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Lowering pH enables duckweed (*Lemna minor* L.) growth on toxic concentrations of high-nutrient agricultural wastewater

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ABSTRACT

The use of duckweed species to remediate nutrient-rich wastewater has grown as a field of research and in industry; however, the need to dilute wastewater to the low ammoniacal-N concentrations tolerated by duckweed represents a barrier to commercially implementing these systems in agriculture. This study investigated the potential for acidifying anaerobically digested cattle slurry (digestate), shifting the NH_4^+:NH_3 equilibrium towards the less toxic ionised form, thus allowing the growth of *Lemna minor* on less dilute wastewater. First, a study was conducted to identify the ammoniacal-N concentrations tolerated by *L. minor* and to confirm the positive effect of lower pH on growth in high nutrient solutions using modified Hutner's solutions at two pH levels (8.2 and 6.5). In Hutner's solution at a pH of 8.2, *L. minor* growth was highest at the lowest ammoniacal-N concentration of 10 mg L^{-1} and decreased with increasing concentrations. At a pH of 6.5, *L. minor* growth remained unaffected with increasing ammoniacal-N up to a concentration of 250 mg L^{-1}. *L. minor* was then grown in digestate concentrations ranging from 5% (65 mg L^{-1} ammoniacal-N) to 30% (350 mg L^{-1} ammoniacal-N), based on its growth in Hutner's solutions. It was hypothesised, that growth would decrease as the digestate concentration increased at pH 8.2, and that acidifying digestate to pH 6.5 would alleviate this effect. On unamended digestate (pH 8.2), *L. minor* growth was prevented even in the most dilute treatment (5%); however, on acidified digestate (pH 6.5), growth rates remained positive and significantly higher than the unamended controls up to the 20% dilution (239.3 mg L^{-1} ammoniacal-N). Higher growth rates in the Hutner's solutions compared to digestate, particularly at pH 8.2 where no growth was recorded in digestate, suggest the presence of additional inhibitory factors in complex, high-nutrient wastewaters, and potentially sub-optimal concentrations of some of the nutrients provided in Hutner's solution. Nevertheless, correlation matrix analysis of digestate chemical properties highlighted the importance of acidification, with a strong negative correlation between pH and *L. minor* growth rate. For the first time, we demonstrate that by lowering pH, *L. minor* could be grown on dilutions of nutrient-rich agricultural wastewater that were otherwise toxic, and which make it feasible as a nutrient removal method. These findings could have important implications for implementing duckweed-based remediation systems in agriculture, increasing water- and land use efficiency, and thus, their commercial viability.

1. Introduction

In response to a growing demand for meat and dairy products (van der Spiegel et al., 2013), agricultural practices for the rearing of livestock have shifted to more intensive methods (Herrero et al., 2016). A major problem arising from the intensification of livestock production is the storage and disposal of the high-nutrient wastewater produced, commonly referred to as slurry (Sońta et al., 2020). Slurry is typically stored in lagoons before being applied onto land as a fertiliser. However, modern farms require large areas of land to dispose of the quantity of slurry typically produced. Over-application of slurry on land leads to an accumulation of nutrients such as nitrate and phosphate in the soil, which subsequently leach into surface- and groundwater (Dungait et al., 2012), whilst runoff slurry entering waterbodies also represents a direct, point source of pollution (Withers and Lord, 2002). Pollution of waterbodies from agricultural sources leads to the death of aquatic species, due to the hypoxic conditions resulting from algal blooms (Johnson and Dawson, 2005) and the high biological oxygen demand (BOD) of slurry

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(Otabbong et al., 2007), as well as the direct toxic effects of ammonia and nitrite (O'Neill et al., 2020). Gaseous ammonia (NH_3) emissions from slurry is also a concern, as it can further contribute towards the eutrophication and acidification of aquatic habitats when deposited via rainfall (Kavanagh et al., 2019), as well as react with other atmospheric pollutants to form particulates that are harmful to human health (Erisman et al., 2007). Slurry is also a substantial source of potent greenhouse gases (nitrous oxide and methane) (Kavanagh et al., 2019). As a result, slurry storage and disposal is highly regulated, with additional restrictions aiming to mitigate the effect of intensive agricultural practices on water quality, such as the Nitrate Vulnerable Zones resulting from European Union directive 91/676/ECC (European Union, 1991), adding to the challenges of effectively utilising slurry.

Due to the challenges of managing agricultural wastewater and preventing environmental pollution, there is a growing need to develop alternative methods of treatment and/or extracting value from these resources. One approach that has become common in the UK in recent years is anaerobic digestion (AD) (Bhogal et al., 2016; Taylor et al., 2011). AD involves anaerobically digesting slurry, along with other waste streams including crop residues and food waste, generating biogas (mainly CH_4 and CO_2) and a residual effluent called digestate (Möller and Müller, 2012). Digestate has high concentrations of readily available nitrogen (RAN) due to the mineralisation of organic matter during the digestion process (Möller and Müller, 2012), and as such it is a valuable fertiliser (Litterick et al., 2016). However, AD alone does not solve the problem of slurry management, as the digestate produced must still be stored and disposed of in the same way as slurry to avoid the same environmental pollution effects (Lamolinara et al., 2022; Litterick et al., 2016). Therefore, there remains a need for additional steps to digestate treatment and valorisation, especially by utilising nitrogen compounds (Fernandes et al., 2020).

Several methods have been developed for nutrient removal from agricultural wastewater and digestate, including ammonia stripping, air scrubbing, membrane filtration and struvite crystallisation (Shi et al., 2022). High nutrient recovery rates can be achieved with these approaches, ranging between 57-86% and 64–87% for N and P removal, respectively, with struvite crystallisation proving the most efficient (Shi et al., 2022). However, they are expensive to set up and require technical expertise to maintain, which is a major barrier preventing their widespread implementation (Shi et al., 2022). In contrast, phytoremediation, where plants are used to recover pollutants from wastewater, represents a relatively low-tech and cost-effective alternative (Landesman et al., 2010). Whilst the potential for wastewater treatment using macrophytes and algae has been known since the 1980s (Zirschky and Reed, 1988), research into its potential use in the agricultural industry is still a fairly recent development (Devlamynck et al., 2021b; Mohedano et al., 2012; Stadtlander et al., 2019). Duckweed species are particularly suitable, given that they can grow under a wide range of climatic conditions and can be harvested with relative ease (Zirschky and Reed, 1988). High rates of nutrient removal in duckweed growing systems have been reported (Mohedano et al., 2012), due in part to its rapid growth rate (Ziegler et al., 2015). In addition, duckweed crude protein content can be as high as 43% dry matter (DM) (Leng et al., 1995) with an amino acid profile comparable to soybean (Appenroth et al., 2017; Rusoff et al., 1980). Thus, the harvested biomass can be used as an animal feed in agriculture (Sońta et al., 2019) and aquaculture (Stadtlander et al., 2019), and potentially processed for human consumption (Appenroth et al., 2017). Therefore, duckweed could not only address the eutrophication risk posed by agricultural wastewater, its potential as a sustainable livestock feed could reduce reliance on costly and often imported feedstocks such as soybean (Sońta et al., 2019).

The prospect of using duckweed as part of a circular economy approach to close the nutrient loop in agriculture has attracted a great deal of attention, with large-scale systems already trialled in a range of countries including the Netherlands (Kroes et al., 2016) and Brazil (Mohedano et al., 2014). However, concerns remain over the commercial viability of implementing such growing systems in agriculture. It is well documented that climatic factors may limit duckweed growth in temperate regions (Paterson et al., 2020). There may also be regulatory barriers to incorporating wastewater-grown duckweed into food chains (van der Spiegel et al., 2013). Another issue is the high concentrations of inorganic nitrogen in agricultural wastewater, mainly in the form of ammoniacal-N ($NH_4^+ + NH_3$) (Nicholson et al., 2016). The ammoniacal-N concentration of livestock slurry-based digestate, for example, typically ranges between 1800 and 2800 mg L^{-1} (Taylor et al., 2011). Whilst ammonium is reported to be its preferred nitrogen form (Fang et al., 2007; Porath and Pollock, 1982), the concentrations found in digestate and other agricultural wastewaters are substantially higher than those tolerated by duckweed; optimal ammoniacal-N concentrations range between 3.5 and 20 mg L^{-1}, whilst growth rates decline above 50 mg L^{-1} (Caicedo et al., 2000). Unamended digestate would therefore require a 40-fold or greater dilution to allow duckweed growth, and previous studies using similar agricultural waste streams also report high dilution rates (Sońta et al., 2020; Stadtlander et al., 2022). This makes using duckweed for wastewater remediation unfeasible on the scale required by the agricultural industry, as dilution increases the water and land area required to treat each unit of wastewater (Sońta et al., 2020). As such, research into ways of growing duckweed on more concentrated wastewater is essential.

One potential approach to growing duckweed on more concentrated wastewater involves lowering the pH of the medium. In solution, ammoniacal-N exists as two forms in equilibrium: the ammonium ion (NH_4^+), and non-ionised free ammonia (NH_3). Their relative concentrations are determined by pH and temperature, with pH being particularly important (Körner et al., 2001). At a pH of 6, most of the ammoniacal-N is in the form of NH_4^+. As the pH increases, the proportion as NH_3 increases. NH_3 is more toxic to duckweed as it is lipid soluble and can therefore easily enter cells through the membranes, whereas the ionised form is less toxic (Körner et al., 2001). Shifting the NH_4^+:NH_3 equilibrium by acidifying media therefore allows duckweed to grow in higher ammoniacal-N concentrations. An isopleth contour map fitted by Körner et al. (2001) suggests that *Lemna gibba* could be grown in ammoniacal-N concentrations of up to 300 mg L^{-1}, provided that the pH is maintained at 7 or below. Similarly, Caicedo et al. (2000) found that for domestic wastewater containing ammoniacal-N concentrations of 50–100 mg L^{-1}, duckweed grew better when the pH was below 7. Therefore, given that the pH of unamended livestock slurry and digestate typically range between 7.3-7.9 and 8.1–8.4, respectively (Taylor et al., 2011), lowering the pH below 7 may represent a way of growing duckweed on higher concentrations of such waste streams.

For the first time, this study assessed the potential for growing *Lemna minor* L. on higher concentrations of agricultural wastewater by lowering pH. *L. minor* was grown in anaerobically digested cattle slurry under control (unamended) and acidified conditions. An initial study was also conducted using modified Hutner's solutions adjusted to the same pH levels, to inform on the dilution range required for the digestate and help with interpreting the results of the experiment using the more complex digestate medium. Two main hypotheses were tested herein. Firstly, it was hypothesised that duckweed growth decreases as the concentration of digestate, and thus ammoniacal-N, increases. Secondly, acidifying the wastewater allows duckweed to maintain growth rates in higher ammoniacal-N concentrations, relative to unacidified controls.

2. Materials and methods

Two complementary experiments were conducted in a controlled environment growth chamber set to a temperature of 22 °C, and light intensity of 60 μmol m^{-2} s^{-1} at a 16:8 h light:dark cycle. The *Lemna minor* L. (Blarney strain - ID 5500 in the RDSC collection, supplied by Dr M. Jansen, University College Cork, Ireland) plants used in these experiments were cultured under the same light and temperature conditions, on a modified 1/5th strength Hutner's solution (Hutner, 1953)

Table 1
Composition of the modified 1/5th strength Hutner's solution used for culturing *Lemna minor*.

Compound	Concentration	
	mg L^{-1}	mM
NH_4NO_3	40	0.4997
$Ca(NO_3)_2{\cdot}7H_2O$	40	0.1694
$MgSO_4{\cdot}7H_2O$	100	0.4057
K_2HPO_4	80	0.4592
Ferric citrate	0.4	0.0016
$Na_2EDTA{\cdot}2H_2O$	1.16	0.0035
$MnCl_2$	0.03	0.0002
$ZnSO_4{\cdot}7H_2O$	0.4	0.0025
H_3BO_3	0.4	0.0065
$NaMoO_4{\cdot}2H_2O$	0.04	0.0002
$CuSO_4{\cdot}5H_2O$	0.12	0.0008

(Table 1). Both experiments were conducted in Magenta plant tissue culture vessels (7.7 cm × 7.7 cm × 9.7 cm), with two 8 mm holes in the lids plugged with cotton wool to allow gas exchange.

2.1. Experiment 1 – L. minor growth in modified Hutner's solutions

Modified Hutner's solutions were used to identify the ammoniacal-N concentrations tolerated by *L. minor* and confirm the positive effects of lowering pH on growth rates in higher concentrations (Caicedo et al., 2000; Körner et al., 2001). The 1/5th strength Hutner's solution (Table 1) was modified so that nitrogen was only supplied in ammoniacal form; the NH_4NO_3 was replaced with varying amounts of NH_4Cl to provide solutions with a range of ammoniacal-N concentrations (10 mg L^{-1}, 50 mg L^{-1}, 100 mg L^{-1}, 150 mg L^{-1}, 200 mg L^{-1}, 250 mg L^{-1}, and 300 mg L^{-1}), whilst 15.68 mg $CaCl_2$ L^{-1} was added to replace the Ca not added as a result of omitting $Ca(NO_3)_2{\cdot}7H_2O$. A 1M NaOH solution was added to alter the pH of the solutions to either 8.2 ± 0.2, a similar pH range to that of the digestate used in experiment 2, or 6.5 ± 0.2, which is within the range considered optimal for many duckweed species (Caicedo et al., 2000; Körner et al., 2001; Leng et al., 1995). The experiment had a 7 × 2 factorial design, with seven ammoniacal-N concentration levels, and two pH levels (low pH and high pH). Each Magenta vessel contained 250 mL of a given solution (n = 6). Ten three-frond colonies of *L. minor* were weighed for fresh weight and then placed in each vessel (Caicedo et al., 2000; Walsh et al., 2020). The nutrient solutions were buffered with 3 mM 3-(4-Morpholino)propane sulfonic acid (MOPS) (Fisher Scientific, UK) and replaced with fresh media on days three, seven, and ten to limit pH shift and nutrient depletion. The experiment was harvested after 14 days, with the final fresh weights recorded, and the relative growth rate (RGR) was calculated using the equation below (Hunt, 1978):

$$RGR = \frac{\ln\left(\frac{FW2}{FW1}\right)}{T}$$

where ln = the natural log, *FW1* = fresh weight (g) at the start of the experiment, *FW2* = fresh weight (g) at the end of the experiment, and *T* = the duration of the experiment in days.

2.2. Experiment 2 – L. minor growth in anaerobically digested cattle slurry

Anaerobically digested cattle slurry (referred to as digestate hereafter) was collected from a dairy farm near Lampeter, Wales, UK. The digestate had been separated into a liquid and solid fraction on the farm using a screw-press and only the liquid fraction was collected. The liquid digestate was analysed for its chemical composition (Table 2), and based on the analysis, different dilutions were prepared with distilled water to provide six treatments (digestate concentrations of 5%, 10%, 15%, 20%, 25%, and 30%) that had a similar ammoniacal-N concentration range to the initial experiment using the inorganic nutrient solution. The pH of the digestate solutions was either left unamended (8.2 ± 0.2 over the course of the experiment) or acidified by incrementally adding 1M H_2SO_4 at a concentration of 3% (v/v, acid/undiluted digestate). The pH gradually increased by ~0.5 units per week over the course of the experiment, most likely due to chemical changes in the medium following exposure to air (Husted et al., 1991). Therefore, more H_2SO_4 was added to the acidified treatment at a concentration of 0.4% (v/v) at the mid-way point, to maintain an average pH of 6.5 (±0.2) over the 14-day duration of the experiment (Fig. 1). This resulted in a 6 × 2 factorial design, with six dilutions and two pH levels (n = 6). As with experiment 1, the fresh weight of 10 colonies were recorded before they were placed on the various solutions. After 14 days, the final fresh weights were recorded and the RGR was calculated.

Table 2
Initial chemical properties of the undiluted digestate sample collected for experiment 2.

	Undiluted digestate
pH	8.64
EC (mS cm^{-1})	18.49
%DM	2.3
Total C (mg g^{-1} DM)	342.33 (34.23%)
Total N (mg g^{-1} DM)	36.01 (3.60%)
C:N ratio	9.51
Total P (mg g^{-1} DM)	10.65
NH_4^+ (mg L^{-1})	1191.8
NO^{2-} (mg L^{-1})	-[a]
NO^{3-} (mg L^{-1})	-[a]
PO_4^{3-} (mg L^{-1})	272.6
K^+ (mg L^{-1})	3450.8
SO_4^{2-} (mg L^{-1})	36.9
Ca^{2+} (mg L^{-1})	265.5
Mg^{2+} (mg L^{-1})	99.0
Mn^{2+} (mg L^{-1})	92.7
Na^+ (mg L^{-1})	725.8
Cl^- (mg L^{-1})	2336.5

[a] Below detection limit.

2.3. Chemical analyses

The digestate was analysed for parameters relevant to water quality and environmental pollution. The concentration of key anions and cations was measured by ion-exchange chromatography (Metrosep C4 250/4.0 and A Supp 5250/4.0 columns, Metrohm, Switzerland). Total carbon and nitrogen content of freeze-dried material was measured using the Dumas method with a Vario MAX cube elemental analyser (Elementar, Germany), whilst total phosphorus was measured by ICP-OES (Varian, USA) following nitric acid digestion. The pH was measured using a benchtop Hydrus 500 pH meter (Fisherbrand, UK), with measurements regularly made over the duration of experiments 1 and 2 to monitor shifts in the pH of solutions. Electrical conductivity (EC) was measured using a SevenMulti EC meter (Mettler Toledo, USA).

2.4. Statistical analysis

All statistical analyses were conducted in R (Version 4.1.0.). Prior to analysis, plots of residuals were inspected and Levene's Test was carried out to ensure the data met model assumptions. Two-way Analysis of Variance (ANOVA) was used to investigate how RGR was affected by ammoniacal-N concentration (experiment 1) or digestate concentration (experiment 2), pH level, and the interaction between these factors. One-way ANOVA was used to investigate the effect of increasing ammoniacal-N concentrations or digestate concentration within specific pH levels. Tukey's HSD test was used for pairwise comparisons for both the one-way and two-way ANOVA models using the *emmeans* package (Lenth, 2020). The relative impact of the chemical parameters of the digestate on *L. minor* growth was assessed with a correlation matrix

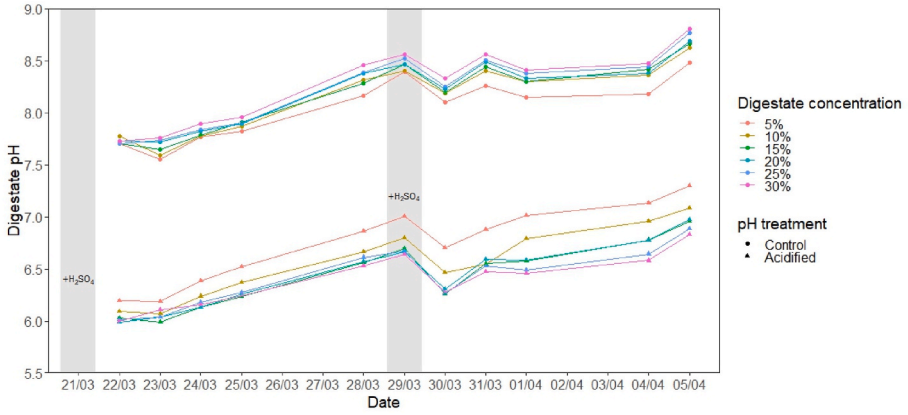

Fig. 1. Mean pH of the various digestate solutions over the duration of experiment 2. Digestate was acidified with 1M H_2SO_4 before the experiment began, and at the mid-point of the experiment.

using the R package "*corrplot*" (Wei and Simko, 2021).

3. Results

3.1. Experiment 1 – L. minor growth in modified Hutner's solutions

In line with previous findings, *L. minor* RGR decreased with increasing ammoniacal-N concentrations in the higher pH modified Hutner's solutions of experiment 1 ($P < 0.001$; Fig. 2). *Post-hoc* analysis detected no significant difference between the 10 and 50 mg L^{-1} treatments ($P = 0.239$), indicating that *L. minor* could tolerate these ammoniacal-N concentrations at a pH of 8.2. However, a significant reduction in RGR was observed when grown in solutions containing 100 mg L^{-1} ammoniacal-N, relative to the 10 mg L^{-1} ($P < 0.001$) and the 50 mg L^{-1} ($P = 0.027$) treatments. RGR was further reduced relative to the 10 mg L^{-1} and 50 mg L^{-1} treatments in concentrations of 150 mg L^{-1} and higher ($P < 0.001$). Toxicity symptoms including chlorosis and plant mortality (Tian et al., 2021; Wang et al., 2014) were also observed in the higher concentration treatments (>200 mg L^{-1}), as reflected in the low and negative RGR values shown in Fig. 2.

Unlike the higher pH solutions discussed above, *L. minor* RGR in experiment 1 remained relatively stable with increasing ammoniacal-N concentrations at a pH of 6.5. Two-way ANOVA revealed significant effects of ammoniacal-N concentration and pH on *L. minor* RGR ($P < 0.001$), as well as a significant interaction between these two factors ($P < 0.001$). *Post-hoc* analysis found no significant differences between treatments in the 10–250 mg L^{-1} range for the solutions with a pH of 6.5 ($P > 0.05$). *L. minor* growth was only reduced in the 300 mg L^{-1} ammoniacal-N treatment, where the RGR was significantly lower relative to the 10 ($P < 0.001$), 50 ($P < 0.001$), 100 ($P = 0.013$) and 200 mg L^{-1} treatments ($P = 0.038$), but not relative to the 150 and 250 mg L^{-1} treatments ($P = 0.226$ and $P = 0.611$, respectively). Furthermore, pairwise comparison between high and low pH solutions with the same ammoniacal-N concentrations show that RGR was consistently lower at the higher pH level, except for the 10 mg L^{-1} ammoniacal-N treatment ($P = 0.921$ for 10 mg L^{-1}, $P < 0.001$ for all other concentrations; Fig. 2).

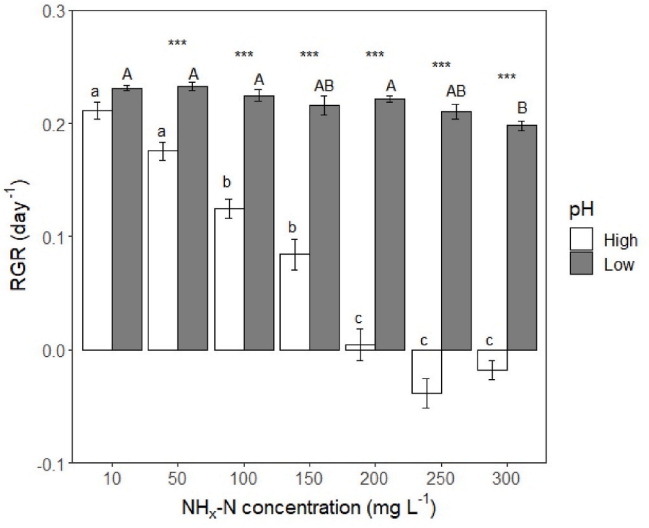

Fig. 2. *Lemna minor* relative growth rate (day^{-1}) in modified Hutner's solutions with a range of ammoniacal-N concentrations and two pH levels (high: 8.2 and low: 6.5). Error bars represent the standard error of the mean. Within each pH treatment, common lower-case letters denote statistically non-significant differences between the high pH solutions of varying ammoniacal-N concentrations, whilst common upper-case letters denote the same for the low pH solutions. Statistically significant differences between pH levels at each ammoniacal-N concentration are denoted by asterisks above the bars (*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$).

3.2. Experiment 2 – L. minor growth in digestate

As with the first experiment, two-way ANOVA showed *L. minor* RGR was significantly affected by the digestate concentration and pH ($P < 0.001$), whilst there was also a significant interaction between these two factors ($P < 0.001$). In contrast to the results in modified Hutner's solution, *L. minor* showed no growth in all concentrations of the control digestate (pH 8.2; Fig. 3), with no significant differences in RGR observed between treatments ($P = 0.718$). Fronds appeared chlorotic and mortality was observed in all concentrations, as reflected in the negative RGR values. In the acidified digestate, *L. minor* grew best in concentrations of 15% and below, with *post-hoc* analysis revealing no significant differences in RGR between 5% and 10% ($P = 0.999$), 5% and 15% ($P = 0.796$), or 10% and 15% ($P = 0.088$). Growth in the acidified 20% digestate remained positive relative to non-acidified controls (pH 8.2) and was not significantly different to the acidified 5% ($P = 0.058$), 15% ($P = 0.928$), 25% ($P = 0.740$) or 30% ($P = 0.721$) concentrations, but was reduced relative to the acidified 10% concentration ($P = 0.034$). In acidified digestate concentrations of 25% and 30%, growth was inhibited relative to the 5% and 10% digestate solutions (all $P < 0.001$), as well as the 15% digestate ($P = 0.044$ and $P = 0.040$, respectively).

Pairwise comparison of the unamended digestate (control) and the acidified digestate of the same concentration showed that RGR was significantly higher in the acidified 5%, 10%, 15%, and 20% digestate ($P < 0.001$ for 5–15%, $P = 0.050$ for 20%; Fig. 3). No difference was observed for the 25% and 30% digestate concentrations ($P = 0.747$ and $P = 0.387$, respectively).

The 20% digestate solutions contained similar NH_4^+ concentrations to

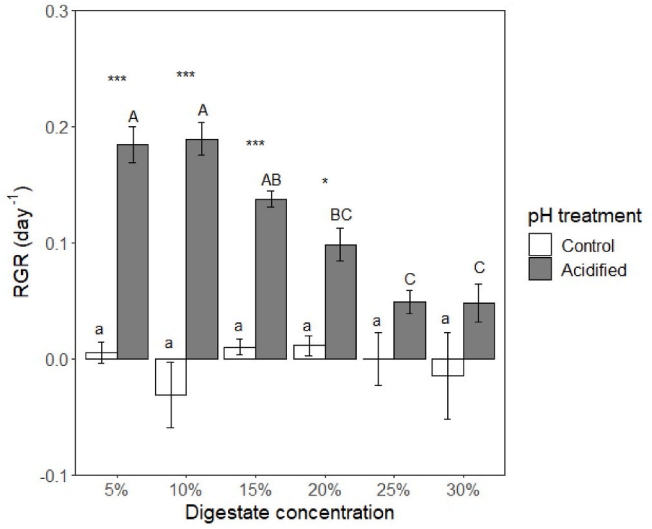

Fig. 3. *Lemna minor* relative growth rate (day^{-1}) in a range of digestate concentrations at two pH levels (unamended control: 8.2, and acidified: 6.5). Error bars represent the standard error of the mean. Within each pH treatment, common lower-case letters denote statistically non-significant differences between the control digestate dilutions, whilst common upper-case letters denote the same for the acidified digestate dilutions. Statistically significant differences between pH levels for each digestate concentration are denoted by asterisks above the bars (*P < 0.05; **P < 0.01; ***P < 0.001).

the upper limit for uninhibited growth at pH 6.5 in Hutner's solution (~250 mg L^{-1}; Table 3). To investigate the importance of digestate ammoniacal-N concentration and pH on *L. minor* growth relative to other key parameters known to influence duckweed growth in wastewater samples (Devlamynck et al., 2021a; Sońta et al., 2020), measurements were also made of EC, K^+, Na^+, Cl^-, Ca^{2+}, Mg^{2+}, and SO_4^{2-} (Table 3). The relationships between these measured parameters and *L. minor* RGR were investigated using correlation matrix analysis (Fig. 4). RGR was significantly and negatively correlated to digestate pH ($R = -0.66$, $P < 0.001$), and weakly correlated to Ca^{2+} ($R = 0.24$, $P = 0.040$) and SO_4^{2-} ($R = 0.28$, $P = 0.015$). No significant correlations were observed between *L. minor* RGR and any of the other chemical parameters measured ($P > 0.05$).

4. Discussion

This study investigated the relationship between the dilution strength and pH of agricultural wastewater and its effect on *L. minor* growth, with the aim of growing *L. minor* on stronger dilutions of digestate by amending the pH. Two hypotheses were tested; firstly, that *L. minor* growth decreases as the concentration of digestate, and thus ammoniacal-N increase, and secondly, that acidifying the media allows duckweed to maintain growth rates in higher ammoniacal-N concentrations relative to unamended controls. In digestate, *L. minor* showed no growth even in the lowest concentration of 5% (65 mg L^{-1} ammoniacal-N) at pH 8.2; whereas in acidified conditions, positive growth was maintained up to a 20% dilution (239.3 mg L^{-1} ammoniacal-N). This contrasted with the initial study using inorganic Hutner's solutions

Table 3
Chemical properties of the digestate solutions of experiment 2. Sub-samples were taken for analysis from bulked solutions prior to beginning the experiment, and from each replicate at the end of the experiment (n = 6).

Time	Digestate concentration	Treatment	EC (mS cm^{-1})	Concentration (mg L^{-1})							
				Cl^-	PO_4^{3-}	SO_4^{2-}	Na^+	NH_4^+	K^+	Ca^{2+}	Mg^{2+}
Start	5%	Control	2.22	83.2	46.4	2.2	45.6	64.8	194.2	30.3	11.3
	5%	Acidified	1.72	92.0	63.6	236.9	45.8	66.1	204.0	35.8	9.0
	10%	Control	2.68	196.2	76.0	1.6	87.8	123.1	388.6	49.4	10.1
	10%	Acidified	3.18	195.4	111.7	514.3	90.2	125.4	386.7	60.9	13.4
	15%	Control	3.21	248.4	83.9	1.4	110.9	150.0	471.6	48.2	10.5
	15%	Acidified	4.20	270.5	156.2	722.4	119.8	167.3	514.9	83.9	14.4
	20%	Control	4.08	361.2	96.8	1.6	193.5	260.9	848.0	61.0	9.9
	20%	Acidified	5.8	402.1	209.9	1052.7	165.3	239.3	736.7	117.6	14.3
	25%	Control	5.45	489.1	112.2	2.5	149.5	207.3	656.9	47.0	9.7
	25%	Acidified	7.39	542.4	262.4	1380.7	214.3	300.5	943.9	142.9	26.8
	30%	Control	7.07	666.2	140.2	2.4	254.7	337.6	1115.4	70.9	11.4
	30%	Acidified	8.81	675.8	306.5	1703.3	261.3	372.6	1141.8	147.4	31.2
End	5%	Control	1.42 ± 0.01	97.2 ± 4.0	41.4 ± 5.3	4.9 ± 1.7	55.5 ± 4.7	61.0 ± 1.9	208.0 ± 6.4	36.0 ± 1.4	10.3 ± 0.9
	5%	Acidified	1.62 ± 0.01	62.8 ± 4.9	32.1 ± 2.0	160.8 ± 10.3	42.6 ± 2.5	53.0 ± 3.4	172.6 ± 9.4	34.8 ± 2.7	9.8 ± 1.3
	10%	Control	2.52 ± 0.02	154.4 ± 20.9	45.9 ± 5.3	19.1 ± 6.9	100.7 ± 5.4	107.4 ± 7.3	404.7 ± 26.8	47.3 ± 3.7	9.8 ± 0.7
	10%	Acidified	3.00 ± 0.02	178.3 ± 16.2	91.0 ± 6.0	460.8 ± 48.0	112.3 ± 8.5	133.7 ± 6.6	430.8 ± 26.2	70.7 ± 4.6	12.7 ± 1.2
	15%	Control	3.72 ± 0.01	335.1 ± 17.5	91.0 ± 4.1	16.5 ± 7.6	152.8 ± 7.2	151.8 ± 7.9	611.9 ± 19.4	47.8 ± 3.3	11.5 ± 1.3
	15%	Acidified	4.38 ± 0.02	321.2 ± 41.3	154.4 ± 19.8	826.5 ± 119.4	179.3 ± 18.3	214.3 ± 21.2	706.8 ± 72.7	95.6 ± 12.6	17.6 ± 0.9
	20%	Control	4.80 ± 0.04	497.6 ± 34.7	108.2 ± 5.1	11.8 ± 7.1	210.4 ± 12.8	207.7 ± 13.4	845.5 ± 58.1	49.7 ± 5.0	11.1 ± 1.1
	20%	Acidified	5.84 ± 0.03	486.4 ± 26.7	204.4 ± 14.7	1278.5 ± 75.0	207.5 ± 11.5	256.7 ± 14.0	856.5 ± 44.3	99.1 ± 6.2	20.4 ± 1.0
	25%	Control	5.72 ± 0.02	441.7 ± 59.0	87.3 ± 11.7	10.8 ± 3.4	182.8 ± 20.5	174.9 ± 19.7	715.2 ± 82.9	41.7 ± 1.9	7.1 ± 1.0
	25%	Acidified	7.12 ± 0.08	538.1 ± 53.2	240.0 ± 21.5	1412.8 ± 135.3	218.5 ± 18.7	270.3 ± 23.0	865.7 ± 73.3	99.5 ± 6.6	23.9 ± 1.9
	30%	Control	6.87 ± 0.06	436.6 ± 71.3	77.9 ± 10.0	4.6 ± 0.5	181.3 ± 25.5	171.4 ± 20.5	719.3 ± 97.4	50.8 ± 4.9	11.4 ± 2.6
	30%	Acidified	8.23 ± 0.10	547.6 ± 49.1	245.0 ± 18.1	1462.2 ± 129.9	217.5 ± 15.5	270.3 ± 20.9	869.0 ± 67.5	92.0 ± 5.9	22.6 ± 1.5

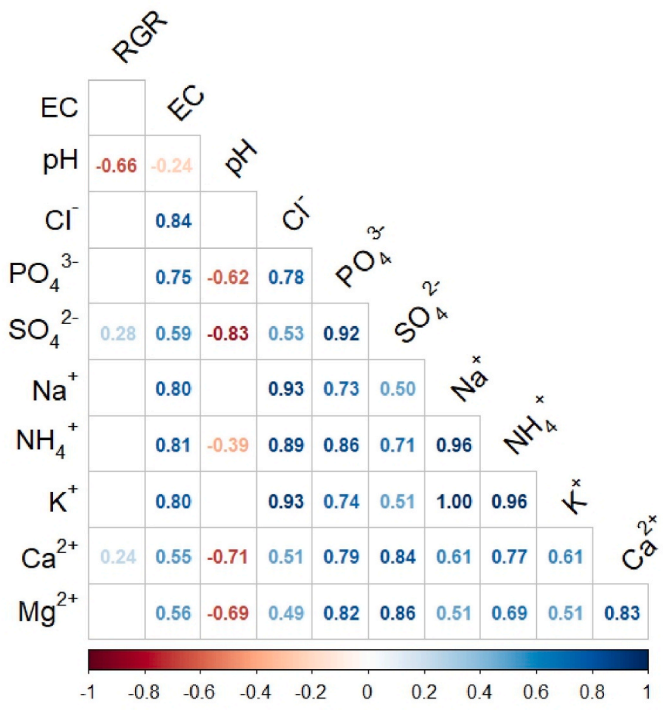

Fig. 4. Correlation matrix showing the results of a regression analysis investigating the relationship between *L. minor* RGR and the various chemical parameters of the digestate concentrations in experiment 2 (n = 6). For significant correlations ($P < 0.05$), the correlation coefficient (*R*) is provided and the strength of the correlation strength is indicated by the colour scale. *R* values are not shown for non-significant correlations ($P > 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(experiment 1). At the higher pH level (8.2), growth was decreased in Hutner's solutions containing ammoniacal-N concentrations of 50 mg L^{-1} and above, but positive growth rates were recorded up to 150 mg L^{-1}. Lowering pH to 6.5 increased RGR in all but the lowest concentration (10 mg L^{-1}) and prevented the negative effects of increased ammoniacal-N concentration up to 250 mg L^{-1}.

The results using modified Hutner's solution are consistent with previous studies investigating the impact of pH on the NH_4^+:NH_3 equilibrium in inorganic nutrient solutions or less concentrated waste streams. For example, Caicedo et al. (2000) state that *Spirodela polyrhiza* could not be grown in a modified Hutner's solution containing 50 mg L^{-1} ammoniacal-N at a pH of 7.9, whereas a RGR of over 0.2 was observed in 100 mg L^{-1} ammoniacal-N at a pH of 5.9. Similarly, Körner et al. (2001) observed a RGR of 0.28 for *Lemna gibba* on modified domestic wastewater containing 10 mg L^{-1} ammoniacal-N at a pH of 6.8, with growth rates declining as the pH and/or ammoniacal-N concentration increased beyond this optimal point (Körner et al., 2001). Whilst this relationship between pH and ammoniacal-N was also seen in experiment 2, our findings suggest that for more complex wastewater samples such as the digestate used, there may also be additional inhibitory factors. This was evident when comparing the upper limits where growth rates were not inhibited in Hutner's solutions (experiment 1) with digestate dilutions (experiment 2) that had similar pH and ammoniacal-N concentrations. For example, in the pH 6.5 Hutner's solutions, growth was uninhibited up to 250 mg L^{-1} ammoniacal-N (RGR of 0.21), whereas growth was lower in the 20% acidified digestate (RGR of 0.1), despite the pH and ammoniacal-N concentration being similar (6.5 and 239.3 mg L^{-1}, respectively; Table 3). Whilst there are no previous experiments on acidifying high-nutrient, farm wastewater prior to growing duckweed, a study by Goopy et al. (2004) grew three duckweed species (*Spirodela polyrhiza*, *Wolffia angusta*, and *Lemna aequinoctialis*) on abattoir effluent derived from the anaerobic digestion of waste animal tissue and blood which contained 184 mg N L^{-1}, 34.2 mg P L^{-1}, and had a pH of 7.86. Prior to duckweed growth, the effluent was diluted to 25% and the pH was set to 7, with one treatment amended with bentonite to adsorb NH_4^+ amongst other cations (Goopy et al., 2004). Because the duckweed tolerated ammoniacal-N concentrations of up to 100 mg L^{-1} when grown in inorganic solutions at pH 7, but could not survive in the 25% effluent (46 mg N L^{-1}) without bentonite addition, they concluded that there were other inhibitory factors which were not identified (Goopy et al., 2004).

One potential inhibitory factor in the digestate of the current study could be the high concentrations of dissolved anions and cations which lower the osmotic potential of the solution, potentially impairing plant water uptake (Sikorski et al., 2013). Duckweed species grow optimally in media with conductivity between 0.6 and 1.4 mS cm^{-1} (Landolt and Kandeler, 1987). The EC of the digestate dilutions in the current study were above this optimal range (Table 3) and that of the inorganic solutions used in experiment 1 (0.32–2.50 mS cm^{-1}, Table A1). However, no significant correlation was observed between RGR and EC (Fig. 4). Additionally, *L. minor* growth was higher in acidified digestate despite the EC also being higher due to H_2SO_4 addition (Table 3), indicating that osmotic stress did not strongly influence *L. minor* growth in digestate. High concentrations of some anions and cations can also have ion-specific effects on *L. minor* growth. Landolt and Kandeler (1987) report the optimal concentration ranges and upper limits tolerated by duckweed species for a range of ions, including Cl^-, K^+, Mg^{2+} and Ca^{2+}, as summarised by Devlamynck et al. (2021a). Of these, all digestate dilutions had Cl^- concentrations above the reported optimal range (0.4–36 mg L^{-1}; Table 3), whilst the dilutions of 20% and higher also had K^+ concentrations above the optimal range (39–780 mg L^{-1}). Additionally, Na^+ in the 20% and higher digestate solutions exceeded 117 mg L^{-1}, which contributed to impaired *L. minuta* growth in Sońta et al. (2020). The concentrations of Cl^-, K^+, and Na^+ were not significantly correlated with *L. minor* RGR (Fig. 4), suggesting that ion-specific toxicity was unlikely to be the main cause of the observed growth inhibition. However, their combined effect cannot be ruled out, as some ions can synergistically impair growth when present together (Simmons, 2012). Additionally, variations in the ratios between certain ions are known to impact duckweed growth (Walsh et al., 2020).

Wastewater samples may also contain sub-optimal concentrations of some ions, also potentially contributing towards reduced duckweed growth relative to standardised media. In the current study, the SO_4^{2-} concentration of unamended digestate was low relative to digestate acidified with H_2SO_4 (Table 3), as well as the modified Hutner's solutions (Table 1). Whilst S is an essential plant nutrient, SO_4^{2-} limitation is unlikely to be a major factor explaining the poor growth in unamended digestate. Low SO_4^{2-} concentrations are not considered to be limiting to duckweed (Landolt, 1986), particularly over relatively short 14-day trials (Sun et al., 2022) such as the current study. Furthermore, SO_4^{2-} depletion was not observed in the unamended digestate, with concentrations in fact higher at the end of the experiment, presumably due to mineralisation of suspended organic matter (Table 3). In addition to the inorganic ions discussed above, digestate also contains organic compounds and humic substances, which may have inhibited the growth of the alga *Chlorella vulgaris* in Fernandes et al. (2020). The effect of such organic compounds on duckweed growth is not well known, however, a *Lemna gibba* bioassay of olive mill effluent by Cayuela et al. (2007) found polyphenols, organic acids and lipids to be inhibitory in high concentrations. Analysis for such organic compounds was beyond the scope of this study, however, it is possible that these too may have contributed to the impaired *L. minor* growth on digestate. Additionally, mineralisation of organic matter during the experiment would have released more inorganic ions, including ammoniacal-N.

Whilst the impaired *L. minor* growth on complex, eutrophic waste streams may be due to a combination of several inhibitory factors, as

discussed above, the fact that growth rates were higher in acidified digestate indicates that ammoniacal-N toxicity was a key determinant. Following acidification, *L. minor* could grow with a relatively high RGR of 0.19 on 10% digestate containing 125.4 mg L^{-1} ammoniacal-N, and no reduction in growth was observed up to 167.3 mg L^{-1}. Without acidification, *L. minor* growth was prevented even in the most dilute digestate solution (5%) containing 64.8 mg L^{-1} ammoniacal-N. These findings are supported by the correlation analysis conducted, which show a strong, negative correlation between *L. minor* RGR and pH.

Our findings have important implications for the implementation of duckweed growing systems to remediate high-nutrient wastewater in the agricultural industry. Duckweed's potential for remediation (Devlamynck et al., 2021a) and as a high-protein feedstock is well known (Stadtlander et al., 2022), however, the need for dilution is currently a major constraint on its practical implementation. A more dilute treatment than 5% digestate was not included in the current study as it was deemed that it would not be practical in industry, but based on previous studies (Sońta et al., 2020; Stadtlander et al., 2022), it is likely that unamended digestate would need to be considerably more dilute for adequate duckweed growth. The optimal ammoniacal-N concentration for duckweed growth on unamended cattle slurry is reported to be 19 mg L^{-1} (Stadtlander et al., 2022). To achieve this concentration, high-nutrient waste streams such as the digestate used in the current study require substantial dilution. This study demonstrates that the extent of dilution required to allow adequate *L. minor* growth can be reduced if the pH is lowered. Less dilution improves water-use efficiency, and in turn, the land footprint of the process. Acidifying wastewater is therefore an important step towards a more widespread adoption of duckweed growing systems in industry. Future research should focus on upscaling this approach to an industrial scale. This may involve using automated systems to maintain a pH within the target range of 6.5–7 (Caicedo et al., 2000; Körner et al., 2001), as well as using waste products such as CO_2 to acidify media (Newnes et al., 2021). Focus should also be placed on optimising nutrient removal and duckweed protein content to maximise the commercial viability of such systems, as well as identifying more ammoniacal-N (Zhang et al., 2014) and salt tolerant strains (Sońta et al., 2020) to further increase yield on more concentrated media.

5. Conclusions

This study highlighted the impact of pH on the NH_4^+:NH_3 equilibrium and the influence this has on *L. minor* growth in a practical wastewater sample (digestate). In the digestate, *L. minor* RGR was strongly influenced by pH, with no reduction in growth observed up to 167.3 mg L^{-1} ammoniacal-N following acidification. Without acidification, growth was prevented even on the most dilute digestate. For the first time, we demonstrate that by acidifying agricultural wastewater, *L. minor* could be grown on stronger dilutions than those used in previous studies using similar, unamended wastewater.

CRediT authorship contribution statement

Gruffydd Jones: Conceptualization, Methodology, implementation, Data curation, data collection, Formal analysis, Writing – review & editing, writing of manuscript. **John Scullion:** Conceptualization, Methodology, Writing – review & editing, writing of manuscript. **Sarah Dalesman:** Conceptualization, Methodology, Writing – review & editing, writing of manuscript. **Paul Robson:** Conceptualization, Methodology, Writing – review & editing, writing of manuscript. **Dylan Gwynn-Jones:** Funding acquisition, Conceptualization, Methodology, Writing – review & editing, writing of manuscript.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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