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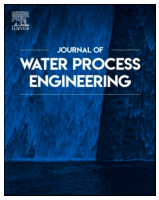

Integration of microbial bioreactors and *Lemna minor* cultivation for sustainable treatment of dairy processing wastewater

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ABSTRACT

A novel technological approach for dairy processing wastewater remediation is presented. This approach combines microbial bioreactor systems with *Lemna minor* plant biomass cultivation is presented. Sequential anaerobic and aerobic microbial batch reactors achieved wastewater remediation efficiencies of 97.5 %, 83 % and 58.5 %, for chemical oxygen demand (COD), total nitrogen (TN) and total phosphorus (TP), respectively. Molecular profiling of the bioreactor communities indicated that phylum Pseudomonadota dominated nitrification/denitrification metabolism. TP removal was largely correlated with PO_4^{3-}-P uptake, with significant redundancy observed among bacterial genera contributing *ppK* and *ppX-gppA* phosphate metabolism genes. Novel dominant roles for members of the genus *Macellibacteriales* and *Rikenellacea* in phosphate uptake are proposed. Integration of *Lemna* cultivation increased wastewater TN and TP remediation efficiencies to 96.5 % and 73 %, respectively. However, relative growth rates of *Lemna* were found to be critically dependent on pH adjustment of effluents from pH 8.9 to pH 4.9–5.1. Phytotoxicity under alkaline conditions was correlated with wastewater NH_3-N concentration ($p < 0.001$). Elevated wastewater sodium and chloride levels did not appear to induce plant stress, with no statistical difference in photosynthetic activity. This study provides valuable, practical insights into the integration of microbial and phyto-remediation technologies, coupling wastewater treatment with opportunities for valorisation.

1. Introduction

Milk and dairy products contribute significantly to human food security due to their high nutritional value and broad health benefits [1]. Global milk production was reportedly 897 million tons in 2022 with future growth projected to reach 1039 million tons within the next decade [2]. In low to middle income countries with high milk production, e.g. India and Pakistan, the majority of domestic product is directly consumed as whole milk. However, in high income dairy production countries such as the USA and several members of the European Union, >50 % of milk output undergoes processing into diverse products such as milk powders, infant formula, cheese and butter. Processed dairy products account for up to 14 % of global agricultural trade, with approximately 10 million tons traded in 2021 [2]. However, intensification of dairy production and processing has seen the industry face increasing scrutiny in the context of environmental sustainability and climate change contributions.

Dairy processing can result in up to 10 fold equivalent wastewater outputs for each unit of milk processed, with the dairy sector representing the largest source of food industry wastewater in some European countries [3]. The scale of this output is compounded by the high organic and inorganic nutrient loadings associated with untreated dairy processing waste streams. These include 2000–7700 mg L^{-1} chemical oxygen demand (COD), 64–270 mg L^{-1} NH_3-N, 9–30 mg L^{-1} NO_3^--N, 20–356 mg L^{-1} PO_4^{3-}-P, as well as a range of trace metals (e.g. Ca, Mg, K, Fe) (6–10). As a result, dairy processing wastewaters are subject to stringent regulatory controls and emission limits and require extensive remediation to minimise pollution/eutrophication risks to receiving

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waterways [4].

The high COD/BOD quotient of dairy processing wastewater makes it amenable to biological secondary treatment, which is a common feature of onsite remediation as described in previous reviews [5,6]. Current commercial systems typically incorporate anaerobic digestion for COD conversion to methane and/or activated sludge systems under oxic/anoxic cycling for COD consumption coupled with nitrogen species transformations. Orthophosphate capture is often achieved with chemical precipitants, e.g. aluminium or calcium salts [7]. However, a recent review of sustainability issues in the dairy sector identified the biological treatment of processing wastewaters as a significant source of GHG emissions (CH_4, N_2O, CO_2) [8]. In addition, activated sludge systems can generate dairy processing sludge (DPS) volumes approaching 20 kg/m^3 of milk processed, with an estimated 3.8 million tonnes generated in the EU per annum [9]. Land spreading of DPS as a management strategy presents inefficiencies in terms of balanced nitrogen and phosphorous delivery and can also contribute to GHG emissions and the risk of soil accumulation of heavy metals [9,10].

There has been significant research in recent years into biological treatment approaches with a capacity to couple wastewater remediation with circular economy goals of sustainable management and resource recovery. These have included a shift from multistage, energy intensive, high cost chemical and biological secondary treatments to single stage, microbial sequencing batch reactor designs for flexible applications. One such technology is aerobic dynamic feeding which can enrich for bacteria in mixed culture systems with a capacity for carbon capture in the form of condensation polyesters [11]. Such mixed culture systems can also achieve efficient nitrogen removal and partial TP removal [12]. In addition, phyco and phytoremedation approaches for industrial wastewater nutrient capture have also been proposed [13]. Research into mixotrophic phycoremediation of dairy processing wastewaters have shown some promise. However, challenges persist with respect to algal strain tolerances to fluctuating nutrient compositions and high salt contents of process effluents [14]. Phytoremediation with fast growing aquatic plants such as members of the *Lemnaceae* (Duckweed) family have also been recently proposed, offering opportunities to couple biomass production with remediation of N, P and other plant nutrients [15,16]. However, such application to dairy processing wastewaters would require pretreatments as *Lemna* species remove relatively small amounts of organic matter in autotrophic and mixotrophic conditions [17].

While novel technologies for dairy processing wastewater remediation and biorecovery are reported, significant knowledge gaps exist regarding potential technical challenges to their direct integration and the capacity for such systems to deliver the robust nutrient remediation performances demanded of the sector. In this study we investigated a novel, potential biorefinery configuration for treating dairy processing wastewater, coupling microbial bioreactors for acidogenic anaerobic digestion and aerobic dynamic feeding with *Lemna minor* cultivation over a 5-month operational period. The study investigated (i) dairy processing wastewater remediation performance of the microbial and phytoremediation steps, (ii) microbial community dynamics and metabolic profiling to evaluate key operational taxa, (iii) growth and stress response indicators of duckweed cultivated on microbially-treated dairy processing wastewater.

2. Materials and methods

2.1. Dairy processing wastewater

Dairy processing wastewater was sourced from a large scale, commercial dairy processing facility in the Munster region in the south of Ireland. This facility produces a range of dairy products including cheese, butter and milk powder. Within the facility the aqueous waste streams from all production-lines, along with water and chemicals used for cleaning, are screened for the removal of large solids and then collected in a balancing tank. During peak season dairy production (March – November), the wastewater is pumped to an on-site anaerobic digester (AD) for COD remediation (~90 %) and biogas is captured for thermal energy applications. The AD digestate is then fed to the main WWTP where a combination of chemical precipitation and activated sludge biological treatment is applied for nitrogen and phosphorus species remediation. In this study, raw processing wastewater from the balancing tank was used as a feed-stock for our investigations. Digestate samples from the industrial AD system were also collected for use in evaluations of duckweed growth.

2.2. AD-ADF laboratory scale treatment system

The laboratory bioreactor system consisted of an acidogenic anaerobic digestor and a sequencing batch reactor operated under aerobic dynamic feeding (ADF) conditions (Fig. 1). Effluent from the ADF reactor was then used as feedstock for the duckweed cultivation trials, as described below. Both microbial systems were seeded with biomass sludge from an upflow AD sludge blanket at the commercial dairy processing site, with an initial mixed liquor suspended solids content of 6 g L^{-1}. The acidogenic reactor consisted of a sealed 2 L continuously stirred tank reactor system subjected to 12 h pulse feeding intervals at the start of which 750 mL dairy processing wastewater was added, totalling 1.5 L per day. At the end of each 12 h cycle, 750 mL effluent was pumped to a buffer tank, before commencement of the next feed cycle. The short hydraulic retention time was chosen to encourage acidogenic microbial activity rather than methanogenic species. The ADF reactor was an open system with an operational volume of 2 L and overhead mixing. The reactor was operated in 8 h automated cycles wherein 450 mL of effluent from the acidogenic AD effluent balance tank was added at the start of each cycle, together with mixing (145 rpm) and aeration (0.5 L min^{-1}) (Fisher Scientific vacuum/aerator pump). After 7 h the mixing and aeration ceased, and the reactor biomass was allowed to settle under gravity for 40 min before withdrawing 450 mL of clarified supernatant over a 15-minute period (Watson Marlow 454S peristaltic pump). Manual biosolid removal was performed periodically at the end of the supernatant withdrawal phase to maintain mixed liquid suspended solids between 3 and 4 g L^{-1}. Samples of influent and effluent were collected for physico-chemical analyses.

2.3. Duckweed strain and cultivation conditions

Duckweed cultivation studies were conducted with a previously characterised strain isolated by our group, namely *L. minor* Blarney strain 5500 RDSC, which exhibits high growth rate, high nutrient uptake and tolerance of dairy processing wastewater conditions [18,19] Routine maintenance of the strain involved cultivation on Hutner's media as previously described [20]. Growth trials of *L. minor* Blarney on untreated wastewater or effluents from the AD-ADF laboratory system and the industrial on-site AD were similarly performed (Fig. 1). Colonies of *L. minor* on Hutner's media were transferred to Magenta vessels (GA-7) containing 100 mL volumes of each, respective, wastewater and allowed to acclimate for a period of 7 days in a controlled growth room (average light intensity 50 μmol m^{-2} s^{-1} PAR, 22 °C, 16 h:8 h light:dark photoperiod). These cultures were then transferred to fresh Magentas with 100 mL of fresh wastewater to generate 50 % plant surface coverage, quantified using EasyLeafArea automated digital image analysis software [21]. Densities were maintained at ±2 % of target surface cover throughout the experiment through the removal of excess plant material every 2–3 days. This process was guided by measurements of duckweed surface area, as determined by EasyLeafArea.

2.4. Effluent pH and [NH_3–N] modification

To explore the potential negative effect of ammonia concentration on duckweed growth, cultivation trials were conducted with ADF effluent

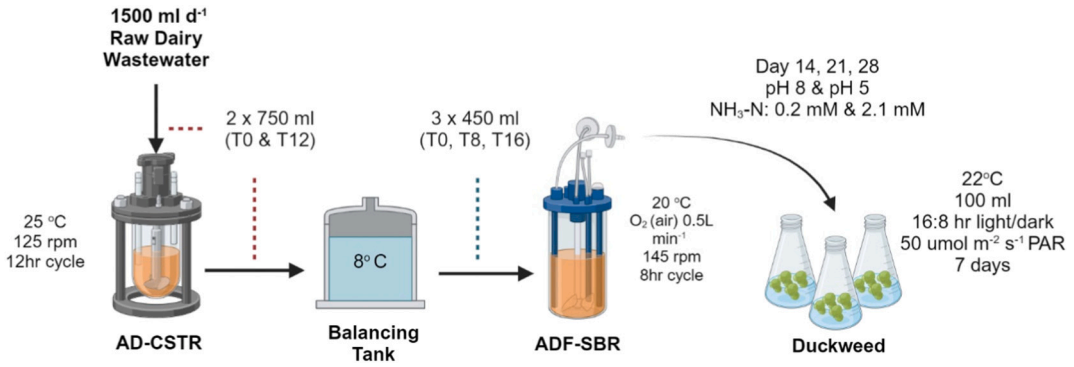


Fig. 1. Overview of the laboratory bioreactor system and operational parameters for combining acidogenic anaerobic digestion, aerobic dynamic feeding and duckweed cultivation trials with and without feedstock modification.

collected at 3 time points over a 28 day period. To optimise growth, pH and ammonia levels were modified. A factorial design with two pH levels (low, pH 5 and high, pH 9) and two ammonia levels (low, 0.21–0.5 mM, and high, 2.1 mM) was used. As the pH of unmodified ADF effluent was routinely pH 8.6–8.9, this was used directly for the high pH evaluations. pH 5 conditions were achieved with 1 M H_2SO_4. The NH_3-N concentration was increased to 2.1 mM (30 mg L^{-1}) through the addition of ammonium sulphate. Cultivations were performed as described above.

2.5. Duckweed growth and stress monitoring

Relative growth rate (RGR) determinations for duckweed on all media were based on changes in biomass over time using the formula, RGR = (ln W_2/W_1)/d, where ln is the natural log, W1 is starting fresh biomass, W2 is the final fresh biomass on the final day of the experiment and d is the time in days. For plants kept at 50 % surface coverage, the removed excess plant biomass was weighed and used to calculate the biomass yield. A 3-day RGR was calculated based on the increase in biomass at the first harvest time point. Yield (g) is the total amount of excess biomass removed over the course of the 7-day experiment. In order to assess photosynthetic performance as an indicator of potential plant stress, chlorophyll *a* fluorescence measurements were taken on randomly selected plants on days 0 and 7, using a pulse amplitude modulated fluorometer (WALZ Imaging fluorometer, Effeltrich, Germany) as described previously by our group [22]. Measurements were used to calculate F_v/F_m, the maximum quantum yield of Photosystem II (PSII); Y(II), the quantum yield of PSII; Y(NPQ), the yield of non-photochemical quenching and Y(NO), the yield of unregulated heat dissipation. The procedure that was followed and the calculations that were made are detailed in Walsh et al. [20]. Statistical analyses were conducted using R (version 3.4.3, R Core Team (2019)). One-way ANOVAs were used to analyse differences between treatments. Two-sample *t*-tests were used to compare chlorophyll fluorescence measurements taken on day 0 and day 7. Normality was assessed through a graphical assessment of the distribution of the residual values for data points (i.e., histogram). Homoscedasticity was assessed with 'residuals vs. predicted values' plots as well as Fligner-Killeen tests. Multiple linear regression was used to analyse the effect of pH and ammonia on duckweed RGR. The presence of multicollinearity was assessed using Variance Inflation Factor (VIF) values.

2.6. Water quality measurements

For full physico-chemical assessment of untreated wastewater, AD effluent, AD-ADF effluent and duckweed reactor effluent, samples of wastewater were analysed by a GLP laboratory (Aquatic Services Unit, Cork, Ireland). BOD, COD, total solids, total nitrogen (TN) and total phosphorous (TP) were measured for whole wastewater samples, i.e. unfiltered sample, as per standard methods for wastewater analysis [23]. Wastewater was filtered (0.45 μm) to determine the dissolved concentrations of ammonia, nitrate, nitrite and orthophosphate using the Lachat QuikChem 8000 by Zellweger Analytics, Inc. Milwaukee, USA (QuikChem Methods 10-107-06-3-D, 10-107-04-1-C, 10-107-04-1-C and 10-115-01-1-B, respectively). Sodium, potassium, calcium, magnesium, zinc and iron were measured in filtered wastewater using a flame AAS (Varian Australia Ply Ltd., 1989). Copper and manganese were measured using a graphite furnace AAS (Varian Australia Ply Ltd., 1989). Chloride was measured using ferricyanide method on filtered wastewater [23].

2.7. Biomass DNA extraction and 16S amplicon library preparation and sequencing

Sludge biomass samples were collected from the ADF system at weeks 2, 4, 13 and 20 for DNA extraction as described by Gil-Pulido and co-workers previously [24]. In summary, 6 mL of ADF mixed liquor was centrifuged for 15 min at 5000 rpm and the supernatant discarded. 300 mg of the resulting biomass pellet was used for DNA isolation using the PowerSoil DNA Isolation Kit (MOBIO Laboratories) as per the manufacturer's instructions. DNA was quantified using a Nanodrop spectrophotometer and visualized for quality/integrity via 1 % TAE gel electrophoresis in the presence of SafeView nucleic acid stain and UV transillumination (BioRad systems). 16S amplicon library preparation for each sample followed the Illumina MiSeq protocol targeting the V3-V4 gene region with the primer pair 341F/805R carrying Illumina adapter overhangs [25]. Reagents and thermocycling conditions were as per the illumina protocol. Amplicons were purified using a spin column, silica-based binding approach (Qiagen), with concentration and purity evaluated spectrophotometrically. A subsequent indexing PCR was performed to add dual index 8 bp tags and sequencing adapters (Nextera XT). Amplicons were purified as above and concentrations determined using a nanodrop, followed by preparation of equimolar pooled samples sent to Eurofins Genomics for sequencing on the MiSeq Illumina platform.

2.8. Bioinformatic analyses

Raw sequences were processed using mothur v1.48.0 [26,27]. First, paired-end sequences were merged into contigs by local alignment through Needleman-Wunsch algorithm, solving disparities in overlapping nucleotides by choosing the possibility with highest Phred score as true. Contigs were then removed if they contained any ambiguous base or 8 bp or longer homopolymers. The remaining sequences were checked for chimeras using de novo method of VSEARCH [28] implemented in mothur. Non-chimeric contigs were then taxonomically classified against the SiLVA nr v132 database through k-nearest neighbor algorithm considering 8 bp k-mer size and a taxonomic cutoff

of 80 %. Contigs that were classified as *Archaea*, *Eukaryota*, or *Bacteria* non identified at phylum level were discarded from the analysis. The remaining contigs were then aligned against the SiLVA SEED v132 database through Needleman-Wunsch algorithm and considering both strands to observe alignment failures. Contigs that did not start and end in the anticipated position of the primers pair used for amplification were discarded from the analysis. The remaining contigs were clustered in a 97 % similarity threshold through the Abundance-based Greedy Clustering of VSEARCH implemented in mothur. Singleton OTUs were deemed as spurious sequences and removed from the analysis. The remaining contigs were considered high-quality sequences and used for computation of microbial ecology. Taxonomic classification for each OTU was obtained by computation of a taxonomic consensus considering all sequences within that OTU.

The most abundant sequence of each OTU was chosen as representative sequence for that OTU. Representative sequences for all OTUs were used for metagenomic prediction using PICRUSt2 software [29]. For this purpose, representative sequences were placed into a reference tree containing 20,000 full 16 rRNA genes of *Bacteria* and *Archaea* organisms retrieved from Integrated Microbial Genomes database [30]. HMMER (http://www.hmmer.org) was used for phylogenetic placement of sequences, while EPA-ng [31] was used to evaluate the fitting of positioning and GAPPA [32] was used for generation of a new phylogenetic tree with the representative sequences added. Finally the phylogenetic tree was used to predict the metagenomes of representative sequences introduced. All sequence data is publicly available on NCBI Database (Bioproject ID: PRJNA732913).

3. Results and discussion

3.1. Wastewater remediation performance of the laboratory AD-ADF system

Table 1 presents physico-chemical wastewater analyses for untreated wastewater together with effluent from the industrial anaerobic digestor, the AD-ADF lab system and the duckweed cultivation system. The composition of the untreated wastewater was representative of typical nutrient loads in the dairy sector, as described in Section 1. Effluent from the industrial AD was characterised as a comparator for the lab system, as this is the first treatment step at the industrial facility. The AD achieved approximately 90 % reduction in COD, and a modest ~10 % removal of both TN and TP. Ammonification activities were evident with a 89 % increase in the mean NH_3-N load of the AD effluent compared with the untreated wastewater. Metal ion concentrations were largely unaltered except for Mg^{2+}, with 87 % mean removal observed in the AD system. Mg^{2+} has previously been linked with enhanced COD removal efficiency in AD systems, although higher concentrations (>250 mg L^{-1}) can prove inhibitory [33]. With respect to the laboratory system, both the AD and ADF reactors were stably maintained over the 20 week evaluation period. Volatile fatty acid profiling of effluent from the acidogenic anaerobic digestor indicated that the 24 h hydraulic retention time was effective in promoting acidogenic and acetogenic metabolism, with total VFA yields of 1500 mg L^{-1}, comprised primarily of acetic (60 %) and propionic (33 %) acids, respectively (data not shown). As per the data in Table 1, nutrient remediation in the combined AD-ADF system achieved mean COD, TN and TP removal efficiencies of 97.9 %, 83 % and 58.5 %, respectively. In relation to NH_3-N, a mean reduction of 73 % was observed, with ammonia oxidation a likely key process as NO_3^--N levels went from below detection limits in raw wastewater to 0.8 ± 0.4 mM in the AD-ADF effluent. PO_4^{3-}-P removal was 53.5 %, similar to TP (58.5 %), indicating orthophosphate accounted for the majority of P removal. Similar phosphate uptake levels in ADF reactor biomass have previously been reported by our group [12] and others [34] and reinforce the potential of ADF systems for significant phosphate recovery without the addition of flocculating agents.

Table 1
Composition of untreated dairy wastewater, Industrial AD effluent, Laboratory AD-ADF effluent and duckweed effluent (post cultivation on AD-ADF effluent).

Parameter	Untreated wastewater (mean ± SD, $n = 5$)	Industrial AD effluent (mean ± SD, $n = 3$)	Lab Scale AD-ADF effluent (mean ± SD, $n = 4$)	Duckweed effluent (mean ± SD, $n = 4$)
pH	6.5 (±0.5)	8.5	8.9 (±0.1)	7.9 (±0.3)
BOD (mg L^{-1})	1496 (±594)	33.2 (±3)	22.3 (±18)	9 (±3.9)
COD (mg L^{-1})	2663 (±459)	292.3 (±181)	60 (±32)	77 (±43)
Total solids (mg L^{-1})	4198 (±659)	3240 (±122)	2825 (±555)	2870 (±0.5)
Total nitrogen (mM)	7.8 (±1.6)	6.7 (±0.8)	1.4 (±0.6)	0.3 (±0.1)
Ammonia-N (mM)	2.7 (±0.8)	5.1 (±0.6)	0.6 (±0.3)	0.001 (±0.0001)
Nitrate-N (mM)	BD (<0.0007)	BD (<0.0007)	0.8 (±0.4)	0.009 (±0.01)
Nitrite-N (mM)	BD (<0.0001)	BD (<0.0001)	NM	NM
Total phosphorus (mM)	1.0 (±0.2)	0.91 (±0.003)	0.5 (±0.1)	0.3 (±0.1)
Orthophosphate-P (mM)	0.8 (±0.2)	0.86 (±0.02)	0.4 (±0.1)	0.3 (±0.1)
Sodium (mM)	36.5 (±7.7)	45.2 (±0.5)	38.7 (±8.7)	36 (±8.5)
Chloride (mM)	26 (±8.6)	26.9 (±1.3)	24.7 (±8.9)	23.5 (±10)
Potassium (mM)	2.7 (±0.6)	2.3 (±0.08)	2.7 (±0.5)	2.5 (±0.7)
Calcium (mM)	2.3 (±1.3)	2.2 (±0.06)	1.2 (±0.3)	1.1 (±0.2)
Magnesium (mM)	1.9 (±3.3)	0.42 (±0.03)	0.4 (±0.1)	0.3 (±0.1)
Iron (mM)	0.006 (±0.004)	0.004 (±0.0003)	0.003 (±0.0008)	0.003 (±0.001)
Zinc (mM)	0.05 (±0.06)	0.0003 (±0.0001)	0.06 (±0.1)	0.009 (±0.006)
Copper (mM)	0.0003 (±0.0002)	0.0001 (±0.0002)	0.0002	0.0007 (±0.0005)
Manganese (mM)	0.007 (±0.01)	0.0003 (±0.00005)	0.0001 (±0.00006)	0.0002 (±0.0001)

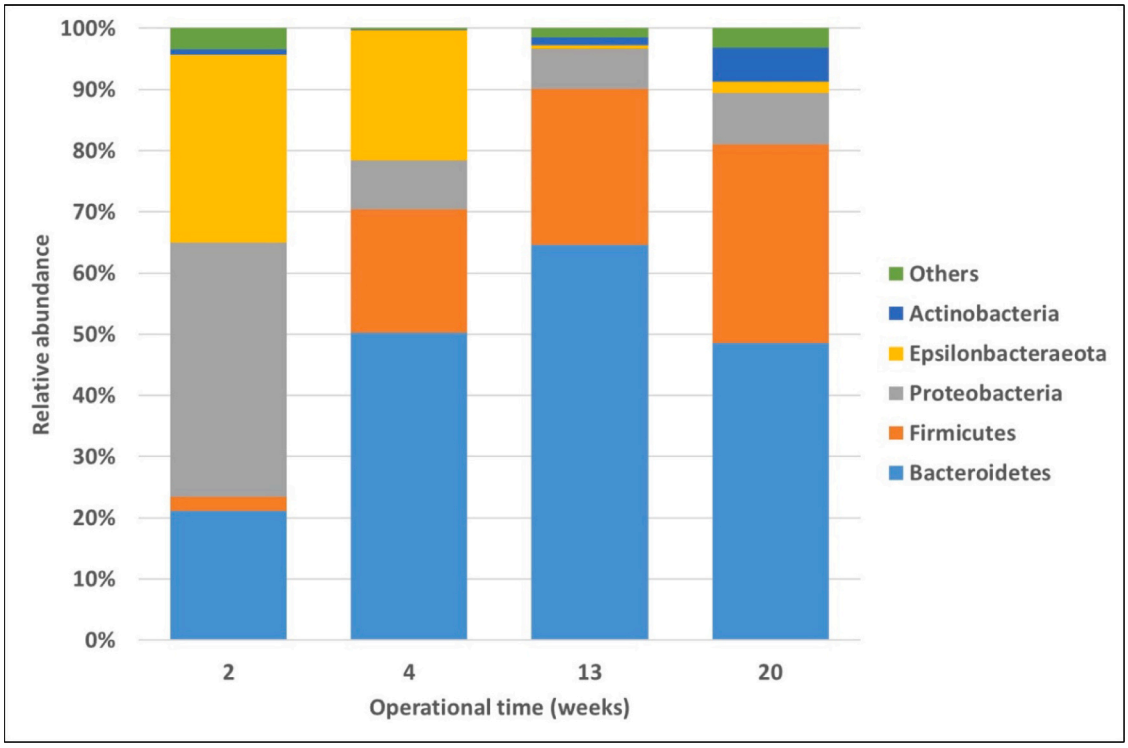
BD – below detection.
NM – not measured.

3.2. ADF microbial community dynamics over time

Biomass samples were collected at various time point across the operation of the ADF system to allow microbial community profiling and phylogenetic prediction of metabolic complexity associated with the final microbial treatment stage. Mean sequence reads across all samples were 130,669 ± 23,025 with 99.6–99.8 % retention post quality control. OTU level rarefaction curve plateaus suggested thorough richness sampling (data not shown). Alpha diversity measures indicated relatively high diversity within the samples (Shannon entropy H = 3.5 ± 0.75, Simpson Index = 0.8 ± 0.07). Fig. 2 presents (a) phylum level and (b) order level relative abundances within the ADF biomass over time. Significant shifts in phylum level relative abundances were observed in biomass samples. Phylum Proteobacteria decreased from 40 % in week 2 to relative abundances of 7–9 % across all samples thereafter. The observed decrease correlated at order level (Fig. 2b), with downward transitions in the order Pseudomonadales and the exclusion of Alteromonadales. Phylum Epsilonbacteraeota representation also decreased substantially from 31 % at week 2 to 1 % by week 13, which was largely attributable at order level to the reduction in Campylobacteriales abundance. In contrast, the phylum Bacteroidetes increased from 21 % in week 2 to 47–65 % across weeks 4 to 20.

This was reflected at order level primarily via increased abundance of order Bacteroidales, while order Flavobacterium members (10 % abundance in week 2) were reduced to below the 1 % cutoff by week 4. In the same period, relative abundance of the Firmicutes phylum increased from 2 % in week 2 to 20–32 % across weeks 4–20,

(a)



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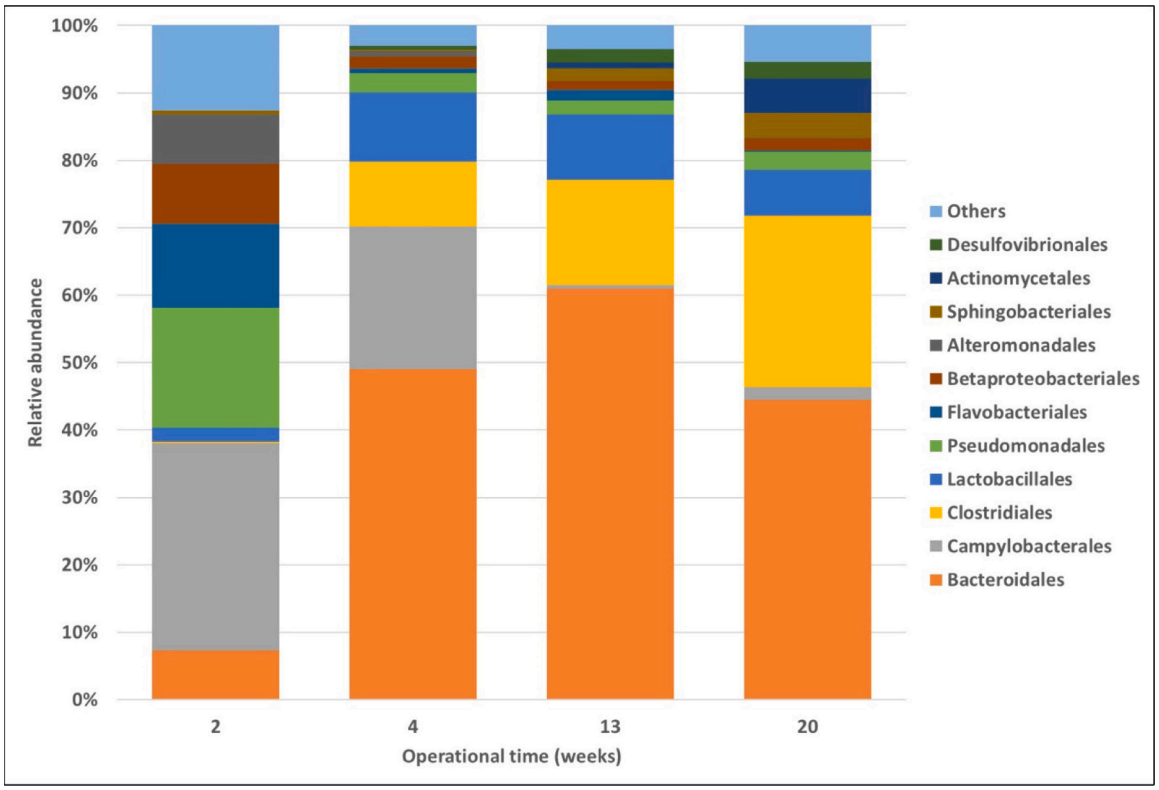

Fig. 2. Relative abundance of bacteria at (a) Phylum and (b) Order levels in ADF reactor biomass samples at weeks 2, 4, 13 and 20 of operation.

respectively. The latter corresponded with elevated relative abundances in the orders Clostridiales (9–24 %) and Lactobacilliales (6–10 %), respectively.

3.3. PICRUST modelling of temporal shifts in community metabolic function related to wastewater remediation

In addition to evaluating the relative ecological diversity, stability and temporal dynamics of the ADF microbial community, we also applied bioinformatic approaches to profile the metabolic functionality underpinning the observed wastewater remediation performance. Phylogenetic investigation of communities by reconstruction of unobserved states (PICRUST) allows reference genome-based interpretations of phylogenetic data to predict metabolic functionality within microbial communities [29,35]. Nitrogen removal in biological wastewater treatment can involve several microbial pathways/genes for nitrification and denitrification, while excess phosphate uptake is primarily associated with polyphosphate kinase activity [36]. Fig. 3 presents an overview of PICRUST predicted, temporal shifts in key representative genes from several, well characterised nitrogen metabolism pathways [37]. The heatmaps show the gene contributions of representative bacterial orders, which were included based on a cutoff of >1 % contribution to one or more nitrogen metabolism pathways. Normalised 16S rRNA gene counts for each order allow correlation of relative abundance with relative contribution to respective pathways. Based on PICRUST analysis it would appear that 3 orders from the phylum Proteobacteria, (Betaproteobacteriales, Campylobacteriales and Pseudomonadales) were the critical contributors to denitrification, dissimilatory nitrate reduction and ammonia oxidation processes in the ADF system. While some redundancy in nitrogen metabolism was observed between these orders, ammonia oxidation appeared to be exclusive to the Betaproteobacteriales, which also dominated the observed contributions to dentrification over the 20 week ADF operational period. Pseudomonadales and Campylobacteriales were associated strongly with dissimilatory nitrate reduction, which can contribute to NH_3-N retention rather than removal in systems. However, in the context of duckweed cultivation (see Section 3.4) this may constitute a positive impact given the critical role of nitrogen in supporting *Lemna* growth. With respect to assimilatory nitrate reduction, the order Micrococcales was the dominant contributor across all samples and has previously been associated with nitrogen fixation roles [38]. It was noted that the relative contributions of nitrogen metabolism genes across the samples did not appear to be directly dependent on the relative abundance of contributing orders. As an example, the order Rhizobiales accounted for 62 % of the ammonia oxidation *amoA* gene content in week 4, despite representing only 0.03 % of 16S rRNA gene content among nitrogen metabolism providers. Thus, while ecological profiling provided insights into the broad structural organisation and dominant community members (Fig. 2), it did not correlate with key functional contributions to system performance as per Fig. 3.

Fig. 4 presents PICRUST predicted, genus level bacterial contributions to luxury phosphate uptake associated genes, *pp*K and *pp*X, across the ADF samples. In contrast to nitrogen metabolism (Fig. 3), there appeared to be greater redundancy in polyphosphate metabolism capacity within the system with wide taxonomic distribution of genes. As a result this system may offer greater adaptability and performance stability when compared to traditional configurations such as EBPR which rely on specialist PAOs and are prone to instability [39]. Indeed, Fig. 4 potentially indicates that the robust orthophosphate removal capacity of the AD-ADF laboratory system (Table 1) does not appear to be dependent on commonly identified PAOs in wastewater treatment settings (e.g. *Tetrasphaera*, *Decholoromonas*, *Microlunatus*, *Candidatus*) [39]. Contrary to nitrogen metabolism predictions, it would appear from Fig. 4 that relative polyphosphate gene contributions correlated closely with 16S rRNA gene abundance of the respective genera. The increasing temporal dominance in the ADF community by phylum Bacteroidetes from week 4 (Fig. 2(a)) correlated with dominant predicted contributions to *ppK* from related genera such as *Macellibacteroides* and *Rikenellaceae* (Fig. 4). This is the first report to our knowledge to propose potential roles for *Macellibacteroides* or *Rikenellaceae* in biological phosphorus uptake. However, further work is required to corroborate in silico predictions with in situ functional roles and to elucidate specific mechanisms. The authors note that *Macellibacteroides* appear to lack the concomitant *ppX-gppA* exophosphatase gene for energy recovery from polyphosphate, while *Rikenellaceae* demonstrated *co*-contribution of both polyphosphate kinase and phosphatase genes.

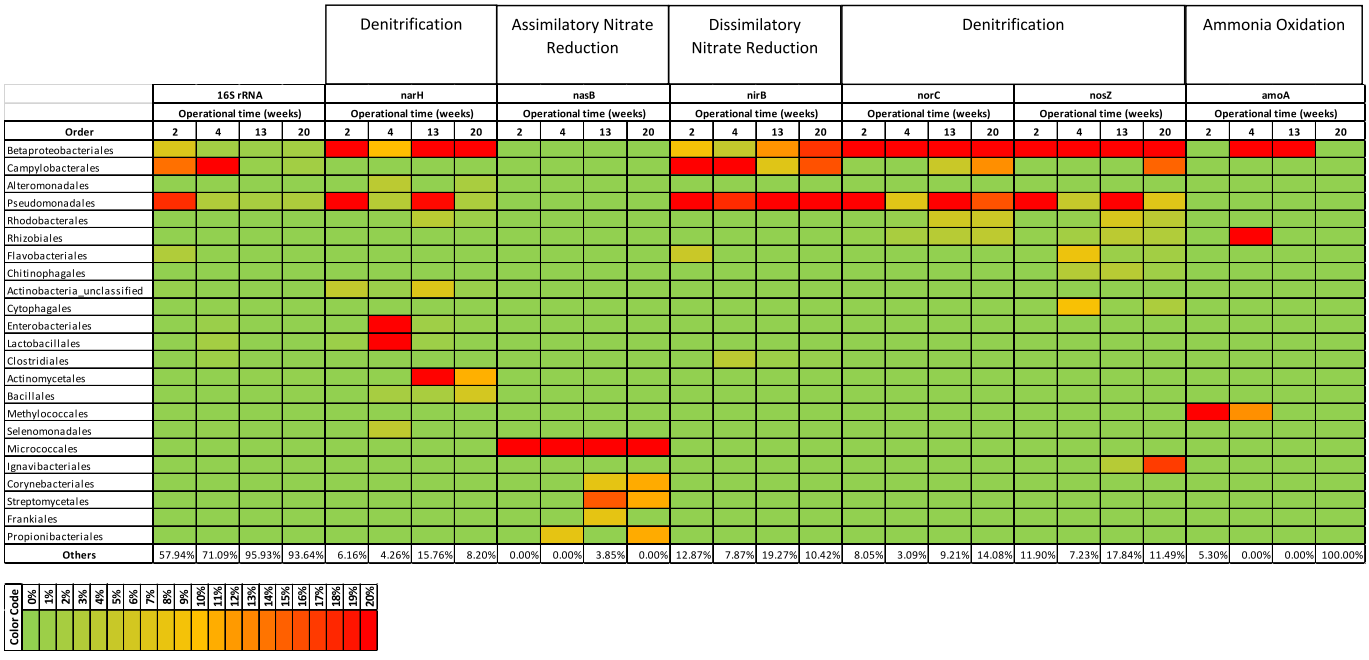

Fig. 3. PICRUST phylogenetic prediction of representative nitrogen metabolism pathway gene abundances and relative (>1 %) contributions at bacterial order level.

		16S rRNA				ppk1				ppx-gppA			
		Operational time (weeks)				Operational time (weeks)				Operational time (weeks)			
OTU	Taxonomy	2	4	13	20	2	4	13	20	2	4	13	20
Otu000005	Arcobacter												
Otu000009	Bacteroidales_unclassified												
Otu000010	Burkholderiaceae_unclassified												
Otu000012	Macellibacteroides												
Otu000015	Rikenellaceae_unclassified												
Otu000016	Arcobacter												
Otu000017	Rheinheimera												
Otu000027	Acinetobacter												
Otu000034	Dysgonomonadaceae_unclassified												
Otu000040	Enterococcus												
Otu000041	Pseudomonas												
Otu000044	Rikenellaceae_uncultured												
Otu000051	Fluviicola												
Otu000059	Bacteroidales_unclassified												
Otu000077	Flavobacterium												
Otu000078	Eubacteriaceae_unclassified												
Otu000116	Dysgonomonadaceae_unclassified												
Otu000118	Actinomyces												
Otu000120	Sphingobacteriales_unclassified												
Otu000122	Paludibacteraceae_unclassified												
Otu000128	Paludibacter												
Otu000139	Petrimonas												
Otu000143	Rikenellaceae_unclassified												
Otu000184	Acetobacterium												
Others		33.49%	31.06%	32.25%	36.83%	38.40%	26.63%	23.62%	28.88%	31.97%	18.50%	24.39%	26.57%

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Fig. 4. PICRUST phylogenetic prediction of representative polyphosphate gene abundances and relative (>1 %) contributions at genus level.

3.4. Optimisation of duckweed growth on wastewater

While the primary focus of the study was on integration of AD-ADF biological treatment with duckweed cultivation, duckweed growth was also comparatively assessed on untreated wastewater and digestate from the industrial anaerobic digestor at the dairy processing site. Relative growth rates on the differing wastewaters, with and without pH modification to pH 5, are presented in Fig. 5. On untreated wastewater, *L. minor* grew with modest success in the initial stages, however by day 7 a thick layer of microbial biofilm routinely formed on the surface of the medium with the plants becoming discoloured and losing viability. As a result the RGR for *L. minor* on untreated wastewater was 0.15 ± 0.05 with the high variability attributed to the rate and extent of biofilm formation.

Initial growth trials on effluent from the ADF laboratory system showed poor *L. minor* growth overall, with considerable variation between samples (Fig. 5). ADF effluent was observed to contain significant solids carry over from the reactor, (Table 1), however filtration (1.2 μm) had no effect on observed growth and was excluded as a significant contributory factor (data not shown). It was observed that direct growth of *Lemna minor* on industrial AD effluent also resulted in relatively low RGRs when compared to cultivation on untreated effluent. Physico-chemical analyses of the wastewaters (Table 1) identified a significant disparity between the pH of untreated wastewater (pH 6.5 ± 0.5) and

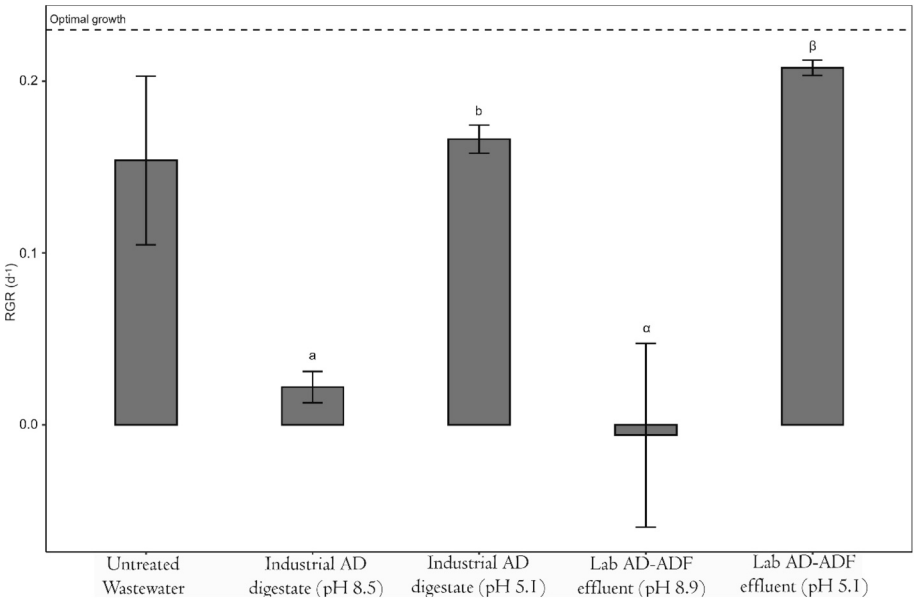

Fig. 5. Mean RGR (d^{-1}) ± SE for *L. minor* cultivation on wastewaters with and without pH modification. The dashed line represents RGR for *L. minor* grown on optimal Hutner's medium [20]. Bars that do not share letters differ significantly from one another ($p < 0.01$).

the industrial AD and laboratory AD-ADF effluents (pH 8.5–8.9), coupled with fluctuations in NH_3-N concentration in the ADF effluent. The combination of high pH and ammonia has previously been identified as a major hindrance for successful duckweed growth [40,41]. As a result, the pH of both the industrial AD and Lab-scale AD-ADF effluents were adjusted to pH 4.9–5.1 with H_2SO_4, which resulted in statistically significant, increased RGRs for the industrial AD effluent and the laboratory AD-ADF effluent, respectively (Fig. 5). In the case of the ADF effluent, potential phytotoxicity impacts of ammonia were also assessed through ammonia addition in tandem with pH modification (Fig. 6). It was observed that a combination of high pH, 8.6–8.8, with an NH_3-N concentration of 2.14 mM (30 mg L^{-1}) negatively affected duckweed RGR. However, when the pH was adjusted to pH 4.9–5.1, no negative effects on RGR were observed, irrespective of the ammonia concentration. A multiple linear regression ($F(3,32) = 46.29$, $p < 0.001$, $R^2 = 0.81$; Fig. 6) suggested that pH and ammonia variables explained a high amount of the variation in RGR observed when plants were grown on the AD-ADF effluent.

Table 1 also highlights the elevated sodium and chloride levels in the AD-ADF effluent compared to other metals. Oukarroum and colleagues previously reported the potential for high NaCl concentrations to inhibit photosynthesis [42]. Therefore, in an effort to investigate broader, negative impacts of ADF effluent on *Lemna minor* cultivation, plant photosynthetic integrity was also quantified by measuring chlorophyll *a* fluorescence parameters at the beginning and end of each 7 day growth trial (Fig. 7). Such measurements facilitate a deeper understanding of the health of plants by assessing the utilisation of light energy. For example, a stressed or damaged plant may have less capacity for photosynthesis, lower F_v/F_m, or use less photosynthetic energy, lower Y (II). Cultivation of *Lemna minor* on pH modified (pH 4.9–5.1), AD–ADF reactor effluent did not result in any statistically significant differences between chlorophyll *a* fluorescence measurements taken on day 0 and day 7; (*t*-test F_v/F_m: $t(6) = 0.37$, $p = 0.72$; t-test Y(II): $t(6) = 1.56$, $p = 0.17$; t-test Y(NPQ): $t(6) = 0.74$, $p = 0.49$; t-test Y(NO): $t(6) = 0.005$, $p = 1.0$ Fig. 6). In summary, cultivation of *Lemna minor* on pH modified ADF effluent did not appear to induce general plant stress in relation to energy capture, use or quenching.

3.5. *Lemna minor* impacts on wastewater nutrient removal

As discussed in Section 3.1, the AD-ADF system alone achieved significant NH_3-N reduction (83 %), but the effluent still retained sufficient, bioavailable nitrogen concentrations to support duckweed growth as outlined above. Fig. 8 presents a comparative graphical summary of nutrient removal impacts of the industrial AD, laboratory AD-ADF system and, *Lemna minor* growth on pH modified AD-ADF effluent. Integration of duckweed cultivation enhanced the remediation performance of the overall system (Table 1) resulting in TN removal of 96.5 %, with 99 % removal of residual NH_3-N and 99.5 % removal of the residual NO_3^--N generated in the AD-ADF system. TP removal was also improved with duckweed incorporation and increased to 73 %. Scaling of the duckweed TN removal rates (mg N m^{-2} d^{-1}) under the conditions tested suggest a potential capacity of 402–931 mg N m^{-2} d^{-1}, which aligns well with previously reported rates 124–4400 mg N m^{-2} d^{-1} in other treatment settings ([43]; J. [44–46]). However, it is likely that our study underestimates the maximal possible rate as the efficiency of the AD-ADF system resulted in limited nitrogen availability overall. This may be of relevance to the demonstrated phosphate uptake capacity also, which is linked to nitrogen metabolism in *Lemna* growth. The duckweed TP uptake rate is normally lower than the TN uptake rate [44]. Indeed, the ratio of N:P concentrations in duckweed is usually 5:1 [47], which reflects the ratio in which plants need these nutrients [48]. However, in the ADF reactor effluent this ratio was closer to 2:1. Thus further work would be required to optimise the ratios to maximise plant biomass yields and corresponding nitrogen and phosphorus uptake. For example, addition of an auxiliary nitrogen source such as urea could accelerate phosphate uptake [49].

3.6. Conclusion

This is the first report on the integration of acidogenic AD and ADF bioreactors with duckweed cultivation for potential, sustainable bioremediation of dairy processing wastewater. The AD-ADF system provides several potential advantages over traditional, multistep biological treatment configurations and chemical sludge precipitation including reduced infrastructural demands, energy input, chemical costs and sludge generation management. When coupled with downstream duckweed cultivation the system also allows for biomass generation

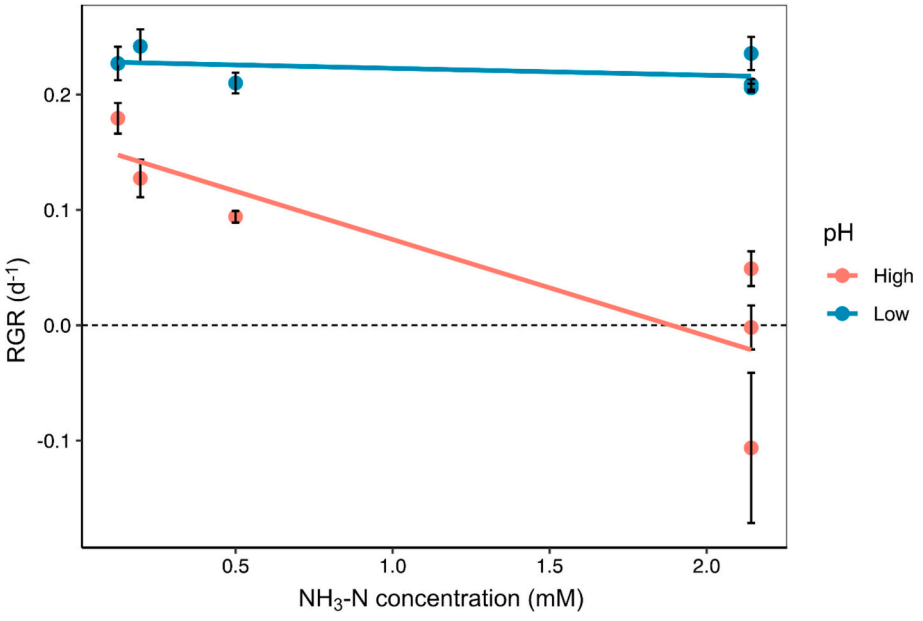

Fig. 6. Mean RGR (d^{-1}) ± SE of *L. minor* grown on AD-ADF wastewater effluent vs. concentration of NH_3-N (mM). 'High' pH denotes values from 8.6 to 8.8 and 'low' pH denotes values from 4.9 to 5.1. Trendlines are fitted as per multiple linear regression analysis.

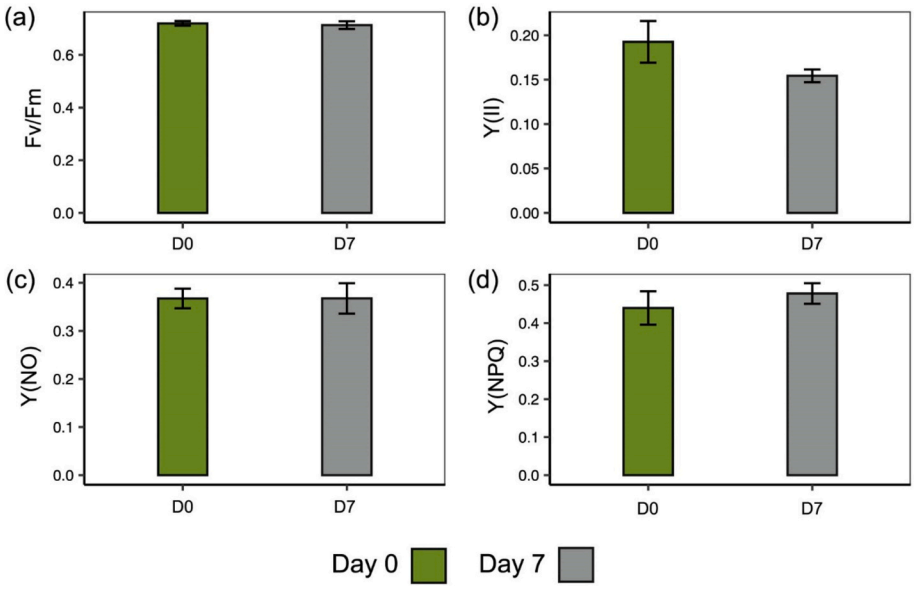

Fig. 7. Mean (±SE) (a) F_v/F_m, (b) Y(II), (c) Y(NPQ), (d) Y(NO) on day 0 and day 7 for *L. minor* grown for 7 days on ADF effluent.

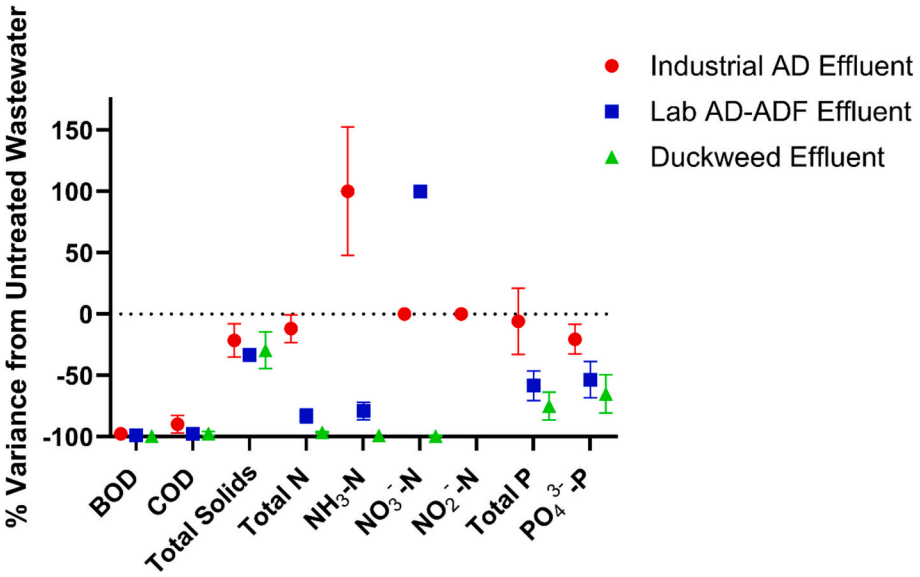

Fig. 8. Impact of industrial AD, Lab AD-ADF and duckweed cultivation on wastewater nutrient composition calculated as % variance from untreated wastewater concentrations. Note duckweed effluent data refers to the collective impact of laboratory AD-ADF and duckweed cultivation systems on untreated wastewater.

with a high protein content suited to food/feed applications [50]. The system demonstrated excellent nutrient remediation with real time wastewater. Microbial community profiling and predictive functional modelling suggested atypical bacterial orders and genera dominated the ADF reactor ecology and nitrogen metabolism performance, with novel associations for genus Macellibacteriales and Rikenellacea in PO_4^{3-}-P metabolism. As this study focused on food grade, dairy processing wastewaters, pathogenic bacteria were not anticipated or observed in community profiles of the industrial or laboratory systems. However, application of this approach to other agri-sector wastestreams with faecal bacterial contamination (e.g. meat processing wastewater) would need to evaluate the potential for pathogen transmission to duckweed or duckweed derived nutrition products. Duckweed cultivation on the AD-ADF system effluent contributed significant residual NH_3-N and NO_3^--N uptake, with more modest impacts on PO_4^{3-}-P levels. However, a key novel insight was established with respect to plant toxicity in response to alkalinity and NH_3-N concentration when cultivated on such effluents, together with positive impacts of pH modification. In conclusion sustainable growth and environmental impact mitigation are major issues facing the global dairy industry. Biotechnological opportunities and approaches to re-envisage dairy processing wastewater as an energy and bioresource reservoir have begun to be widely explored. However, the key evolutionary step for such research is the integration of compatible, biorecovery technologies to provide platforms for biorefinery design and development. Here we provide valuable, practical insights into the performance capacity and technical challenges to effective integration of microbial bioreactor systems with duckweed cultivation. While our study presents a laboratory scale, proof of concept evaluation, the combined nutrient remediation impact of this novel technological approach shows great potential. Next stage, pilot-scale trials, incorporating industrially relevant parameters, will further establish the feasibility of this technology to deliver the stringent wastewater remediation demands faced by the sector with opportunities for value added biorecovery.

CRediT authorship contribution statement

Éamonn Walsh: Writing – original draft, Methodology, Investigation, Formal analysis, Data curation. **Lekha Menon Margassery:** Investigation, Data curation. **Alejandro Rodriguez-Sanchez:** Writing – original draft, Visualization, Software, Data curation. **David Wall:** Resources, Funding acquisition, Conceptualization. **Paul Bolger:** Project administration, Funding acquisition, Conceptualization. **Marcel A.K. Jansen:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization. **Niall O'Leary:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

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

Link to Next generation sequencing data has been provided in the methods section of the manuscript.

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