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Impact of photobioreactor design on microalgae-bacteria communities grown on wastewater: Differences between thin-layer cascade and thin-layer raceway ponds

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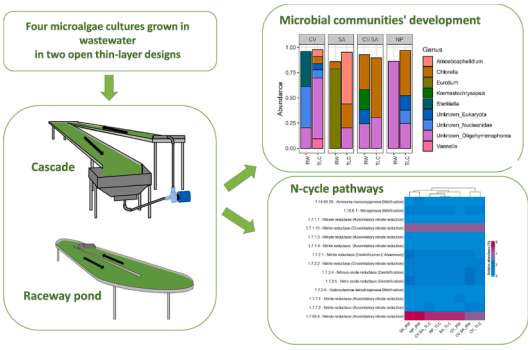
HIGHLIGHTS

- *Chlorella* is a robust strain often acting as an "invasive" species.
- Eukaryotic microalgae inocula led to a more robust and stable community.
- RWs favoured a higher community variability than TLCs.
- *Nostoc* was maintained but the prokaryotic community was variable.
- TLC design enhanced nitrification while RW denitrification.

GRAPHICAL ABSTRACT



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ABSTRACT

Thin-layer (TL) photobioreactors (PBRs) are characterised by high productivity. However, their use is limited to lab/pilot-scale, and a deeper level of characterisation is needed to reach industrial scale and test the resistance of multiple microalgae. Here, the performance and composition of eight microalgal communities cultivated in the two main TLs design (thin-layer cascade (TLC) and thin-layer raceway pond (RW)) were investigated through Illumina sequencing. *Chlorella vulgaris* showed robustness in both designs and often acted as an "invasive" species. Inoculum and reactor type brought variability. Eukaryotic microalgae inocula led to a more robust and stable community (higher similarity), however, RWs were characterised by a higher variability and did not favour the eukaryotic microalgae. The only cyanobacterial inoculum, *Nostoc piscinale*, was maintained, however the community was variable between

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designs. The reactor design had an effect on the N cycle with the TLC and RW configurations, enhancing nitrification and denitrification respectively.

1. Introduction

Recently, microalgae have aroused considerable interest worldwide due to their extensive bio-industry applications for biomass production, bioremediation, CO_2 capture and the extraction of various added-value products (Dagnaisser et al., 2022).

Microalgae mass cultivation is mostly carried on outdoors in constructed, large-scale, bioreactors with partial control of some physiological conditions (e.g. pH, biomass density, dissolved oxygen concentration, nutrition, mixing) (Zittelli et al., 2013). Open and closed bioreactors can be used for cultivation, but only in open, large-scale systems, might construction, production and maintenance costs be significantly reduced (Morales-Amaral et al., 2015). Recently, two main types of open system have been employed for mass production: raceway ponds and thin-layers, with the latter showing high efficiencies in biomass production (Grivalský et al., 2019). In these two systems, different circulation devices, paddle wheels or pumps, are usually used, which can partly determine the selection of strains (Grivalský et al., 2019). Therefore, the suitability of the particular cultivation system has to be validated for each selected strain before application to large-scale cultivation plants.

Commercial demand has pushed microalgal production towards the use of synthetic growth media to increase yields. The use of wastewater however has emerged as a valid low-cost alternative medium for biomass production, further reducing wastewater (WW) related quality problems (Suparmaniam et al., 2019). Numerous microalgae, such as *Scenedesmus*, *Chlorella* and *Nostoc*, have been grown efficiently in WW, demonstrating their importance for bioremediation of nutrients (López-Sánchez et al., 2022). *Chlorella* and *Scenedesmus* cultivation is especially economically favourable, since their biomass can be used as biofertilisers enriched by biostimulants and biopesticides produced by the microalgae themselves (Ronga et al., 2019). However, when using WW in open cultivation systems, microalgae cultures are inevitably invaded or co-cultured, to a certain degree, with other microorganisms.

The co-culturing of microalgae-bacteria consortia might improve yield and robustness of cultivations, as in some cases microalgae monocultures are not required for the production of target compounds. Recently, co-culturing has been the growing field in microalgal biotechnology, and it may be an alternative to the more difficult 'monoculture' approach which faces problems with contamination as well as low biomass productivity (Ramanan et al., 2016). In co-cultures, microalgae release dissolved organic matter and oxygen, which are used by bacteria, and these in turn release other important metabolites that can be used by their partners such as CO_2, micronutrients, growth stimulants, etc.

The investigation of the role of bacteria and other microorganisms in microalgae cultures, ranging from laboratory flasks to outdoor units, is difficult, as these systems are rather variable and unstable. Uncovering the correlations between microalgae and associated microorganisms, mostly bacteria, is considered necessary to establish the functional relationships. Therefore, studies of microbial communities in bioreactors are of interest to identify the invading species and their effects, positive or negative, on microalgae (Lian et al., 2018).

In this context, the amount of available information on TLs remains limited when compared to other PBRs, with only one study partially dealing with community characterisation (Villaró et al., 2022). Here, different growth media were tested and *Tetradesmus* (i.e. *Scenedesmus obliquus*) was grown alternatively on freshwater, WW and diluted pig slurry. *Tetradesmus* growth was reduced on WW, with other spontaneous microalgae dominating, possibly due to the presence of algal predators and grazers. More information on the characterisation of TLs is therefore needed to reach full-scale dimensions at an industrial level, test the resistance of multiple microalgae and to understand the positive interactions within the consortium (both between different microalgae and between microalgae and bacteria).

Within this study, 16S and 18S rRNA genes amplicon next generation sequencing (NGS) analyses were performed to investigate the difference that two different PBRs configuration, 1) thin-layer cascade (TLC) and 2) thin-layer raceway pond (RW), and their operational modes, respectively: 1) overnight tank storage and 2) continuous operation, might introduce on 1) composition and development of the bacterial-microalgal consortia and 2) *N*-cycle metabolism of cultures inoculated with three different microalgae species characterised by high adaptation to the local environment and economic relevance.

2. Materials and methods

2.1. Microalgae production and biomass analyses

The microalgae within this study were all selected due to their biopesticide and bio-stimulant activity (Carneiro et al., 2021; Ranglova et al., 2021; Grivalský et al., 2022). The same culture of starting inoculum for each microalga strain was grown on the same WW medium in parallel within TLC and RW set up under non-limiting conditions in terms of nutrients and conditions.

Microalgae production was established at the Algatech centre (48°59′15″ N; 14°46′40.630″ E), Institute of Microbiology of the Czech Academy of Science (Trebon, Czech Republic). The TLC and RW outdoor cultivation units (5 m^2), differing in the circulation device (i.e. paddle wheel or centrifugal pump) were placed side by side in a greenhouse following an east–west orientation. Cultivation was done between June and September 2019, and both designs, inoculated with the same microalgae, ran simultaneously. RWs (volume: 100 L, water level: 18 mm, speed: 0.2 ms^{-1}, CO_2 supply based on pH set point, pH: 7.8–8.2) were operated continuously. TLCs (volume: 70 L, water level: 10 mm, speed: 0.5 ms^{-1}, CO_2 supply, pH: 7.8–8.2) were operated only during day-time (7:30 a.m. – 7:30p.m.) and the culture was stored in a retention tank during the night-time (mixed via air bubbling, light:dark ~ 12:12 h). Flow speed was around 0.2 m s^{-1} for RWs while it was 0.5 m s^{-1} for TLCs. A 25% dilution rate was used. Evaporation was compensated by adding tap water daily which contributed to total nutrients with an additional 20 μg L^{-1} of NO_3^- and 28 μg L^{-1} of PO_4^{3-} at maximum (a negligible amount) (Carneiro et al., 2021; Ranglova et al., 2021; Grivalský et al., 2022).

Microalgae were grown on wastewater taken after secondary aerobic digestion from the municipal wastewater treatment plant in Trebon (Czech Republic) with a total nitrogen (TN) content similar to that of the synthetic medium (i.e. BG-11) and a total phosphorus (TP) content > 20x higher than that of BG-11 (Carneiro et al., 2021; Ranglova et al., 2021; Grivalský et al., 2022); in detail the wastewater features were: biochemical oxygen demand (BOD): 180 mg L^{-1}; chemical oxygen demand (COD): 1000–1100 mg L^{-1}; total organic carbon (TOC): 310–560 mg L^{-1}; TN: 230–260 mg L^{-1}; TP: 150–170 mg L^{-1}; TN:TP: 1.5).

Selected strains of microalgae (obtained from Prof. Vince Ördög, from the Algal Culture Collection of the Szechenyi Istvan University, Mosonmagyarovar, Hungary) were: 1. *Chlorella vulgaris* MACC-1 (CV), 2. *Scenedesmus acutus* (*Tetradesmus obliquus*) MACC-677 (SA) and 3. the cyanobacteria *Nostoc piscinale* MACC-612 (NP). All these strains were inoculated as pure cultures, plus a mix of *C. vulgaris.* and *S. acutus* (CV. SA). Cultures were initially grown in BG-11 medium in 10 L Pyrex bottles (28–30 ∘C, 200 μmol photons $m^{-2}s^{-1}$, air-bubbling 1% CO_2 (v/v) to maintain a constant pH ranging between 7.8 and 8.2). PBRs were

inoculated at the biomass density of 0.7 g of dry weight (DW) L^{-1} (a sufficient level to ensure a first prevalence of the selected strains if competitive (Clagnan et al., 2022a)) and were grown with a batch regime for seven days to reach the steady state, then semi-continuously for another five days, by harvesting 25% of the culture and replacing it with WW (Carneiro et al., 2021; Ranglova et al., 2021; Grivalský et al., 2022).

Culture temperature and irradiance were recorded using a meteorological station (modular control system ADiS-AmiT) with a solar radiation sensor located next to the PBRs, and temperature sensors in the cultures. Across the experimentation period, temperatures within the cultures ranged between 12 and 37°while solar irradiation reached peaks of 1800 μmol photons $m^{-2}s^{-1}$ (Carneiro et al., 2021; Ranglova et al., 2021; Grivalský et al., 2022).

2.2. Nutrients and biomasses analyses

Nutrient analyses were carried out as by Ranglova et al. (2021) while biomass densities and growth rates were calculated as by Ranglova et al. (2019).

Biomass samples of about 250 mg were used to detect the N concentration (% m/m), using an elementary analyser (Elementar Rapid max N exceed) based on the analytical method of combustion by Dumas and equipped with a thermal conductivity detector (TCD).

2.3. NGS, bioinformatics and statistics

Microalgal biomasses were collected between June and September 2019, at the end of the experimental period. Samples (~20 mg) of freeze-dried biomass of the assayed microalgae strains were processed as described by Clagnan et al. (2022a). Briefly, DNA extractions were performed using the Biosprint 96 One-For-All Vet Kit (Qiagen) together with the semiautomatic extractor BioSprint 96 (Qiagen) and MagAttract technology in three technical replicates. DNA yield was quantified using Qubit (Invitrogen, Italy), purity through Nanodrop (Invitrogen, Italy) and possible fragmentation with gel electrophoresis 1% (w/v) 1 × TAE agarose gels. DNA was then stored at −80 °C. Library for 16S and 18S marker gene were prepared following the Illumina Protocol. For the 16S, 341F and 805R primers were used (Herlemann et al., 2011) while for 18S, 1389F and 1510R primers (Piredda et al., 2017). Nucleotide sequences generated and analysed are available at the NCBI SRA repository (BioProject accession number: PRJNA913110).

Amplicons were processed as by Dumbrell et al., (2016) for 16S rRNA while a slightly modified protocol was used for the 18S rRNA (Bani et al., 2021).

All statistical analyses were performed on R studio (version 4.1.2) as in Clagnan et al. (2022a). The prokaryotic pathway of the enzyme profile for N metabolism was investigated through iVikodak (Nagpal et al., 2019).

3. Results and discussion

3.1. Biomass characterisation and nitrogen analysis

The microalgae strains in this study were selected as being biotechnologically promising in terms of bioremediation, biostimulants, biomass and agricultural biofertilizer production. Samples of microalgae biomass from microalgae cultures were collected at the end of the 4-day semi-continuous growth phase (at a dilution rate of 0.25 d^{-1}).

On collection day, growth rates were similar between both reactors for the same microalga while productivity showed a trend of higher values in the TLC (except for the CV.SA culture) (see e-supplementary materials). Highest growth and production were seen for CV and SA while they were lowest for the cyanobacterial cultures NP. However, when considering the whole experimental period, TLC showed significantly higher growth than RW for all cultures (Carneiro et al., 2021; Ranglova et al., 2021; Grivalský et al., 2022).

Considering the performances of the two systems, the nutrient removal efficiency was higher in the TLC than in the RW in almost all cases (see e-supplementary materials). This trend is consistent with the higher growth rate within TLCs. The levels of chemical oxygen demand (COD) generally increased throughout trials, reflecting the production of novel biomass by photosynthesis, which in turn supports non-autotrophic microbial metabolism and the increase of organic compounds dissolved in the medium. Thus, the calculated nutrient removal efficiency was negative.

CV.SA and NP biomasses showed a similar N content in TLC and RW while CV showed a higher N in TLC and SA in RW ($p < 0.05$) (see e-supplementary materials).

In both reactors, $N\text{-}NH_4$ reached concentrations below 5 mg L^{-1} while $N\text{-}NO_3$ was below 20 mg L^{-1} with a noticeable influence of denitrification and ammonium stripping (Carneiro et al., 2021; Ranglova et al., 2021).

Although microalgae can utilise different forms of N (i.e. NO_3^-, NO_2^-, NH_4^+ or organic N), NH_4^+ is preferred, as its uptake requires less energy, and microalgae can also inhibit the uptake of other N forms, favouring NH_4^+ (Kumar and Bera, 2020). In terms of N bioremediation, the stripping of ammonia (a fast reaction occurring spontaneously due to chemical equilibrium) is expected to be between 10 and 30% of the initial N, considering 1) the initial concentration of N in WW at 230–260 mg L^{-1}, 2) the average biomass concentration at the time of collection of ~ 2.2 g DW L^{-1} and 3) the sum of $N\text{-}NH_4$ and $N\text{-}NO_3$ in the outlet medium at 20 mg L^{-1}. Additionally, since the total Kjeldahl nitrogen is at ~ 200 mg L^{-1} including the N fixed by the biomass, it can be assumed that microalgae consumed mostly $N\text{-}NH_4$ due to the low concentration of NO_3-N, and that therefore the remaining $N\text{-}NH_4$ was subjected to stripping while the remaining NO_3-N could be involved in nitrification–denitrification within the reactors.

Most of the N that is lost from the mass balance is therefore stripped as ammonia. However, since NO_3 in these systems (even though at low concentrations) is constantly present, denitrification can occur, with the release of N_2O, considering the likelihood of the presence of denitrification genes and also thanks to functional redundancy (Ferrón et al., 2012; Bauer et al., 2016).

3.2. Eukaryotic communities

The total number of assembled reads for the eukaryotic communities was between 5,262 ± 658 and 17,495 ± 3,401 with a number of inputted reads ranging from 10,806 ± 1,342 to 35,758 ± 6,961 (see e-supplementary materials).

Within the eukaryotic community, the dominant phyla were *Chlorophyta* (green algae) and *Ciliophora* (aquatic unicellular microorganisms) with *Ascomycota* especially present in the SA cultures in RW (see e-supplementary materials).

In terms of eukaryotic genera, *Chlorella* was the most common across all samples (Fig. 1). CV cultivation in TLC showed the presence at high abundance of *Vannella* (9–11%), an ameboid protist, followed by *Amoeboaphelidium protococcarum* (6–8%), an algal parasitoid (Hoeger et al., 2022), and as expected, *Chlorella* (5–8%). On the other hand, CV cultures in RW showed a predominant contamination of the ciliate *Sterkiella* that feeds on microalgae (33–39%) which, together with the absence of *Chlorella*, might identify a "failed culture" with respect to the inoculum introduced.

Chlorella was also present in SA cultures in both RW (7–8%) together with the fungus *Eurotium* (70–87%), and in TLC (23–32%) together with *Amoeboaphelidium* (42–54%). The presence of *Amoeboaphelidium*, probably feeding on the cultivated microalgae, might point to an unstable system that could potentially be subject to failure risks (Molina-Grima et al., 2022).

Mixed cultures CV.SA showed a high abundance of *Chlorella* (30–37% in RW and 53–60% in TLC) together with the microalga

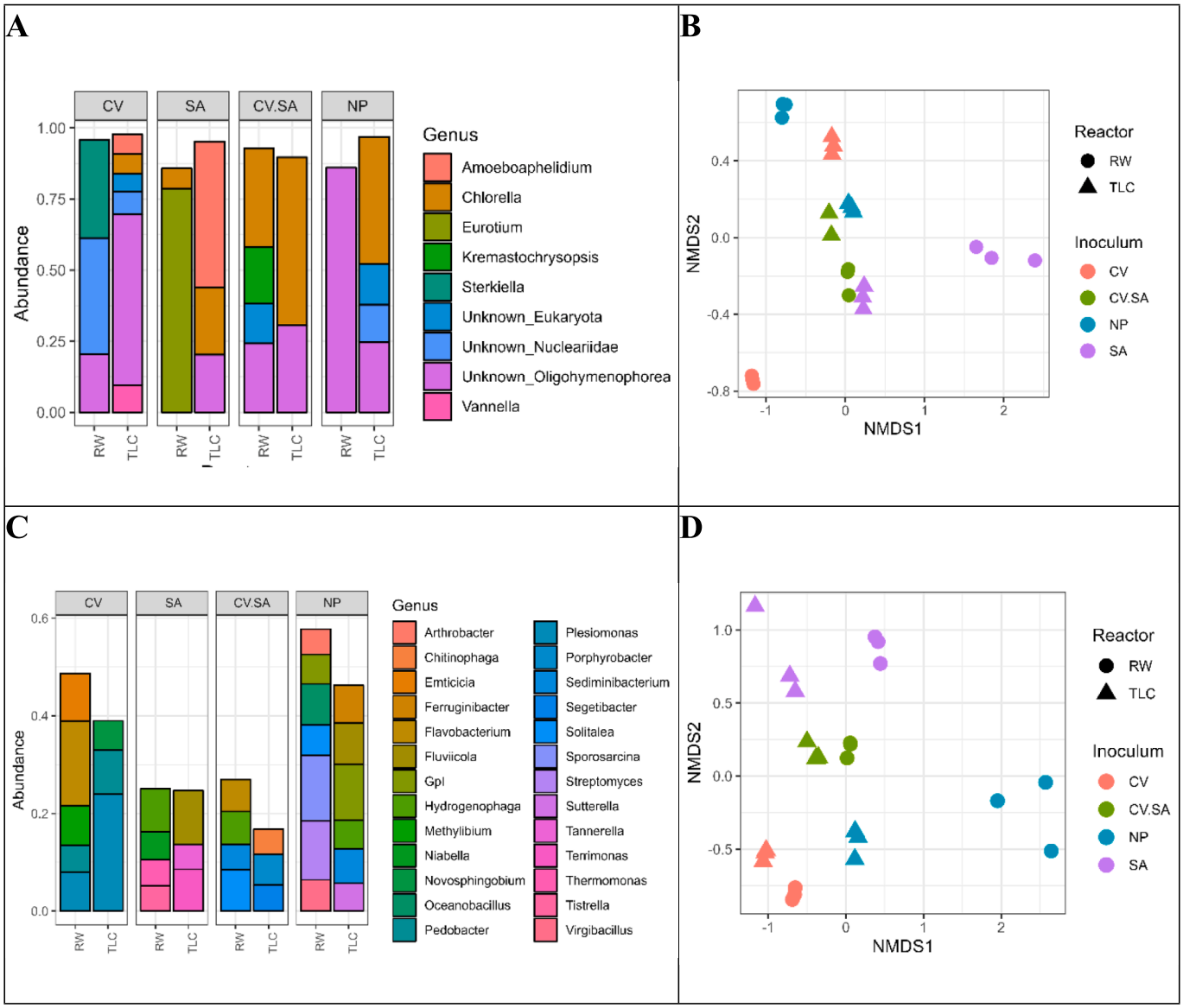

Fig. 1. Taxonomic composition at genus level of eukaryotic (A) and of bacterial (C) abundances (cut-off > 5%) in each photobioreactor configuration. Average values of three replicates are shown for each bar. NMDS plot for the eukaryotic (B) and bacterial (D) community.

Kremastochrysopsis (18–21%) in RW. Even though present at the start of the experimental set up, no *Scenedesmaceae* were found in *Scenedesmus acutus* cultures. However, this is not surprising as 1) cultures are in open systems and therefore prone to external contamination (Bani et al., 2021), 2) the use of WW is a source of contamination (although homogeneous to all our trials) (Clagnan et al., 2022b) and 3) *Chlorella,* a rapid growing microalga (Galès et al., 2019), might be more resilient to contamination, pollution or variation in environmental fluctuation than *Scenedesmus* and might overcome the initial mixed inoculum and establish itself as the main microalga within the community. *Chlorella* is, in fact, a genus renowned for its robustness, and is almost invasive, due to its fast growth and tolerance to various environmental conditions. Additionally, *Chlorella* is an eurythermal microorganisms with an ubiquitous character, as it can inhabit all type of aquatic and terrestrial environments, further being a high-performance primary producer (Krienitz et al., 2015). However, although the primers used have previously been shown to amplify *Scenedesmus* sp., *Tetradesmus* sp., and other genera commonly used in PBRs (Su et al., 2022), it is important to note that an accurate characterisation of the composition of microalgal biomass is not straightforward as, although DNA barcoding enable a rapid and reliable identification of organisms (Hebert and Gregory, 2005), it has the disadvantage of being a PCR-based approach and as such it is inherently biased by both DNA extraction and PCR complications.

The cyanobacterial NP cultures in RW were dominated by an unknown *Oligohymenophorea* while the same inoculum in TLC showed the main presence of *Chlorella* (41–52%).

Other microalgae were retrieved at low abundance (<5%), such as a *Spumella*-like flagellate, *Monoraphidium*, *Chlamydomonas*, *Pteromonas*, *Tetraselmis*, *Chromulina*, *Ochromonas*, *Coelastrella* and *Desmodesmus*.

Richness was similar across all samples (Table 1). Eukaryotic alpha-diversity indexes, species diversity within a system, showed in general a higher diversity in CV.SA culture grown in RW than in TLC (Shannon: $p < 0.05$) while the opposite occurred for NP (Shannon and Simpson: $p < 0.05$).

NMDS and PERMANOVA analyses on the eukaryotic communities indicate an influence of the inoculum, the reactor type and their interaction on the shaping of the communities (Permanova: $p = 0.001$) (Fig. 1). When doing pairwise analyses, communities among all cultures were different between TLC and RW configurations ($p = 0.001$), also

Table 1
Eukaryotic and bacterial alpha diversity community indexes (Observed and Chao1 richness, Shannon, and Simpson diversity, Pielou's evenness). Each number is the average of three replicates (Av. ± St. Dev.). Letters indicate significant differences for each index.

	18S rRNA					16S rRNA				
	Observed	Chao1	Shannon	Simpson	Evenness	Observed	Chao1	Shannon	Simpson	Evenness
RW										
CV	23 ± 4 a	32 ± 11 a	1.3 ± 0.0 abc	0.7 ± 0.0 ab	0.4 ± 0.0 ab	279 ± 75 a	458 ± 111 a	3.9 ± 0.0 d	0.9 ± 0.0 abc	0.4 ± 0.0 ab
SA	31 ± 5 a	40 ± 8 a	0.9 ± 0.3c	0.4 ± 0.1b	0.3 ± 0.1c	456 ± 59 a	642 ± 53 a	4.6 ± 0.0 ab	1.0 ± 0.0 abc	0.3 ± 0.1c
CV.SA	46 ± 5 a	60 ± 6 a	1.8 ± 0.0 a	0.8 ± 0.0 a	0.5 ± 0.0 ab	529 ± 97 a	681 ± 84 a	4.7 ± 0.1 ab	1.0 ± 0.0 ab	0.5 ± 0.0 ab
NP	43 ± 8 a	54 ± 12 a	0.9 ± 0.3c	0.3 ± 0.1b	0.2 ± 0.1c	197 ± 81 a	345 ± 76 a	3.6 ± 0.1 d	1.0 ± 0.0 bc	0.2 ± 0.1c
TLC										
CV	27 ± 4 a	33 ± 1 a	1.6 ± 0.0 ab	0.6 ± 0.0 ab	0.5 ± 0.0 ab	388 ± 37 a	543 ± 69 a	4.0 ± 0.0 cd	1.0 ± 0.0c	0.5 ± 0.0 ab
SA	36 ± 9 a	50 ± 12 a	1.3 ± 0.0 abc	0.6 ± 0.0 ab	0.4 ± 0.0 bc	314 ± 168 a	481 ± 172 a	4.6 ± 0.3 ab	1.0 ± 0.0 abc	0.4 ± 0.0 bc
CV.SA	34 ± 12 a	40 ± 15 a	1.2 ± 0.0 bc	0.6 ± 0.0 ab	0.3 ± 0.0 bc	463 ± 134 a	621 ± 118 a	5.0 ± 0.1 a	1.0 ± 0.0 a	0.3 ± 0.0 bc
NP	28 ± 4 a	43 ± 8 a	1.8 ± 0.0 a	0.8 ± 0.0 a	0.5 ± 0.0 a	440 ± 134 a	593 ± 111 a	4.4 ± 0.1 bc	1.0 ± 0.0 abc	0.5 ± 0.0 a

communities differed among inocula, with only CV and NP culture showing similarities ($p > 0.05$). When looking at pairwise analyses for beta-diversity, variability in species diversity among sampling units emphasizing the role of rare species, while when combining both species and reactor types, no significant differences were found (most likely due to the small sample size). However, when looking at the NMDS plot, the RW reactor design introduced a higher variability with a lower abundance of eukaryotic microalgae. TLC units usually had higher biomass productivity, due to a shallower depth, than RW, while higher biomass density can also be reached within TLCs, this could also possibly be a factor linked to a higher richness of eukaryotic microalgae found in TLCs reactors.

Furthermore, looking at the most influential species that accounted for > 70% of differences between samples, we can see that between the two CV cultures, the main difference ($p < 0.005$ across groups) is given by the presence of an uncultured *Oligohymenophorea*, an uncultured *Nucleariidae* and *Sterkiella multicirrata*, for SA by *Eurotium* sp. and *Amoeboaphelidium protococcarum*, for NP by uncultured *Oligohymenophorea*, *Chlorella* sp. and *Chlorella sorokiniana* ($p < 0.05$) while for the CV.SA culture by *Chlorella* sp., *Kremastochrysopsis austriaca* and by an uncultured eukaryote.

3.3. Bacterial communities

Bacterial sequencing resulted in a total number of assembled reads between 9,119 ± 2,945 and 27,088 ± 6,427 starting from 19,630 ± 6,017–57,418 ± 13,858 inputted reads (see e-supplementary materials).

Samples were dominated by the Bacteroidetes (8–47%) and Proteobacteria (9–55%) phyla (see e-supplementary materials). As expected, Cyanobacteria were present in all NP samples (5–57%) accompanied by Actinobacteria (12–31%) and Firmicutes (28–57%) in the RW set up. Actinobacteria (4–9%) were also present in SA samples with the addition of Acidobacteria (5–6%) in the TLC set up. No Cyanobacteria were retrieved in the PBRs inoculated with a eukaryote.

CV cultures in RW, showed as dominant genera *Flavobacterium* (17–18%); *Emticicia* (9–10%), a microalgal growth-promoting bacteria (Toyama et al., 2019); *Methylibium* (7–9%), a genus involved in biodegradation of siloxanes (Boada et al., 2020); the human pathogen *Plesiomonas* (7.9–8.2) and *Pedobacter* (5–6%), both environmental superbugs with generally multiple antibiotic resistance mechanisms (Viana et al. 2018) (Fig. 1). The same culture in TLC, showed the presence again of *Plesiomonas* (23–24%), *Pedobacter* (9–10%) plus *Novosphingobium* (6–7%), a genus known for its metabolic versatility and bioremediation potential (Liu et al., 2021).

In RW SA cultures, the main genera found were *Hydrogenophaga* (8.5–9.0%), a bacteria often found in microalgal-bacterial consortia and able to participate in sulfamethoxazole degradation (Xie et al., 2020), *Niabella* (5–6%) and *Thermomonas* (5–7%), often isolated from similar environmental samples, and *Tistrella* (5–6%) which is involved in *N*-fixation and has been shown to impair (possibly actively killing) *Chlorella* due to micronutrients limitation or the generation of secondary metabolites (Haberkorn et al., 2020). Research on the genus *Tistrella* is however scarce, and its effect on different microalgae needs to be explored (Collao et al., 2022). Whereas the same culture grown on TLC showed a different bacterial profile with a prevalence of *Fluviicola* (11–12%), often present in WW utilising carbohydrates (Rodriguez-Gonzalez et al., 2021), the heterotrophic denitrifier *Terrimonas* (8–9%) and *Tannerella* (2–6%).

Similarly to CV cultures in RW, CV.SA cultures grown on RW showed *Flavobacterium* (6–7%), mostly commensal or pathogenic bacteria, as dominant genera accompanied by *Hydrogenophaga* (6–7%), *Sediminibacterium* (5–6%), an ubiquitous taxa of freshwater bacterioplankton (Ogata et al., 2022), and the autotrophic denitrifiers *Solitalea* (8–9%). The same mix grown on TLC showed a peculiar composition consisting of *Porphyrobacter* (6–7%) which has been shown to be a key player in microalgae culture by producing a broad spectrum of B vitamins (Astafyeva et al., 2022), *Segetibacter* (5–6%), and the endohyphal bacterium *Chitinophaga* (5–6%).

The cyanobacteria *Nostoc piscinale* (labelled as GpI genus) was maintained in both RW (5–7%) and TLC (8–14%) designs. The RW set-up had high abundances of specific genera such as *Sporosarcina* (10–20%), the antibiotic producer and plant growth promoter *Streptomyces* (4–17%), the halotolerant and bioflocculant producer *Oceanobacillus* (4–17%), *Virgibacillus* (4–12%), a genus able to mediate mineralisation processes (Abdel Samad et al., 2020), *Lentibacillus* (3–13%), *Solitalea* (5–7%) and *Arthrobacter* (4–6%), a genus often used for useful for bioremediation or commercial applications (Busse and Wieser, 2014). The same cultures in TLC bioreactor, showed a different composition of *Fluviicola* (8–9%) and *Ferruginibacter* (7–9%), known to decompose long-chain fatty acids, monomers, and oligomers (Kwon et al., 2019); *Hydrogenophaga* (5–8%), *Sediminibacterium* (7.0–7.2) and *Sutterella* (5–6%), a common inhabitant of the human gastrointestinal tract (Hiippala et al., 2016).

Similarly to the eukaryotic community, prokaryotic richness was similar across all samples (Table 1). Bacterial diversity indexes showed in general a lower diversity in both CV and NP cultures than in CV.SA and SA (Shannon: $p < 0.005$). Additionally, NP cultures showed a higher diversity when grown in TLC rather than in RW.

Again, NMDS and PERMANOVA analyses on the eukaryotic communities indicated an influence of inoculum, reactor type and their interaction in shaping the communities (Permanova: $p = 0.001$) (Fig. 1). When doing pairwise analyses, communities among all cultures were different between TLC and RW configuration and between inocula species, similarly to the eukaryotic communities. When looking at the NMDS, CV.SA communities showed higher similarity between reactors similarly to its eukaryotic communities. Similarly, CV prokaryotic communities showed higher similarity between reactors than SA, while NP had the highest variability between reactors.

Looking at the most influential species that account for > 70% of differences between samples (and are present at an abundance > 5% in

at least one sample), we can see that between the two CV cultures, the main difference is given by the presence of *Flavobacterium, Plesiomonas, Emticicia, Novosphingobium, Methylibium, Pedobacter, Terrimonas, Hydrogenophaga, Sutterella, Porphyrobacter* and *Sediminibacterium*. For NP, the main species found were *Streptomyces, Sporosarcina, Fluviicola, Virgibacillus, Sediminibacterium, Sutterella, GpI, Solitalea, Arthrobacter* and *Hydrogenophaga.* For CV.SA, the main differences were related to the presence of *Solitalea, Flavobacterium, Chitinophaga, Hydrogenophaga, Porphyrobacter, Plesiomonas, Methylibium, Terrimonas, Sediminibacterium, Tistrella* and *Fluviicola*; while for SA it was due to the presence of *Fluviicola, Terrimonas, Hydrogenophaga, Niabella, Tistrella, Tannerella, Solitalea, Thermomonas, Lentibacillus, Emticicia, Fluviicola* and *Methylibium.*

3.4. Co-occurrences

Interactions between microalgal genera and both the eukaryotic and prokaryotic communities were investigated in terms of co-occurrence (Fig. 2). Among the whole eukaryotic communities, the highest number of positive interactions were detected for *Nostoc* (*GpV*) and *Chlamydomonas*, 19 and 17 respectively. *Chlorella* showed the highest number of negative interactions with a *Spumella*-like flagellate (a genus feeding on algae, fungi, and starch grains (Jeong et al., 2021)), the ciliate and microalgal predator *Sterkiella* (Hue et al., 2018), and uncultured Ciliates, while it showed positive interactions with the mould *Hagiwaraea; Myzocytiopsis,* and an uncultured Eukaryote.

When looking at the interaction between microalgae and the bacterial community, *Chlorella* showed again the highest number of interactions, 2 negatives (with *Plesiomonas* and *Pedobacter*) and 13 positives including the vitamin B producer *Porphyrobacter*; *Zooglea,* the growth of which is known to be promoted by algae organic matter (Wang et al., 2016), the denitrifiers *Caldilinea* and *Methyloversatilis*, and the microalgal growth-promotion bacteria *Achromonobacter* (Zhou et al., 2021). *Achromonobacter* was the bacterial genus showing the highest number (7) of positive interactions with microalgae, together with *Sphingopyxis* (5), another microalgal growth promoter (Haberkorn et al., 2020). On the other hand, *Nostoc* had 8 positive interactions, one in particular with *Exiguobacterium* that when found in co-culture with *Chlorella* can stimulate the secretion of *N*-related enzymes in the photosynthesis pathways of *Chlorella* and increase its enzymatic activities (Wang et al., 2020).

3.5. N-cycle pathways

A main operational difference between RW and TLC is that RW is always agitated while TLC stops at night and the culture is stored in a tank overnight where it is mixed via air bubbling. It was therefore hypothesised that at night within the RW there is only a slight drop in the oxygen concentration as, even though microalgae stop O_2 production, this drop is limited by the large gas exchange surface. On the other hand, the TLC O_2 concentration could increase more at night than in the RW, as the surface for the gas exchange within the tank is increased thanks to air bubbling. This could be supported by the retrieval of a higher $N\text{-}NO_3^-$ concentration, as the result of a higher nitrification and lower denitrification within the TLC (Ranglova et al., 2021), while in the RW the lower O_2 could have supported a higher degree of denitrification and production of nitrous oxide (N_2O). Different metabolic pathways might be therefore selected for the two reactors' designs. The prokaryotic enzyme profile for the *N*-metabolism was therefore investigated through iVikodak and multiple *N*-pathways of the *N*-cycle were retrieved (Fig. 3).

In accordance with what was hypothesised, bacterial communities cultivated in RWs showed a small but significantly ($p < 0.05$) higher abundance of genes coding for denitrification enzymes than in TLCs, with the exception of NP cultures, in which the opposite was achieved.

When considering nitrification, NP and SA cultures did not show any differences, while both CV and CV.SA cultures showed a higher abundance of nitrification genes in the TLC configuration, in agreement with Carneiro et al. (2021), where the significant drop in $N\text{-}NH_4$ concentration, accompanied by an increase in $N\text{-}NO_3$, was connected to nitrification in both CV.SA cultures, but at a higher degree within the TLC set-up, possibly linked to the higher dissolved oxygen. For assimilatory nitrate reduction, both CV and NP showed a higher abundance in RWs while no differences were reported for CV.SA and SA. Dissimilatory nitrate reduction was similar across all cultures except for CV which showed higher abundances in TLC.

4. Conclusions

Chlorella strain proved to be a robust strain in both designs, often acting as an "invasive" species. Inoculum and reactor type brought variability. Unfortunately, it was not possible to quantify the variability introduced by the external environment (open design and WW). More robust and stable community (higher similarity) was seen between reactors when inoculated with eukaryotic microalgae. RWs, when compared to TLCs, did not favour eukaryotic microalgae and supported a higher variability. For the prokaryotic community, *Nostoc* was maintained, however the community was variable between designs. The reactor design influenced the N cycle: TLC enhanced nitrification while RW, denitrification.

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CRediT authorship contribution statement

Elisa Clagnan: Conceptualization, Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing. **Marta Dell'Orto:** Investigation, Writing – review & editing. **Karolína Štěrbová:** Investigation, Writing – review & editing. **Tomáš Grivalský:** Investigation, Writing – review & editing. **João Artur Câmara Manoel:** Investigation, Writing – review & editing. **Jiří Masojídek:** Conceptualization, Funding acquisition, Resources, Writing – review & editing. **Giuliana D'Imporzano:** Conceptualization, Supervision, Writing – review & editing. **Francisco Gabriel Acién-Fernández:** Conceptualization, Supervision, Funding acquisition, Project administration. **Fabrizio Adani:** Conceptualization, Supervision, Funding acquisition, Resources, Writing – review & editing.

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Elisa Clagnan reports financial support was provided by University of Milan. Marta Dell Orto reports financial support was provided by university of Milan. Giuliana D Imporzano reports financial support was provided by University of Milan. Fabrizio Adani reports financial support was provided by University of milan. Karolina Sterbova reports financial support was provided by Centre Algatech, Laboratory of Algal Biotechnology. Tomas Grivalsk reports financial support was provided by Centre Algatech, Laboratory of Algal Biotechnology. João Artur Câmara Manoel reports financial support was provided by Centre Algatech, Laboratory of Algal Biotechnology. Jiri Masojidek reports financial support was provided by Centre Algatech, Laboratory of Algal Biotechnology. Francisco Gabriel Acien Fernandez reports financial support was provided by University of Almeria.

Data availability

Data will be made available on request.

Fig. 2. Co-occurrence based on Spearman rank correlation index of microalgal genera against eukaryotic genera for the statistically significant interactions (p value < 0.05) (A) and of microalgal genera against the most abundant (>2% in at least one sample) prokaryotic genera for the statistically significant interactions (p value < 0.05) (B).

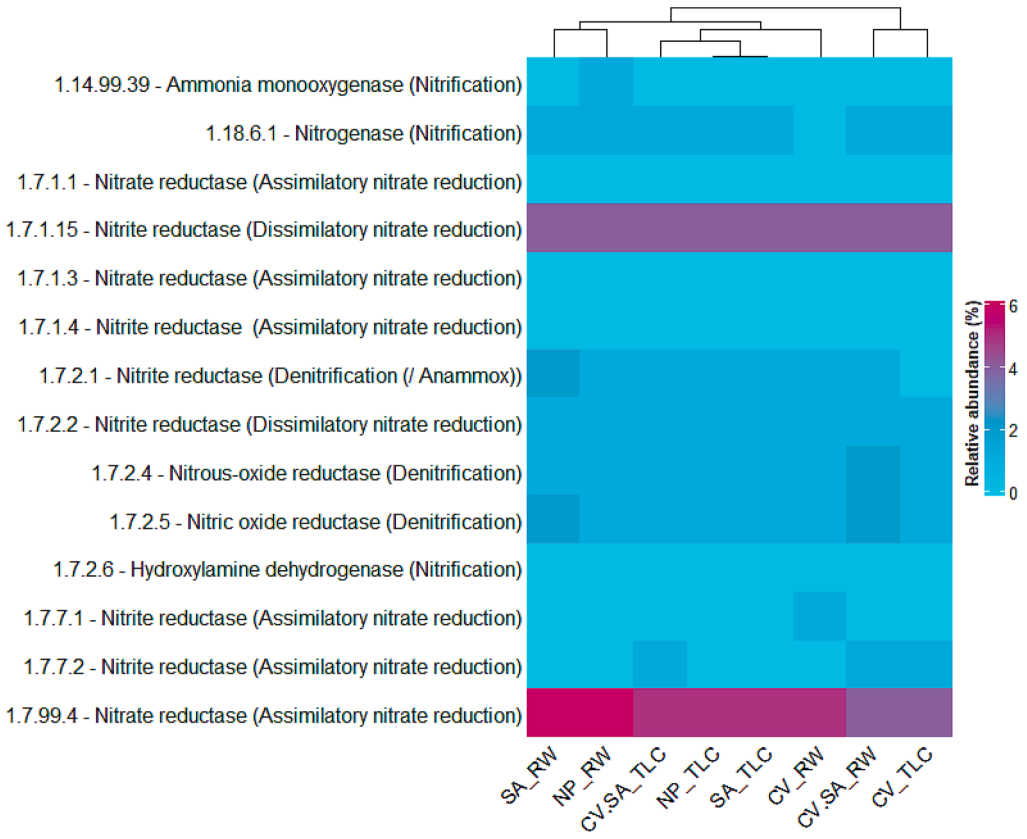

Fig. 3. Enzyme abundance profile inferred by iVikodak for the N metabolism.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2023.128781.

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