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Greenhouse gas and ammonia emissions from duckweed cultivation systems using diluted liquid manure

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Protein from duckweed (Araceae, subfamily Lemnoideae) grown on diluted animal slurries for nutrient upcycling could potentially replace plant-derived feed proteins which would be more efficiently used as human food. However, little information is available on greenhouse gas (GHG) emissions from slurry-grown duckweed, and previous studies have not reported methane emissions from a similar system. Here, we report on GHG (methane, carbon dioxide and nitrous oxide) and ammonia emissions from duckweed grown on diluted cattle slurry measured in daylight and darkness, compared with emissions from diluted slurry without duckweed. We observed (i) initially high but rapidly declining methane emissions, independent of lighting or treatment, (ii) a net carbon dioxide fixation by duckweed, independent of lighting, (iii) high nitrous oxide emissions, independent of lighting, and (iv) a > 80% reduction of ammonia emissions by duckweed, independent of lighting. Our data shows potential of duckweed protein as a sustainable protein with 3.54 to 6.54 CO_2eq kg^{-1} protein, compared to faba bean (3.61 kg CO_2eq kg^{-1} protein) or barley protein (5.35 CO_2eq kg^{-1} protein). But despite the potential of slurry-grown duckweed as sustainable protein source, swapping ammonia volatilization for nitrous oxide emissions represents a limitation of the current system and mitigation strategies are needed.

Keywords Methane, Carbon dioxide, Nitrous oxide, Slurry, Protein carbon-dioxide equivalents

Biogeochemical fluxes (reactive nitrogen and phosphorus) and greenhouse gas (GHG) emissions are two of the six critical Earth System processes for which boundaries of the regulating capacity of our planet were transgressed[1]. Animal production strongly contributes to GHG emissions and to reactive nitrogen (N) and phosphorus (P) losses to the environment. Ruminants are a critical source of methane (CH_4) emissions[2], ammonia (NH_3) and nitrous oxide (N_2O) are primarily emitted from management of animal manures and slurries[3,4] and their application on agriculturally utilized soils[5].

The supply of protein for monogastric animal feed or human food is challenging because of high inefficiencies along the production chain, from synthetic N fertilizer production using the Haber–Bosch process to human consumption. Nitrogen losses were estimated between 86% for plant-based and 96% for animal-based products[6].

Reducing N losses, for example by nitrate (NO_3^-) leaching into groundwaters[7,8], NH_3 volatilization from slurry pits or N_2O emissions from soils[9] is therefore a key task in the endevour of making global agriculture sustainable. In particular livestock production contributes to environmental pollution via feed production and global transport[10], in addition to direct physiological emissions from the animals. Furthermore, animal slurries are rich in N compounds such as undigested protein and the sum of ammonium (NH_4^+) and NH_3 known as total ammoniacal nitrogen (TAN), which contribute to N losses and GHG emissions.

Growing duckweed, small aquatic floating plants of the family Araceae, subfamily Lemnoideae, on diluted animal slurries has recently gained increased attention due to opportunities for up-cycling N into duckweed protein for animal feeding. Different duckweed species frequently showed their ability to produce protein in larger amounts and similar or better quality compared to other crops such as beans, peas or soy[11–16]. Duckweeds

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have successfully been tested as feed ingredients for pigs[17], poultry[18,19], small ruminants[20] and different commercially important fish species such as carnivorous rainbow trout[15,21,22], herbivorous tilapia[23,24] and omnivorous carps[25,26].

Since duckweed species are highly versatile plants, they have been tested for several applications besides animal feed. These include use as human food[13,27], phyto-remediation for different pollutants including heavy metals[28] or pharmaceuticals[29,30] and as an energy crop for bioenergy or biofuel production[31,32]. Duckweed also contains relatively high levels of vitamin B_{12}, a critical micronutrient that is typically deficient in vegetarian or vegan diets[33].

Diluted slurry-duckweed production systems have shown promising results in producing duckweed protein from animal manure[16,34,35]. Yet, little is known about the GHG and NH_3 emissions from such systems. So far, few studies investigated CO_2 and CH_4 emissions from duckweed grown on domestic wastewater and none quantified N losses via N_2O or NH_3[36,37]. One of the studies, Mohedano et al. 2019, provided evidence that duckweed ponds used for wastewater treatment are net carbon (C) sinks while no CH_4 emissions were detected. Other studies on GHG emissions from duckweed ponds have investigated natural stormwater treatment ponds in the United States and found significant levels of CH_4 and N_2O emissions[38–40].

The aim of this study was to assess GHG (CH_4, CO_2 and N_2O) and NH_3 emissions of an outdoor slurry-fed duckweed (*Lemna minor*) production system during one experimental week under different light conditions. Light and dark conditions during experimental GHG measurements were chosen in order to clearly distinguish between CO_2 respiration rates during the night and CO_2 fixation rates during the day. Therefore, we report CH_4, CO_2 and N_2O fluxes, NH_3 volatilisation, dynamics of mineral nitrogen forms and protein production from two different experiments: i) under dark conditions (Experiment 1, simulating emission dynamics during night) and ii) under light conditions (Experiment 2, simulating emission dynamics during day). Emissions from duckweed grown on diluted cattle slurry (slu + dw) were compared to baseline emissions from duckweed-free diluted slurry (slu). We measured CO_2, CH_4, N_2O and NH_3 fluxes over six consecutive days with up to three measurement timepoints a day. This is the first time that GHG and NH_3 emissions are reported for a duckweed protein production system, including CO_2eq per mass of duckweed protein.

Results

Duckweed composition

In both experiments, the initial duckweed composition was similar (Table 1). It had a dry matter (DM) content of around 10%, a carbon (C) content of 41% of DM, a nitrogen (N) content of around 5.5% of DM, a resulting C:N ratio of 7.5 and a CP content of around 34% of DM.

At the end of experiment 1, the duckweed DM content was reduced to 6.5%, C content slightly increased to 42% of DM, N content slightly reduced to 5% of DM, resulting in an increased C:N ratio of 8.35 while the crude protein (CP) content was reduced to 31.5% of DM.

The differences between initial and final duckweed were less pronounced for experiment 2. Duckweed sampled after the one-week period showed a similar C and N content, C:N ratio and CP content as the initial duckweed. Only the DM content was reduced to 7% between initial and final sampling.

Biomass and protein production

The fresh and dry duckweed biomass and protein production as well as the extrapolated protein production and CO_2eq per kg of CP produced differed between experiments (Table 1). During experiment 1, the relative growth rate (RGR) of *L. minor* was 0.088 day^{-1}, with a fresh duckweed production of 81.8 g m^{-2} d^{-1}, DM production of 5.31 g m^{-2} d^{-1} and CP production reaching 1.67 g m^{-2} d^{-1}, which corresponded to 6.49 t ha^{-1} a^{-1}, assuming a continuous growth over a full year. The RGR in experiment 2 (0.056 day^{-1}) was lower than in experiment 1, as was fresh duckweed production (45.2 g m^{-2} d^{-1}), DM production (2.93 g m^{-2} d^{-1}), CP production (0.92 g m^{-2} d^{-1}) and the extrapolated protein production (3.36 t ha^{-1} a^{-1}). Taking the GHG emissions and the protein production into account, 3.54 kg CO_2eq kg^{-1} CP were produced during experiment 1, and 6.45 kg CO_2eq kg^{-1} CP produced during experiment 2.

Slurry N compounds and pH

In experiment 1, the pH in both treatments ranged between 7.60 and 8.08 for slu and between 7.55 and 8.36 for slu + dw, but did not show any larger fluctuations. Total N (N_{tot}) concentrations declined in both treatments from initially 25 mg l^{-1} to around 18 mg l^{-1} (Fig. 1). Total ammoniacal nitrogen also decreased in both treatments, although the reduction was more pronounced in slu + dw. The faster decline of TAN in slu + dw lead to a widening gap between N_{tot} and TAN in slu + dw compared to a parallel decline of N_{tot} and TAN concentrations in slu. Nitrite (NO_2^-) concentrations in slu were mostly close to zero and increased to 0.36 mg l^{-1} only on the last sampling day. In comparison, NO_2^- in slu + dw increased steadily from initially 0.05 mg l^{-1} to 8.88 mg l^{-1} on the last sampling day. Nitrate (NO_3^-) fluctuated less in slu with values between 4.17 mg l^{-1} on the first and 4.52 mg l^{-1} on the last sampling day, and only a low increase in slu + dw from 4.38 mg l^{-1} on the first to 5.58 mg l^{-1} on the last sampling day.

In experiment 2 (with light) the pH fluctuated slightly more compared to experiment 1. In slu, the minimum pH was 7.30, the maximum 7.92 while for slu + dw the minimum pH was 7.25 and the maximum 8.08. Similar to experiment 1, N_{tot} of slu declined from initially around 25 mg l^{-1} to 18.9 mg l^{-1}, while N_{tot} declined to 14.7 mg l^{-1} in slu + dw. Total ammonia nitrogen declined again less in slu from 21 mg l^{-1} to 15.2 mg l^{-1} on the last sampling day. In slu + dw, the decline in TAN was more pronounced from an initial 21.5 mg l^{-1} to 5.1 mg l^{-1} at the end. Nitrite concentrations during experiment 2 were basically close to zero in slu, but showed a strong increase in slu + dw, again similar to experiment 1, with NO_2^- concentration reaching 5.25 mg l^{-1}on the last

	Experiment 1 (dark)		Experiment 2 (light)	
	Initial	**Final**	**Initial**	**Final**
Duckweed				
DM (% of FM)	9.89 ± 0.65	6.49 ± 0.13	10.3 ± 0.59	6.96 ± 0.37
C (% DM)	41.0 ± 0.15	42.1 ± 0.14	41.0 ± 0.74	41.1 ± 0.09
N (% DM)	5.41 ± 0.05	5.05 ± 0.06	5.50 ± 0.04	5.45 ± 0.05
C:N ratio	7.58 ± 0.07	8.35 ± 0.09	7.45 ± 0.12	7.53 ± 0.06
Substrate				
TDS slu (mg l^{-1})	1234 ± 11.0	1221 ± 4.46	1244 ± 11.5	1158 ± 11.7
TDS slu + dw (mg l^{-1})	1241 ± 11.4	1131 ± 8.73	1249 ± 11.4	1082 ± 7.48
Corg slu (% DM)	19.2 ± 2.4	13.2 ± 1.0	15.4 ± 1.0	14.3 ± 0.6
Corg slu + dw (% DW)	18.8 ± 1.9	11.6 ± 0.7	16.1 ± 2.2	11.7 ± 1.1

Productivity parameters		
Inoculum (g drip weight)	750	750
Inoculum (g FM)[a]	537 ± 9.70	530 ± 8.68
Harvest (g drip weight)	1391 ± 26.0	1109 ± 18.7***
Harvest (g FM)	995 ± 7.56	784 ± 7.84***
Biomass gain (g FM)	458 ± 11.5	253 ± 10.1***
RGR (day^{-1})	0.088 ± 0.003	0.056 ± 0.002***
FM production (g m^{-2} d^{-1})	81.8 ± 2.06	45.2 ± 1.80***
DM production (g m^{-2} d^{-1})	5.31 ± 0.16	2.93 ± 0.11***
Protein production (g m^{-2} d^{-1})	1.67 ± 0.05	0.92 ± 0.03***
Extrapolated protein production (t ha^{-1} a^{-1})	6.09 ± 0.18	3.36 ± 0.12***
CO_2eq (kg kg^{-1} CP)	3.55 ± 0.10	6.45 ± 0.23***
PAR transmission (%)	n.a	54.8 ± 1.79***

Table 1. Composition and productivity of duckweed and substrate TDS in both experiments, extrapolated protein production and CO_2eq for duckweed protein. Values = mean ± SEM (N = 5). [a]: Inoculum (g drip weight) corrected for loss of water after centrifuging duckweed in salad spinner, *** = highly significant difference between experiments ($p < 0.001$; Student's t-test).

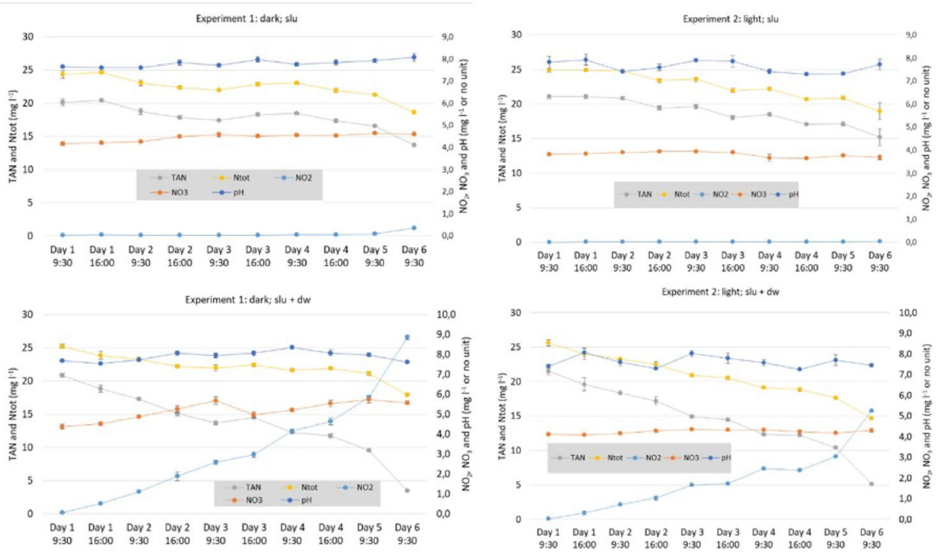

Fig. 1. Concentrations of mineral N forms during experiment 1 (left) and experiment 2 (right) in the substrates of both treatments (top = slu, bottom = slu + dw) and pH. TAN = total ammoniacal nitrogen ($NH_3 + NH_4^+$), N_{tot} = total N, NO_2^- = nitrite, NO_3^- = nitrate. Values = mean ± SE (N = 5).

sampling day. Nitrate concentrations showed only little fluctuation in both treatments but were on average lower in slu (3.64–3.94 mg l^{-1}) compared to slu + dw (4.09–4.34 mg l^{-1}).

Ammonia emissions

In both experiments, NH_3 volatilization was significantly reduced by duckweed presence acting as a live cover compared to diluted slurry without duckweed (Fig. 2).

In experiment 1, NH_3 emissions from slu fluctuated during the day, with typically highest emissions around midday (01:00 pm). For slu + dw, NH_3 emissions fluctuated less and were several-fold lower compared to slu. Peak NH_3 emissions from slu were 50.8 µg NH_3-N m^{-2} h^{-1} on sampling day 4, while peak NH_3 emission from slu + dw were 4.54 µg NH_3-N m^{-2} h^{-1} on sampling day 3.

In experiment 2, the NH_3 emissions were also significantly reduced in slu + dw compared to slu only. However, emissions from slu were highest on the afternoon of the first sampling day (05:00 pm, 44.4 µg NH_3-N m^{-2} h^{-1}), while emissions from slu + dw were highest in the morning of the first sampling day (09:00 pm, 15.05 µg NH_3-N m^{-2} h^{-1}). The emission patterns of the slu treatment were different between experiments 1 and 2. In experiment 1, NH_3 emissions from slu increased during the experimental period, while in experiment 2, the highest NH_3 emissions occurred at the beginning and decreased toward the end of the week.

Greenhouse gas emissions

Methane

In both experiments, substrates showed highest CH_4 emissions within 12 h from the start (Fig. 3). After 24 h from the start, CH_4 fluxes had decreased by 60–70%. From the third day of the experiments onward, CH_4 fluxes were negligible. When slurries were exposed to light during experiment 2, CH_4 fluxes were higher, reaching peaks of 445 µg CH_4–C m^{-2} h^{-1} (slu) and 421 µg CH_4–C m^{-2} h^{-1} (slu + dw). Methane fluxes showed diurnal dynamics, with lowest values in the morning and highest values in the evening. The average CH_4 fluxes of experiments 1 and 2 were 125 µg C m^{-2} h^{-1} for slu and 117 µg C m^{-2} h^{-1} for slu + dw, respectively.

Carbon dioxide

Carbon dioxide (CO_2) fluxes showed strong daily dynamics (Fig. 4), with lower values in the morning and higher values in the evening. No considerable variation of values measured in the mornings was observed during the experiments: differences between the lowest and the highest fluxes ranged from 17 mg CO_2–C m^{-2} h^{-1} (slu, without light) to 31 mg CO_2–C m^{-2} h^{-1} for slu + dw in experiment 2 (with light). In contrast, diurnal differences of up to 62 mg CO_2–C m^{-2} h^{-1}were found for slu + dw in experiment 1 (without light). Differences in CO_2 flux between treatments and experiments were considerable. In experiment 2, when exposed to light during GHG

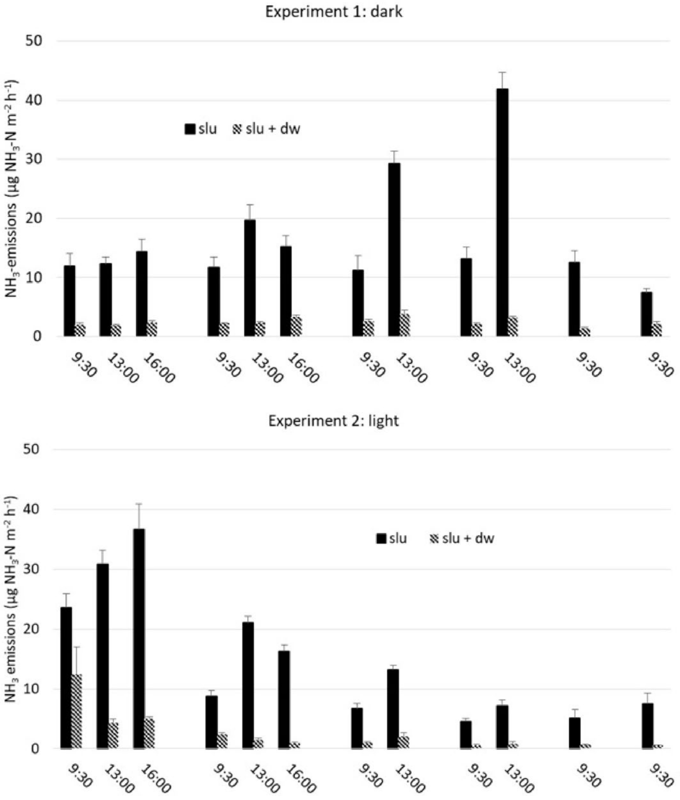

Fig. 2. NH_3-emissions (µg NH_3–N m^{-2} h^{-1}) for both experiments (mean ± SE; N = 5). slu: diluted slurry; dw: duckweed.

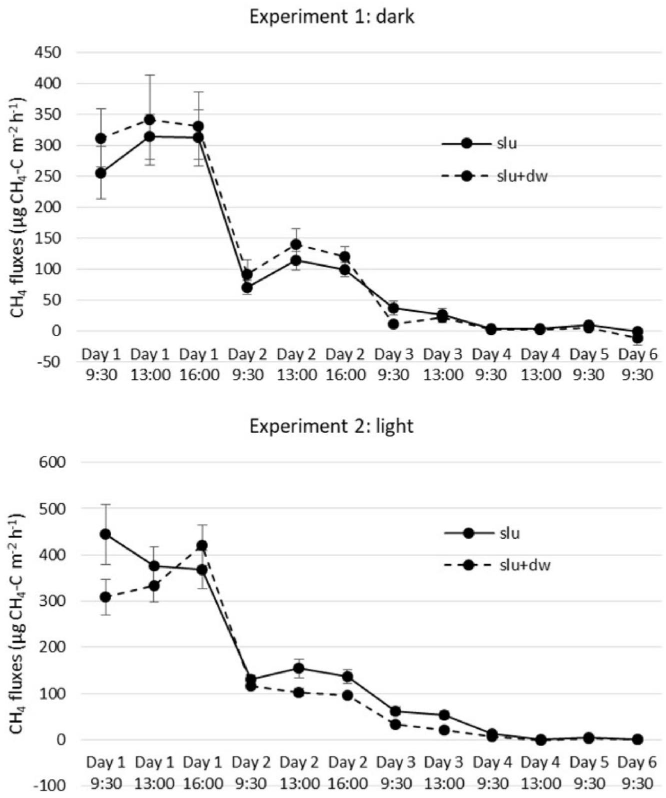

Fig. 3. Mean CH_4 fluxes (µg CH_4–C m^{-2} h^{-1}) in experiments 1 and 2 (mean ± SE; N = 5). slu: diluted slurry; dw: duckweed.

sampling, slu + dw showed negative CO_2 fluxes, but when sampling occurred in the dark during experiment 1, the same treatment exhibited higher CO_2 emissions than slu. Carbon dioxide fluxes from slu were comparable between experiments. The average CO_2–C fluxes of experiments 1 and 2 were 34.1 µg C m^{-2} h^{-1} for slu and 12.8 µg C m^{-2} h^{-1} for slu + dw, respectively.

Nitrous oxide

In both experiments, slu showed negligible N_2O fluxes (Fig. 5). However, slu + dw showed highly elevated N_2O fluxes between the first and third day of experiment when N_2O was measured in the dark (experiment 1). This effect was weaker when the substrate was exposed to light during GHG measurements, and lasted only until the second day after the start of the experiment. Particularly in the experiment without light, the N_2O fluxes of slu + dw showed a strong diurnal dynamic, with higher values in the afternoon compared to the morning. This dynamic was not as strong when the substrate was exposed to light during N_2O measurements. The average N_2O fluxes of experiments 1 and 2 were 8.30 µg N_2O-N m^{-2} h^{-1} for slu and 235 µg N_2O-N m^{-2} h^{-1} for slu + dw, respectively.

Discussion

Comparing the productivity of both experiments to earlier reports showed that experiment 1 (5.31 g DM m^{-2} d^{-1}) performed slightly lower than reported in other studies with duckweed DM production ranging between 5.7 and 10.1 g DM m^{-2} d^{-1}[12,16,40] while experiment 2 (2.93 g DM m^{-2} d^{-1}) clearly underperformed. The large difference was probably due to the different weather during experiments, which was sunnier and warmer during experiment 1 compared to more rain and cloudy conditions during experiment 2. The higher temperatures during experiment 1 is refleceted in an overall higher metabolic activity in duckweed, as reflected by higher growth. Due to space and resource limitations, the experiments had to be separated. The duckweed itself originated in both experiment from the same stock culture. Although the duckweed culture itself was older in experiment 2, the management of the stock culture was identical during the whole time, ensuring a similar age structure of duckweed in both experiments.

Some older studies[11,42] reported duckweed DM production rates as high as 12.7–14.8 g DM m^{-2} d^{-1}. A comparison of the RGR in both experiments (experiment 1: 0.088 day^{-1} and experiment 2: 0.056 day^{-1}) with the published maximum RGR for *Lemna minor* (0.375–0.426 day^{-1})[43] shows that the duckweed performance in our experiments was rather low and that there is a large potential for optimization. The CP content in both experiments (31.5% to 34.3% of DM) was in the typical range of 25–40%, but it can also reach values above 40%[34,35,44]. Similarly, the chemical compositions (DM, CL, CA, CF and P) of produced duckweed were in the expected and previously reported ranges (Table 1)[13,15,16,44].

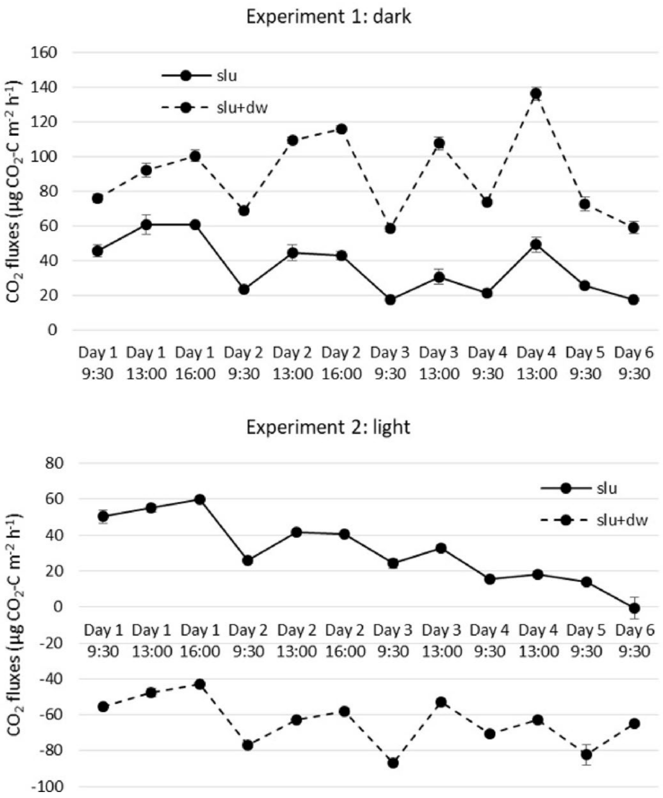

Fig. 4. Mean CO_2 fluxes (µg CO_2–C m^{-2} h^{-1}) in experiments 1 and 2 (mean ± SE; N = 5). CO_2 emissions shown here are not included in CO_2eq for protein production, as they do not only include respiration and photosynthesis, but also microbial activity of the slurry. slu: diluted slurry; dw: duckweed.

While pH, NO_3^- and N_{tot} showed similar patterns between experiments and treatments, in both experiments NO_2^- increased steadily in slu + dw but not in slu (Fig. 1). Although we did not investigate the microbial communities in slu and slu + dw, a likely explanation is the activity of NH_3-oxidizing bacteria (e.g. *Nitrosomonas* spp., *Nitrosococcus* spp., *Nitrosospira* spp. etc.)[45]. They may have colonized surfaces provided by the duckweed canopy including the rhizome in slu + dw faster compared to NO_2^- oxidizing bacteria[46], thus potentially leading to increasing NO_2^- concentrations in slu + dw but not in slu, where surface area is restricted to the side walls and the bottom of the boxes. For efficient nitrification, NH_3 oxidizing bacteria need sufficient and stable oxygen (O_2) availability, which however, was not measured in our experiments. Previous O_2 measurements in 3 cm depth of an experiment in a greenhouse using diluted chicken slurry covered with duckweed showed highly hypoxic to anoxic conditions[45] while average oxygen concentrations of 6.6 mg l^{-1} were reported for surface water (depth not reported) of duckweed covered natural ponds[40]. While a natural pond is totally subject to ambient weather and the greenhouse experiment with duckweed on diluted chicken slurry was not, the boxes in this experiment were also subject to ambient weather. Wind had probably a lower impact with the steep sides of the box protecting the duckweed, rain would reach the surface mostly unhindered and was a likely source of oxygen enrichment in the top substrate layer, except during the 1-h sampling periods when boxes were covered by lids. Furthermore, microbe density and associated oxygen consumption is probably a lot higher in diluted slurry compared to natural pond waters. Thus, it can be assumed that sufficient O_2 for associated NH_3-oxidizers was present in the top layer where fronds and roots provide surface areas for bacteria to colonize. Own but unpublished measurements of O_2 concentration gradients in similar slurry-duckweed systems in the top 10 mm of diluted slurry, resolved in 1 mm steps, support this assumption. A strong decline was observed from 1 mm depth (2.79 mg l^{-1} O_2) to 4 mm depth (1.30 mg l^{-1} O_2) after which it was more or less stable down to 50 mm at 1.26 mg l^{-1} O_2. Therefore, for future experiments on GHG emissions and N-dynamics in duckweed–diluted animal manure systems, we recommend including detailed and highly sensitive O_2 measurements with high resolution in the top layer, stretching as deep as the duckweed roots or at least 10 mm.

The TAN fractions of slu + dw also declined faster compared to slu, since duckweed species prefer NH_4^+ as N source over NO_3^-[47]. Thus, part of the TAN decline can be explained by uptake through duckweed. Since TAN also declined in slu, although not as much, microbial conversion to other N fractions can be assumed and loss as volatile NH_3 was observed in both treatments. Covering the diluted slurry with a duckweed canopy reduced NH_3 emissions by 85% (experiment 1) and 82% (experiment 2), regardless of the presence or absence of light (Fig. 2). During experiment 1 the NH_3 emissions were lower compared to experiment 2. The likely explanation is the higher metabolic activity of duckweed during experiment 1 and N uptake rates as reflected in the higher growth and protein production. However, the NH_3 emissions were measured with acid traps in

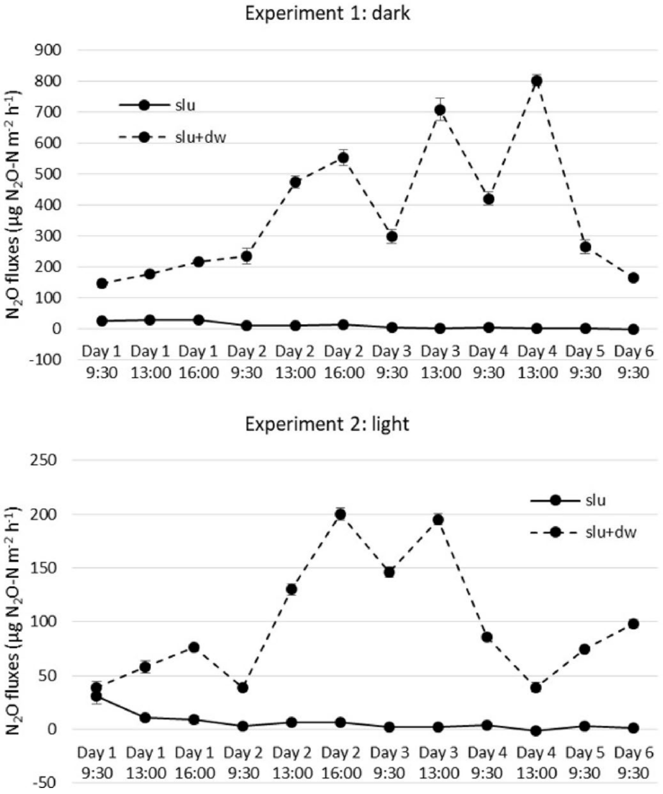

Fig. 5. Mean N_2O fluxes (µg N_2O–N m^{-2} h^{-1}) in experiments 1 and 2 (mean ± SE; N = 5). slu: diluted slurry; dw: duckweed.

the closed chamber, which underestimates volatilization compared to the dynamic flow-through chamber or portable ammonia detector methods[48]. However, the general efficiency of duckweed to reduce NH_3 emissions by 34.2–54.2% has previously been shown for emissions from paddy rice fields[49,50]. At the same time, N_2O emissions from rice fields covered by duckweed increased significantly by 187–210%[50].

No differences were observed for CH_4 emissions between both experiments of our study, both without and with light. They were initially high in both experiments, probably due to system start-up and agitation during filling of the boxes on day 1, before rapidly declining until day 4, with almost no emissions until end of both experiments. No CH_4 emissions have been detected for duckweed grown on domestic wastewater[36], which was probably due to the detection method used not being sensitive enough (LANDTEC® GEM 2000 portable analyser equipped with an infrared probe). In lab-scale duckweed ponds dedicated to storm water treatment, CH_4 emissions between 502 to 1900 mg m^{-2} d^{-1} were reported and attributed to diurnal dissolved O_2 patterns[38]. That was 70 to 176-fold higher compared to the maximum CH_4 emissions detected in both our experiments, ranging from 300 to 450 µg m^{-2} h^{-1} or around 7.2 to 10.8 mg m^{-2} d^{-1} (Fig. 3). Emissions from natural ponds, covered with duckweed were on average 1150 mg CH_4–C m^{-2} d^{-1} (corresponding to 1987 mg CH_4 m^{-2} d^{-1}),[40]. Simultaneously, duckweed ponds also had a significantly larger anoxic fraction and lower surface O_2 concentration compared to ponds without duckweed[40]. In experimental stormwater treatment ponds inoculated with duckweed (*L. minor*), the CH_4 fluxes were between 180 and 299 mg m^{-2} d^{-1} with duckweed present and increased to 329 and559 mg m^{-2} d^{-1} after removal of duckweed[39]. While most reports indicated average CH_4 emissions much higher than those observed in the two experiments of our study, the most striking fact was that the CH_4 fluxes dropped to zero within a few hours after the start of the experiments and apparently no new CH_4 was released. The reason might be in the relatively shallow depth of our system and the little sedimentation rate during the short experimental periods, as observed at the end of the experiment after draining the boxes. In natural ponds, high organic loading and anoxic conditions in and above the sediment provide suitable conditions for methanogenic archaea to constantly resupply CH_4 to the system[36] which was not the case for our system.

Carbon dioxide emissions in our experiments followed expected and previously reported patterns for duckweed growing on domestic wastewater[36]. For example, CO2 was released in the absence of light (respiration), while CO_2 was fixed in the presence of light (photosynthesis).

A net CO_2 fixation for duckweed wastewater treatment plants has also been reported previously with emission rates of 831–1641 mg CO_2–C m^{-2} d^{-1} and CO_2 fixation rates of 5343–11,469 mg CO_2–C m^{-2} d^{-1}, showing high C-fixation potential of duckweed biomass[36].

In stormwater treatment ponds, CO_2 fixation rates were significantly negatively correlated with duckweed cover. Ponds with high duckweed coverage had lower fixation rates (25.9 mg CO_2–C m^{-2} d^{-1}) but were still a net

C sink[40]. In lab-scale stormwater treatment ponds, emissions were between 626 and 1472 mg m^{-2} d^{-1} CO_2–C with duckweed which declined to 395–492 mg m^{-2} d^{-1} after removal of duckweed[39].

Of course, C-fixation by duckweed depends strongly on duckweed growth. A production of 3.3–5.2 g DM m^{-2} d^{-1} was reported from Brazil[36], which was similar to the DM production in our experiments. Optimization of biomass production thus has the potential to also increase the C-fixation capacity.

The difference in N_2O emissions between slu and slu + dw was striking and independent of light in both experiments. Slurry + duckweed emitted significantly more N_2O compared to slu (Fig. 5). The emissions measured in our experiments were similar to reported ranges of N_2O emissions from lab-scale duckweed wastewater ponds (0.40–2.55 mg N_2O–N m^{-2} d^{-1})[38]. In constructed wetlands, temperature was found to be a major factor influencing N_2O emissions. High sediment temperatures (> 20 °C) resulting in high N_2O fluxes which were higher in plots with plants (605 ± 209 µg N_2O–N m^{-2} d^{-1}) compared to plots without plants (− 55 ± 209 µg N_2O–N m^{-2} d^{-1})[50]. In line with this, emissions from 12.7 to 605 mg N_2O–N m^{-2} d^{-1} have been reported for artificial sludge treatment wetlands with higher emissions in summer compared to winter[52]. While temperature might explain generally higher N_2O emissions in experiment 1 compared to experiment 2, higher emissions from slu + dw compared to slu was observed in both experiments and cannot be explained by temperature alone. Instead, elevated N_2O emissions from slu + dw might be the result of higher mineralisation rates as suggested by greater decrease of total C during the experiments (Table 1) and larger CO_2 fluxes in the experiment without PAR (Fig. 4). The combination of increased ammonification and decreased NH_3 volatilisation might have stimulated nitrification and denitrification resulting in increased N_2O emissions.

Results from rice paddies covered with another floating plant, the water fern *Azolla* spp., were somewhat contradictive. On the one side, significantly higher N_2O emissions (615 µg N_2O–N m^{-2} d^{-1}) were reported when *Azolla* spp. was used as cover crop compared to no cover (182 µg N_2O–N m^{-2} d^{-1})[53]. Contrary to that, only initially high N_2O emissions (163 µg N_2O–N m^{-2} d^{-1}) rapidly declining to close to the detection limit (< 0.01 µg N_2O–N m^{-2} d^{-1}) were reported for paddies covered with *Azolla*[54]. They attributed the initially high emissions to high soil solution NO_3^- concentrations subject to denitrification, after which no N_2O was newly formed. Incomplete nitrification and denitrification processes under changing microaerobic and anaerobic conditions were considered as major factors responsible for high N_2O emissions[38,51]. This suggests that diffusion of O_2, possibly from duckweed respiration, might have enabled transformation of NH_4 via nitrification and thus delivered substrate for N_2O forming processes in the slu + dw treatment. Raising NO_2^- concentrations indicate enhanced nitrification in slu + dw compared to slu, but future experiments should include O_2 measurements via microsensors to confirm this hypothesis.

Conclusion

Duckweed–slurry systems are efficiently taking up N and P and are highly productive protein producers[34,35]. Yet, they are severely understudied in terms of GHG and NH_3 emissions under production conditions. As previously reported, duckweed produced on slurry acts as a net CO_2 sink, with initially high CH_4 emissions, which quickly drop to negligible fluxes. However, the high N_2O emissions give rise to concern, and their causes need to be studied in detail. This includes the microbial community present in diluted cattle slurry, both alone and with growing duckweed, as well as oxygen profiles over time and at different depths. These results will lead to possible measures for mitigating N_2O emissions. Nitrous oxide contributes massively to the CO_2eq of the duckweed protein produced in these experiments. However, higher productivity reduces the CO_2eq for duckweed protein by almost half, showing that system productivity also needs to be optimized. Nevertheless, despite high N_2O emissions and only average productivity, the CO_2eq of duckweed protein can compete with other plant-based proteins from optimized agricultural systems such as faba beans or barley.[55] This provides evidence of the large potential of duckweed protein as a sustainable new source of protein. We acknowledge that certain limitations of our experimental design, especially the lack of oxygen measurements and the time lag between experiments, introduce uncertainties in the results. However, while the amplitude of the measured GHG and NH_3 emissions under dark and light conditions may be affected by the time lag between experiments, the general emission dynamics will most likely not.

Methods

Duckweed culture

Before the experiment, duckweed was collected from a wild population in Switzerland and initially cultured as described elsewhere[44]. The duckweed species *Lemna minor* was genetically identified by the team of Dr. Laura Morello (Italian National Research Council – Institute of Agricultural Biology and Biotechnology) based on the method described by Braglia et al.[56]. *Lemna minor* is not an endangered plant species (IUCN category: least concern (LC)) and thus not subject to either the IUCN Policy Statement on Research Involving Species at Risk of Extinction (https://portals.iucn.org/library/efiles/documents/PP-003-En.pdf) nor the Convention on the Trade in Endangered Species of Wild Fauna and Flora (https://cites.org/).

Experimental conditions

Meteorological conditions where different during experiment 1 (dark, without photosynthetic active radiation PAR) and experiment 2 (light, with PAR) (Fig. 6). Experiment 1 was conducted from 22.08.2022 to 29.08.2022. During this period the weather was mostly sunny with mean air temperatures (5 cm above ground) of 21.6 °C, with a highest value of 42.7 °C on day 2, and a lowest value of 12.6 °C, in the night between day 1 and day 2. The solar radiation peaked on day 2 with 902 W m^{-2} at 13:00 and dropped to 253 W m^{-2} on day 5 at 15:00. Precipitation was limited to days 4 and 5 with a maximum of 3.4 l m^{-2} on the evening of day 5.

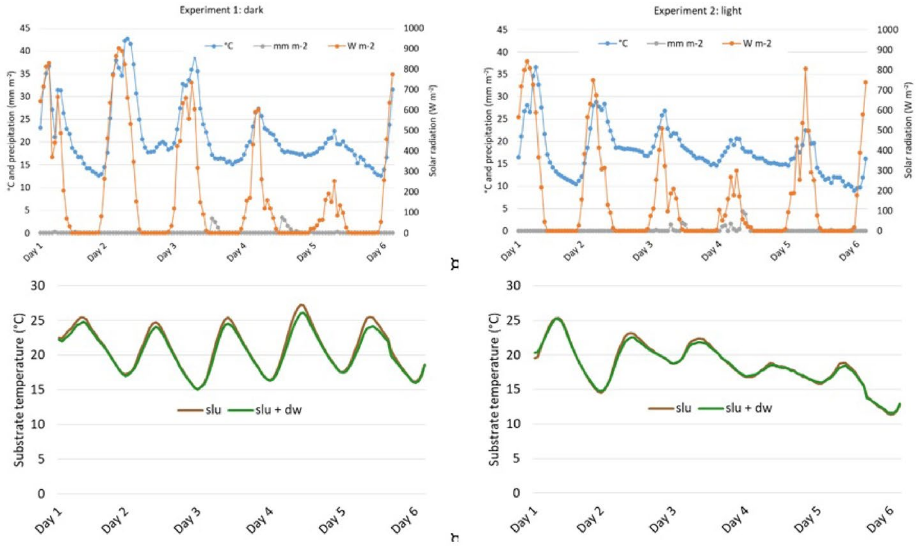

Fig. 6. Air temperatures, precipitation and solar radiation (upper figures) and substrate temperatures (lower figures) shown for experiment 1 (left) and experiment 2 (right). slu: diluted slurry; dw: duckweed.

Experiment 2 was conducted from 12.09.2022 to 19.09.2022 with only the first day having comparable weather conditions to experiment 1 (hot and sunny). The following days were cloudy with some heavy rains in between. Mean air temperature during experiment 2 was 17.8 °C, peaking on day 1 at 36.6 °C, followed by a sudden drop to 10.4 °C in the early morning of day 2. The solar radiation during experiment 2 peaked on day 1 with 841 W m^{-2} at 13:00 and dropped to 299 W m^{-2} on day 4 at 14:00. Precipitation was highest on day 4 with 4.4 l m^{-2} d^{-1}. Due the heavy rainfall on day 3 sampling point 8 (day 3, 13:00) could not be addressed.

Slurry temperatures were identical in both experiments between boxes without (slu) and with duckweed stocked (slu + dw) (Fig. 6). In experiment 1, average daily values were around 25 °C with a maximum of 27.3 °C during the day and a minimum of 15.0 °C during the night. In experiment 2, the temperatures were slightly lower overall than in experiment 1, but also identical between the slu and slu + dw treatments. The substrate temperatures reached their maximum (25.3 °C) on the first day and declined during the week. The minimum substrate temperature was 11.3 °C on the last day. The weekly average fraction of photosynthetic active radiation (PAR) reaching the duckweed through the transparent lids of experiment 2 was 54.8% or 480 ± 268 µmol m^{-2} s^{-1}, with a maximum PAR of 1105 µmol m^{-2} s^{-1} reaching the duckweed on day 1, and a minimum of 128 µmol m^{-2} s^{-1} on day 3.

Experimental setup

Two different experiments were conducted, one simulating night and one simulating day conditions during assessment of gaseous emissions. For experiment 1 (dark) grey PVC lids were used preventing light penetration. For experiment 2 (light) transparent lids were used, allowing a certain percentage of photosynthetic active radiation (PAR, µmol m^{-2} s^{-1}) to pass. A total of 10 boxes (116 × 73 × 41.5 cm; 0.85 m^2; 331.7 l total volume) were placed outdoor at the Research Institute of Organic Agriculture in Switzerland. They were each filled with 8.5 l organic cow slurry and diluted with tap water to a level of 21 cm to reach a total volume of 170 l in the box, leaving a headspace volume of 160 l. This dilution was chosen to initially reach around 20 mg/l NH_4^+-N which was identified as the optimum for protein production[16].

Each box was prepared identically (Fig. 7), except that in two boxes, one with and one without duckweed, thermometers were installed to enable temperature measurements of headspace air. The top edge of each box was sealed by e-profile soft-rubber seals (tesa SE, Norderstedt, Germany). Inside each box, two battery-powered brushless fans were attached in opposite corners using Velcro in order to guarantee headspace homogeneity during each measurement cycle of gaseous emissions. In the centre of each lid, a strip of Velcro was glued for later attachment of acid traps. In one corner of each lid, a 3-way valve for headspace sampling was installed (Gardena micro-drip, Gardena, Germany), which was tested for gas tightness beforehand.

In both experiments, two different treatments were tested, treatment 1 consisted of diluted slurry only (slu) while treatment 2 consisted of diluted slurry inoculated with 750 g of duckweed (slu + dw).

Experimental procedures

Before stocking duckweed onto the diluted slurry, three 250 g samples of fresh duckweed were taken from the stock culture for determination of fresh and dry matter and C and N content in initial duckweed. Then, five boxes were stocked each with 750 g of fresh *L. minor* while the substrate of the other five boxes was not inoculated. The fresh matter of duckweed was determined in two ways, a gentle and a precise way[44]. For inoculation, duckweed was harvested from a stock culture with a net and left dripping for around 10 min until no more water drops were visible (drip-weight). For precise fresh matter determination of the three initial duckweed samples and at

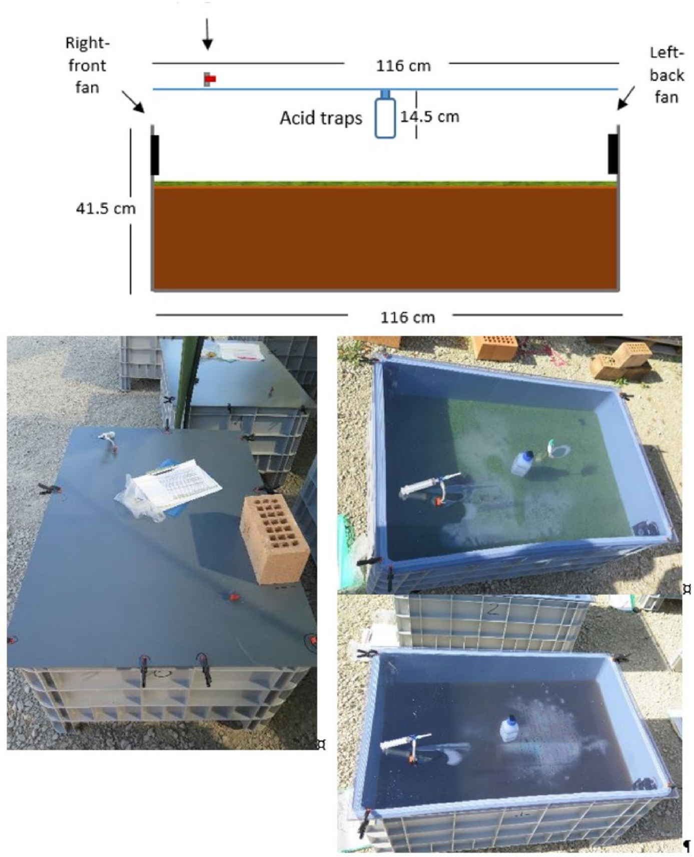

Fig. 7. Experimental setup of the boxes with a schematic drawing (upper) and pictures of boxes with opaque (experiment 1, left) and transparent lids (experiment 2, right). Through the transparent lid, both fans are visible in opposite corners, the acid trap in the centre besides an installed thermometer and the sampling valve with attached 20 ml syringe in the lower left corner. The picture on the bottom right shows a box with only diluted slurry and no duckweed. Source: T. Stadtlander/D.M. Gomez.

the end of both experiments, duckweed was harvested and the respective biomass centrifuged 2 times each for 30 revolutions in a common household salad spinner. In both experiments measurement cycles were started on a Monday morning and GHG and NH_3 volatilization measurements were conducted Monday and Tuesday at around 9:30, 13:00 and 16:00, Wednesday and Thursday at 9:30 and 13:00 and Friday and Monday at 9:30. For each measurement cycle, the lids were placed on the boxes and sealed airtight for 60 min using spring clamps. Before closing the lids, the acid traps (rectangular 100 ml plastic bottles with 4-cm diameter wholes in each of the four sides, see Fig. 7) were filled with 10 ml 0.05 M H_2SO_4 and attached in the centre of the lids by Velcro just before lids were closed. The 10 ml of 0.05 M H_2SO_4 used in each trap were sufficient for binding a maximum of 17 mg gaseous NH_3 per measurement cycle. During the first 30 min after lids were closed, the headspace of each box was sampled four times at regular intervals (0 min, 10 min, 20 min, and 30 min). Before each headspace sampling the 20 ml disposable syringes were rinsed three times with headspace air. Afterwards, 20 ml headspace air was transferred to pre-evacuated 12 ml vials (Labco, UK). The timepoint of each sampling was recorded using stop watches for each box. After headspace sampling, boxes were kept close for another 30 min in order to guarantee sufficient contact time between mixed air in the headspace of boxes and acid traps. After 60 min, the lids were opened, the H_2SO_4 from acid traps was quantitatively transferred into 50 ml sample tubes using a 10 ml precision pipette and stored at − 20°C until further analysis. All collected GHG samples were stored at room temperature until analysis. Every day before the first cycle and, if more than one measurement cycles were conducted, after the last cycle, substrate depth was measured with a precise ruler in order to determine evaporation, evapotranspiration and head space volume during the experimental week.

Before the lids were closed for the first measurement cycle of the day, substrate temperature and total dissolved solids (TDS, mg/l) were determined using a WTW Multi 3420 equipped with a TetraCon 925 probe (Xylem Analytics Germany, Weilheim, Germany). For continuous slurry temperature measurements during each experiment, six Thermochron iButton temperature loggers (iButtonLink Technology, Whitewater, USA) were sealed into water tight bags and stored on the bottom of three boxes each per treatment, respectively. A 50 ml substrate sample was collected from every box before the first measurement and after the last measurement cycle, except for Fridays and Mondays, when there was only one measurement cycle. The slurry samples were analysed for TAN (total ammonia nitrogen), total N (N_{tot}), nitrite (NO_2^-), nitrate (NO_3^-) and pH and were kept frozen at − 20 °C until analysis. To calculate light transmission of PAR (µmol m^{-2} s^{-1}) through the transparent lids in experiment 2 PAR was measured with a MQ-610 handheld PAR detector (Apogee Instruments Inc., Logan, USA) directly above and below the lids after each cycle in experiment 2 only.

Duckweed growth and biomass production was calculated as fresh and dry biomass gain and growth was calculated as relative growth rate (RGR, day^{-1})[43]:

$$\mathrm{RGR}\ (\mathrm{day}-1) = (\ln\ (\mathrm{W_{end}}) - \ln\ (\mathrm{W_{start}}))\,/\mathrm{d}$$

With W_{end} being duckweed biomass at the end, W_{start} being duckweed biomass at the start of the growth period, and d the elapsed time in days (d) between both measurements.

Analyses of duckweed and substrates

Duckweed analysis

Frozen duckweed samples were analysed after thawing. Dry matter (DM) was determined in a 2 g fresh matter (FM) sub-sample by drying at 105 °C for 6 h. The remaining duckweed was dried at 40 °C for two days. After the two days, the dried sample was finely ground using an electrical household coffee grinder. Total carbon and nitrogen were determined in pulverized and dried duckweed by the Dumas method with a C/N elemental analyser (vario Max CUBE, Elementar Analysensysteme GmbH, Langenselbold, Germany) and a N to CP conversion factor of N * 6.25 was used.

Substrate analysis

For total N (N_{tot}; TAN + NO_2^- –N + NO_3^- –N) analysis, total ammoniacal nitrogen including that from the acid traps (TAN; $NH_3 + NH_4^+$), nitrite (NO_2^-) and nitrate (NO_3-) determination samples were thawed and 2 ml subsamples centrifuged for 3 min at 20.000 rpm (13.000 × g). The supernatants were analysed using a Smartchem 450 discrete analyser (AMS Alliance, Frepillon, France). Total C was determined according to the Dumas method by total oxidation of 2.5 g substrate fresh weight at 900° C followed by CO_2 quantification by thermal conductivity detection. The pH was determined using the WTW pH 7110 pH meter (Xylem Analytics Germany, Weilheim, Germany) with an attached Mettler Toledo Expert pT 1000 pH probe (Mettler Toledo Schweiz GmbH, Greifensee, Schweiz).

GHG analysis and calculations

Gas samples were analysed for CH_4, CO_2 and N_2O simultaneously on a double-channel gas chromatograph (7890A, Agilent Technologies, CA) equipped with HayeSep Q 80/100 UM columns and configured according to Wang[57]. To reduce the total measurement duration, oven temperature was adjusted to 100° C. N_2O was quantified on an electron capture detector (μECD), CH_4 and CO_2 were quantified on a flame ionization detector (FID) coupled with a methanizer for the reduction of CO_2 to CH_4. Samples were injected to the instrument by an autosampler (MPS 2XL, Gerstel AG, Switzerland).

Sample concentrations of measured GHG were calculated based on the peak area of each signal as integrated by Open Lab Chemstation Software (Agilent Technologies, CA) and a three-point calibration. GHG fluxes were obtained using the R package "gasfluxes"[58] considering chamber headspace temperature, GHG emission area, GHG concentrations at four different sampling time points and time interval between sampling time points. The "kappa.max" selection algorithm was applied to choose between linear, robust linear and Hutchinson–Mosier regression (HMR) models for GHG flux calculation[59].

To calculate the CO_2eq (kg) per kg crude protein produced in both experiments, each emitted kg CH_4 was calculated as 27 kg CO_2eq and each emitted kg N_2O was calculated as 273 kg CO_2eq[60].

Statistical comparison of productivity parameters (Table 1) between experiments 1 and 2 were conducted by Student's t-test ($p = 0.05$) after testing for homogeneity of variance (Levene test) and normal distribution (Shapiro-Wilks test) using SPSS version 24 (IBM Corporation, Armonk, USA).

Data availability

All relevant data is available in the manuscript, tables and figures.

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Author contributions

T.S.: Design of study, data acquisition, data analysis, writing of manuscript, revision of manuscript. D.M.G.: Design of study, data acquisition, data analysis. R.M.: Design of study, data acquisition, data analysis. C.B.: Data acquisition. N.B.: Design of study, revision of manuscript. F.L.: writing of manuscript, revision of manuscript. H.M.K.: data analysis, writing of manuscript, revision of manuscript. L.A.: Design of study, data acquisition, data analysis, writing of manuscript, revision of manuscript.

Competing interests

The authors declare no competing interests.

Declaration of plant material procurement

Since *Lemna minor* is endemic to Switzerland and is not a protected species (IUCN status: least concern) and it is naturally growing on our own institute's premises in rainwater ponds, it is not subject to any type of permission process (e.g. such as the Nagoya protocol) necessary for this type of research project. No voucher specimen has been deposited in a publicly available herbarium.

Additional information

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