Wastewater treatment using *Scenedesmus almeriensis*: Effect of operational conditions on the composition of the microalgae-bacteria consortia

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Abstract

Primary urban wastewater was treated in outdoor raceways using a microalgae-bacteria consortia dominated by *Scenedesmus almeriensis*. The current study aimed at assessing the effect of operational conditions, namely culture depth and dilution rate, on: (i) biomass productivity; (ii) the nutrient removal capacity, and (iii) the composition of the microalgae-bacteria consortium and the presence of unwanted microorganisms. Optimum dilution rates to process large quantities of wastewater during summer and achieve high biomass productivities were 0.3-0.5 day^{-1}. Under the optimum operational conditions, nitrogen and phosphorus removal rates were higher than 90% while removal of chemical oxygen demand was 70%. Operating at different culture depths had a striking effect on the composition of the microalgae-bacteria consortium. The relative abundance of nitrifiers increased with culture depth and was minimised at 0.05 m: larger culture depths led to enhanced nitrifying activity and therefore to nitrate production and accumulation in the system. Results demonstrate the potential of microalgae-based wastewater treatment processes and the importance of selecting suitable operational conditions to maximise both, biomass production and nutrient removal by minimising the occurrence of nitrifying bacteria.

Keywords: Primary wastewater, microalgae, raceway reactor, taxonomic classification, nitrification, industrial production.

1. Introduction

Conventional wastewater treatment processes have restrictions concerning their environmental impact (due to greenhouse gas emissions) and are facing challenges to meet nutrient discharge standards (Balkema et al. 2002; Muga and Mihelcic 2008). Microalgae-based wastewater treatment processes are one of the most promising sustainable strategies for the advanced treatment of wastewater (Craggs et al. 2013; Mehrabadi et al. 2017). The feasibility of using microalgae to process wastewater has been suggested by a large number of research groups worldwide. However, the number of studies validating microalgae-based processes outdoors using large reactors is scarce. One of the main advantages of processing wastewater using microalgae, besides the removal of nutrients that represent an environmental hazard, is the production of valuable microalgal biomass.

Microalgae are produced in controlled facilities using photobioreactors. Raceways are the most widespread bioreactors used for microalgae production mainly because of their flexibility, ease of operation and scale-up, low construction costs, and low energy consumption for mixing (Jorquera et al. 2010). Moreover, their low surface-to-volume ratio is especially interesting for wastewater treatment processes because this allows to process large volumes of water per surface area. Among the thousands of microalgal strains currently available in culture collections, only a few demonstrated potential for being produced in wastewater, being the genus *Chlorella* and *Scenedemus* the most promising. *Phormidium*, *Arthrospira*, *Chlamydomonas*, *Nannochloropsis*, *Galdieria*, or *Synechocystis* have also been used to recover nutrients from wastewater (Chai et al. 2021). *Scenedesmus* species are especially relevant because they are candidates for the production of biofuels and biomaterials (Yang et al. 2018; Nag Dasgupta et al. 2018) or biofertilisers (Puglisi et al. 2018, 2020) and many species are capable of enhanced production of secondary metabolites such as carotenoids (that can be used for the formulation of animal feeds) when produced using wastewater (Msanne et al. 2020).

Microalgae based wastewater treatment processes involve the association of microalgae with aerobic and anaerobic microorganisms naturally present in the wastewater and in the environment. Microalgae provide *in situ* aeration via photosynthetic release of oxygen, supporting the growth of heterotrophic microorganisms and the degradation of organic compounds. In turn, certain bacterial groups can synthesise valuable compounds such as micronutrients, siderophores, growth stimulants, and antibiotics that promote growth and protect algae from

pathogenic microorganisms (Lian et al. 2018). In addition, bacterial respiration produces carbon dioxide, which is needed by microalgae for photosynthesis (Sánchez-Zurano et al. 2021) avoiding or reducing the need for introducing carbon dioxide into the system. Mmicroalgae-bacteria interactions can negatively affect microalgal growth as certain bacterial groups can synthesise secondary metabolites that are strong algicidal agents (Lian et al. 2018). The presence of pathogens could also be a drawback as these could limit the number of end applications of the biomass. Therefore, it is of key importance to improve knowledge on the presence and concentration of bacterial communities involved in microalgae-based wastewater treatment processes. It is known that environmental fluctuations can affect the composition of microalgae-bacteria consortia (Sánchez Zurano et al. 2020; Collao et al. 2021). However, to date, little is known on how operational conditions can affect the composition of these consortia.

The aim of the current study was to assess the effect of operational conditions, namely culture depth and dilution rate, of outdoor raceways on: (i) biomass production; (ii) the nutrient removal capacity of the system, and (iii) the composition of the microalgae-bacteria consortia and the presence of unwanted pathogenic microorganisms. Up to the best of the authors' knowledge, the effect of operational conditions on the composition of the microalgae-bacteria consortia formed during wastewater treatment using raceway reactors larger than 10 m^2 has not been studied. This information is of key importance as biomass productivity and quality as well as the nutrient removal efficiency of the system depend on the composition of the consortia.

2. Materials and methods

2.1. Microorganisms and culture conditions

The freshwater *Scenedesmus almeriensis* (CCAP 276/24) strain was used to inoculate the raceway ponds. The strain was obtained from the culture collection of the Department of Chemical Engineering of the University of Almería in Spain. It was selected because it is highly productive and is particularly adapted to stressful conditions and to the environmental conditions of the region (high solar radiation and temperatures). Briefly, the inocula were produced in a 3.0 m^3 capacity industrial tubular photobioreactor with modified Arnon medium (Allen and Arnon 1955). The culture medium was prepared using commercial-grade fertilisers instead of pure chemicals.

The primary wastewater used in the present study was collected from an urban wastewater treatment plant operated by FCC AQUALIA S.A. in Almeria (Spain). The composition of the wastewater was variable with N-NH_4^+, N-NO_3^-, P-PO_4^{3-}, and COD values that ranged within 58.2-136.9, 1.5-5.6, 7.9-27.7, and 296.5-858.3 mg·L^{-1}.

2.2. Experimental set-up

Experiments were conducted in three outdoor 80 m^2 raceways located at the Andalucian Institute of Agricultural and Fisheries Research and Training (IFAPA) in Almería, Spain. Each pond was considered an experimental unit. All of the experiments were conducted during summer (**Fig.1**). To perform the experiments, the depth of the microalgae-bacteria cultures was set at either 0.20, 0.12, or 0.05 m (culture volumes were 17.0, 13.0, and 5.0 m^3, respectively). A SCADA system monitored and controlled the overall operation of the raceways including pH (#5333, Crison, Spain), temperature (#PT1000, Crison, Spain), and dissolved oxygen concentration (#9336, Crison 9336, Barcelona, Spain). The pH of the cultures was kept constant at 8.0 by on-demand injection of CO_2 and the raceway ponds were operated 24 h per day.

The raceway ponds were inoculated with a 10% of their total volume with *S. almeriensis* culture and were filled up with wastewater from primary treatment until the desired culture depth was reached. Evaporation was compensated daily by addition of freshwater. The reactors were initially operated in batch mode until no variations in biomass concentration were observed, and after this period, they were operated in semi-continuous mode at different dilution rates (0.15, 0.30, 0.40, or 0.50 day^{-1}) until the total volume of the reactor was replaced (at least) twice. The dilution rate indicates the amount of culture media that is daily harvested and replaced with fresh culture media. For example, a dilution rate of 0.5 day^{-1} means that 50% of the volume of the culture was daily harvested, and therefore, the total volume of the reactor was replaced in two days. When operating at a dilution rate of 0.3 day^{-1}, 30% of the reactors volume was replaced with fresh culture media every day, and it took 7 days to completely replace the cultures' volume twice.

The composition of the primary wastewater was not constant and varied from week to week - although the same batch, which was constant, was used for each dilution rate. Samples were taken when the raceways achieved the steady-state, and characterisation and metagenomic analysis were performed as described below.

2.3. Analytical methods

Biomass concentration was measured by dry weight by filtering 100 mL aliquots of the culture through Macherey-Nagel glass fiber MN 85/90 (Thermo Fisher Scientific, Madrid, Spain) and drying in an oven at 80 ºC for 24 h. The biomass produced was a combination of microalgae and other microorganisms as the production was conducted in open reactors using wastewater. Cell status was assessed daily by measuring the maximum quantum yield of the photosystem II complex (PSII) chemistry (F_v/F_m) using an AquaPen AP 100 fluorometer (Photon System Instruments, Czech Republic). Microalgal cells were dark-adapted for 5 min prior to the determination of F_v/F_m values. Biomass productivity was calculated as the product of biomass concentration and the imposed dilution rate using the equation:

$$Biomass\ productivity\ \left(\frac{g}{m^2 \cdot day}\right) = \frac{C_b \cdot D \cdot V}{A}$$

where C_b is the biomass concentration ($g \cdot L^{-1}$), D is the dilution rate (day^{-1}), V is the cultures' volume (L), and A is the area of the reactor (m^2).

Standard official methods approved by the Spanish Ministry of Agriculture were used to analyse the composition of the primary wastewater (BOE 1982). Briefly, the $P\text{-}PO_4^{3-}$ content was measured by visible spectrophotometry through the phospho-vanado-molybdate complex. $N\text{-}NO_3^-$ was quantified at 220-275 nm using a GENESYS 10S UV-Vis spectrophotometer (Thermo Fisher Scientific, Spain) and $N\text{-}NH_4^+$ was measured using the Nessler reactive method. The chemical oxygen demand (COD) was determined spectrophotometrically using LCI-400 commercial kits (Hach Company, Manchester, UK). Three technical replicates were conducted per natural replicate.

2.4. DNA extraction and 16S rRNA gene amplicon sequencing library preparation.

Bacterial genomic DNA was extracted from the culture samples collected to assess the microbial community present in the cultures. One determination was conducted per natural replicate and results shown are the average of three determinations. Samples were collected at the end of the stationary phase, when the biomass concentration was constant for at least three consecutive days. Illumina MiSeq system was used to perform the metagenomic analyses. The V3 and V4 variable regions of the 16S rRNA gene were amplified from the bacterial DNA obtained from the biomass samples. Then, PCR reactions were performed using specific primers as described previously (Sánchez Zurano et al. 2020). Subsequently, the size of the products from the PCR was

evaluated using an Agilent 2200 TapeStation (Agilent Technologies, Italy). The PCR products were purified by discarding primers and primer dimers using the Agencourt AMPure XP Kit (Beckman Coulter Genomics, Italy). Dual indices and Illumina sequencing adapters (P5 and P7) were then attached to the amplicons using the Nextera XT Index Kit (Illumina, San Diego, CA) to develop the final libraries using the Illumina 16S Metagenomic Sequencing Library preparation protocol.

The PCR reaction conditions were as follows: 95 °C for 3 min, 8 cycles of 95 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s with a final extension step at 72 °C for 5 min. The final libraries were quantified as absolute using KAPA Library Quantification Kits for Illumina® platforms (Kapa Biosystems Ltd., UK). Using the absolute quantification, the diluted library sample concentrations were calculated against the standard curve. The final libraries were pooled in equimolar amounts, denatured, and diluted before being loaded onto the MiSeq flow cell and sequenced on the Illumina MiSeq platform (Illumina, San Diego, CA) with MiSeq reagent Kit v3 (Illumina, San Diego, CA) (Rimoldi et al. 2018).

2.4.1 Bioinformatics analysis

The QIIME pipeline was used to process the sequencing data using the "closed-reference" OTU picking strategy (Caporaso et al. 2010). Quality of raw reads was checked using FastQC v0.11.2. The quality control was performed using QIIME, and the reads were collected into OTUs (Operational Taxonomic Units (with an identity ≥97%) using the QIIME closed-reference OTU picking strategy against the QIIME-formatted Greengenes v.13.8 reference database (http://greengenes.lbl.gov). Finally, the sequences were clustered into the 4 main taxa ranks: phylum, class, order, and family.

2.5 Data analysis

Microalgae were produced using three independent and identical photobioreactors (located inside the same greenhouse) being each photobioreactor the experimental unit. Three technical replicates at the steady state phase were performed per natural replicate. Normality and homoscedasticity of the variables within each group were checked. Results shown are the average of three independent experiments (three different reactors) ± standard deviation. Differences between culture depths and dilution rates were assessed using one-way analysis

of variance (ANOVA) with JMP 13 (SAS Institute Inc., USA). A Tukey pairwise comparison of the means was conducted to identify where sample differences occurred. The criterion for statistical significance was $p<0.05$.

3. Results

3.1 Biomass productivity

Environmental conditions that reached the cultures during the different experiments are shown in **Fig.1**. The average solar radiation that reached the cultures during the experiments was 679.6 ± 85.2 μmol of photons·m^{-2}·s^{-1}, while the average temperature was 27.7 ± 1.8 °C. Maximum solar radiation and temperature values ranged within 1,700-2,500 μmol of photons·m^{-2}·s^{-1} and 28-35 °C while minimum temperature values reached during microalgae production were within the range 17-24 °C. Biomass productivity, calculated as the product of biomass concentration and the dilution rate, was affected by dilution rate ($p<0.001$), culture depth ($p<0.05$), and the combination of both factors ($p<0.05$). Maximum biomass productivities, which ranged between 20 and 30 g·m^{-2}·day^{-1}, were obtained when operating at a dilution rate of 0.3 day^{-1} (**Fig.2a**). Culture depth also had a significant effect on biomass productivity. In the current study, higher biomass productivities were obtained when the culture depth was 0.05 m, the lowest studied ($p<0.05$). Moreover, **Fig.2b** shows the effect of culture depth and dilution rate on the F_v/F_m ratio. In the current study, F_v/F_m values ranged between 0.4 and 0.5.

3.2 Nutrient uptake

As the composition of wastewater is not constant and not all the experiments were performed on the same day, the content of N-NH_4^+ in the culture (inlet) varied between 60 and 140 mg·L^{-1} between different experiments – **Table 1**. The N-NH_4^+ content in the outlets was significantly lower and ranged between 1 and 10 mg·L^{-1} ($p<0.05$; **Table 1**). Overall, operating at different culture depths and dilution rates had no effect on the N-NH_4^+ content of the outlets, as approximately all of the N-NH_4^+ present in the inlet was removed from the wastewater. Consumption values shown in **Table 1** correspond to N-NH_4^+ depuration efficiencies higher than 90%.

The N-NO_3^- content in the inlet and outlet of the raceway reactors is shown in **Table 1**. The N-NO_3^- content of the wastewater (inlet) was relatively low and around 1-5 mg·L^{-1} for all the experimental runs, being higher in the

outlets than in the inlets for all the studied culture depths and dilution rates (p<0.05). $N\text{-}NO_3^-$ production by the microalgae-bacteria consortia (**Table 1**) was significantly affected by dilution rate (p<0.05) and culture depth (p<0.05). Overall, no $N\text{-}NO_3^-$ production was observed when the culture depth was 0.05 m while 5-20 $mg \cdot L^{-1} \cdot day^{-1}$ (0.5-4.0 $g \cdot m^{-2} \cdot day^{-1}$) of $N\text{-}NO_3^-$ were produced when the depth of the culture was 0.20 or 0.12 m. After analysing the content of nitrogen in the inlet and outlet (both in the treated water and the biomass), results suggest that not all the nitrogen removed from the wastewater was used by microalgae and bacteria. Nitrogen losses caused by stripping were influenced by both culture depth (p<0.05) and dilution rate (p<0.05). No nitrogen loss was observed when operating the reactors at a culture depth of 0.05 m, while up to 66.4 ± 3.8 $g \cdot day^{-1}$ were lost when operating the reactors with a culture depth of 0.2 m and a dilution rate of 0.5 day^{-1}.

The potential of microalgae of the *Scenedesmus* genus to remove not only nitrogen but also phosphorus from wastewaters has been demonstrated previously (Raeisossadati et al. 2019; Ye et al. 2020; Mantovani et al. 2020; Morillas-España et al. 2021). The $P\text{-}PO_4^{3-}$ content in the inlet and outlet of the reactors as well as the daily removal of $P\text{-}PO_4^{3-}$ is shown in **Table 1**. Differences in the inlet can be attributed to the variability in the composition of the wastewater used – $P\text{-}PO_4^{3-}$ content varied within 8 and 30 $mg \cdot L^{-1}$. The $P\text{-}PO_4^{3-}$ content in the outlet was lower when compared to the inlet for all the studied culture depths and dilution rates (p<0.05), demonstrating the potential of the microalgae-bacteria consortia to remove/consume the $P\text{-}PO_4^{3-}$ present in wastewater. Consumption values correspond to $P\text{-}PO_4^{3-}$ removal efficiencies between 20 and 100% and were affected by both dilution rate and culture depth (p<0.05). The highest $P\text{-}PO_4^{3-}$ volumetric removal capacity was observed when operating at higher dilution rates and lower culture depths, in this case, 0.05 m (p<0.05). When operating at a dilution rate of 0.5 day^{-1}, as the initial content of $P\text{-}PO_4^{3-}$ was very high (25-30 $mg \cdot L^{-1}$), not all the phosphorus was used for microalgal and bacterial growth (**Table 1**). Operating at 0.5 day^{-1} and 0.20 m allowed the highest $P\text{-}PO_4^{3-}$ removal because of the larger volume of wastewater processed. The most relevant effect was the larger $P\text{-}PO_4^{3-}$ removal when reducing the water depth, achieving values up to 9.1 $mg \cdot L^{-1} \cdot day^{-1}$ (0.6 $g \cdot m^{-2} \cdot day^{-1}$) when operating at a culture depth of 0.05 (**Table 1**).

The COD was also determined at the reactors' inlets and outlets. Differences between the inlets and the outlets were significant (p<0.05). No major differences were observed when operating at different culture depths and dilution rates. COD values in the harvested culture ranged between 50 and 100 $mg \cdot L^{-1}$, demonstrating a huge

potential of microalgae and bacteria to remove organic matter and reduce COD concentrations as those evaluated in the current study – 600-900 $mg \cdot L^{-1}$. COD removal was significantly affected by both dilution rate (p<0.05) and culture depth (p<0.05). COD removal values shown in **Table 1** correspond to depuration efficiencies greater than 70%. When operating at the highest dilution rate and culture depth studied (0.5 day^{-1} and 0.20 m respectively) the COD removal capacity of the system was 63.8 $g \cdot m^{-2} \cdot day^{-1}$.

3.3 Taxonomic classification of bacterial communities

The phylogenetic diversity was evaluated in the biomass samples after filtering for quality, trimming length, and assigning taxonomies. All the samples were successfully categorised into their phylum, class, order, and family level. Considering only the taxa that had a relative abundance of more than 1%, the microbial community overall was mainly comprised of 11 phyla, 20 classes, and 28 orders. Related to the family level, only the taxa that had a relative abundance of more than 0.1% were considered (40 families). Results shown in **Fig.3a** suggested that the most abundant phyla identified during the whole summer season were Proteobacteria (45-60%), Bacteroidetes (10-42 %), and Actinobacteria (2-15%). Various other minority phylum classifications, such as Verrucomicrobia and Planctomycetes were present although at a lower percentage. Within the Proteobacteria phylum, the classes Alpha- Beta-, Gamma- and Delta-proteobacteria were detected in all the reactors at different dilution rates and culture depths. Alphaproteobacteria (16-26%) were the most abundant class, with the major families Rhodobacteraceae and Sphingomonadaceae (**Fig.3b**). The second major class detected in the samples was Gammaproteobacteria, (8-22%) with Xanthomonadaceae as the most predominat family. Betaproteobacteria was the following major class (6-19%), being Comamonadaceae and Rhodocyclaceae the most dominant families. A relationship was observed between the Rhodocyclaceae family and the dilution rate (p<0.05). Regarding the phylum Bacteroidetes, bacteria belonging to the classes Saprospirae (2-15%), Flavobacteriia (1-14%), Cytophagia (1-10%) and Sphingobacteriia (1-5%) were identified in all samples. The third most abundant phylum (Actinobacteria) included the classes Acidimicrobiia (3-10%) and Actinobacteria (1-4%).

Results described in the previous section suggested an irregular distribution of $N\text{-}NO_3^-$ production in different experiments. To gain a deeper understanding of the nitrification process in the raceways, the main ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB) families were identified in cultures produced at different dilution rates and depths (**Fig.3c**). The primary AOBs identified belonged to the family Chromatiaceae,

which includes the genus *Nitrosococcus*. The family Nitrosomonadacea, which includes the genera *Nitrosomonas*, *Nitrosospira*, and *Nitrosovibrio*, was also identified. Two common NOBs in wastewater treatment plants were detected in the raceways: Nitrospiraceae and Bradyrhizobiaceae. Within these families, *Nitrospira* and *Nitrobacter* were the main genera detected.

4. Discussion

Environmental conditions of the cultures are shown in **Fig.1**. Different results are expected during different seasons as environmental conditions have a striking effect on biomass productivity (de Godos et al. 2016). Light availability is the most important parameter for any photosynthetic organism (Acién Fernández et al. 2013). In addition to environmental conditions, the most relevant operational conditions in open large-scale raceway reactors are the depth of the culture and the dilution rate (as well as the flow rate, which depends partially on the depth of the culture) (Collao et al. 2021). The dilution rate refers to the daily volume of culture harvested and directly affects biomass concentration and productivity. By replacing part of the culture with fresh culture medium: (i) biomass concentration decreases and light availability increases and (ii) fresh nutrients are introduced into the system, leading to higher nutrient availability. Moreover, lower culture depths improve light availability and biomass productivity. This is one of the main reasons why thin-layer reactors are highly productive (Masojídek et al. 2015) and the reason why a culture depth of 0.05 m, which is lower than the average 0.15-0.20 m for raceway reactors was studied.

As highlighted previously, maximum biomass productivities were achieved when operating the reactors at a dilution rate of 0.3 day^{-1} (**Fig.2a**). No differences were observed in biomass productivity when operating the reactor with a culture depth of 0.05 m and dilution rates of 0.3 or 0.5 day^{-1}. The advantage of operating the reactors at a lower dilution rate is that the volume of culture that is replaced every day is lower and therefore harvesting and processing costs are lower. Results are consistent with previous studies that reported similar productivities and an optimum dilution rate of 0.3 day^{-1} for these same reactors in summer (Morillas-España et al. 2020). Results were also in line with a long-term study using modular offshore floating reactors that allowed biomass productivities of 23 $g \cdot m^{-2} \cdot day^{-1}$ during semi-continuous operation (Novoveská et al. 2016). The authors

of that study concluded that productivity was mainly driven by temperature and harvesting frequency, which is consistent with the results obtained in the current study. Similarly, biomass productivities of 17 $g \cdot m^{-2} \cdot day^{-1}$ were reported in 8.33 m^2 raceway reactors operated using a dilution rate of 0.3 day^{-1} and a culture depth of 0.15 cm (Posadas et al. 2015). It is accepted that the irradiance inside the cultures is negatively affected by water depth and biomass concentration because of the known self-shading or shadow effect of microalgae. Previous reports of our research group demonstrated that current raceway designs do not allow to optimise light utilisation (Barceló-Villalobos et al. 2019). Results reported herein were higher than those obtained in previous studies where higher culture depths were evaluated. For example, the production of *Chlorella* spp., *Scenedesmus* spp., and *Chlamydomonas* spp. using wastewater and 5.78 m^2 raceway reactors and a culture depth of 0.2 m led to biomass productivities of 3-8 $g \cdot m^{-2} \cdot day^{-1}$ (Mantovani et al. 2020). The high content of $N\text{-}NH_4^+$ in the wastewater used in that study together with the different environmental conditions in the north of Italy and the south of Spain could have partially contributed to the lower productivity values. Similarly, higher biomass productivities were achieved in South Korea when operating high rate algal ponds at 0.2 m that was the lowest culture depth studied. In this case, not only the productivity was higher at lower culture depths but also the nutrient requirements and the energy consumption were lower (Kim et al. 2018).

The optimal F_v/F_m value for any microalgal strain is 0.6-0.7 (Santabarbara et al. 2019). This value is constant in non-stressed cultures, thus lower values indicate certain type of stress such as nutrient limitation (He et al. 2015) or excess of light (Acién Fernández et al. 2003). The F_v/F_m ratio of the samples was in the range 0.4-0.5 (**Fig.2b**). The relatively low vales obtained in the current study suggested that the cultures were subjected to some type of stress. Reasons for these values could be a relatively high $N\text{-}NH_4^+$ concentration or the potential presence of toxins or other compounds present in wastewater that affect microalgal growth. This hypothesis is supported with previous results that reported F_v/F_m values in the range 0.4-0.5 when microalgae were produced using pre-treated pig urine (Zou et al. 2020) or wastewater (Sánchez Zurano et al. 2020). Low F_v/F_m values can also be partially attributed to the fact that microalgae were produced in summer and that F_v/F_m values can be lower during this season because of photoinhibition caused by (reversible) damage to key PSII components (Acién Fernández et al. 2003). Irradiance reached maximum values of approximately 2,500-2,600 µmol of

photons·m^{-2}·s^{-1} during this study (**Fig.1a**). Overall, despite the low F_v/F_m values, biomass productivities were comparable to those reported in previous works.

In terms of nutrient recoveries, microalgal growth inhibition caused by high N-NH_4^+ concentrations has been reported at concentrations ranging within 100 mg·L^{-1} (Collos and Harrison 2014) and 160 mg·L^{-1} (Scarponi et al. 2021). However, the inhibitory effect of N-NH_4^+ on microalgal growth is strain-dependant (Rossi et al. 2020) and therefore, it is important to select a robust strain capable of adapting to the variable composition of wastewater. The strain used in the current study demonstrated a high tolerance to N-NH_4^+ (169 mg·L^{-1}) previously (Morales-Amaral et al. 2015). N-NH_4^+ removal values were in line with previous reports. Comparable values were obtained in Italy (Mantovani et al. 2020). The percentage of nutrients recovered gives an estimate of the performance of the system. However, this value depends largely on the concentration of the inlet and therefore it is more accurate to compare removal capacities. In the current study, operating at different dilution rates significantly affected the daily removal of N-NH_4^+ (p<0.001; **Table 1**). Indeed, when operating at a dilution rate of 0.15 day^{-1}, removal of N-NH_4^+ was around 8 mg·L^{-1}·day^{-1} for all the studied culture depths while daily N-NH_4^+ removal rates ranged between 60 and 70 mg·L^{-1}·day^{-1} when operating at a dilution rate of 0.5 day^{-1}. The larger volume of water being processed when operating at 0.5 day^{-1}, caused the observed increase. The same trend was observed if considering the removal capacity per surface area. Higher culture depths led to higher N-NH_4^+ areal removal capacities because of a higher volume of water being processed per surface unit. In this sense, when operating the reactors with a culture depth of 0.20 m and a dilution rate of 0.5 day^{-1}, the N-NH_4^+ removal capacity was 14.3 g·m^{-2}·day^{-1}, higher than that obtained when the depth of the culture was 0.05 m, that is 4.8 g·m^{-2}·day^{-1} (p<0.05). Not all the N-NH_4^+ was used for microalgal growth. Part of the N-NH_4^+ available in the reactor was transformed into N-NO_3^- and part was stripped into the atmosphere. The production of N-NO_3^- during microalgae production is caused by nitrification, that is the biological oxidation of ammonia to nitrite by AOB, followed by the oxidation of nitrite to nitrate by NOB, also known as nitrifying bacteria or nitrifiers (Siripong and Rittmann 2007). As highlighted before, no N-NO_3^- production (nitrification) was observed when the culture depth was 0.05 m. This can be mainly attributed to the absence (or lower content) of nitrifying bacteria when the biomass was produced using a culture depth of 0.05 m. The reason for this could be a higher light availability at lower culture depths, which favours microalgal growth and N-NH_4^+ consumption by microalgae. This hypothesis is supported by

previous reports on thin-layer cascade reactors (0.02 m culture depth), where nitrifying bacteria had an effect on $N\text{-}NO_3^-$ production but it was almost negligible (Sánchez Zurano et al. 2020). Raceway and thin-layer cascade reactors are different but the 0.05 m culture depth studied herein is closer to the depth of thin-layer cascade reactors than to the 0.15-0.30 m depth commonly used in raceways. Moreover, the stripping phenomena such as $N\text{-}NH_4^+$ volatilisation was reported previously as part of nutrient removal processes based on microalgae (Delgadillo-Mirquez et al. 2016). Results obtained herein support previous research that suggested that the depth of the culture has a striking effect on nitrogen losses caused by stripping, being higher at higher culture depths.

The higher $P\text{-}PO_4^{3-}$ removal rate achieved at higher dilution rates and lower culture depths can be attributed to a higher volume of wastewater processed and to higher biomass productivities at lower culture depths, which is in line with previous studies that reported an almost complete removal of $P\text{-}PO_4^{3-}$ when using *S. almeriensis* and a culture depth of 0.02 m (Sánchez Zurano et al. 2020). Results are also consistent with those reported in a previous study where $P\text{-}PO_4^{3-}$ removal improved with increased nitrogen supply (Beuckels et al. 2015). $P\text{-}PO_4^{3-}$ removal values reported herein are much higher than those obtained using a biofilm photobioreactor with a maximum phosphorus uptake of 0.16 $g \cdot m^{-2} \cdot day^{-1}$ (Sukačová et al. 2015). This lower value can be attributed to the lower productivity of that reactor, which was around 12 $g \cdot m^{-2} \cdot day^{-1}$ (Sukačová et al. 2015). Indeed, when compared to a more productive raceway design, values were similar. For example, a previous study reported a total phosphorus uptake of 0.6 $g \cdot m^{-2} \cdot day^{-1}$ when processing centrate from anaerobic digestion as the nutrient source in *S. almeriensis* cultures produced in 7.2 m^2 raceways (Jebali et al. 2018). The reactors used herein are approximately 10-fold larger than those used in the above-mentioned study. Results suggest that the treatment of wastewater using microalgae shows potential for further up-scaling.

As discussed previously, microalgae-based wastewater treatment is performed by complex microalgae-bacteria consortia. The composition of the consortia vary as a function of the environmental and operational conditions (Posadas et al. 2014). Results shown in Fig. 3 demonstrated the presence of several bacterial phyla in the microalgal cultures. Proteobacteria, Bacteroidetes, and Actinobacteria are ubiquitously found both in traditional activated sludge processes (Ferrera and Sánchez 2016) and microalgae-based wastewater treatments (Ferro et al. 2020). The Verrucomicrobia phylum, composed of bacteria with heterotrophic aerobic or facultative

metabolism, has been identified in algal–bacterial photobioreactors processing piggery wastewaters, which is in accordance with the high concentrations of oxygen and organic matter available in these systems (Ferrero et al. 2012). The presence of the Rhodobacteracea family, grouped within the Rhodobacterales order, was reported in microalgal cultures previously. Some bacteria of this family are heterotrophic and consume organic carbon during and after algal blooms - their presence has been linked to phytoplankton blooms (Cooper and Smith 2015; Hogle et al. 2017). Special attention must be given to the members of the genus Cytophaga and Saprospira because of their algicidal capacity, capable to degrade algal cell surface macromolecules (MAYALI and AZAM 2004). Beyond the possible negative effects of some Bacteroidetes groups, one of the most abundant families detected (in all samples) was Chitinophagaceae, a xenobiotic degrading family relevant in wastewater treatment processes, whose activity is promoted by microalgae co-culture (Hassan et al. 2019). In the current study, relative abundance of Chitinophagaceae was affected by the dilution rate used (p<0.05), showing the highest abundance when operating at 0.15 day^{-1}. This suggests that lower dilution rates could enhance the activity of biodegrading bacteria leading to an enhanced wastewater treatment efficiency. Lower dilution rates gives the bacterial populations in the culture more time to degrade organic matter and promotes their growth by reducing light availability (because of higher biomass concentrations) and minimising their wash-out from the reactor. In addition, several bacteria genera related to the families Comamonadaceae and Rhodocyclaceae are described as acetate-utilizing denitrifiers in activated sludge. A previous work demonstrated that environmental variables like temperature and radiation could affect the Rhodocyclales order (Sánchez Zurano et al. 2020). It would be interesting to conduct a similar study such as the current one during one complete annual production cycle and in different locations.

Previous reports suggested that the presence and content of nitrifiers (AOB and NOB) could be influenced by factors such as dissolved oxygen concentration, $N\text{-}NH_4^+$, hydraulic retention time, or temperature (Prosser 1990; Mehrani et al. 2020). Results reported herein suggest, for the first time, that the relative abundance of nitrifiers can also be affected by the culture depth, increasing the abundance of nitrifiers when operating using culture depths of 0.12 and 0.20 m (p<0.05). Results are in accordance with the $N\text{-}NO_3^-$ concentration measured in the outlet of the reactors: larger culture depths lead to enhanced nitrifying activity and therefore to $N\text{-}NO_3^-$ production and accumulation in the system. Results could be explained by the different interactions between microalgae and

nitrifiers in terms of $N\text{-}NH_4^+$ availability that occur when microalgae are introduced into wastewater treatment. On the one hand, it has been proposed that microalgae may stimulate the nitrification process by increasing the dissolved oxygen concentration by oxygenic photosynthesis and thereby stimulating $N\text{-}NH_4^+$ oxidation. Other authors have suggested that microalgae could suppress nitrification by reducing $N\text{-}NH_4^+$ availability (Risgaard-Petersen et al. 2004). The raceway reactor with 0.05 m culture depth can be compared with thin-layer cascade reactors, where the low depth of the culture allows high ratios of exposed surface to total culture volume - improving biomass productivity when compared to mixed ponds or raceways (Grivalský et al. 2019). Assuming the hypothesis that microalgae and AOB compete for $N\text{-}NH_4^+$, the ammonia-oxidizing activity in the 0.05 m depth reactor could be suppressed by a higher microalgal $N\text{-}NH_4^+$ assimilation. However, a recent study suggested that the depth of the culture does not affect the structure of bacterial communities (Collao et al. 2021). More studies are needed to fully clarify how operational conditions affect the composition of the microalgae-bacteria consortia.

5. Conclusions

S. almeriensis and raceway reactors can be effectively used to process primary urban wastewater and results suggested that biomass productivities achieved when producing microalgae in wastewater can be comparable to those obtained using commercial-grade chemicals and fresh water. Selecting a robust microalgal strain is of key importance because (i) the composition of the wastewater is variable and can contain high concentrations of ammonia, which is can limit microalgal growth, and (ii) the selected strain must cope with variable environmental conditions throughout the year. Culture depths and dilution rates must be optimised for each process and reactor design and location, as the consumption of nitrogen and phosphorus is largely influenced by operational and environmental conditions. The depth of the culture has a striking effect on the composition of the microalgae-bacteria consortia and on the presence and abundance of nitrifying microorganisms. Therefore, it is important to optimise operational conditions not only to promote biomass production, but also to optimise the composition of the microalgae-bacteria consortium inside the reactor. The present study described for the first time the relationship between the culture depth of raceway reactors and the abundance of nitrifying bacteria, highlighting the key importance of culture depth when designing a wastewater treatment process. Further studies on the effect of operational and environmental conditions on the composition of microalgae-bacteria consortia are

needed. In the current study, culture depths over 0.05 m allowed the growth of nitrifying bacteria and to the production of $N\text{-}NO_3^-$ that accumulate in the system, leading to lower nitrogen removal. It is necessary to up-scale current processes outdoors to promote the industrial implementation of microalgae based processes.

Declaration

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Conflicts of interests/Competing interests

There are no potential financial or other interests that could be perceived as influencing the outcome of the research. No conflicts, informed consent, human or animal rights issues are applicable. All the authors confirmed authorship of the manuscript and agreed to submit it for peer review.

Availability of data and material

Data from the SABANA Project are available at http://www2.ual.es/sa-347 bana/data-center-2/.

Authors' contributions

A. Sánchez-Zurano: Investigation, Formal analysis, Writing – Original draft; **T. Lafarga:** Formal Analysis, Visualisation, Writing – Original draft; **M.M. Morales-Amaral:** Investigation; **C. Gómez-Serrano:** Investigation, Supervision; **J.M. Fernández-Sevilla:** Conceptualisation, Supervision, Writing – Review & Editing; **F.G. Acién-Fernández:** Supervision, Conceptualisation, Writing – Review & Editing, Funding Acquisition; **E. Molina-Grima:** Conceptualisation, Supervision, Writing – Review & Editing.

Table 1. Nutrient recovery capacity as a function of culture depth and dilution rate. Microalgae were produced in triplicate using three independent photobioreactors. Three technical replicates at the steady state were performed per natural replicate. Values represent the average of these independent determinations ± S.D. Different capital letters indicate differences between culture depths while different lower case letters indicate differences between dilution rates. The criterion for statistical significance was $p<0.05$.

		Inlet ($mg \cdot L^{-1}$)	Outlet ($mg \cdot L^{-1}$)			Removal* ($mg \cdot L^{-1} \cdot day^{-1}$)			Removal* ($g \cdot m^{-2} \cdot day^{-1}$)		
Nutrient	**Dilution rate**	**0.05-0.20 m**	**0.20 m**	**0.12 m**	**0.05 m**	**0.20 m**	**0.12 m**	**0.05 m**	**0.20 m**	**0.12 m**	**0.05 m**
$N\text{-}NH_4^+$	**0.15 (day^{-1})**	59.1 ± 4.3	2.2 ± 0.2 ^{Ab}	0.5 ± 0.2 ^{Ba}	0.4 ± 0.3 ^{Ba}	8.5 ± 0.6 ^{Ad}	8.8 ± 0.6 ^{Ad}	8.8 ± 0.6 ^{A}	1.9 ± 0.1 ^{Ad}	1.3 ± 0.1 ^{Bd}	0.7 ± 0.0 ^{Cd}
	0.30 (day^{-1})	58.9 ± 1.9	0.3 ± 0.1 ^{Ac}	0.5 ± 0.4 ^{Aa}	0.5 ± 0.1 ^{Aa}	17.6 ± 0.5 ^{Ac}	17.5 ± 0.5 ^{Ac}	17.4 ± 0.5 ^{A}	3.9 ± 0.1 ^{Ac}	2.5 ± 0.1 ^{Bc}	1.3 ± 0.0 ^{Cc}
	0.40 (day^{-1})	123.7 ± 2.3	1.8 ± 0.2 ^{Ab}	0.7 ± 0.4 ^{Ba}	0.7 ± 0.2 ^{Ba}	48.8 ± 0.8 ^{Ab}	49.2 ± 0.8 ^{Ab}	47.3 ± 0.8 ^{A}	10.9 ± 0.2 ^{Ab}	7.1 ± 0.1 ^{Bb}	3.5 ± 0.1 ^{Bb}
	0.50 (day^{-1})	137.1 ± 2.2	9.3 ± 0.4 ^{Aa}	0.5 ± 0.3 ^{Ba}	0.7 ± 0.4 ^{Ba}	63.9 ± 0.9 ^{ABa}	68.2 ± 0.9 ^{Ba}	64.6 ± 0.6 ^{A}	14.3 ± 0.2 ^{Aa}	9.8 ± 0.1 ^{Ba}	4.8 ± 0.0 ^{Ca}
$N\text{-}NO_3^-$	**0.15 (day^{-1})**	1.4 ± 0.3	53.5 ± 3.5 ^{Aa}	41.4 ± 2.9 ^{Ba}	42.1 ± 2.2 ^{Ba}	-7.8 ± 0.5 ^{Ca}	-6.1 ± 0.3 ^{Ba}	-0.6 ± 0.0 ^{A}	-1.7 ± 0.1 ^{Ca}	-0.9 ± 0.0 ^{Ba}	-0.05 ± 0.01 ^{Ac}
	0.30 (day^{-1})	3.5 ± 0.2	36.5 ± 3.5 ^{Ad}	35.7 ± 3.6 ^{Aa}	35.2 ± 1.4 ^{Ab}	-9.9 ± 1.0 ^{Bb}	-9.5 ± 0.4 ^{Bb}	0.6 ± 0.1 ^{A}	-2.2 ± 0.2 ^{Cb}	-1.4 ± 0.1 ^{Bb}	0.04 ± 0.01 ^{Ab}
	0.40 (day^{-1})	5.4 ± 0.4	46.0 ± 1.4 ^{Ab}	45.6 ± 5.9 ^{Aa}	44.8 ± 4.9 ^{Aa}	-16.3 ± 0.4 ^{Bc}	-15.8 ± 1.8 ^{Bc}	1.8 ± 0.1 ^{A}	-3.6 ± 0.1 ^{Cc}	-2.3 ± 0.3 ^{Bc}	0.13 ± 0.00 ^{Aa}
	0.50 (day^{-1})	5.6 ± 0.4	42.5 ± 3.5 ^{Ac}	40.5 ± 6.8 ^{Aa}	41.2 ± 1.3 ^{Aa}	-17.8 ± 0.5 ^{Bd}	-17.7 ± 0.5 ^{Bd}	0.4 ± 0.4 ^{A}	-4.1 ± 0.4 ^{Cc}	-2.6 ± 0.1 ^{Bc}	0.03 ± 0.02 ^{Ab}
$P\text{-}PO_4^{3-}$	**0.15 (day^{-1})**	7.8 ± 0.5	6.1 ± 0.4 ^{Ab}	3.4 ± 0.8 ^{Bb}	3.5 ± 0.6 ^{Bb}	0.3 ± 0.0 ^{Cc}	0.6 ± 0.0 ^{Bc}	1.1 ± 0.1 ^{Ac}	0.1 ± 0.0 ^{Ad}	0.1 ± 0.0 ^{Ac}	0.1 ± 0.0 ^{Ad}
	0.30 (day^{-1})	14.4 ± 0.3	1.9 ± 0.6 ^{Ac}	1.0 ± 0.3 ^{Bc}	1.1 ± 0.4 ^{Bc}	3.8 ± 0.1 ^{Cb}	4.0 ± 0.0 ^{Bb}	4.2 ± 0.0 ^{Ab}	0.8 ± 0.0 ^{Ac}	0.6 ± 0.0 ^{Bb}	0.3 ± 0.0 ^{Cc}
	0.40 (day^{-1})	13.3 ± 2.8	2.3 ± 0.5 ^{Ac}	1.4 ± 0.5 ^{Bc}	1.4 ± 0.4 ^{Bc}	4.4 ± 0.9 ^{Ab}	4.8 ± 1.0 ^{Ab}	5.2 ± 1.1 ^{Ab}	1.0 ± 0.2 ^{Ab}	0.7 ± 0.1 ^{Ab}	0.4 ± 0.1 ^{Bb}
	0.50 (day^{-1})	26.3 ± 1.3	12.9 ± 2.0 ^{Aa}	8.8 ± 0.9 ^{Ba}	13.6 ± 1.0 ^{Aa}	6.7 ± 0.3 ^{Ba}	6.3 ± 0.2 ^{Ba}	8.5 ± 0.5 ^{Aa}	1.5 ± 0.1 ^{Aa}	0.9 ± 0.1 ^{Ba}	0.6 ± 0.1 ^{Ca}
COD	**0.15 (day^{-1})**	618.2 ± 1.5	77.4 ± 2.9 ^{Ab}	70.8 ± 2.6 ^{Bb}	72.3 ± 4.2 ^{ABc}	81.1 ± 0.2 ^{Bd}	81.9 ± 0.4 ^{Ad}	77.1 ± 0.9 ^{Cd}	18.1 ± 0.0 ^{Ad}	11.8 ± 0.1 ^{Bd}	5.7 ± 0.1 ^{Cd}
	0.30 (day^{-1})	855.6 ± 1.8	102.2 ± 10.0 ^{Aa}	100.0 ± 9.0 ^{Aa}	99.4 ± 5.9 ^{Aa}	226.0 ± 2.4 ^{Ac}	226.9 ± 1.2 ^{Ac}	212.7 ± 8.6 ^{Bc}	50.5 ± 0.5 ^{Ac}	32.7 ± 0.2 ^{Bc}	15.8 ± 0.6 ^{Cc}

	0.40 (day^{-1})	692.2 ± 9.8	57.2 ± 8.5 ^{Bc}	84.1 ± 8.8 ^{Aa}	84.7 ± 6.6 ^{Ab}	254.0 ± 0.5 ^{Ab}	243.0 ± 1.3 ^{Bb}	244.0 ± 2.8 ^{Bb}	56.7 ± 0.1 ^{Ab}	35.0 ± 0.2 ^{Bb}	18.2 ± 0.6 ^{Cb}
	0.50 (day^{-1})	670.4 ± 11.5	98.8 ± 4.5 ^{Aa}	81.7 ± 12.6 ^{Ba}	83.7 ± 3.7 ^{Bb}	285.8 ± 3.5 ^{Aa}	293.4 ± 7.6 ^{Aa}	277.2 ± 1.9 ^{Ba}	63.8 ± 0.8 ^{Aa}	42.3 ± 1.1 ^{Ba}	20.6 ± 0.1 ^{Ca}

***** Negative values indicate nitrification and nitrate production

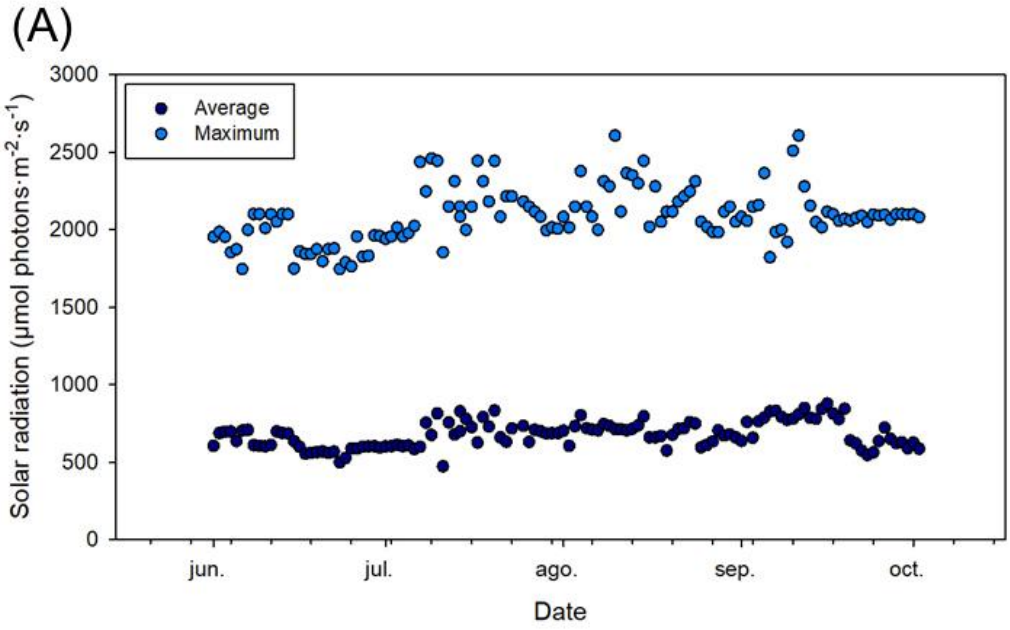
Figure legends:

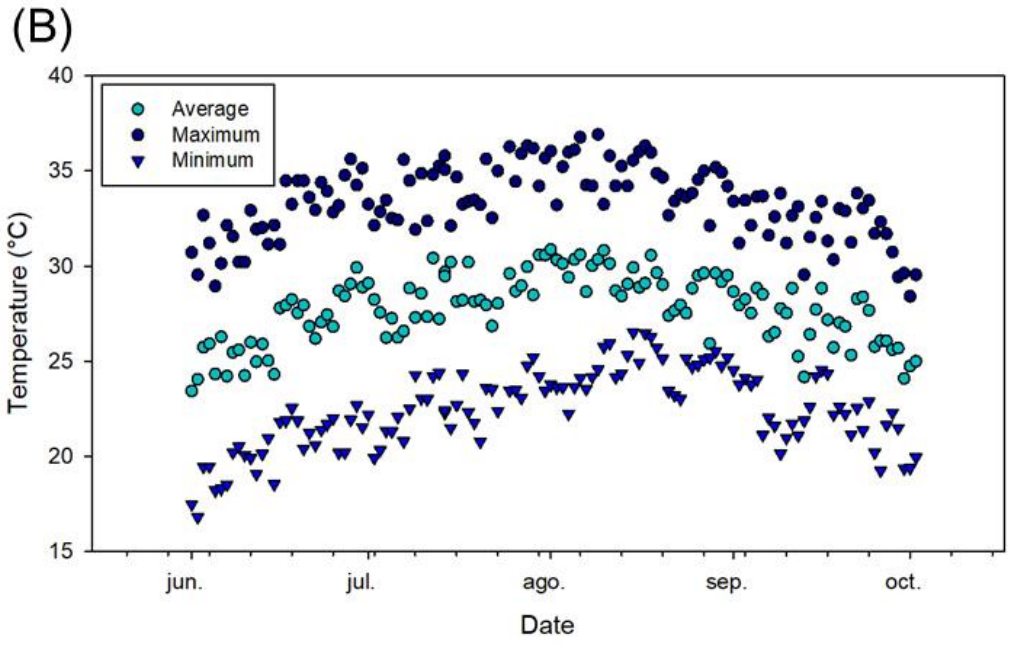
Figure 1. (A) Solar radiation and (B) temperature during wastewater treatment and (C) raceway reactors used

Figure 2. Effect of dilution rate and culture depth on (A) biomass productivity and (B) fluorescence of chlorophylls in outdoor cultures of *Scenedesmus* almeriensis Different capital letters indicate differences between dilution rates. Different lower case letters indicate differences between culture depths.

Figure 3. Taxonomic classification of the bacterial communities in the raceway reactors operated at different dilution rates and culture depths. Relative abundance of bacterial (A) phylum and of (B) major bacterial classes. (C) Main AOB and NOB families identified in the raceway reactors.

Figure 1


(A)
Average
Maximum
Solar radiation (µmol photons·m-2·s-1)
3000
2500
2000
1500
1000
500
0
jun.
jul.
ago.
sep.
oct.
Date


(B)
Average
Maximum
Minimum
Temperature (°C)
40
35
30
25
20
15
jun.
jul.
ago.
sep.
oct.
Date

(C)

Figure 2

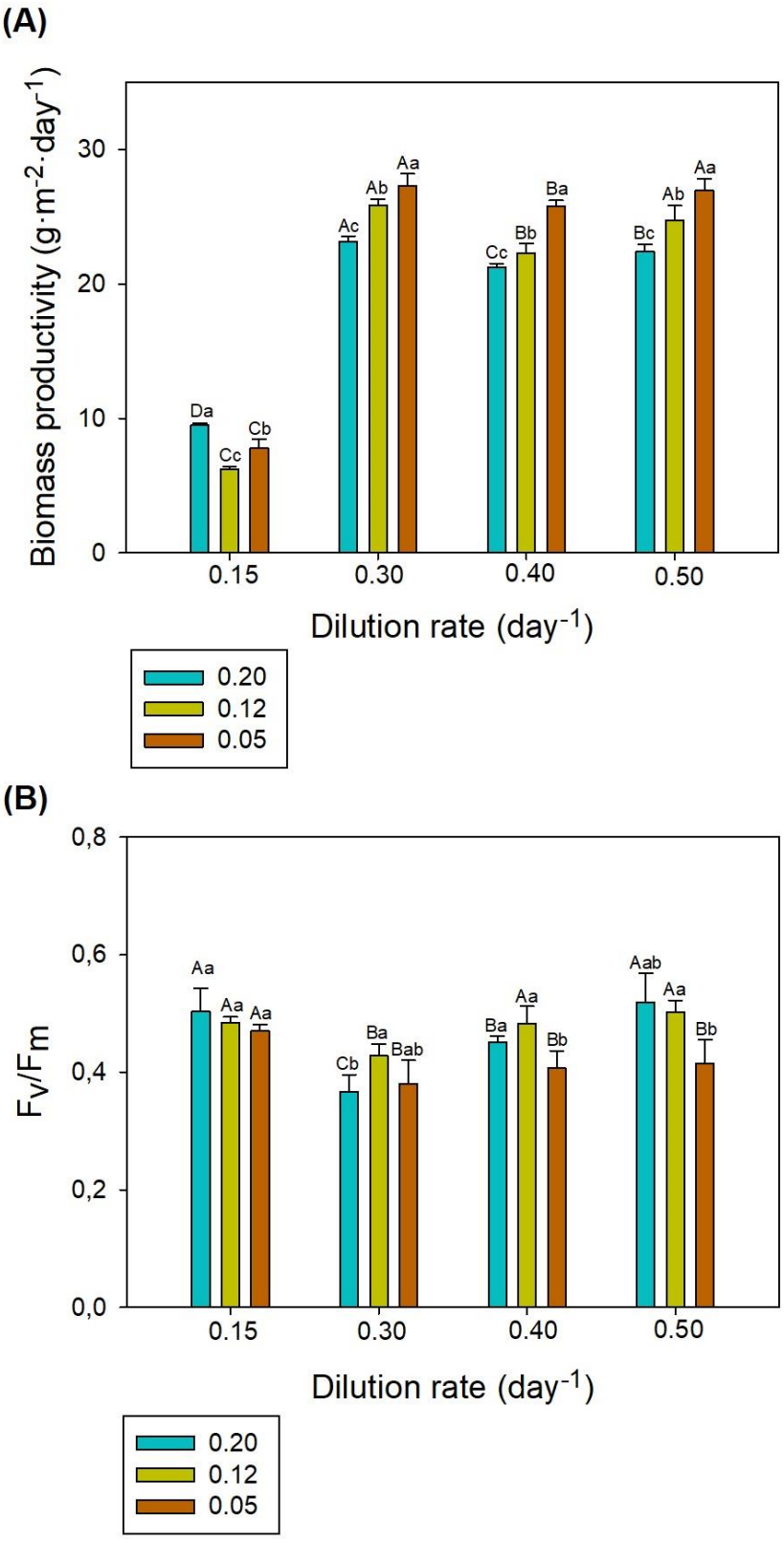
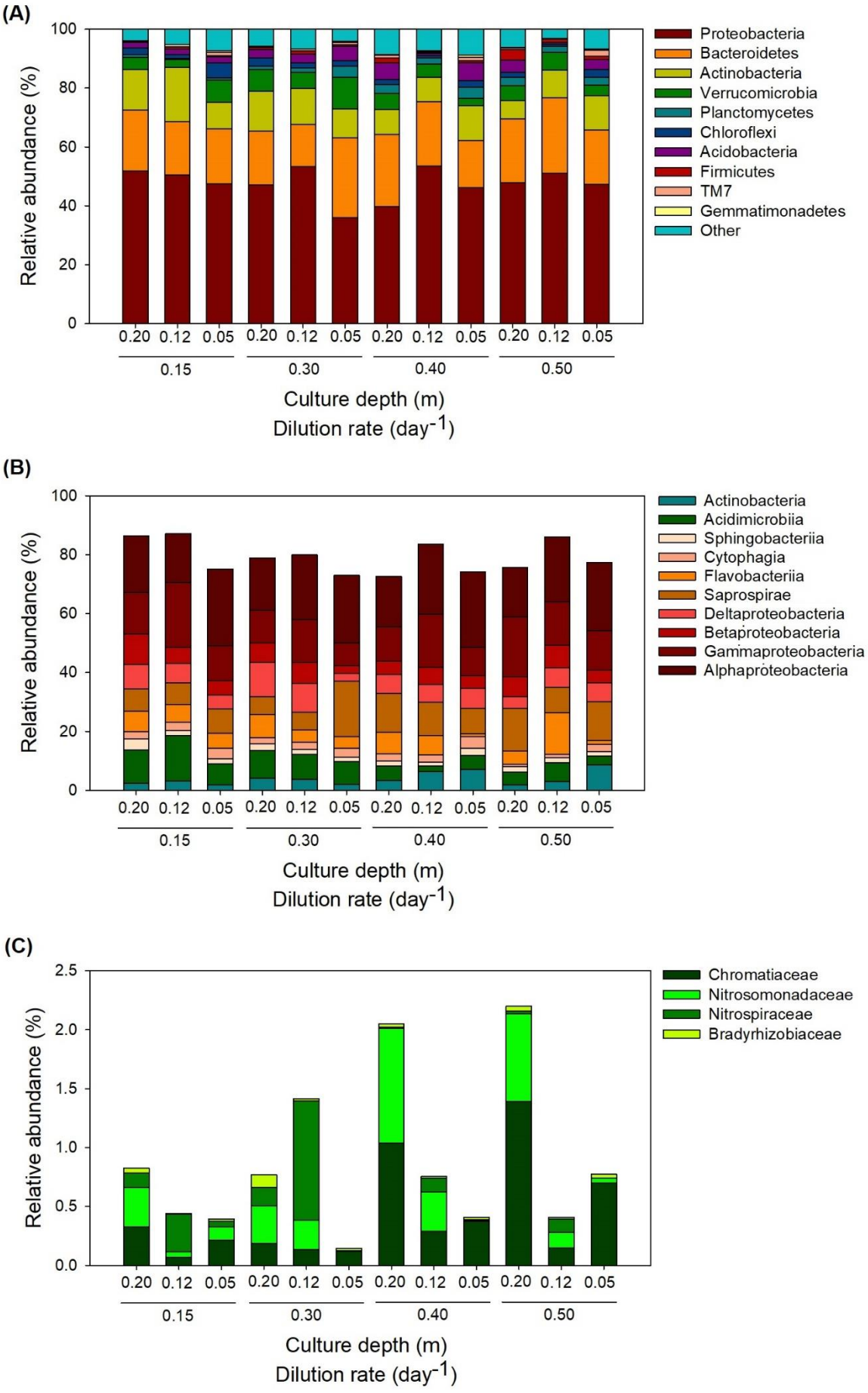

(A)
Biomass productivity (g·m-2·day-1)
30
20
10
0
Da
Cc
Cb
Ac
Ab
Aa
Cc
Bb
Ba
Bc
Ab
Aa
0.15
0.30
0.40
0.50
Dilution rate (day-1)
0.20
0.12
0.05
(B)
Fv/Fm
0,8
0,6
0,4
0,2
0,0
Aa
Aa
Aa
Cb
Ba
Bab
Ba
Aa
Bb
Aab
Aa
Bb
0.15
0.30
0.40
0.50
Dilution rate (day-1)
0.20
0.12
0.05

Figure 3


(A)
Relative abundance (%)
100
80
60
40
20
0
Proteobacteria
Bacteroidetes
Actinobacteria
Verrucomicrobia
Planctomycetes
Chloroflexi
Acidobacteria
Firmicutes
TM7
Gemmatimonadetes
Other
0.20 0.12 0.05 0.20 0.12 0.05 0.20 0.12 0.05 0.20 0.12 0.05
0.15
0.30
0.40
0.50
Culture depth (m)
Dilution rate (day-1)
(B)
Relative abundance (%)
100
80
60
40
20
0
Actinobacteria
Acidimicrobiia
Sphingobacteriia
Cytophagia
Flavobacteriia
Saprospirae
Deltaproteobacteria
Betaproteobacteria
Gammaproteobacteria
Alphaproteobacteria
0.20 0.12 0.05 0.20 0.12 0.05 0.20 0.12 0.05 0.20 0.12 0.05
0.15
0.30
0.40
0.50
Culture depth (m)
Dilution rate (day-1)
(C)
Relative abundance (%)
2.5
2.0
1.5
1.0
0.5
0.0
Chromatiaceae
Nitrosomonadaceae
Nitrospiraceae
Bradyrhizobiaceae
0.20 0.12 0.05 0.20 0.12 0.05 0.20 0.12 0.05 0.20 0.12 0.05
0.15
0.30
0.40
0.50
Culture depth (m)
Dilution rate (day-1)

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