Influence of Photobioreactor Set-up on the Survival of Microalgae Inoculum

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ABSTRACT

Cultivation of specific microalgae is still difficult in an industrial setup as contamination and balancing the economic cost are not always possible. Understanding the ecology of cultivation of microalgae is therefore necessary to implement stable production. The aim of the study was to understand how different types of photobioreactors and types of culture medium influenced the survival of a specific microalgae inoculum, *S. almeriensis*. The bacterial and microalgae community were studied using Illumina sequencing. Only the closed configuration was able to maintain the inoculated species while all the other systems developed a different eukaryotic community due to contamination and the higher fitness of contaminants. Photobioreactor configuration was more important than medium in shaping the eukaryotes community, while the bacterial community was influenced strongly by both. Results showed that even a well-adapted strain is maintained only in the closed reactor while the open reactors are colonized by a multispecies consortium.

1. INTRODUCTION

Although a large number of microalgae-related applications have been previously reported, only a few of them are effectively used at a commercial scale, and less than ten microalgae strains are commercially available (Raja et al., 2008). One of the reasons for that is the high production cost and limited production capacity when using closed photobioreactors and also the contamination problems which occur by the utilization of open reactors. Moreover, to increase the sustainability of microalgae production, the integration of wastewater treatment processes has been recommended, and in this case control of contamination becomes more difficult. For example, high amounts of N-rich and P-rich wastewaters are produced by the agriculture and livestock sectors (D'Imporzano et al., 2018), and these can provide the nutrients l to produce microalgae biomass (Monlau et al., 2015). Additionally, algae biomass holds an intrinsic value for its potential use for feedstock production in aquaculture or as biofertilizer (Acién et al., 2012; del Mar Morales-Amaral et al., 2015). This strategy is are not merely convenient economically because it leads to a cost reduction, but is also beneficial at the environmental scale due to a lowering of CO_2 emissions (D'Imporzano et al., 2018). Often, in a biorefinery process, bioreactors are limited to open pond raceways, due their relatively low cost, easy maintenance and large volume (Acién et al., 2012). Nevertheless, thin layer reactors could give equivalent results or perform more optimally than raceway reactors and as a result thin layer reactors would potentially be more economically viable (del Mar Morales-Amaral et al., 2015).

Open reactors are easily contaminated from the surrounding environment, including by other algae species (Fulbright et al., 2014), bacteria (Ganuza et al., 2016) or predators (Deruyck et al., 2019) that could outcompete the desired target species.

To overcome the contamination problem, or at least limit it so that the production is not depressed, while keeping the associated costs within the limits for economic success, several strategies have been implemented. Extreme environmental conditions were applied to the production of different strains of economic value, this included a high level of salinity for *Dunaliella* sp. or alkalinity for Spirulina (Lee, 2001). High pH has been demonstrated to reduce the contamination in open ponds where Chlorella sp remain the main microalga (above 90% of the species present) over a 16 day period (Bell et al., 2016). Modulation of pH has also been applied to prevent complete loss of a Chlorella culture due to bacterial contamination, using a pulse change in the pH that strongly affected the contaminant but not the microalga which was better able to regulate the internal pH (Ganuza et al., 2016). However the solutions mentioned are not always feasible, since many microalgae would not be able to survive or to produce the optimal biomass under such stressful conditions and thereby meet the industrial goals.

Interactions between the different organisms and their modulations could help in maintaining species purity and the microalgae production even in environments prone to contamination such as open reactors. Furthermore, the study of the interactive mechanisms between bacteria and microalgae should contribute to the improvement of production by allowing the selection of optimal combinations of microalgae and bacteria for different applications such as the production of fatty acids for biodiesel production (Mooij et al., 2015). Bacterial cells could easily outnumber algae cells and biological relationships range from positive to negative (Lian et al., 2018). Bacteria could be harmful, as some species could release toxins (Lian et al., 2018), although it should not be disregarded that they are also essential for algal growth as they provide vitamin B12 to algae that are not able to synthesise B12 intrinsically or they may help in the mediation

of nutrient solubilisation (Krohn-Molt et al., 2017). To monitor this system, classical microbiological/morphological techniques are time consuming, laborious and incapable of identifying a high percentage of bacterial genera. Molecular techniques can overcome some of these limitations, providing quick monitoring solutions for pathogens (Ganuza et al., 2016), and also help in identifying a higher number of taxa, including many unculturable bacteria, and can shed light on the interactions between the different microorganisms present in the community. Results from previous trials to compare the influence of different types of bioreactors on the microalgae/bacterial composition of the culture, cultivated under the same conditions (such as using the same algae species inoculum) remain limited. Pilot scale volume studies (De Vree et al., 2015) are especially underexplored, in contrast to the lab scale, in which studies are well established (A. M. Lakaniemi et al., 2012; Zevin et al., 2016).

Selection of the desired strain should be a key point especially if open bioreactors are going to be used. Two approaches can lead to a successful operating system: using a consortium in which different microalgae occupy slightly different niches and do not compete for the same nutrients and have different optima for temperature and light; or to use a microalga that is known to be well adapted to the local environment so that in theory it should be able to colonize and maintain dominance over possible contaminants (Mooij et al., 2015; Narala et al., 2016).

This study aims to test the performance and composition of the microbial community of microalgae cultures, cultivated in different types of photobioreactors and different growth media, inoculated with conspecific algal species that should be able to be maintained as pure cultures due to their high adaptation to the local environments. The starting hypotheses were that i) the microalgae inoculum would survive and grow in all of the

109 photobioreactors as the strain fits the different conditions set up, while ii) the bacterial
110 communities would be more susceptible to the changes in composition in relation to the
111 different growth conditions and bioreactors' configuration.

112 2. MATERIAL AND METHODS

113 2.1 Experimental design

114 The microalgae production was established at the research centre of "Estación
115 Experimental Las Palmerillas", property of Fundación CAJAMAR (Almería, Spain).
116 Three different pilot scale outdoor photobioreactors were developed and subsequently
117 used to grow the microalgae: a tubular reactor (closed system, referred to as T samples);
118 a raceway reactor; and a thin layer reactor (open systems, referred to as RW and RH for
119 raceway, and RI for thin layer). All the reactors were inoculated with the same algal
120 specie, *S. almeriensis*, with 20% of their volume, and then operated in batch mode for one
121 week, and were later operated in continuous mode for five weeks at 0.3 day^{-1} dilution rate,
122 to achieve a stable steady state.
123 Experiments were performed in summertime, from May to July, using different culture
124 media prepared with tap water plus fertilizers, pig manure, and wastewater.
125 In detail, T samples derived from a culture grown in a tubular reactor (3 m^3) operated in
126 continuous mode using fertilizers and clean water as culture medium; sample RW comes
127 from a raceway reactor (20 m^3) also operated in continuous mode using fertilizers and
128 clean water as culture medium. Fertilizers used in the trials were: $NaNO_3$, $MgSO_4$ and
129 KH_2PO_4, in order to have a concentration of 200 mg L^{-1} N and 50 mg L^{-1} of P
130 approximately.
131 Sample RH comes from a raceway reactor (4 m^3) on which microalgae are produced in
132 continuous mode using wastewater as culture medium, thus supplying an average

concentration of N as NH_3 and P equal to 60 mg^{-1} and 10 mg L^{-1} respectively. Sample RI comes from a culture grown in continuous mode using a thin-layer reactor (1.5 m^3) using clean water plus manure (10%) to provide nutrients, with average concentrations of NH_4 and P at 100 and 20 mg L^{-1}. All the culture media supplied had no limiting amount of N and P for algae growth in the specific irradiance conditions.

Whatever the bioreactor, the cultures were regulated at pH8 by on-demand injection of CO_2. Samples were taken at a one-off sampling event on one single day for all the configurations, after more than three weeks in steady state. Samples were freeze-dried and stored at -80°C until extraction.

The specific growth rate μ (day $^{-1}$) was calculated from the Equation (1):

$$\mu = 1/t \ln(Xf/Xo) \qquad [1]$$

in which *Xo* and *Xf* are the concentrations of cells (g L^{-1}) at the beginning and at the end of the batch run, respectively, and *t* (days) is the duration of the run.

Daily biomass productivity (*Dp* as mg L^{-1} d^{-1}) during the culture period was calculated by the Equation (2):

$$Dp = (Xf - Xo)/t \qquad [2]$$

2.2 Sampling and DNA extraction

All samples were collected on the same day after three weeks of steady state and immediately freeze-dried. The DNA was extracted from ~ 20 mg of lyophilized algae per sample by using the Biosprint 96 One-For-All Vet Kit (QIAGEN), in association with the semiautomatic extractor BioSprint 96 (Qiagen) and MagAttract technology, following the user manual. DNA quantity was measured with Qubit (Invitrogen, Life Technologies,

Monza, Italy) and the 260/280 ratio evaluated with Nanodrop (Invitrogen, Life Technologies, Monza, Italy). For each reactor, three technical replicates were obtained. Illumina sequencing was performed on all samples for bacteria and eukaryotic communities. For bacteria the V3-V4 hypervariable region on the 16S gene was selected and amplified with the following primers: 341F-805R (Ferris et al., 1996), while for eukaryotes the ITS region with ITS1-ITS4 primers (White et al., 1990) was targeted for amplicon sequencing. Illumina sequencing was performed by IGATech company (IGA Technology Services s.r.l., Udine, Italy).

Sequences were submitted to the National Center for Biotechnology Information (NCBI) Short Read Archive (SRA) with the following accession numbers: PRJNA666427 and PRJNA666428.

2.3 Bioinformatics analysis

Amplicons were processed following the same protocol as in Bani et al. (2019) for 16s while for the ITS a slightly modified protocol was used. For 16s and ITS sequences were trimmed ($q < 20$) with Sickle (Joshi and Fass, 2011) and error corrected using SPAdes (Bankevich et al., 2012) implemented within BayesHammer (Nikolenko et al., 2013). For 16S only sequences were paired-end aligned using the PEAR algorithm (Zhang et al., 2013). VSEARCH (Rognes et al., 2016) was used for chimera checks and picking operational taxonomic units (OTUs) based on 97% similarity via the QIIME2 pipeline (Bolyen et al., 2019). The resultant OTU sequences were assigned taxonomy using the Naïve Bayesian Classifier (Wang et al., 2007) against the RDP database. ITS sequences were not paired-end because no overlap was possible between the two reads and only the forward reads were retrieved for the following analysis. Sequences were clustered in OTU on 97% of similarity using the VSEARCH algorithm inside QIIME2 pipeline (Bolyen et

al., 2019). The taxonomic annotation was performed using blastn (version 2.8.1) (Zhang et al., 2000) against the NCBI nucleotide database (Morgulis et al., 2008) and taxonomy retrieved using taxdump repository (version 11 March 2019).

2.4 Quantitative Real Time PCR

qPCR was used to quantify 16S rRNA gene copy numbers for bacteria. Alongside general quantification of the microbial community, the study focused on the quantification of specific groups of interest such as *S. almeriensis* and the ammonia oxidizing bacteria (AOB). qPCR was performed with 1× POWRUP SYBR Green Master Mix (Applied Biosystems, Life Technologies, Monza, Italy) on Applied Biosystems 7500 Fast Real Time PCR System (Applied Biosystems, Life Technologies, Monza, Italy), used in combination with Applied Biosystems software. Each reaction took place in a 20 µl final volume containing 1× POWRUP SYBR Green Master Mix, forward and reverse primers (200 nM each primer, see below), 0.4 mg mL^{-1} Bovine Serum Albumin (BSA), distilled water (RNase/DNase free, Life Technologies, Monza, Italy) and 2 µL of DNA-extracts, and ten-fold diluted standard DNA. Primers, thermal condition and source of standards are described in Table 1, when the standard was a plasmid with the interesting gene insert it was produced by GeneArt Synthesis services (Life Technologies, Monza, Italy). Qubit was used to determine the stock concentration (gene copies μL^{-1}) and standard curves were freshly prepared with ten-fold dilutions ranging from 10^2 to 10^9 copies μL^{-1}. All standards and samples were run in triplicate. To check for product specificity and potential primer dimer formation, all runs were completed with a melting analysis starting from 65°C to 95°C with temperature increments of 0.25°C and a transition rate of 5 s.

2.5 Statistical analysis

All statistical analyses were performed on R studio. Gene copy numbers from qPCR were tested to check the assumption of ANOVA with Levene test (Car package) (Fox et al., 2012) and Shapiro test. The assumptions were respected if data were log-transformed and ANOVA was applied to test the importance of the reactor type on the abundance of bacteria. When ANOVA results showed a statistically significant effect of the type of reactor on data, an HSD test was applied to determine the groups. When data did not follow a normal distribution a Kruskal-Wallis test was applied (agricolae package) (de Mendiburu, 2017). Taxonomic summaries were performed using the phyloseq library (McMurdie and Holmes, 2013). Ordination plots were created applying Constrained Analysis of Principal Coordinates (CAP) based on Bray Curtis distance at genus level for both bacteria and fungi (capscale function) (vegan) and to test the effect of the reactor factor PERMANOVA was used on an OTU table clustered at 97% similarity (Oksanen et al., 2007).

3. RESULTS AND DISCUSSION

The aim of the study was to understand how different photobioreactors in contrasting configuration systems (open vs. closed) and different growth media (fertilizers and wastewaters), could influence the microbiota of a culture started with the same inoculum over time. To provide clear evidence of how the differential operation conditions affected the microbial communities, it was necessary to use exactly the same inoculum, as even the same species grown in different laboratories could harbour different microbial communities that would compromise the comparison between different cultivation systems (Zevin et al., 2016). The inoculated microalgae *S. almeriensis* had been isolated in the surrounding area of the experimental set-up under high levels of irradiance and temperature. The strain was also selected since it was expected to be well adapted for

cultivation in open reactors (raceways and thin layer), thus providing it with a possible ecological advantage over other non-desired microalgae that could enter the open systems as contaminants. This algal species has an important economic value as it holds the ability to accumulate lutein, an important antioxidant (Sánchez et al., 2008). In order to prevent an initial potential contamination of the culture that would alter the experiment, the inoculum used for the trials was identical for all the photobioreactors and it had been growing in the tubular system of the experiment.

3.1 Reactor performance and chemical parameters

Reported in Table 2 are the growth performances of the four trials, data were in line with productivity reported in the literature (Barceló-Villalobos et al., 2019; del Mar Morales-Amaral et al., 2015). RI (thin layer and 10% of pig manure) showed the best performance while the T (tubular and fertiliser) resulted in the lowest daily productivity (YD). Thin layer reactors usually have the highest productivity (del Mar Morales-Amaral et al., 2015) compared to the other reactors. High levels of productivity are also achieved when using wastewater as medium and not only fertiliser, reducing the overall cost of the system. Productivity for *S. almeriensis* in thin layer reactors is reported to vary between 45 g m^{-2} d^{-1} and 11.7 g m^{-2} d^{-1} in summer and winter, respectively (Barceló-Villalobos et al., 2019; del Mar Morales-Amaral et al., 2015) (RI productivity is 38.4 g m^{-2} d^{-1}) (Table 2). In line with other productivity data for *S. almeriensis* (del Mar Morales-Amaral et al., 2015), productivity for raceway photoreactors was lower (31.5 g m^{-2} d^{-1} at RW (raceway and fertiliser) and 24 g m^{-2} d^{-1} at RH (raceway and wastewater) (Table 2). The best performance, exhibited by thin layer reactors, is usually obtained as a result of the shallower depth of the culture vs the greater depths in raceways, that allowed a better penetration of the light (del Mar Morales-Amaral et al., 2015). In addition, the use of thin

layer reactors appeared to offer further advantages such as a relatively minor loss of biomass. It has been estimated that there is a loss of 30% of algae biomass in thin layer against a complete culture collapse in open ponds in case of rotifer contamination, which is relatively common in open set-ups (Deruyck et al., 2019). However, recent studies have highlighted that thin layer reactors are still far from providing the optimal running parameters. The net photosynthesis rate could be increased to more than 60% if differential fluxes of pH and oxygen are corrected within the system (Barceló-Villalobos et al., 2019).

3.2 Bioinformatics results

Sequencing of the 16S amplicon library resulted in 299,186 ± 121,384 sequences for samples. After trimming, an average of 295,031 ± 120,048 sequences were retained and 131,450 ± 49,524 assembled (Table 3). For the ITS library, a total of 131,188 ± 78,449 sequences for samples were found. After trimming, 119,320 ± 79,002 sequences were retained for samples (Table 3). The 16S amplicon library resulted in a total of 6,693 OTUs already excluding singletons and OTUs that were assigned to Chloroplast [is that the right word?] at the order levels while retained the OTUs assigned to Cyanobacteria (Table 3). For ITS, the total number of OTUs was 3056 without singletons (total including singletons was 10,579 OTUs, Table3).

3.3 Community composition and structure

The main phyla and genera found in this study (Figure 1A,C) have been reported in other studies on microalgae consortia in wastewater treatment processes or high rate algae ponds (Ibekwe et al., 2017; Lian et al., 2018). The main phylum was Proteobacteria (42% ± 9), this phylum includes many generalist bacteria that are known to colonise different environments including wastewater or microalgae cultivation. Within this phylum are

275 the genera *Roseicyclus*, which accounted for 8% of the bacterial community composition
276 (Figure 1C), genera which are usually found connected with microalgae (Tang et al.,
277 2018). Proteobacteria was followed by Bacteroidetes (25% ± 22), Planctomyces (8% ±
278 6), Actinobacteria (5% ± 3) and Verrucomicrobia (4% ± 2) (Figure 1A). At the genus
279 level, differences were more marked between the closed reactor and the other open
280 reactors with the first dominated by *Flavobacterium* (12% ± 21 across the 4 treatments,
281 but in T samples this genus accounted for 45% of the reads) which is usually found in
282 association with many microalgae (Lian et al., 2018). The open configurations have a
283 more complex composition without a clear bacterial dominance (Figure 1C). The
284 presence of a complex community could be due to the species arriving in the outside
285 environment due to the movement of air/rain and also to their presence in the wastewater
286 or in the pig slurry that were being used as sources of nutrients. Complex bacterial
287 communities could also be the results of strict species-specific interactions. For example,
288 Eigemann et al. (2013) reported that the same inoculum of *Desmodesmus sp.*, exposed to
289 different environmental conditions, had the same bacterial community (> 80 and 90%
290 similarity). This finding leads to a supposition that microalgae may determine or, at least,
291 affect the microbial community present. Similar findings, that a microalgae community
292 can determine the composition of a bacterial community, are reported by Krohn Molt et
293 al. (2017), highlighting a mechanism of innate immunity of some microbes that are thus
294 selected in the phycosphere of specific algae. Then, it is not surprising then to find the
295 high relative abundance of the Planctomycetes phylum, found in all the open reactors
296 (Figure 1C, *Rhodopirellula* (6% ± 5)) as this phylum is able to establish a positive
297 feedback between bacteria and algae. Higher presence of harmful bacteria will result in a
298 negative effect on the microalgae fitness therefore microalgae could have recruited this

299 phylum to help in regulating the associated microbiota as it could produce small
300 antibiotics molecules while obtaining carbon compounds from the algae (Lage and
301 Bondoso, 2014; Wiegand et al., 2018).
302 In our case, both the algal and bacterial community were affected by environmental
303 conditions (open vs closed reactors) and, even if it is possible that algae community
304 influenced the composition of a bacterial community, data from this work cannot be
305 conclusive on this point.
306 *Gemmatimonas*, the second most abundant genus (7% ± 5), could result in a positive
307 impact on N_2O emission, usually a by-product of incomplete ammonia oxidation or an
308 intermediate in denitrification (Park et al., 2017) in many microalgae production system,
309 as this genus is able to reduce nitrous oxide even in anoxic conditions (Park et al., 2017).
310 Other common genera found in this study were: *Roseicyclus* (8% ± 1), *Rhodopirellula*
311 (6% ± 5), *Porphyrobacter* (5% ± 4), *Hydrogenophaga* (4% ± 2) and *Oligoflexus* (3% ±
312 5).
313 For Eukaryote communities, Chlorophyta dominated all the reactors (>90% of the
314 sequences were assigned to this phylum), while on the species level, different mixtures
315 of species could be found based on the photobioreactor set up (Figure 1D).
316 This study provides evidence that after 5 weeks of continuous mode-culturing the
317 inoculum was retrieved only in a closed reactor (Figure 1D and 3B). The tubular reactor
318 was dominated by the inoculated species *S. almerienis* (95% ± 1.3), while in the other
319 three reactors this alga could be found only at a low percentage (3.6% ± 2.3) as it was
320 almost exclusively replaced by the genus *Desmodesmus* sp. In particular, *D. armatus*
321 (24% ± 23), *D. opoliensis* (35% ± 33) and *D. communis* (8% ± 9) were found as the
322 dominant species in the three open bioreactors. Each of the species dominated a different

reactor: *D. armatus* was the dominant specie in RH reactor (44% ± 27) while *D. opoliensis* dominated in RI reactor (76% ± 7). RW configuration did not show a clear dominance as *D. armatus*, *D. opoliensis* and *D. communi*s were all present in similar concentrations (17% ± 15, 26% ± 11 and 18% ± 14 respectively) (Figure 1D). It is well known that open outdoor photobioreactors are prone to contamination, but the presence of a native species inoculated in the culture at high concentrations should hold competitive ecological advantages then enable survival even in open reactors; however, this was not the case (Narala et al., 2016). Typically, the use of native species is more robust as they are acclimated to operational conditions and environmental variables and this should be enough to maintain the monoculture (García et al., 2018), in alignment with other studies reported in the literature (Petrini et al., 2020). The results found corroborated the difficulties of maintaining a monoculture within economically advantageous conditions (open configuration systems) that is possible only with a few species of microalgae that require specific environmental conditions such as high pH or salinity such as *D. salina or A. platensis* (Mooij et al., 2015). .

The origin of the contaminant microalgae could be tracked to a low abundance in the inoculum and then a proliferation in a complex community in the open reactor; however, it is more probable that contaminant algae were present in the growth media or were recruited by dispersal from the surrounding environment and then by ecological advantages, replaced the selected strain (Bohutskyi et al., 2016). Species such as *D. opoliensis* or *D. armatus* replaced *S. almeriensis* in all the reactors as they have higher fitness, better ability to retrieve nutrients and differential growth, and also an ability to tolerate high organic pollution, as in the case of the wastewater growth medium (Palmer, 1969).

Eukaryotic alpha diversity indexes (Figure 2E-H) were lower when compared to measures for bacterial alpha diversity (Figure 2A-D). Eukaryotic communities in all reactors usually include a few species that dominate the community while bacterial communities are more diverse and a single dominant species does not become established. For all diversity indices, the RW reactor had the highest alpha diversity followed by the other two open reactors, RH and RI, while the tubular reactor had the lowest alpha diversity (Figure 2E, F). T samples showed the lowest Shannon diversity index as they were clearly dominated by a single species (Figure 2G), in this case the inoculum.

All alpha diversity indices for bacterial communities showed the same trend, with the RH configuration having the highest diversity, followed by RI, RW and, finally by T with a lower diversity bacterial community (Figure 2A, B). Shannon's index (Figure 2C) showed that the bacterial communities in T samples were more uneven, as T samples were clearly dominated by *Flavobacterium* (Figure 1C) unlike the other samples.

The lower diversity and low Shannon index in T samples for both communities could be the results of a more stable system in which contamination from the environment was reduced to zero and the number of microniches available was limited, due to the smaller fluctuations in temperature and light than those which the open systems experienced daily. The higher diversity in the bacterial communities could also be explained, since different phyla have been shown to use different compounds released by the microalgae in the environment. For example, *Flavobacterium* only uses high molecular weight carbon sources, while *Rhodobacteriales* (*Roseicyclus)* prefer low molecular weight metabolites (Ferrer-González et al., 2020).

3.4 Community structure and interaction

Bacterial community structure for the four configurations tested was clearly different as shown in Figure 3A. All samples clustered based on the reactor type (PERMANOVA p-value 0.001, df= 3, F= 17.131, R^2= 0.865) with no overlap; T samples were the most dissimilar as they were the most distant from all the other reactors (Figure 3A). In particular, the large distance between T and RW (different bioreactor and same growth media), revealed that the bacterial community would be strongly influenced by the configuration system of the bioreactors (open system vs. closed system) rather than culture medium composition (clean medium vs. wastewater, PERMANOVA p-value 0.002, df= 2, F= 5.3067, R^2= 0.54113). For the eukaryotic community, reactor set-up also had a strong influence on composition (PERMANOVA p-value 0.01, df=3, F= 18.169, R^2=0.872) (Figure 3B). However, for eukaryotic data, there were only three distinct clusters as samples from RW and RH exhibited overlap. It is not possible to completely understand the relative importance of the growth medium and the reactor type, as the experimental design does not allow a full comparison between the treatments. Based on the results of the raceway reactors, it is clear that reactors set-up seems more important than the growth medium, as the samples from raceways were very similar not only in community structure but also composition, while microalgae communities, which shared the same growth medium but different set-ups, were clearly different (T vs RW). If it makes sense that the use of clean medium in a contamination-prone set-up would result in a different community, it is less intuitive to explain why similar communities should be detected in clean medium and wastewater raceways. Probably both systems resulted in a highly competitive environment due to, on one hand, the high resource availability, and on the other hand, an already complex community present in the medium and high

complexity in the substrate available, resulting in similar microalgae communities with higher fitness/ability to establish themselves as dominant (Bohutskyi et al., 2016).

Differently from the eukaryotic communities, bacterial communities were completely distinct in all the set-ups. That closed photobioreactors have limited bacterial diversity is due to the impossibility of spatial dispersion and further limited by the use of clean fertilizer. Variability within the bacterial community is, in the closed photobioreactor, connected with the microalga inoculated, as each microalgae harbors a different microbiota that is host specific and different from the surrounding environment (Jackrel et al., 2020). However, multiple microalgae could be present at the same time, as in all the open systems, and a trade-off between host selection and environmental effects is in action, with growth media providing multiple types of carbon source and other nutrient forms. In this study, for bacterial communities, environmental filtering and dispersal from outside sources are stronger than selection from the host, so if the host selection had been the primary source of the diversity, the resulting bacterial communities in the raceways should have been similar, as for the microalgae communities.

As the two communities of eukaryotes and bacteria were not confined in two different environments, their interactions between each other could provide information of ecological importance (Figure 3C). Co-occurrences, based on the Spearman index, were investigated for the most common genera (abundance above 5% threshold) and the most common eukaryotic species (above 1% threshold). *Gemmatimonas* had the highest numbers of positive interactions (4) while *Chrysolinea* had the highest number of negative interactions with *Oligoflexus* (3 each). *Flavobacterium* was the genus with most interactions in general (6 in total). *Flavobacterium* is commonly found in many microalgal environments both as a free-living organism but also closely associated in the

phycosphere. *Chlorella* sp. have been demonstrated to actively recruit this genus if an axenic culture is exposed to environmental bacteria (Jackrel et al., 2020). Bacterial cells could help with *Chlorella* fitness by providing additional CO_2, as this is often a limiting factor in photobioreactors, as by-products of the degradation of organic compounds that the *Chlorella* itself could be providing (Lian et al., 2018). The interaction between *Flavobacterium* and *Chlorella* sp. could also result in other economic advantages for microalgae production as not only the bacteria could help the fitness of the algae but also help in flocculation of the culture. Even if not all the species within the genera have the same properties, it has been shown that when *Flavobacterium* sp. were removed from a *Chlorella* culture the flocculation activity was reduced by 3% (Lee et al., 2013). This has important economic implications, as harvesting of the culture represents one of the most expensive steps. Further study should clarify the active role of the bacteria.

3.5 Community quantifications

Each different reactor type exhibited different bacterial abundances as shown in Figure 4A and C. The tubular reactor (T) had the highest abundance of bacteria followed by the thin layer (RI) reactor and the two raceway reactors with waste medium and synthetic medium (RH and RW), respectively. The differences were also supported by the result of ANOVA and the following HSD test. When focusing on the specific group of ammonia oxidizing bacteria (AOB), similar results were obtained to the total quantification (Figure 4C). T reactor had the highest proportion of AOB followed by RI and finally RH and RW, respectively. Results were also confirmed by the Kruskal-Wallis analysis. To support the metabarcoding data, *S. almerienis*, the inoculated species, was also quantified across the different samples. As Figure 4B clearly shows, it could be found in abundance in T

samples and, in lower quantity, also in RW samples. In the other two reactors, RI and RH, no amplification was detected for any sample.

4. CONCLUSION

This study demonstrated how photobioreactors' set-up and growth media have effected microalgae inoculum dynamics. The inoculum was only retrieved in the closed photobioreactor. Algal community was strongly influenced by the bioreactors configurations (open vs. closed systems) rather than by growth medium composition. Microalgae communities cultivated in the same configuration system using different growth media were similar, while microalgae sharing the same medium, but grown in different configurations were clearly different. Possibly shifts in algal communities influenced the bacterial composition and productivity, data from this work cannot be conclusive although, beneficial in implementing the planning and large production of microalgae.

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Figure 1. Taxonomic composition of the bacterial and eukaryotic community in each of the photobioreactor configurations. Bacterial community composition at Phylum level (A) and Genus level (most common above 1% abundance threshold) (C). Eukaryote community composition at Phylum level (B) and Species level (D). Each barplot is the average of the three replicates.

Figure 2. Diversity index bacterial community. A) Richness, B) Chao1, C) Shannon index and D) Simpson index. Each boxplot is the results of the three replicates. Diversity indices for eukaryotes community. E) Richness, F) Chao1, G) Shannon index and H) Simpson index. Each boxplot is the results of the three replicates. Different letters are for different groups based on Kruskal-Wallis (non normal distributed samples, Shannon and Simpson index) or LSD post hoc analysis (normal distributed samples, Richness and Chao1).

Figure 3. Ordination plot CAP scale and Spearman correlation. A) Bacterial community and B) eukaryotic community. Different colours are used for the different experimental set-up. For each treatment are presented the three technical replicates. C) Co-occurrence based on Spearman rank correlation index. Interactions are shown only if statistically significant (p-value < 0.05). The blue dots are for positive interaction while red dots are for negative interactions. Only most abundant.

Figure 4. A) 16S gene copy number, B) *S. almerienis* gene copy number and C) AOB gene copy number. Different colours are used for each experimental set up. Each box plot is representing the three technical samples.

641 Table 1. qPCR details for each target gene is specified: the standard origins, primers,

642 thermal protocol and references

Target group	Standard origin	Primers	Thermal protocol	Reference
Bacteria	*N. Communis* (DSMZ number 28436) PCR product	1055f/1392r (Ferris et al., 1996)	95 C-10 m/95 C– 20 s/58 C–15 s/72 C–30 s	(Bani et al., 2019)
Ammonia oxidising bacteria AOB	plasmid with fragment of *N. Eutropha* (GenBank KU747123.1)	AmoA1F/AmomA2R (Rotthauwe et al., 1997)	95 C-2m/94 C-45 s/56 C-30 s/72 C-60s	(Bellucci and Curtis, 2011)
S. almeriensis	plasmid with fragment of *S. Almeriensis* (GenBank MF977406.1)	SalmF/SalmR (Beatrice-Lindner et al., 2018)	95 C-10 m/95 C– 15 s/63 C– 1m/72 C -15s	(Beatrice-Lindner et al., 2018)

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646 Table 2. Performance of the different reactor set-ups. Cb: biomass concentration, D
647 dilution rate for continuous culturing, YD daily productivity, N and P supplied to each
648 reactor.

	Cb[a] ($g\ L^{-1}$)	D[a] (d^{-1})	YD[a] ($g\ L^{-1} d^{-1}$)	YD[a] ($g\ m^{-2}\ d^{-1}$)	N $mg\ L^{-1}$	P $mg\ L^{-1}$
T	2.1±0.1	0.3	0.63±0.05	23.6±0.5	200	50
RW	0.7±0.1	0.3	0.21±0.05	31.5±0.5	200	50
RI	3.2±0.1	0.3	0.96±0.05	38.4±0.5	100	20
RH	0.6±0.1	0.3	0.18±0.05	24.0±0.5	60	10

649 [a]Cb: biomass concentration; D dilution rate for continuous culturing; YD daily
650 productivity

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652 Table 3. Information on the number of sequences retained after the trimming (first two rows) and on the number of assembled sequences. Only

653 bacterial sequences were assembled but was not possible for eukaryotic sequences (see Material and Methods section)

	Bacteria				Eukaryotes			
	T	RW	RI	RH	T	RW	RI	RH
Total input	282,791 ± 45,178	258,099 ± 47,701	290,915 ± 89,202	364,938 ± 243,395	108,164 ± 31,378	141,638 ± 108,280	123,814 ± 55,753	126,474 ± 112,116
Total trimmed	277,249 ± 44,366	253,604 ± 47,195	288,003 ± 87,503	361,268 ± 240,223	107,967 ± 31,147	141,085 ± 107,608	123,501 ± 55,575	126,162 ± 111,994
Total assembled	123,879 ± 15,148	114,432 ± 19,744	127,194 ± 32,414	160,296 ± 100,118	N/A	N/A	N/A	N/A
Not assembled	13,894 ± 7,720	11,664 ± 7,203	16,265 ± 11,079	19,680 ± 19,495	N/A	N/A	N/A	N/A

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