Title: 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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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* showed robustness in both designs and often acted as an "invasive" species. Inoculum and rector 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 when compared to TLCs. The only cyanobacterial inoculum, Nostoc, was maintained however the community was variable between designs. The reactor design had an effect on the N cycle with the TLC and RW configuration enhancing nitrification and denitrification respectively.

Keywords: Microalgae, Thin-Layer Cascade, Photobioreactor, Next Generation Sequencing, Raceway pond, Wastewater, Bacterial Community.

Introduction

Recently, microalgae have obtained considerable interest worldwide due to their extensive bio-industry application for biomass production, bioremediation, CO_2 capture and the extraction of various added-value products (Dagnaisser et al., 2022). Microalgae mass cultivations are mostly carried out 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 construction, production and maintenance cost might be significantly reduced (Morales-Amaral et al. 2015). Recently, mostly two types of open systems have been employed for mass production, raceway ponds and thin-layers. 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 low-cost alternative 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 showing their importance for bioremediation of nutrients (Lopez-Sanchez 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 may be an alternative to the more difficult 'monoculture' approach which faces problems with contaminations 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 problematic as these systems are rather variable and unstable. Uncovering the correlations between microalgae and associated microorganisms, mostly bacteria, is considered necessary to find out the functional relationship. Therefore, studies of microbial communities in bioreactors are of interest to identify the invading species and their effect, 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 (Villarò 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 the understand the positive interaction within the consortium (both between different microalgae or 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), 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 relevancy.

2. Materials and methods

The microalgae within this study were all selected due to their biopesticide and bio-stimulant activity (Carneiro et al., 2021; Ranglova et al., 2021). 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 (5m^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 occurred between June and September 2019. 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 (25% dilution rate). TLCs (volume: 70 L, water level: 10 mm, speed: 0.5 ms^{-1}, CO_2 supply, pH: 7.8-8.2) was operated only during day-time and the culture stored in a retention tank during the night-time (mixed via air bubbling, light:dark ~12:12 h). Evaporation was compensated by adding tap water (Carneiro et al., 2021; Ranglova et al., 2021).

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 the synthetic medium (i.e. BG-11) and a total phosphorous (TP) content >20x higher than BG-11 (Carneiro et al., 2021; Ranglova et al., 2021); 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 (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)). PBRs were inoculated at the biomass density of 0.7 g of dry weight (DW) L^{-1} 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 centrate (Carneiro et al., 2021; Ranglova et al., 2021).

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

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).

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143 **2.3 NGS, bioinformatics and statistics**

144 Microalgal biomasses were collected between June and September 2019, at the end of
145 the experimental period. Samples (~20 mg) of freeze-dried biomass of the assayed
146 microalgae strains were processed as per Clagnan et al. (2022). Briefly, DNA
147 extractions were performed using the Biosprint 96 One-For-All Vet Kit (Qiagen)
148 together with the semiautomatic extractor BioSprint 96 (Qiagen) and MagAttract
149 technology in three technical replicates. DNA yield was quantified using Qubit
150 (Invitrogen, Italy), purity through Nanodrop (Invitrogen, Italy) and possible
151 fragmentation with gel electrophoresis 1% (w/v) 1×TAE agarose gels. DNA was then
152 stored at -80 °C. Library for 16S and 18S marker gene were prepared following
153 Illumina Protocol. For the 16S, 341F and 805R primers were used (Herlemann et al.,
154 2011) while for 18S, 1389F and 1510R primers (Piredda et al., 2017). Nucleotide
155 sequences generated and analysed are available at the NCBI SRA repository (BioProject
156 accession number: PRJNA913110).
157 Amplicons were processed as per Dumbrell et al., 2016 for 16S rRNA while a slightly
158 modified protocol has been used for the 18S rRNA (Bani et al., 2021).
159 All statistical analyses were performed on R studio (version 4.1.2) mainly with the
160 package vegan (Oksanen et al., 2020) while taxonomic summaries were performed
161 using the phyloseq package (McMurdie and Holmes, 2013). Observed and Chao1
162 richness, Simpson and Shannon diversity index and Pielou's evenness were calculated,
163 following a Shapiro-Wilk test to test normality, differences among samples of normally
164 distributed data were tested by one-way analysis of variance (ANOVA), followed by a
165 Tukey's post hoc test, while non-normal data were analysed through a non-parametric
166 Kruskal-Wallis test followed by Dunn's Test for multiple comparisons. For pairwise
167 comparison, T-test and Wilcoxon signed-rank test was used for normal and non-normal

data respectively. Multivariate analyses were performed on Operational Taxonomic Unit (OTUs) relative abundances. To test the effect of reactor design and inoculum on beta diversity, first, a nonmetric multidimensional scaling (NMDS) based on Bray-Curtis distances was applied and then results were confirmed through a PERMANOVA test. Furthermore, pairwise comparisons were carried out with the package 'pairwiseAdonis' (Martinez, 2020). The betadisper function was further used to understand variance followed by the simper function to understand the main differences in composition. Co-occurrences were Investigated through the package cooccur (Griffith et al., 2016) to reveal intra- and inter-kingdom interaction.

The prokaryotic pathway of the enzyme profile for N metabolism was investigated through iVikodak (Nagpal et al., 2019).

3. Results and discussion

3.1 Nitrogen analysis in biomass

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 (a dilution rate of 0.25 d^{-1}).

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 all reactors, N-NH_4 reached concentration below 5 mg L^{-1} while N-NO_3 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 require less energy and microalgae can furthermore 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 10-30% 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-NH_4 and N-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-NH_4 due to the low concentration of NO_3-N, and that therefore the remaining N-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 concentration) is constantly present, denitrification can occur with the release of N_2O considering the likeliness of the presence of denitrification genes also thanks to functional redundancy. Usually in agriculture it is estimated that the N to N_2O ratio is about 4:1 (Fagerstone et al., 2011; 14. 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 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

parassitoid (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" 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 be potentially subjected 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 *Kremastochrysopsis* (18-21%) in RW. Even if 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) and 2) *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 microalgae within the community. However, although the primers used have previously 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 abundances (<5%), such as *Spumella-like flagellate*, *Monoraphidium*, *Chlamydomonas*, *Pteromonas*, *Tetraselmis*, *Chromulina*, *Ochromonas*, *Coelastrella* and *Desmodesmus*.

Richness was similar across all samples (see e-supplementary materials). Eukaryotic diversity indexes 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 a pairwise analyses, communities among all cultures resulted different between TLC and RW configuration ($p=0.001$), also communities differed among inocula with only CV and NP culture showing similarities ($p>0.05$). When looking at pairwise analysis for beta-diversity, variability in species composition among sampling units, 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.

Further looking at the most influential species that account for >70% differences between samples, we can see that between the two CV cultures 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 an 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%), 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 shown to impair (possibly actively killing) *Chlorella* due to micronutrients limitation or the generation of secondary metabolites (Haberkorn et

al., 2020). Research on genus 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 bacteria 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 sl., 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 resulted having 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%), *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 eukaryotic community, prokaryotic richness was similar across all samples (see e-supplementary materials). 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 a pairwise analyses, communities among all cultures resulted different between TLC and RW configuration and 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% differences between samples (and are present at an abundance >5% in at least one sample), we can see that between the two CV cultures main difference is given by the presence of *Flavobacterium, Plesiomonas, Emticicia, Novosphingobium, Methylibium, Pedobacter, Terrimonas, Hydrogenophaga, Sutterella, Porphyrobacter, Sediminibacterium*, for NP by *Streptomyces, Sporosarcina, Fluviicola, Virgibacillus, Sediminibacterium, Sutterella, GpI, Solitalea, Arthrobacter and Hydrogenophaga*; as per CV.SA main differences where 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 *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 mold *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* which growth 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 the latter 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-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 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 were 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 accordance with Carneiro et al. (2021) where the significant drop in N-NH_4 concentration accompanied by an increase in N-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 culture except for CV which showed higher abundances in TLC.

Conclusions

Chlorella strain proved to be a robust strain in both designs, often acting as an "invasive" species. Inoculum and rector 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 seemed to support a higher variability. For procaryotic community, *Nostoc* was maintained however the community was variable between designs. The reactor design influenced the N cycle, TLC enhanced nitrification while RW denitrification.

E-supplementary data

E-supplementary data for this work can be found in e-version of this paper online.

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589 **Figure captions**

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

594

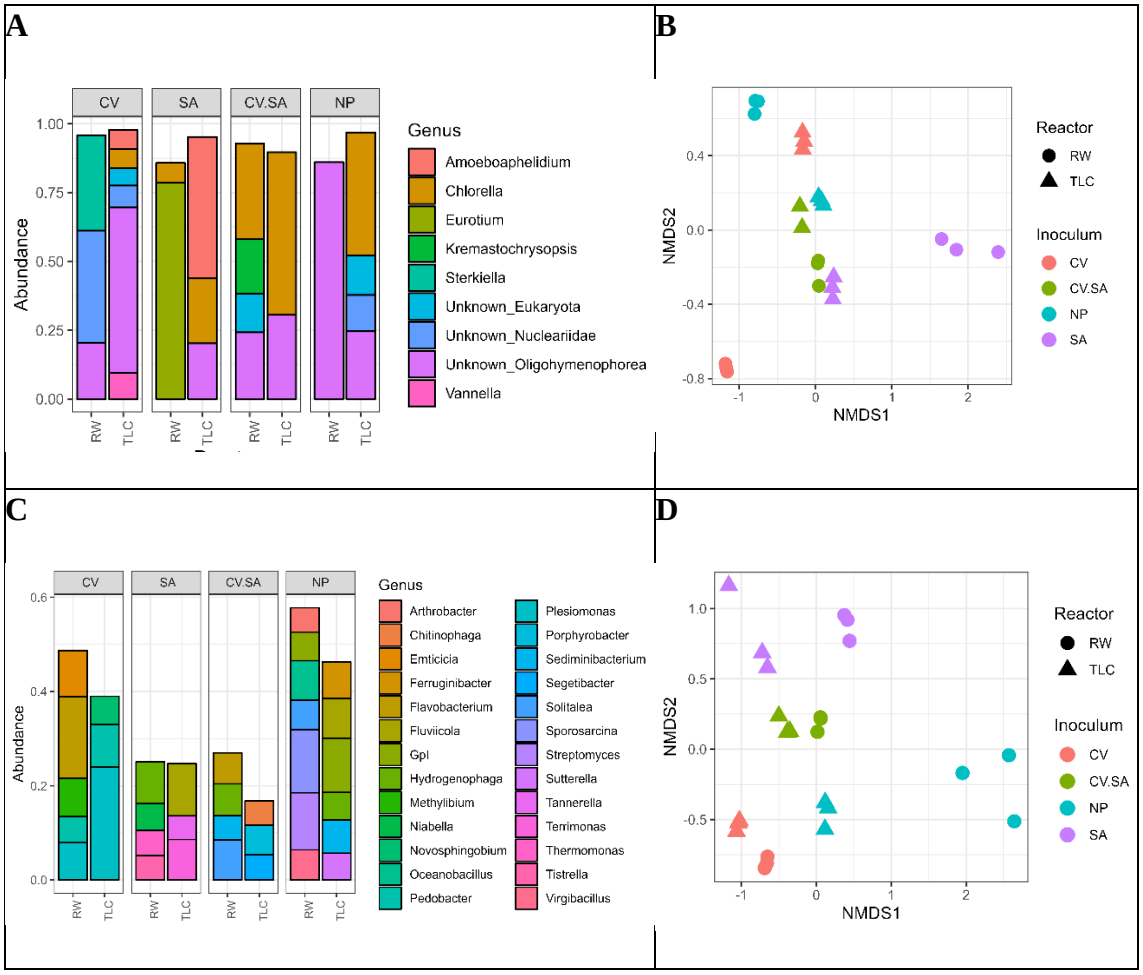
595 **Fig. 2.** Co-occurrence based on Spearman rank correlation index of microalgal genera
596 against eukaryotic genera for the statistically significant interactions (p value< 0.05) (A)
597 and of microalgal genera against the most abundant (>2% in at least one sample)
598 prokaryotic genera for the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

599

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

601 **Figures**

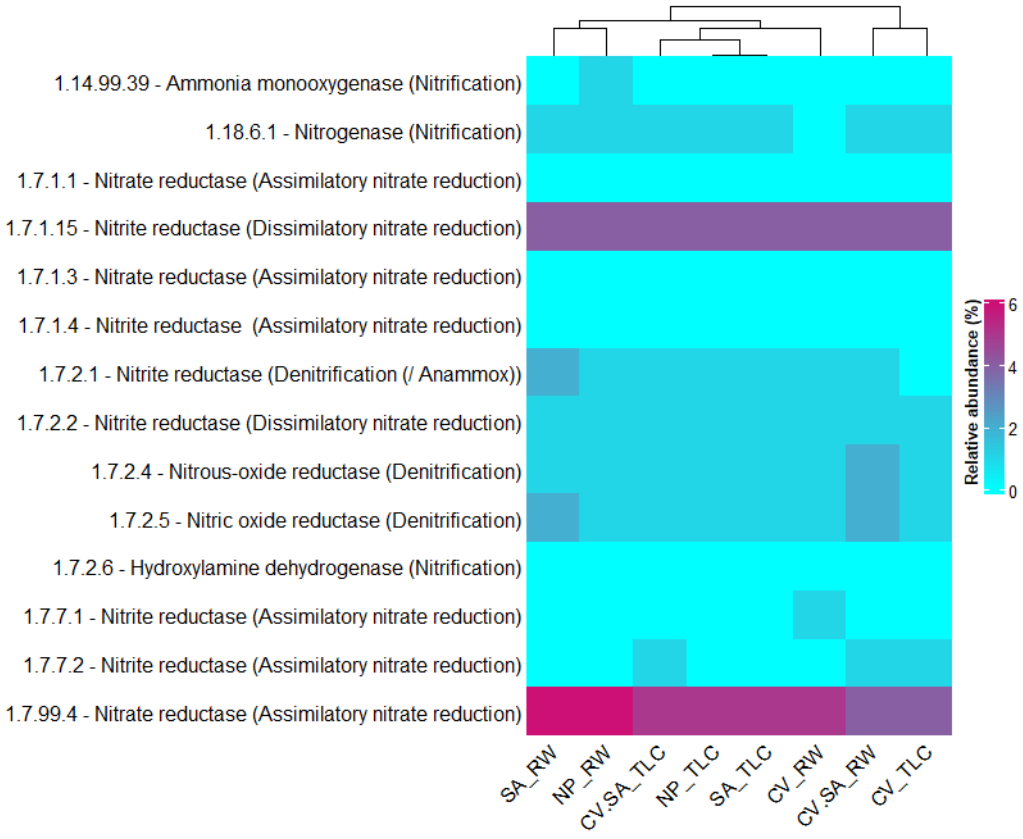
602



603 **Fig. 1.**


A
GpV
Gpl
Uncultured.Trebouxiophyceae
Tetraselmis.sp.
Ochromonas.sp.
Kremastochrysopsis.austriaca
Desmodesmus.sp.
Coelastrella.sp..QW.2019c
Chromulina.cf..nebulosa
Chlorella.sp.
Chlamydomonas.incerta
Allantion.parvum
Alternaria.mali
Amoeboaphelidium.protococcarum
Aspergillus.cervinus
Bodo.saltans
Bodonidae.sp..river.Thames
Cladosporium.halotolerans
Clydonella.sp.ATCC.50884
Epicoccum.sorghinum
Filamoeba.sp..COHH.101
Fungal.sp.
Fusarium.graminearum
Gonostomum.strenuum
Haguwarasea.radiculata
Hyphozyma.variabilis
Malassezia.restricta
Metschnikowia.sp..JJW.2009a
Mucor.sp.
Myzocytiopsis.humicola
Nucleariа.sp.
Orchitophryidae.environmental.sample
Parabodo.caudatus
Penicillium.sp.
Pharyngomonas.sp..RL
Phoca.vitulina
Piptocephalis.cruciata
Pompholyxophrys.sp.
Porphyridium.purpureum
Quercus.lobata
Rhabdostyla.sp..5.PPS.2010
Rhizopus.oryzae
Rhodotorula.sp.
Ripella.sp.
Spizellomycetales.sp.
Spongomonas.minima
Spumella.like.flagellate.JBC.S23
Stenamoeba.limacina
Sterkiella.multicirrata
Tokophrya.lemnarum
Tremula.longifila
Uncultured.ciliate
Uncultured.eukaryote
Uncultured.fungus
Uncultured.Oligohymenophorea
Uncultured.stramenopile
Uncultured.zygomycete
Vorticella.sp.
Corr
-1.0
-0.5
0.0
0.5
1.0
B
GpV
Gpl
Uncultured.Trebouxiophyceae
Tetraselmis.sp.
Pteromonas.protracta
Ochromonas.sp.
Monoraphidium.sp..HDMA.11
Kremastochrysopsis.austriaca
Desmodesmus.sp.
Coelastrella.sp..QW.2019c
Chromulina.cf..nebulosa
Chlorella.sp.
Chlamydomonas.incerta
Plesiomonas
Hydrogenophaga
Flavobacterium
Sediminibacterium
Ferruginibacter
Fluviicola
Solitalea
Terrimonas
Filimonas
Pedobacter
Porphyrobacter
Segetibacter
Sporacarcina
Streptomyces
Runella
Craurococcus
Opitutus
Peredibacter
Gp4
Sphingopyxis
Oceanobacillus
Sutterella
Virgibacillus
Chitinophaga
Lentibacillus
OP10_genera_incertae_sedis
Larkinella
Clostridiisalibacter
Zoogloea
Caldilinea
Achromobacter
Arthrobacter
Tannerella
Methyloversatilis
Anaeromyxobacter
Plesiocystis
Rudanella
Orientia
Exiguobacterium
Brevibacterium
Corr
-1.0
-0.5
0.0
0.5
1.0

604 **Fig. 2.**


1.14.99.39 - Ammonia monooxygenase (Nitrification)
1.18.6.1 - Nitrogenase (Nitrification)
1.7.1.1 - Nitrate reductase (Assimilatory nitrate reduction)
1.7.1.15 - Nitrite reductase (Dissimilatory nitrate reduction)
1.7.1.3 - Nitrate reductase (Assimilatory nitrate reduction)
1.7.1.4 - Nitrite reductase (Assimilatory nitrate reduction)
1.7.2.1 - Nitrite reductase (Denitrification (/ Anammox))
1.7.2.2 - Nitrite reductase (Dissimilatory nitrate reduction)
1.7.2.4 - Nitrous-oxide reductase (Denitrification)
1.7.2.5 - Nitric oxide reductase (Denitrification)
1.7.2.6 - Hydroxylamine dehydrogenase (Nitrification)
1.7.7.1 - Nitrite reductase (Assimilatory nitrate reduction)
1.7.7.2 - Nitrite reductase (Assimilatory nitrate reduction)
1.7.99.4 - Nitrate reductase (Assimilatory nitrate reduction)
SA_RW
NP_RW
CV.SA_TLC
NP_TLC
SA_TLC
CV_RW
CV.SA_RW
CV_TLC
Relative abundance (%)
6
4
2
0

605

606 **Fig. 3.**