of the experiment (Figure 1(b)), with differences in timing and removal efficiency between treatments. This resulted in a small initial increase, rapidly followed by a decrease in total dissolved inorganic nitrogen (TDIN) concentrations (Figure 3(a)). In fact, water treatment with *Lemna* resulted in 100% removal of TDIN, while the other treatments were less efficient, with *Azolla* having little to no N removal.

PO_4^{3-}-P concentrations were below our detection limit (<0.05 μmol/l) after 8 days for *Lemna*, *Azolla* and the control treatment (Figure 1(c)). *Ceratophyllum* treatments started with higher PO_4^{3-}-P concentrations, yet after 10 days all PO_4^{3-}-P was removed. Treatment with *Callitriche* resulted in PO_4^{3-}-P increase in the first 3 days, followed by PO_4^{3-}-P uptake. However, after 2 weeks still, a considerable amount of PO_4^{3-}-P (average 0.75 μmol/l) was present. Total dissolved phosphorus (TDP) concentrations were lowered from 9.0 (±1.3 sd.) to 3.3 (±0.8 sd.) μmol/l in treatments with *Azolla*, *Lemna* and *Ceratophyllum*, while *Callitriche* initially showed an increase and later a decrease in TDP concentration, but plateaued around 7.8 (±4.7 sd.) μmol/l (similar as the start concentration) after 2 weeks (Figure 3(b)).

GHG flux measurements started on day 3 (after 49 h). Fluxes of CH_4 were low in all treatments (max. CH_4 flux 0.04 mg/m^2/day), and from day 4 onwards no CH_4 fluxes were observed (Figure 2(a)). At the start of the measurements, only *Lemna* and *Azolla* were taking up CO_2 and had highest CO_2 uptake during the whole experiment (Figure 2(b)). Control treatments had similar CO_2 uptake as *Ceratophyllum* and *Callitriche*. CO_2 uptake only took place after day 4 for these treatments, which was at the onset of algal growth. N_2O emissions were low overall, with only a small peak after day 4 for *Callitriche* and the

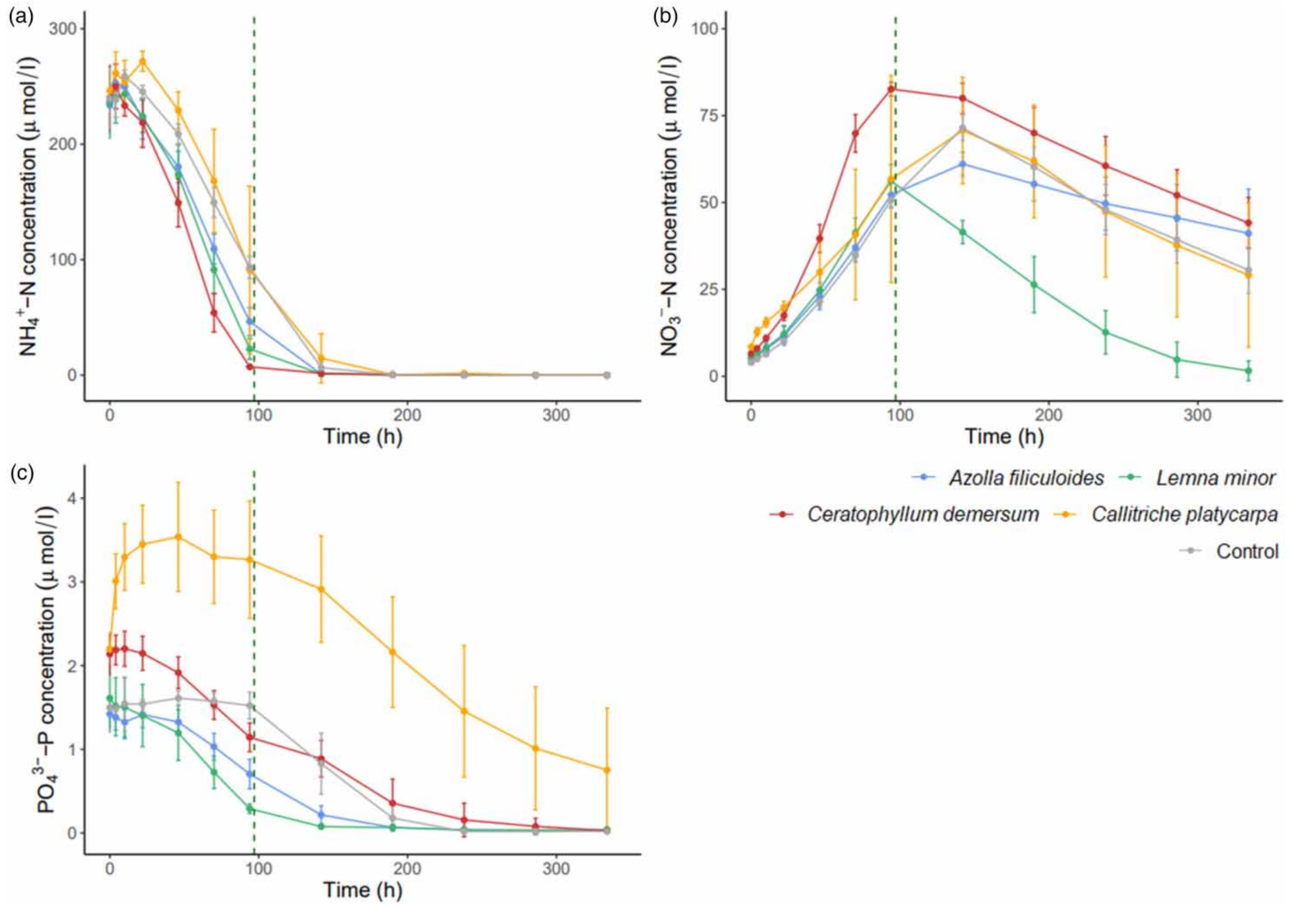


Figure 1 | NH_4^+-N, (a) NO_3^--N (b) and PO_4^{3-}-P (c) concentration over time for the different treatments (mean values ± sd). The vertical dashed green line indicates the date in which algae started to appear in treatment *Ceratophyllum*, *Callitriche* and Control. Note the difference in *y*-axis scale. Please refer to the online version of this paper to see this figure in colour: https://dx.doi.org/10.2166/wst.2023.203.

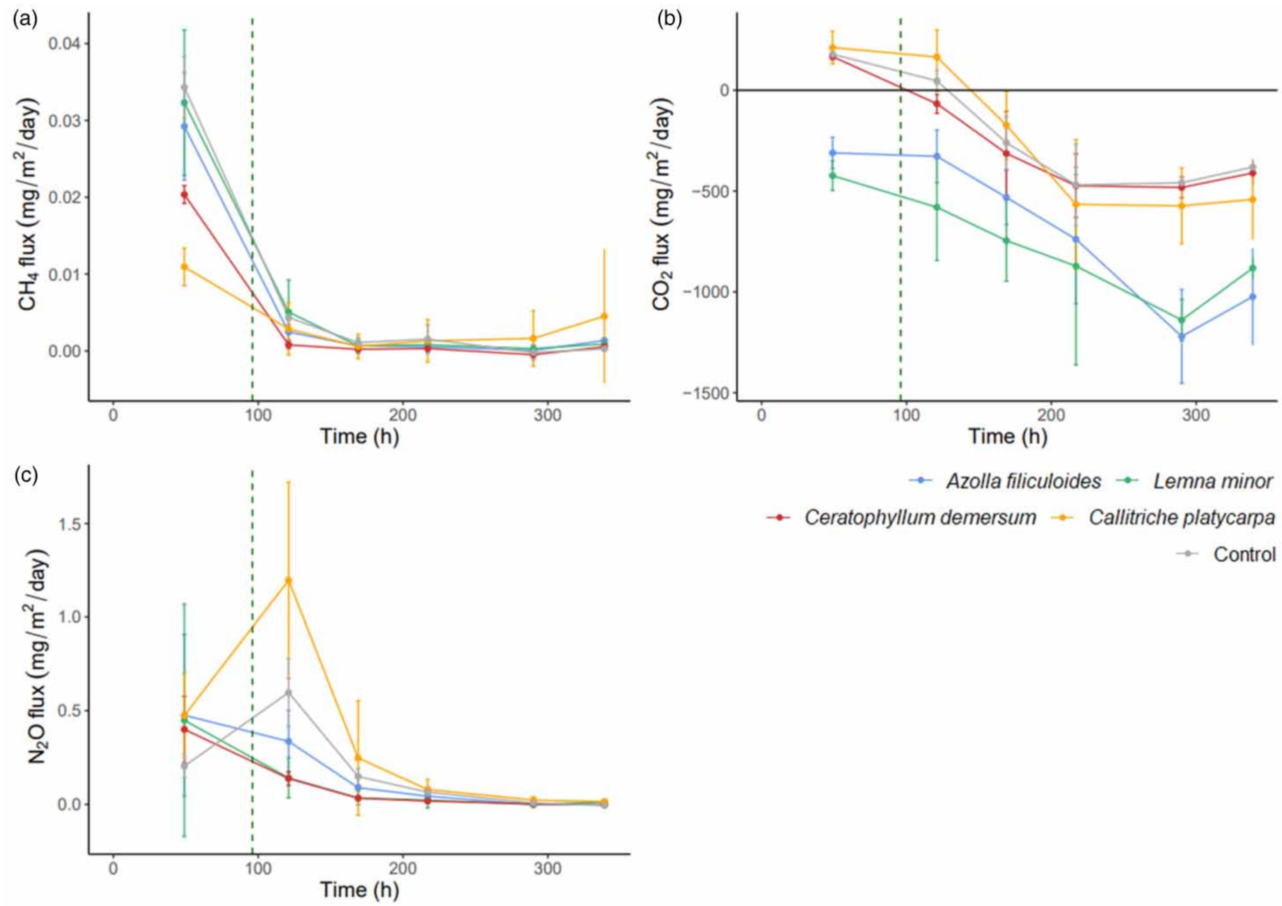


Figure 2 | CH_4, (a) CO_2 (b) and N_2O (c) fluxes over time for the different treatments (mean values ± sd). The vertical dashed green line indicates the date in which algae started to appear in treatment *Ceratophyllum*, *Callitriche* and Control. Note the difference in *y*-axis scale. Please refer to the online version of this paper to see this figure in colour: https://dx.doi.org/10.2166/wst.2023.203.

control treatment (Figure 2(c)). *Lemna* and *Azolla* showed a net GHG uptake, having negative fluxes in CO_2 equivalents, while the other three treatments first emitted GHGs, followed by net uptake (Figure 3(c)).

The plants differed significantly in how well they grew on wastewater effluent ($P < 0.001$; Figure 3(d)). *Azolla* and *Lemna* had the highest biomass increase, although *Lemna* did not differ significantly from *Ceratophyllum* ($P = 0.06$). The *Callitriche* treatment had little to no growth, with algae accounting for 46 (11–75)% of its total biomass gain. In control treatment aquaria, an increase in algal biomass was observed as well (up to 0.15 g).

Nutrient removal efficiency

The plants significantly differed in N and P removal efficiency ($P = 0.002$ and $P = 0.04$, respectively). *Lemna* was most efficient in removing TDIN, on average removing 99.2 (97.0–100)%, even though it was not significantly different from the *Callitriche* treatment ($P = 0.08$). All treatments resulted in high TDIN removal (ranging from average 81.6% in *Ceratophyllum* treatment to 88.8% in *Callitriche* treatments) after 2 weeks (Figure 4(a)). Treatment with *Ceratophyllum* resulted in highest TDP removal (77.0 (64.5–82.4)%) but was not significantly higher than *Azolla*, *Lemna* and the control treatment ($P = 0.94$, $P = 0.95$ and $P = 0.56$, respectively; Figure 4(b)).

Elemental plant uptake

The plant species differed in the way they incorporated amounts of C, N and P in their tissues ($P < 0.01$; Figure 5). *Azolla* and *Lemna* showed highest sequestration of all three elements, including N fixation by *Azolla*. *Azolla* did not differ significantly from *Ceratophyllum* in C uptake ($P = 0.05$). Both submerged plants had significantly lower elemental sequestration than the floating plants ($P < 0.001$).

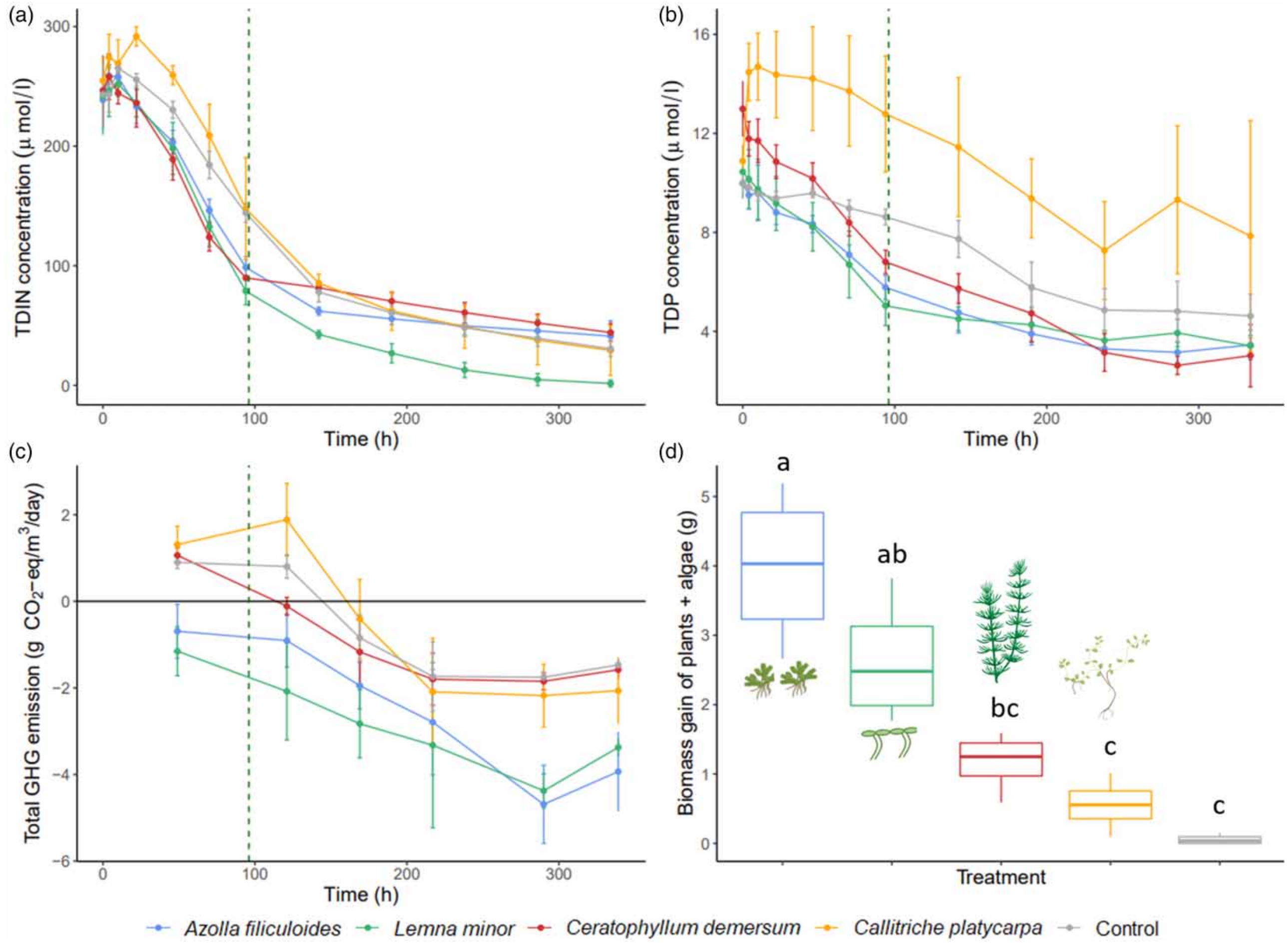


Figure 3 | Total dissolved inorganic N (TDIN) concentration (a), total dissolved phosphorus (TDP) concentration (b) and total GHG fluxes (in g CO_2-eq/m^3/day) (c) over time for the different treatments (mean values ± sd.), and average growth of the plants (d) ($P < 0.001$ (one-way ANOVA), letters indicate significant differences between the treatments, Tukey honestly significant difference (HSD) $P < 0.05$). The dashed green line indicates the date on which algae started to appear in treatment *Ceratophyllum*, *Callitriche* and Control. Note the difference in *y*-axis scale. In (d), boxplots show the median values and 25th and 75th percentiles, whiskers indicate largest and smallest values.

N and P sequestration by the different plant species corresponded with N and P removal from the water column for the floating plants, whereas N and P removal in the submerged macrophyte treatments can only for a small part be explained by plant uptake (Table 1).

DISCUSSION

In this study, we tested the nutrient removal efficiency of floating and submerged macrophytes grown on WWTP effluent, and their potential to capture CO_2 and suppress CH_4 and N_2O emission. We compared the effects of two floating plants, *Azolla filiculoides* and *Lemna minor*, and two submerged macrophytes, *Ceratophyllum demersum* and *Callitriche platycarpa*. In this experiment, systems covered by the floating plants *Azolla* or *Lemna*, had the highest N and P removal efficiency – resulting from plant uptake – and captured most CO_2 while emitting the least CH_4 and N_2O, thus resulting in net GHG uptake. Submerged plants *Ceratophyllum* and *Callitriche* did not grow well on WWTP effluent, and therefore contributed less to both nutrient removal and CO_2 uptake.

Effects of floating and submerged plants on nutrient removal and GHG emission

All treatments, including unvegetated controls, caused TDIN concentrations to decrease to on average ≈30 µmol/l, which is well below the average concentrations observed in the waterbodies to which WWTPs discharge their effluent (≈285 µmol/l;

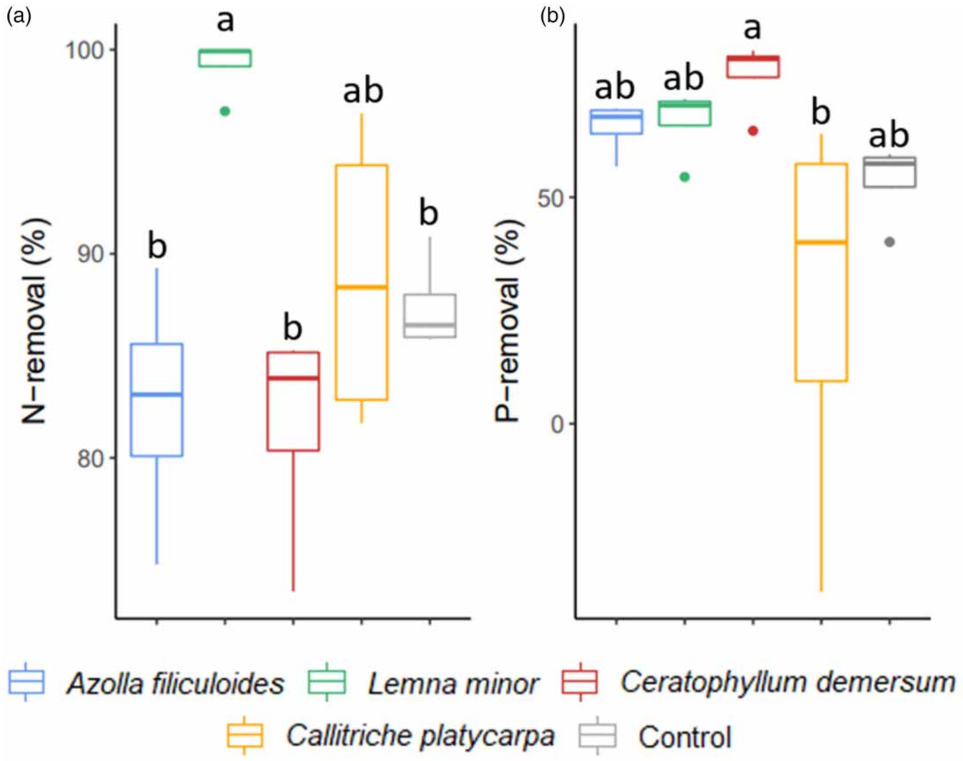


Figure 4 | Nutrient removal efficiency of nitrogen ($P = 0.002$ (one-way ANOVA), letters indicate significant differences between the treatments, Tukey HSD $P < 0.05$) (a) and phosphorus ($P = 0.04$ (one-way ANOVA), letters indicate significant differences between the treatments, Tukey HSD $P < 0.05$) (b) for the different treatments. Note the difference in *y*-axis scale. Boxplots show the median values and 25th and 75th percentiles, whiskers indicate largest and smallest values.

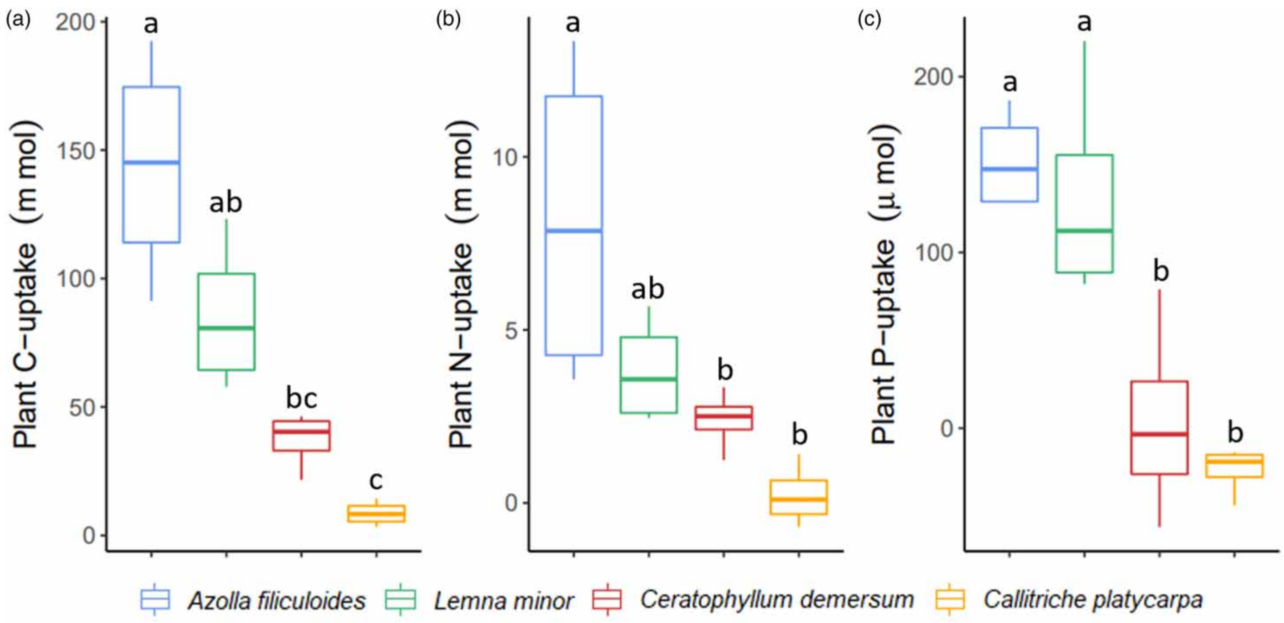


Figure 5 | Total carbon (C) (a), total nitrogen (N) (b) and total phosphorus (P) (c) uptake by the different plant species at the end of the experiment ($P < 0.01$ for all elements (one-way ANOVA), letters indicate significant differences between the treatments, Tukey HSD $P < 0.05$). Note the difference in *y*-axis scale. Boxplots show the median values and 25th and 75th percentiles, whiskers indicate largest and smallest values.

Table 1 | Efficiency of N and P removal by plant uptake for the different plant species

	N efficiency (mean % (min–max %))	P efficiency (mean % (min–max %))
Azolla filiculoides	236.8 (112.6–404.2)	155.8 (120.6–184.8)
Lemna minor	137.1 (74.0–234.6)	142.3 (68.5–304.1)
Ceratophyllum demersum	77.1 (55.4–102.5)	2.1 (– 34.6–47.9)
Callitriche platycarpa	9.4 (– 17.7–50.0)	–23.3 (– 78.1–15.3)

A negative percentage shows a net release of N or P. A percentage of above 100% can be explained by sampling variation when measuring plant uptake. Note that for *Azolla*, N efficiency also includes N-fixation, which explains why an uptake percentage of over 100% is reached.

Carey & Migliaccio 2009; Puijenbroek *et al.* 2010). However, coverage by *Lemna* caused the largest decrease, resulting in almost complete TDIN removal after 2 weeks (Figure 2(a)).

For water treated with *Azolla, Lemna* and *Ceratophyllum*, P concentrations were reduced to on average 3.3 ($\pm$0.8 sd.) μmol/l P after 2 weeks, which is similar to P concentrations occurring in the potential receiving waterbodies (Carey & Migliaccio 2009; Puijenbroek *et al.* 2010). In both floating plant treatments, plant P uptake resulted in immediate P removal from the water column, whereas *Ceratophyllum* P uptake could not explain all P removal from the water column. PO_4^{3-}-P concentrations increased in systems with *Callitriche*, likely due to plant senescence, observed from its lack of growth and visible signs of decay.

While submerged macrophytes were hampered in their growth by algal dominance, and presumably also by the high pH leading to very low CO_2 concentrations in the water layer, floating macrophytes showed high growth rates of 4.9 ($\pm$1.2 sd.) and 3.3 ($\pm$1.0 sd.) g/m^2/day for *Azolla* and *Lemna*, respectively. This is in line with, and for *Azolla* even in the high range of, maximum growth rates found for these species (Reddy & DeBusk 1985).

After 6 days, all treatments resulted in net GHG uptake, with systems covered by *Azolla* or *Lemna* showing the highest uptake (Figure 2(c)). In treatments containing *Ceratophyllum* and *Callitriche*, CO_2 uptake only took place after 4 days, similar to the control treatment and starting at the moment filamentous algae became visible. Combined with the poor growth of these submerged plant species, we expect at least part of the CO_2 uptake to be due to algal growth rather than macrophyte growth. Little to no CH_4 emission was detected in all treatments, which can be explained by the high O_2 concentrations in the water and lack of sediment. A small peak in N_2O emission occurred at the time when NO_3^--N concentrations were at its highest and NH_4^+-N was depleted. Yet, the highest emission of 1.88 mg N_2O/m^2/day (occurring in *Callitriche* treatments) was still well below emissions observed in constructed wetlands, which can reach 3.12 mg N_2O/m^2/day (e.g. Mander *et al.* 2014).

The importance of nitrification-denitrification in nitrogen removal

After 4–5 days, in all plant treatments all NH_4^+-N was removed. It was expected that due to their larger surface area and thus expected higher biofilm production, submerged plants would facilitate a higher NH_4^+-N removal, which was not the case. Because similar NH_4^+-N removal rates were found for the control treatment, in the absence of algae, the NH_4^+-N removal is most likely caused by nitrification performed by microorganisms in the water column and in biofilms on the aquaria walls, rather than by plant uptake. The coincidence with an increase in NO_3^--N in these first days confirms this. This is in line with other hydroponic systems, in which nitrification was also the predominant process of NH_4^+-N removal (Vaillant *et al.* 2003).

Our calculations show that all NO_3^--N removal from aquaria treated with *Lemna* as well as those with *Azolla* can be explained by plant N uptake (Figure 5), which is contrasting to other studies where *Lemna* and *Azolla* species only take up a fraction of NO_3^--N (Singh *et al.* 1992). In our systems, denitrification was not significantly contributing to N removal from the effluent. Moreover, *Azolla* coverage resulted in higher plant N uptake than N removal from the effluent, which indicates N_2 fixation from the atmosphere by the *Azolla–Nostoc* symbiosis.

N fixation causes less efficient N removal by Azolla

Whereas *Lemna* had a 100% NO_3^--N (and thus TDIN) removal after 2 weeks, *Azolla* hardly removed any of the produced NO_3^--N. This is most likely because of its symbiosis with the cyanobacterium *Nostoc azollae* that fixates nitrogen from the atmosphere (Brouwer *et al.* 2018). Normally, N fixation is a costly process which only takes place when N is limited. Yet, it is found that N fixation by the microbial symbiont occurs even when *Azolla* is grown on water containing substantial amounts of inorganic N, and N fixation is only inhibited by much higher concentrations of nitrogen than present in our experiment (Ito & Watanabe 1983). *Azolla* showed the highest N plant uptake (Figure 5) combined with the lowest TDIN removal, suggesting that almost all N that *Azolla* took up was derived from N-fixation from the atmosphere.

Algal growth affected the performance of submerged plants, and facilitated nutrient removal

In treatments containing submerged plants, as well as the unvegetated controls, algae started to appear after 4 days, which was facilitated by the abundance of light and nutrients in these treatments. Likely, light-limitation suppressed algal growth in the floating plant treatments. As a result, N and P uptake by submerged plants was negligible (Figure 5 and Table 1). Most likely, in these treatments, N removal took place via algal uptake and coupled nitrification-denitrification by the microbial community, while P removal was mostly caused by algal uptake, especially in the *Callitriche* treatments.

High nutrient removal efficiency and GHG reduction by floating macrophytes

Our systems including *Azolla* and *Lemna* were more efficient in the removal of N and P than other hydroponic systems (Shah *et al.* 2014) as well as constructed wetlands (Tang *et al.* 2017; Hernández *et al.* 2018), and are performing better than, or similar to floating treatment wetlands (Prajapati *et al.* 2017). In line with these findings, floating macrophytes were more efficient in removing nitrogen and phosphorus than emergent macrophytes in floating treatment wetlands (Prajapati *et al.* 2017) and are therefore considered good candidates for the treatment of wastewater effluent.

Where constructed wetlands can emit up to 500 mg/m^2/day CH_4 and 25 mg/m^2/day N_2O (Hernández *et al.* 2018), our systems did not show any significant CH_4 emissions (lower than 0.04 mg/m^2/day) and N_2O emissions of only 1.5 mg/m^2/day at one specific point in time. While some studies also indicate CO_2 emission in constructed wetlands (Badiou *et al.* 2019), our treatments showed CO_2 uptake, resulting in a total net uptake of GHG. Although our measurements are based on treated wastewater effluent, while constructed wetlands often deal with untreated wastewater – inherently having a higher potential for GHG emission – our data show the potential to mitigate part of the WWTP emissions in the process of hydroponic effluent polishing. Moreover, nutrient reduction in WWTP effluent most likely lowers GHG production in receiving waterbodies, by decreasing eutrophication effects (Beaulieu *et al.* 2019).

Use of floating plants to contribute to a circular economy

Ideally, plants used in effluent polishing are used in added-value applications, to contribute to the circular economy. One prerequisite for growing plants on wastewater effluent is that algal growth should be limited unless algae are the main product to be cultured. Floating plants that prevent light penetration in the water column can suppress algal growth. When using other plant types, algal growth can be suppressed by using UV light or by adding aquatic animals such as snails to counteract the formation of floating algae beds; while zooplankton or mussels can be used to minimise phytoplankton density. But, it remains to be tested if such animals can also be used in wastewater effluent polishing systems.

Both floating plants tested in our experiment have economic value. *Azolla* and *Lemna* are rich in proteins and amino acids, potentially containing even more protein than soybeans (Brouwer *et al.* 2018). However, non-food applications are preferred because plants grown on domestic wastewater may contain contaminants such as heavy metals and traces of pharmaceuticals. *Azolla* can be used to produce potting soil for ornamental plants, substituting peat, thereby contributing to the protection of C-storing peatland ecosystems (Khomami *et al.* 2019). Both species can be digested into bioethanol or biogas as well. Although this would offset the negative carbon footprint of phytoremediation, saving on fossil fuels is always beneficial.

CONCLUSIONS

Based on our results, we conclude that the floating plants *Azolla* and *Lemna* are promising for use in effluent polishing, due to their ability to lower nutrient concentrations in the effluent while at the same time sequestering carbon and limiting the emission of other greenhouse gases. Where the growth of submerged macrophytes was strongly affected by competition with algae, both of the floating plants showed the highest biomass production, and were most efficient in removing nitrogen and phosphorus from the water column. Note, however, that nutrients taken up by the plants are only permanently removed after harvesting. When combining *Azolla* with *Lemna*, or other high value floating plants, excess P and N can be removed from wastewater effluent, while taking up GHGs and producing plant biomass with commercial value, contributing to a circular economy. Moreover, by lowering the nutrient load derived from discharged WWTP effluent, effluent polishing can also contribute to the mitigation of eutrophication and GHG emissions from natural waterbodies.

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DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

CONFLICT OF INTEREST

The authors declare there is no conflict.

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