Potential of the cyanobacteria *Anabaena* sp. and *Dolichospermum* sp. for being produced using wastewater or pig slurry: Validation using pilot-scale raceway reactors

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ABSTRACT

Indoor trials using 0.3 L bubble columns were conducted to demonstrate the potential of two cyanobacterial strains, *Anabaena* sp. and *Dolichospermum* sp., for being produced using nutrients obtained from waste streams (wastewater or pig slurry). Filtered pig slurry diluted at a concentration of 5% (v/v) led to a biomass productivity comparable to that of the standard medium (0.72 ± 0.05 and 0.66 ± 0.09 $g \cdot L^{-1} \cdot day^{-1}$ for *Anabaena* sp. and *Dolichospermum* sp. respectively). When produced using wastewater, the productivity was around 0.40 $g \cdot L^{-1} \cdot day^{-1}$ for both strains. The process was up-scaled using a 1.04 m^3 raceway reactor located outdoors and operated at a culture depth of 0.12 m. Dilution rates of 0.1-0.3 day^{-1} were evaluated, achieving maximum biomass productivities when operating at a dilution rate of 0.3 day^{-1}: 20.9 and 28.0 $g \cdot m^{-2} \cdot day^{-1}$ for *Anabaena* sp. and *Dolichospermum* sp., respectively. The maximum total nitrogen ($N\text{-}NH_4^+$ plus $N\text{-}NO_3^-$) removal rates for *Anabaena* sp. and *Dolichospermum* sp. were 2,471 and 3,621 $mg \cdot m^{-2} \cdot day^{-1}$, achieved when operating at a dilution rate of 0.3 day^{-1}. Nitrification and stripping contributed to the $N\text{-}NH_4^+$ removal. When operating at 0.3 day^{-1}, maximum $P\text{-}PO_4^{3-}$ removal rates for cultures of *Anabaena* sp. and *Dolichospermum* sp. were 81.5 and 87.1 $mg \cdot m^{-2} \cdot day^{-1}$. Illumina sequencing results revealed that both strains dominated the prokaryotic community of both cultures with other notable eukaryotic and prokaryotic genus detected including human pathogens. However, annual studies are needed to assess the potential of producing biomass rich in these strains outdoors.

Keywords: cyanobacterium, waste management, biorefinery, nutrient recovery, wastewater treatment, biomass production.

1. INTRODUCTION

Cyanobacteria are a bacterial phylum capable of performing photosynthesis, and for this reason these microorganisms are generally included into the term “microalgae” [1]. Microalgae, including cyanobacteria, are gaining increased importance in the context of bioeconomy and circular economy. Their ability to fix atmospheric carbon dioxide and produce organic matter is being industrially exploited for a wide range of applications. Currently, microalgae are mainly used for food applications [2] but their ability to use nutrients from wastewater or other residues led to an increased interest in the utilisation of microalgae as biological systems to improve current waste and wastewater treatment processes. Most of the studies reported to date were conducted indoors in controlled photobioreactors, but microalgae-based wastewater treatment processes are being demonstrated and validated at industrial scale in several locations including Christchurch (New Zealand), Cadiz (Spain), and Mérida (Spain). Some added advantages of producing microalgae using wastewater or manure, besides the obvious environmental benefits, are lower production costs and the production of biomass that can be further used for valuable applications such as agriculture or feed production. Thus, depending on the end application of the produced biomass, the utilisation of wastewater or manure as the sources for nutrients can be not only a sustainable but also a profitable option.

The most well-known genus of cyanobacteria is *Arthrospira* (commercially known as *Spirulina*), which is used mainly for food applications because of its high protein content. However, other strains showed potential for being mass cultured due to their possible use in agriculture as biostimulants or biofertilisers or as ingredients in animal feed, mainly aquafeeds. For example, the genus *Anabaena* and *Dolichospermum* recently showed potential phytostimulating effects [3] and the former also demonstrated positive effects when used in the manufacture of animal feeds [4]. Both cyanobacteria grow naturally in freshwater bodies but can also be produced using seawater, which has the advantage of difficulting the growth of bacteria and fungi thus promoting microalgal growth [5]. Microalgae are industrially

produced in controlled facilities using photobioreactors. These can be divided into systems that are either open or closed to the environment. Despite being less productive, the former are the most commonly used because of their ease of operation, ease of scale-up, lower energetic requirements, and lower production costs. Raceways are the most common design and are especially interesting as their low surface-to-volume ratio allows to process large volumes of water per surface area.

One of the main challenges of producing microalgae in raceway reactors is the presence of contaminants such as grazers, fungi, bacteria, viruses, or other photosynthetic microorganisms that can limit the growth of the target strain or even lead to the crash of the culture within a few days. Although it is impossible to produce a mono-culture in raceway reactors, when microalgae are produced using wastewater or slurry as the nutrient source the microbial diversity inside the reactor is especially high. This is not always negative as a microalgae-bacteria consortia is desired to remove a larger amount of nutrients from the water. Complex and not yet fully understood interactions exist between microalgae or cyanobacteria and bacteria including nutrient exchange, signal transduction, and gene transfer [6]. For example, the oxygen produced by microalgae/cyanobacteria is used by bacteria to oxidise organic matter into inorganic compounds. These include carbon dioxide that is further consumed by microalgae/cyanobacteria to produce biomass [7]. The composition of the consortia depends on environmental and operational conditions. The quality of the biomass can be negatively affected if the target strain is not the majoritarian, and this generally occurs when weed microalgal or cyanobacterial species that grow faster than the target strain enter the reactor [8].

The aim of the current study was to assess the potential of producing the *Anabaena* sp. and/or *Dolichospermum* sp. using waste streams that represent an environmental problem: wastewater or pig slurry. Both wastewater and pig slurry were assessed as nutrient sources for microalgae production indoors in controlled photobioreactors and further validated outdoors using 7.1 m^2 pilot-scale raceways. Genetic analyses were conducted to assess the

robustness of both strains when produced outdoors under real environmental conditions. Moreover, a secondary aim was to assess the nutrient removal efficiency of both strains and to identify the optimum operational conditions to improve biomass productivity.

2. Materials and methods

2.1 Strains, chemicals and reagents

Selected strains were *Anabaena* sp. (BEA-0912B) and *Dolichospermum* sp. (BEA-0866B) obtained from the Spanish Bank ok Algae (Las Palmas de Gran Canaria, Spain). Both strains were selected because of their robustness, adaptation to the environmental conditions of Almería (Spain), and phytostimulant potential which was described previously [3]. Analytical-grade reagents used for culture media formulation were $Ca(NO_3)_2$, $MgSO_4$, KH_2PO_4, $C_6H_5FeO_7 \cdot 5H_2O$, $CoCl_2 \cdot 6H_2O$, $CuSO_4 \cdot 5H_2O$, Cr_2O_3, $MnCl_2 \cdot 4H_2O$, $NaMoO_4 \cdot 2H_2O$, SeO_2, and NaCl and were purchased from Sigma-Aldrich (Barcelona, Spain). Commercial-grade chemicals were Karentol®, $NaNO_3$, $MgSO_4$, KH_2PO_4, (commercial fertilisers) and NaCl. All other chemicals used were purchased from Sigma-Aldrich (Barcelona, Spain)

2.2 Indoor laboratory-scale trials

Indoor trials were conducted using 300 mL bubble-column photobioreactors with temperature, pH, light, and agitation control as described previously [9]. The cultures were aerated at 0.2 v/v/min. Temperature and pH values were kept constant at 25 °C and 8.0 respectively. The pH was controlled by on-demand injection of carbon dioxide at 0.02 v/v/min. Light was controlled using Daylight T5 fluorescence tubes 28 W (Philips, Madrid, Spain) and programmed to mimic outdoor conditions. The irradiance inside the reactors in the absence of cells was 1850 $\mu E \cdot m^{-2} \cdot s^{-1}$, measured using a spherical quantum sensor SQS-100 (Walz GmbH, Effeltrich, Germany). The reactors were inoculated with 10% of the cultures' volume (initial biomass concentration was 0.5 $g \cdot L^{-1}$) with a standard inoculum and four different media were studied:

- Medium A, standard medium formulated using analytical-grade chemicals: 0.80 $g \cdot L^{-1}$ $Ca(NO_3)_2$, 0.35 $g \cdot L^{-1}$ $MgSO_4$, 0.10 $g \cdot L^{-1}$ KH_2PO_4, 30.0 $g \cdot L^{-1}$ NaCl, 0.10 $mL \cdot L^{-1}$ of a stock solution of oligoelements, and 0.02 $g \cdot L^{-1}$ Karentol®. This medium was used to maintain the stock cultures and therefore both cyanobacteria were adapted to its composition.
- Medium B, pig slurry: 30.0 $g \cdot L^{-1}$ NaCl in 5% filtered pig slurry and 95% tap water. The $N\text{-}NH_4^+$, $N\text{-}NO_3^-$, and $P\text{-}PO_4^{3-}$ concentration of the diluted slurry was determined as 66.2 ± 12.1, 12.4 ± 1.3, and 11.3 ± 0.5 $mg \cdot L^{-1}$. The chemical oxygen demand (COD) was 526 ± 29 $mg \cdot L^{-1}$.
- Medium C, wastewater: 30.0 $g \cdot L^{-1}$ NaCl in 100% urban wastewater. The wastewater was collected from an urban wastewater treatment plant in Almería (Spain) operated by FCC AQUALIA S.A. The $N\text{-}NH_4^+$, $N\text{-}NO_3^-$, and $P\text{-}PO_4^{3-}$ concentration of the wastewater was determined as 76.5 ± 3.1, 4.1 ± 2.1, and 12.3 ± 3.1 $mg \cdot L^{-1}$. The COD of the wastewater was 574 ± 42 $mg \cdot L^{-1}$.
- Medium D, medium formulated using commercial-grade chemicals (commercial fertilisers): 1.00 $g \cdot L^{-1}$ $NaNO_3$, 0.25 $g \cdot L^{-1}$ $MgSO_4$, 0.30 $g \cdot L^{-1}$ KH_2PO_4, 30.0 $g \cdot L^{-1}$ NaCl, and 0.02 $g \cdot L^{-1}$ Karentol®. This medium was used to produce microalgae using large photobioreactors in previous reports [10,11].

The stock solution of oligoelements consisted of 5.26 $mg \cdot L^{-1}$ $C_6H_5FeO_7 \cdot 5H_2O$, 0.02 $mg \cdot L^{-1}$ $CoCl_2 \cdot 6H_2O$, 0.03 $mg \cdot L^{-1}$ $CuSO_4 \cdot 5H_2O$, 0.15 $mg \cdot L^{-1}$ Cr_2O_3, 1.98 $mg \cdot L^{-1}$ $MnCl_2 \cdot 4H_2O$, 0.24 $mg \cdot L^{-1}$ $NaMoO_4 \cdot 2H_2O$, and 0.01 $mg \cdot L^{-1}$ SeO_2, while Karentol® (Kenogard, Spain) is a commercial solid mixture of micronutrients including copper, boron, iron, manganese, molybdenum, and zinc.

Experiments were conducted in triplicate using three independent photobioreactors that were the experimental units. The location of the reactors inside the illuminated chamber was selected randomly. The reactors were operated in batch mode until the biomass concentration was constant (6-7 days) and after this period they were operated in semi-continuous mode. The experiments were stopped when the biomass concentration was constant for at least

three consecutive days (approximately 8-9 days of semi-continuous operation). The dilution rate used was 0.3 day^{-1}, which means that every day, 30% of the culture was harvested and replaced with fresh culture media. Biomass and culture media were used for analytical determinations as described in the following sections.

2.3 Outdoor pilot-scale trials

Pilot-scale production of both strains was conducted at the pilot plant facilities of the University of Almería located at IFAPA (Almería, Spain). The inocula were produced using 80 L pH-controlled bubble column photobioreactors placed inside a greenhouse. The inocula were produced using Medium D, described above. The pH was controlled by on-demand injection of CO_2 at 8.0 ± 0.1. Once the bubble column photobioreactors reached the desired concentration (1.0 ± 0.1 $g \cdot L^{-1}$), these were used to inoculate raceway reactors (1.04 m^3, 7.13 m^2, and 0.12 m culture depth) that were located outdoors and outside of the greenhouse. Test were conducted on semi-continuous mode and the reactors were operated using different dilution rates (0.1, 0.2, or 0.3 day^{-1}). The reactors were operated in semi-continuous mode until the total volume of the reactors was replaced at least twice and the biomass concentration was constant for at least three consecutive days. All the raceways were operated 24 h per day and the pH, temperature, and dissolved oxygen concentration of the cultures were online monitored using 5083T and 5120 probes (Crison Instruments, Spain) connected to a MM44 control-transmitter unit (Crison Instruments, Spain) and Labview data acquisition software (National Instruments, US). Evaporation was compensated daily by addition of fresh water and the pH was controlled by on-demand injection of CO_2. Experiments were conducted during June-July in triplicate using three natural replicates, that is three different (but identical) raceway reactors.

2.4 Analytical determinations

Biomass concentration was calculated by dry weight by filtering 100 mL of culture through 1 µm filters followed by drying in an oven at 80 °C for 24 h. Biomass productivity was calculated

as the product of biomass concentration and the dilution rate, which was either 0.1, 0.2, or 0.3 day^{-1}. The chlorophyll fluorescence ratio (Fv/Fm) was checked daily with an AquaPen AP 100 fluorometer (Photon System Instruments, Czech Republic). Cells were dark-adapted for 5 min prior to the determination of Fv/Fm values.

The concentration of ammonium, nitrates, and phosphates at the inlet and outlet of the raceways was determined using standard official methods approved by the Spanish Ministry of Agriculture [12]. Briefly, $P\text{-}PO_4^{3-}$ and $N\text{-}NO_3^-$ were measured spectrophotometrically using a Genesys™ 10S UV-Vis spectrophotometer (Thermo Fisher Scientific, Spain) through the phosphor-vanado-molybdate complex and the concentration of $N\text{-}NH_4^+$ was determined using the Nessler reactive method. Removal efficiencies were calculated based on the concentration of $N\text{-}NH_4^+$, $N\text{-}NO_3^-$, and $P\text{-}PO_4^{3-}$ in the inlets and outlets and the amount of water daily removed from the system using the equation:

$$Removal\ (mg \cdot m^{-2} \cdot day^{-1}) = \frac{(C_0 - C_f) \cdot V \cdot D}{A}$$

where C_0 and C_f are the concentrations in the inlet and outlet effluents ($mg \cdot L^{-1}$), V is the volume of the reactors (1,040 L), A is the area of the reactors (7.1 m^2) and D is the dilution rate (0.1, 0.2, or 0.3 day^{-1}).

Moreover, the microbial composition of the samples was also determined. One sample was collected from each reactor (n=3) and analysed in triplicate. Samples were collected during the steady state phase of the production when operating the raceways at a dilution rate of 0.3 day^{-1}. From each sample, DNA was extracted using the Biosprint 96 One-For-All Vet Kit (Qiagen, Hilden, Germany) and a semiautomatic extractor BioSprint 96 with MagAttract technology (Qiagen, Hilden, Germany), following the manufacturers' instructions. Approximately 20 mg of lyophilized biomass were used and experiments were conducted using three technical replicates. The yield of the purified DNA was quantified using Qubit (Invitrogen, Thermo Fisher Scientific, Italy) while DNA purity was measured through Nanodrop (Abs_{260}/Abs_{280} and $Abs_{260}/Abs2_{30}$) (Invitrogen, Thermo Fisher Scientific, Italy). Possible

fragmentation was determined through gel electrophoresis 1% (w/v) 1×TAE agarose gels. DNA was then stored at -80 °C until further analysis. Library for 16S and 18S rRNA gene were prepared following the Illumina Protocol. For the 16S, the hypervariable V3-V4 region was amplified using the 341F and 805R primers [13] while for the 18S, the V9 region was amplified using the 1389F and 1510R primers [14] both modified with the required Illumina sequencing adaptors. The PCR amplification of 16S and 18S was performed as follows: 12.5 µL of appTaq RedMix (Appleton Wood Limited, Birmingham, UK), 1.0 µL of forward and 1.0 µL of reversed primers modified with Illumina over-hanger (10 µM) (IDT, Leuven, Belgium), 2.5 µL of extracted DNA and 8 µL of PCR grade water (Merck, Darmstadt, Germany). The thermal protocol for the 16S gene was 95 °C for 3 min followed by 30 cycles at 95°C for 15 s, 57 °C for 15 s, 72 °C for 30 s, and a final extension step at 72 °C for 7 min. For the 18S rRNA gene, the thermal protocol was 98 °C for 3 min followed by 30 cycles at 98 °C for 10 s, 56 °C for 30 s and 72 °C for 15 s with a final extension step at 72 °C for 7 min. PCR products were cleaned using Agencourt AMPure XP PCR Purification beads (Beckman Coulter, CA, USA). Then, 2.5 µL of purified PCR product were used in a short secondary PCR, to attach Nextera XT indices, in the presence of 2.5 µL of Nextera i5 and i7 index, 12.5 µL Appletonwood Taq, and 5.0 µL of PCR water. Thermal cycling conditions were an initial denaturation step of 3 min at 95°C followed by 8 cycles each of 30 s at 95°C, 30 s at 55 °C and 30 s at 72 °C and a final extension step of 5 min at 72 °C. PCR products were purified again using Agencourt AMPure XP PCR Purification beads. PCR products were quantified using PicoGreen® dsDNA quantification assays (Thermo Fisher Scientific, Rome, Italy), on a POLAR Star Omega plate reader (BMG Labtech, Ortenberg, Germany). Nextera XT amplicons were then pooled in equimolar concentration. The length of amplicons was verified with Agilent bioanalyzer DNA kit (Agilent Technologies, CA, USA). Final quantification of the pooled amplicon library was determined with the NEBNext® Library Quant Kit for Illumina® (New England BioLabs, MA, USA) prior to sequencing on the Illumina MiSeq (2 × 300 bp) at the University of Essex (UK). Amplicons were processed using a previously described protocols for 16S [15] and 18S [16]. The resultant OTU sequences were assigned taxonomy using the Naïve Bayesian Classifier

against the RDP database. The 18S sequences followed the same bioinformatic workflow as above with the only difference that classification was done using Blast (version 2.8.1) against NCBI nucleotide database and taxonomy retrieved using the Taxdump Repository.

2.5 Statistical analysis

All the data shown were determined during the steady state, after the complete volume of the cultures was replaced at least twice and the biomass concentration was constant for at least three consecutive days. Each photobioreactor was considered the experimental unit and three natural replicates were used. Data shown are the mean values of three independent values ± standard deviations. Normality and homoscedasticity of the variables within each group were checked. Differences between determinations were analysed using analysis of variance with JMP 13 (SAS Institute Inc., USA). A Tukey pairwise comparison of the means was carried out to identify where sample differences occurred with a criterion of $p<0.05$.

3. Results and discussion

3.1 Indoor laboratory-scale production of Anabaena sp. and *Dolichospermum* sp.

Both *Anabaena* sp. and *Dolichospermum* sp. are robust strains with potential for being mass cultured using inexpensive culture media based on waste streams or commercial fertilisers as nutrient sources. Indeed, both strains that were being produced (using commercial fertilisers) in raceway reactors at the pilot plant facilities of the University of Almería and naturally contaminated nearby reactors that were being operated using waste streams. For this reason, both strains were produced using pig slurry (Medium B), wastewater (Medium C), or commercial fertilisers (Medium D) and results were compared to those obtained when using a standard medium formulated using analytical-grade chemicals (Medium A). Figure 1A shows the maximum concentration reached during the batch phase when both strains were produced using the different selected media. Briefly, when produced using wastewater or commercial fertilisers, biomass concentration was lower in both strains when compared to the standard

medium (p<0.05). However, no differences were observed when the strains were produced using pig slurry as the nutrient source. Several reports highlighted the potential of microalgae and cyanobacteria for being produced using pig slurry as the main nutrient source [17–20]. The interactions that occur between microalgae and the bacteria naturally present in pig slurry could have contributed to the high efficiency of the system. Moreover, *Anabaena* sp. showed a higher adaptation to the medium prepared using pig slurry while *Dolichospermum* sp. was able to achieve higher concentrations when produced using commercial fertilisers as the nutrient source (p<0.05). Results obtained for biomass productivity were in line with those obtained for biomass concentration. No major differences were observed between the productivity obtained when the strains were produced in standard medium and in medium formulated using pig slurry. Again, when the cyanobacteria were produced using wastewater or commercial fertilisers, biomass productivity was negatively affected (Figure 1B; p<0.05).

The F_v/F_m values of the cultures are shown in Figure 1C. This parameter is a non-invasive measurement of the activity of the photosystem II and the high sensitivity of the photosystem II to abiotic and biotic factors made the F_v/F_m value a common technique to assess how photosynthetic organisms respond to environmental conditions [21]. The optimal F_v/F_m value for any microalgal strain is 0.6-0.7, while the optimal value is around 0.5 for cyanobacteria. Lower values indicate that the culture is being subjected to certain stress condition. In the current study, F_v/F_m values in *Anabaena* sp. cultures were lower in all cases when compared to the standard medium (p<0.05), suggesting that the culture was subjected to some kind of stress when produced using pig slurry, wastewater, or commercial fertilisers instead of analytical grade chemicals. Still, no negative effects on biomass productivity were observed when the biomass was produced using pig slurry as the source of nutrients. Although the same trend was observed for *Dolichospermum* sp. cultures, differences were not statistically significant.

The first goal of the current study was to identify the potential of producing *Anabaena* sp. and/or *Dolichospermum* sp. using either wastewater or pig slurry. Pig slurry allowed to achieve

comparable biomass productivities when compared to the standard medium. A secondary goal was to assess the nitrogen and phosphorous removal capacity of both strains as both nitrogen and phosphorous are the main nutrients of concern in eutrophication and are limiting factors in most growth scenarios [22]. Knowing the consumption rate of nitrogen and phosphorous is important as the outlets of microalgae photobioreactors cannot exceed 1-2 $mg \cdot L^{-1}$ of phosphorous and 10-15 of $mg \cdot L^{-1}$ of nitrogen according to Spanish regulations [23]. Therefore, this information is useful to design a suitable process that allows to meet strong regulations. Figure 2 shows the nitrogen and phosphorous concentration in the inlets and outlets of the photobioreactors when the biomass was produced using pig slurry as the sole nutrient source. The content of inorganic nitrogen, calculated as the sum of $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$, was lower in the outlets than in the inlets for both strains (p<0.05) achieving a 70.5 and 63.3% removal for *Anabaena* sp. and *Dolichospermum* sp., respectively. It is important to highlight that other nitrogen sources such as nitrite or urea could be present in the pig slurry and therefore a more in-depth analytical determination of the effluents would be interesting. The main nitrogen source in the inlets was $N\text{-}NH_4^+$. $N\text{-}NH_4^+$ removal values were higher than 90% in both cases and were responsible for the high total inorganic nitrogen removal. The high $N\text{-}NH_4^+$ removal efficiency was caused not only by microalgal growth but also by the action of nitrifying bacteria, as the $N\text{-}NO_3^-$ concentration in the outlets was higher than in the inlets for both strains (p<0.05). Nitrification is the biological oxidation of ammonia to nitrite followed by oxidation of nitrite to nitrate by ammonia-oxidising and nitrite-oxidising bacteria respectively. This phenomenon has been reported in microalgae-based wastewater treatment processes previously. Most of the $N\text{-}NH_4^+$ was assimilated by the cyanobacteria to produce biomass, probably caused by the conditions of the reactors that favoured cyanobacterial growth. In terms of $P\text{-}PO_4^{3-}$, the concentration in the outlets was lower than in the inlets (p<0.05) suggesting that approximately 30.3 and 43.1% of the $P\text{-}PO_4^{3-}$ present in the media was assimilated by *Anabaena* sp. and *Dolichospermum* sp. to produce biomass. A mass balance conducted to the system, assuming a phosphorus content in the biomass of 1%, revealed that all the phosphates that entered the system were either used for the production

of biomass or let unused in the outlet effluent. Results suggested that no phosphorous precipitation occurred and the good pH control of the system.

3.2 Process up-scaling: outdoor pilot-scale trials

When produced outdoors using open raceway bioreactors, biomass productivity is highly influenced by environmental conditions, namely solar radiation and temperature [11]. Figure 3 shows the maximum, minimum, and average temperature and solar radiation values during the current study. Briefly, average and maximum solar radiation values varied within 636.2-1298.6 and 1986.9-2598.3 $\mu E \cdot m^{-2} \cdot s^{-1}$. Maximum and minimum environmental temperature values were 27.5-33.7 and 16.3-19.5 °C respectively, with average daily temperatures of 20.2-25.6 °C. These values are suitable for the production of both *Anabaena* sp. [24] and *Dolichospermum* sp. [25]. In the current study, biomass production outdoors was conducted within approximately 30 days and temperature variations were not significant. However selecting a robust strain that can resist larger seasonal variations is of key importance when microalgae are produced outdoors as the temperature of the culture can reach 35-40 °C in summer in Almería, Spain [10]. One of the advantages of producing cyanobacteria is that these microorganisms are (generally) better adapted to higher temperatures than eukaryotic microalgae and can potentially resist higher culture temperatures [25]. Values of pH were also online monitored during the whole duration of the study with values being recorded every 1 s. The minimum and maximum pH values recorded during the study were 6.8 and 9.1, demonstrating the good pH control of the system. A good pH control is a critical factor when producing microalgae: the pH influences microalgal growth and an inadequate pH control can lead to the precipitation of phosphorous and other inorganic nutrients, limiting nutrient availability.

Besides environmental conditions, one of the most relevant operational condition influencing biomass productivity is the dilution rate imposed. The most commonly used dilution rates for raceway reactors in the south of Spain are 0.1-0.3 day^{-1} being generally 0.2-0.3 day^{-1} the optimum in spring/summer [10]. The optimum dilution rate depends not only on the reactor

design and environmental conditions but also on the produced strain. The current study aimed to identify the optimum dilution rate for both *Anabaena* sp. and *Dolichospermum* sp. In this sense, the effect of the dilution rate imposed on biomass productivity and nutrient removals is shown in Figure 4. Briefly, the maximum productivity was achieved for both strains when operating at a dilution rate of 0.3 day^{-1}. *Dolichospermum* sp. was more productive than *Anabaena* sp., except when operating at a dilution rate of 0.1 day^{-1}, when both achieved an approximate productivity of 10 $g \cdot m^{-2} \cdot day^{-1}$. These results were obtained using pig slurry as the sole nutrient source. It would be interesting to assess the productivity of these strains when produced using freshwater and fertilisers as the nutrient sources at pilot-scale. In the current study, no differences were observed between F_v/F_m values, which were lower than the optimum for cyanobacteria (0.5) suggesting that the cultures were subjected to some kind of stress factor. Still, these relatively low F_v/F_m values did not affect biomass productivity which was relatively high for a raceway reactor. Values were higher than those obtained when producing *Scenedesmus* sp. and *Chlorella* sp. in a 11 m^2 (1,500 L) raceway reactor operated at a dilution rate of 0.3 day^{-1} (4.2 $g \cdot m^{-2} \cdot day^{-1}$) [26]. Data on the productivity of cyanobacteria in raceway reactors is limited, most of the studies conducted using *Arthrospira* species [27–29]. Results were in line with those reported when producing the halophilic diatom *Amphora* sp. (24.0 $g \cdot m^{-2} \cdot day^{-1}$) [30] and eukaryotic microalgae such as *Scenedesmus* sp. in a 63 m^2 raceway reactor with a culture depth of 0.13 m (21.5 $g \cdot m^{-2} \cdot day^{-1}$) [10] and in a 100 m^2 raceway reactor with a culture depth of 0.15 m (25.0 $g \cdot m^{-2} \cdot day^{-1}$) [31].

Indoor trials demonstrates that the consortia microalgae-bacteria could remove a large percentage of the inorganic nitrogen (70.5 and 63.3% for *Anabaena* sp. and *Dolichospermum* sp. cultures, respectively) and phosphorous (30.3 and 43.1% for *Anabaena* sp. and *Dolichospermum* sp. cultures, respectively) present in pig slurry. Results, shown in Figure 4, were comparable to those observed indoors demonstrating the robustness of the system and of the selected strains. The inlet content of $N\text{-}NH_4^+$, $N\text{-}NO_3^-$, and $P\text{-}PO_4^{3-}$ varied between 97.2-156.7, 0.1-4.8, and 4.4-10.9 $mg \cdot L^{-1}$, respectively. Consumption of $N\text{-}NH_4^+$ varied within 1,714-

2,659 $mg \cdot m^{-2} \cdot day^{-1}$ in cultures of *Anabaena* sp. and within 1,604-3,800 $mg \cdot m^{-2} \cdot day^{-1}$ in cultures of *Dolichospermum* sp., with higher values in *Dolichospermum* sp. cultures when the reactor was operated at a dilution rate of 0.3 day^{-1} ($p<0.05$). *Dolichospermum* sp. was able to consume 92.5-95.8% of the $N\text{-}NH_4^+$ present in the medium when produced at 0.1-0.2 day^{-1}. The higher $N\text{-}NH_4^+$ content in the inlet when the system was operated at 0.3 day^{-1} led to a removal of 55.5% of the $N\text{-}NH_4^+$. Cultures of *Anabaena* sp. led to a removal of 62.6-75.2% of the $N\text{-}NH_4^+$, which was in line with the results reported indoors. Not all the $N\text{-}NH_4^+$ consumed was used for microalgal growth, as the content of $N\text{-}NO_3^-$ in the outlets was higher than in the inlets suggesting that nitrification occurred cause by nitrifying bacteria. Part of the $N\text{-}NH_4^+$ could have been lost by desorption (stripping). Indeed, a mass balance conducted to the system (assuming a nitrogen content of the biomass of 10%) revealed that approximately 300-400 and 100-800 $mg \cdot m^{-2} \cdot day^{-1}$ of $N\text{-}NH_4^+$ were stripped into the atmosphere during the production of the *Anabaena* sp. and *Dolichospermum* sp. respectively. No clear trend was observed between the dilution rate imposed and the amount of $N\text{-}NH_4^+$ that was desorbed when producing *Anabaena* sp., mainly because of the different $N\text{-}NH_4^+$ concentration in the inlets and the presence of other nitrogen sources that were not considered in the current study. Moreover, cyanobacteria produce nitrogenase, which is an enzyme involved in nitrogen fixation by reducing atmospheric nitrogen to ammonia [32] and part of the $N\text{-}NH_4^+$ could have been produced by the microalgae. In the case of *Dolichospermum* sp., higher dilution rates led to a higher amount of $N\text{-}NH_4^+$ stripped out into the atmosphere, probably because of the larger volume of slurry processed when operating at higher dilution rates. Results were lower than those reported in a previous study using microalgae to process pig slurry. In that study, the authors used a 3 L reactor operated at its optimal temperature and illumination and the inocula, containing *Chlorella vulgaris* and *Scenedemus obliquus*, was obtained from a photobioreactor processing pig slurry. Collecting the inocula from a photobioreactor operated using pig slurry suggests that the strains were already adapted to the medium [18]. In terms of $P\text{-}PO_4^{3-}$ removal, *Anabaena* sp. cultures efficiently removed 52-58% of the $P\text{-}PO_4^{3-}$ content of the medium, which is in line with that observed indoors. $P\text{-}PO_4^{3-}$ removal rates varied within

75.3-81.2 $mg \cdot m^{-2} \cdot day^{-1}$ in cultures of *Anabaena* sp. and 73.4-86.7 $mg \cdot m^{-2} \cdot day^{-1}$ in cultures of *Dolichospermum* sp. Operating at higher dilution rates led to a higher removal of $N\text{-}NH_4^+$ and $P\text{-}PO_4^{3-}$, caused by a larger volume of water processed per day and to a higher biomass productivities.

3.3 Microbial community composition and structure

One of the main limitations of open reactors such as those used herein is that the culture is exposed to the environment and therefore to biological contaminants such as rotifers, ciliates, amoebae, or bacteria [8]. These can cause a poor control of the process, limit microalgal growth or even cause a culture crash within a few days [33]. It is not possible to produce a mono-culture in raceway reactors especially if microalgae are produced using pig slurry as the nutrient source. Although microalgae and bacteria generally form a stable and beneficial consortia, antagonistic relationships between microalgae and bacteria (competition for nutrients, production of algicidal compounds, or direct lysis of algal cells) have been reported [34]. Moreover, the dominant species in steady state microalgal cultures are those that grow the fastest, and controlling microalgae that outcompete the target specie is a challenge. For this reason, the current study assessed the microbial populations present in the culture.

Results, shown in Figure 5, represent the average abundance of three reactors operated simultaneously under the same environmental conditions. The analysed biomass was collected during the steady-state phase. A total of 7 phyla were detected. The most dominant phyla identified in both *Anabaena sp.* and *Dolichospermum* sp. cultures were Cyanobacteria, Bacteroidetes, and Proteobacteria. Cyanobacteria phyla was dominant with a relative abundance of 55 and 61% in *Anabaena sp.* and *Dolichospermum* sp. cultures, respectively. Results suggested that both *Anabaena sp.* and *Dolichospermum* sp. were successful as predominant strains when produced outdoors under real environmental conditions. The validation of these results during an annual production would be interesting as different environmental and operational conditions can affect the composition of the microalgae-bacteria consortia [35]. Different abundance of Bacteroidetes and Proteobacteria phyla was

detected between *Anabaena sp.* and *Dolichospermum* sp. cultures (p<0.05). Bacteroidetes phyla represented the 26 and 11% of the total reads in *Anabaena sp.* and *Dolichospermum* sp. cultures, respectively, while the Proteobacteria phyla showed a relative abundance of 17 and 27%, respectively. Proteobacteria and Bacteroidetes have been described as the most predominant phyla in various wastewater treatment plants [36] and as the main phyla (together with Firmicutes) in pig manure and slurry samples collected from commercial pig farms in South Korea [37]. Obligate anaerobic bacteria belonging to the genus Bacteroides, phyla Bacteroidetes, as well as Firmicutes are the dominant bacteria in the human and animal gastrointestinal tract. It is important to highlight that although the majority of the bacterial species of the phylum Bacteroides are crucial for healthy gut functions in humans and animals, some species can be pathogenic to humans [38].

To take a closer look at the microbial community structure under different experimental set-ups, the genetic data was further analysed and compared at genus level. *Anabaena sp.* and *Dolichospermum* sp. cultures presented GpI as the dominant genus, with a relative abundance higher than 50% in both cases (55 and 59% for *Anabaena* sp. and *Dolichospermum* sp., respectively). In the biomass of *Anabaena sp*., the second and third most abundant genera were Gaetbulibacter (13%), only present at 3% in *Dolichospermum* sp. cultures, and Lewinella (5%) also detected in *Dolichospermum* sp. (3%). Data on the role of microorganisms of the Gaetbulibacter genus is scarse. This genus was described as a new member of the family Flavobacteriaceae to accommodate Gram-negative, yellow-pigmented, non-flagellated and rod-shaped cells devoid of flexirubin-type pigments. Some species from this genus are chemoheterotrophic and obligated aerobic species [39]. Their presence in cyanobacterial cultures can be attributed to the high content of dissolved oxygen in these systems and the high amount of organic matter in pig slurry. Members of the Lewinella genus, belonging to the family Saprospiraceae play a role in the breakdown of complex organic compounds in the environment. These organisms are frequently found in activated sludge wastewater treatment systems [40,41].

Results demonstrated the presence of possible pathogens at genus level, probably introduced into the system through pig slurry. Legionella (0.2%), Vibrio (0.14%), Clostridium (0.1%) and Bordetella (0.1%) were found in samples of *Anabaena* sp. and Vibrio genus was detected in *Dolichospermum* sp. samples (0.8%). Despite their low abundance, the presence of pathogenic bacteria could be a problem if the produced biomass is used for feed production or as an agricultural product that will be in contact with food. For example, Clostridium and Vibrio can cause diseases in the intestines and are indications of faecal contamination [42]. The genus Enterobacter was not detected in any sample. The presence of possible pathogens in the microalgal biomass should be evaluated as a function of the biomass' applications. Further studies will include the assessment of the origin (water, slurry, environmental, etc) of the different microorganisms identified as this information could allow developing strategies to avoid or promote their presence in the produced biomass. Inactivation of bacteria by means of a thermal treatment or any other non-thermal technology (high pressure processing, sonication, pulsed electric fields, etc.) will be needed before the utilisation of the produced biomass in field trials. The additional advantage of these technologies is that they also disrupt the cell wall of microalgae allowing the release of the intracellular content into the medium and therefore, facilitate the availability of the bioactive compounds contained inside microalgal cells. It is important to highlight that for feed applications, dried algal biomass is used and the heat treatment that is applied during the extrusion process further allows to reduce bacterial load. This would probably eliminate the presence of pathogens, although this would need to be assessed *in vitro*. The use of microalgal biomass produced in biorefineries processing wastewater does not seem to particularly affect the microbiological quality of aquafeeds, except for the presence of *Clostridium perfrigens* spores which need to be further investigated [43].

For the eukaryotic community, the most abundant genus in *Anabaena* sp cultures was *Chlorella* (51.2%) followed by *Cafeteria* (20.9%), while the genus *Euplotes* (65.1%) followed by *Chlorella* (24.4%) was the most abundant in cultures of *Dolichospermum* sp. Microalgae

and cyanobacteria can be transported in aerosols [44] and a recent study demonstrated that *Chlorella* strains are present in varied aerosols that were collected on land and at the sea [45]. Both reactors were located next to larger reactors that were being used to produce *Chlorella*. Thus, the presence of *Chlorella* species in both cultures suggests that the cultures could have been contaminated by this other larger reactor and that further research on the aerosols surrounding microalgae reactors are necessary to prevent potential diseases and avoid unwanted contaminations.

Conclusions

Within the present study both, *Anabaena* sp. and *Dolichospermum* sp. could be produced outdoors using pig slurry as the sole nutrient source. Nitrogen and phosphorous removal values were comparable to those reported for other microalgal strains and biomass productivity values were in line with those expected for a raceway reactor. Both strains managed to be the predominant bacteria in the reactors. However, longer studies are needed to see the effect of environmental and operational conditions on the composition of the microalgae-bacteria consortia. Other prokaryotic and eukaryotic microorganisms were detected in the culture including human pathogens that were probably introduced into the reactors though pig slurry. Species of the genus *Chlorella* were also detected, and their presence was attributed to aerosols generated in a large reactor located nearby. Because of the presence of other contaminating prokaryotic and eukaryotic microorganisms in the culture, it would be interesting to assess the evolution of the community and determine the potential of both strains for being the predominant strains during one complete year. Long-term studies are scarce and necessary, and studies on this topic are ongoing.

Acknowledgements

This work forms part of the SABANA Project funded by the EU H2020 Research and Innovation Framework Programme (Grant Agreement 727874). Ana Sánchez-Zurano thanks the Spanish Ministry of Education (FPU16/05996). Tomas Lafarga thanks the Spanish Ministry of Science, Innovation, and Universities (IJC2018-035287-I) and the BBVA Foundation for the 2020 Leonardo Grant for Researchers and Cultural Creators.

CRediT authorship contribution statement

Morillas-España, A.: Investigation, Formal analysis, Writing – original draft; **Sánchez-Zurano, A.:** Investigation, Formal analysis; **Gómez-Serrano, C.:** Investigation, Formal analysis; **Ciardi, M.:** Investigation, Formal analysis; **Acién, G.:** Supervision, Funding Acquisition; **Clagnan, E.:** Investigation; **Adani, F.:** Investigation; **Lafarga, T.**: Visualization, Formal analysis, Writing – original draft.

Declaration of competing interest

Authors have no conflicts to declare

Statement of informed consent, human/animal rights

No conflicts, informed consent, human or animal rights applicable.

Figure legends

Figure 1. Effect of culture media on (A) biomass concentration, (B) biomass productivity, and (C) F_v/F_m values. Different capital letters indicate differences between culture media and different lower case letters indicate differences between strains (p<0.05). All the samples were collected and analysed in triplicate during the steady state.

Figure 2. Inlet and outlet concentration of (A) total inorganic nitrogen, (B) N-NH_4^+, (C) N-NO_3^-, and (D) P-PO_4^{3-}. Different capital letters indicate differences between strains and different lower case letters indicate differences between inlets and outlets (p<0.05). All the samples were collected and analysed in triplicate during the steady state.

Figure 3. (A) Solar radiation that reached the culture, (B) environmental temperature, and (C) pH of the culture.

Figure 4. Effect of dilution rate on (A) biomass productivity and (B) N-NH_4^+, (C) N-NO_3^-, and (D) P-PO_4^{3-} inlet concentration and removal capacity. All the samples were collected and analysed in triplicate during the steady state.

Figure 5. Taxonomic classification of prokaryotic communities at the (A) phylum level and (B) genus level assessed in the biomass produced operating the reactors at a dilution rate of 0.3 day^{-1}

Figure 1

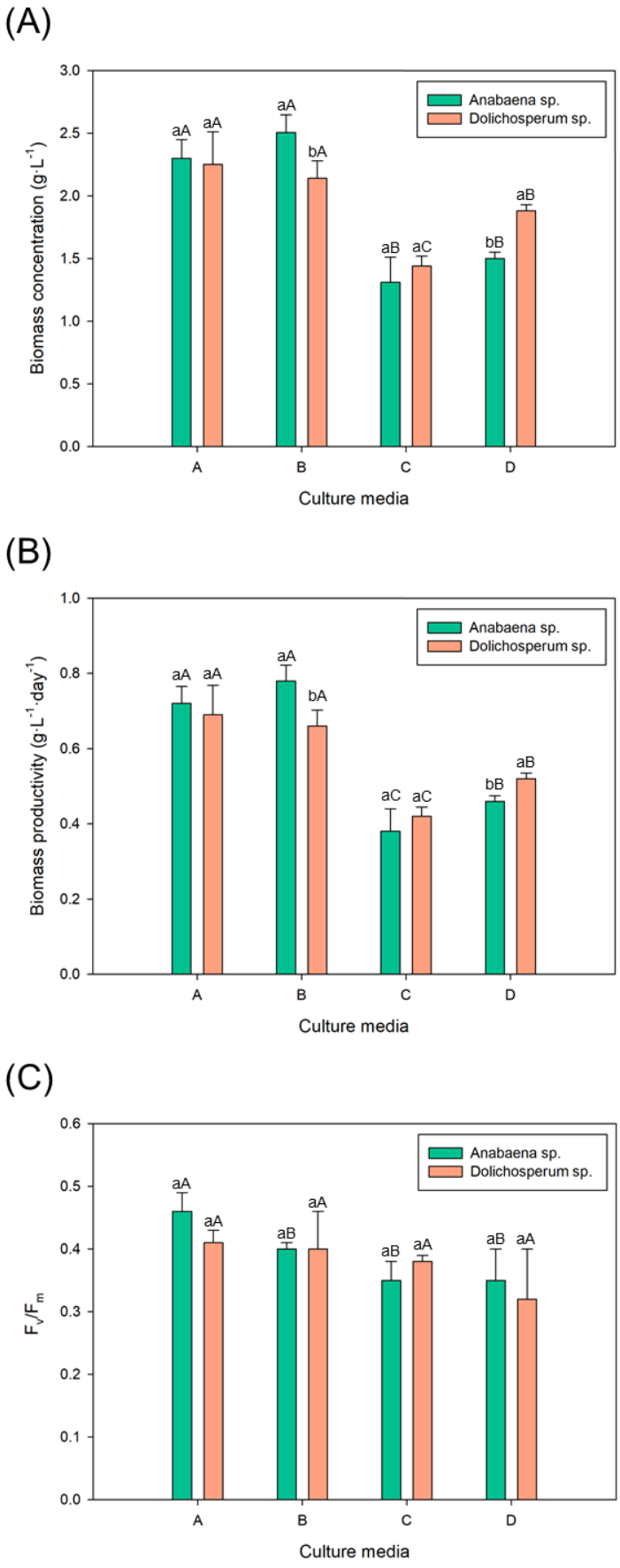

(A)
Biomass concentration (g·L-1)
Anabaena sp.
Dolichosperum sp.
aA aA aA bA aB aC bB aB
A B C D
Culture media
(B)
Biomass productivity (g·L-1·day-1)
Anabaena sp.
Dolichosperum sp.
aA aA aA bA aC aC bB aB
A B C D
Culture media
(C)
Fv/Fm
Anabaena sp.
Dolichosperum sp.
aA aA aB aA aB aA aB aA
A B C D
Culture media

Figure 2

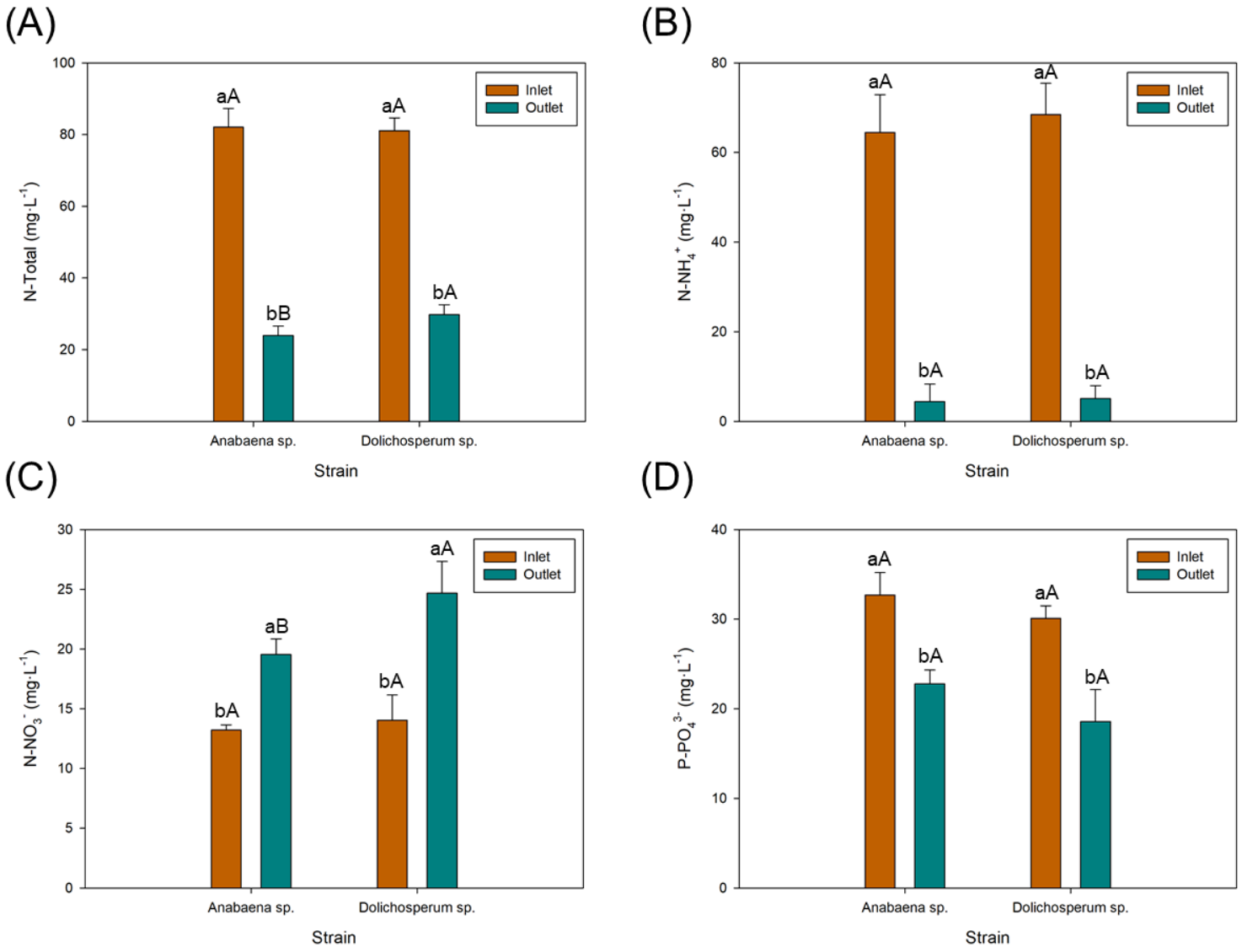

(A)
N-Total (mg·L-1)
100
80
60
40
20
0
aA
bB
aA
bA
Inlet
Outlet
Anabaena sp.
Dolichosperum sp.
Strain
(B)
N-NH4+ (mg·L-1)
80
60
40
20
0
aA
bA
aA
bA
Inlet
Outlet
Anabaena sp.
Dolichosperum sp.
Strain
(C)
N-NO3- (mg·L-1)
30
25
20
15
10
5
0
bA
aB
bA
aA
Inlet
Outlet
Anabaena sp.
Dolichosperum sp.
Strain
(D)
P-PO43- (mg·L-1)
40
30
20
10
0
aA
bA
aA
bA
Inlet
Outlet
Anabaena sp.
Dolichosperum sp.
Strain

Figure 3

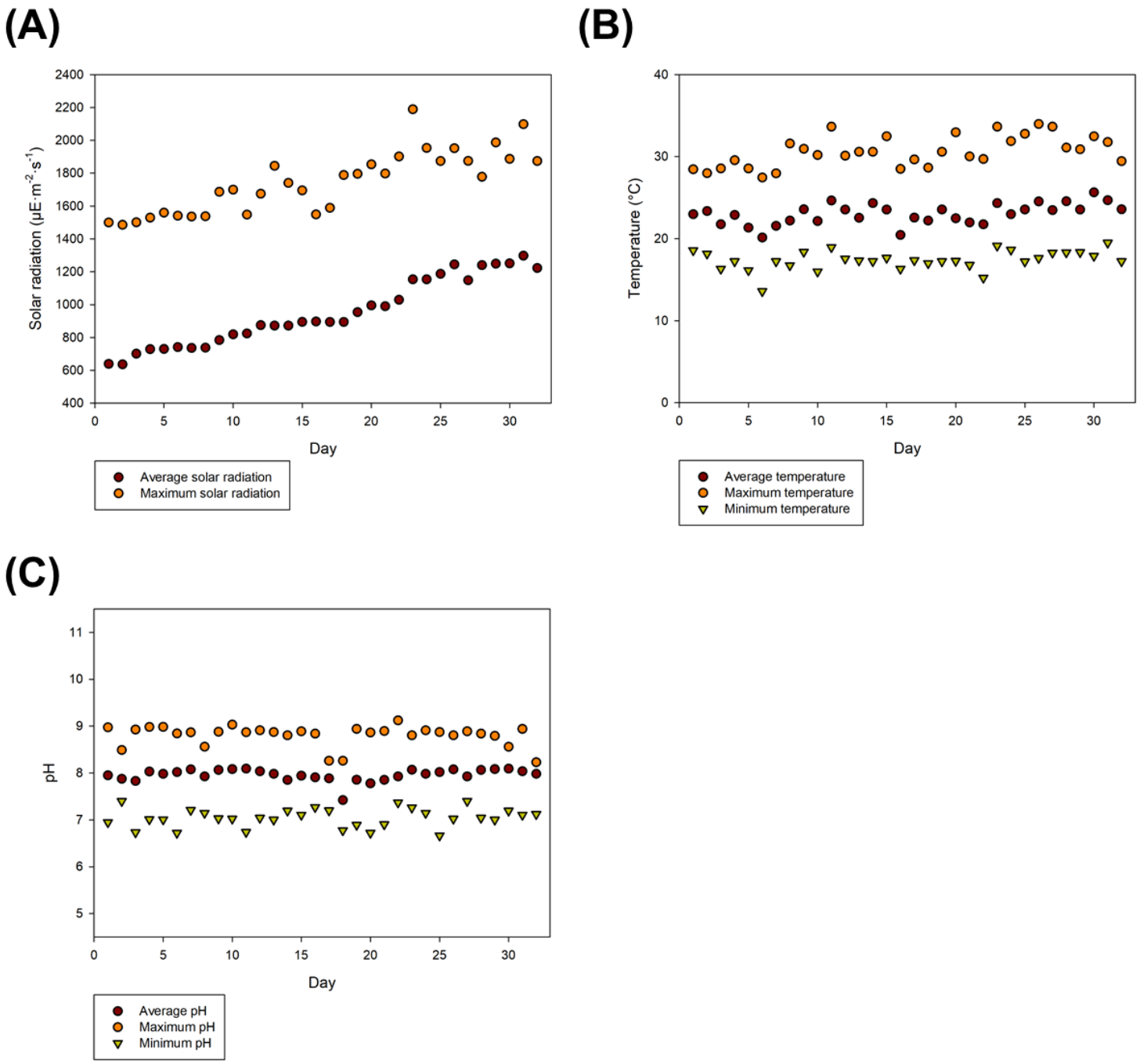

(A)
Solar radiation (μE·m-2·s-1)
2400
2200
2000
1800
1600
1400
1200
1000
800
600
400
0
5
10
15
20
25
30
Day
Average solar radiation
Maximum solar radiation
(B)
Temperature (°C)
40
30
20
10
0
0
5
10
15
20
25
30
Day
Average temperature
Maximum temperature
Minimum temperature
(C)
pH
11
10
9
8
7
6
5
0
5
10
15
20
25
30
Day
Average pH
Maximum pH
Minimum pH

Figure 4

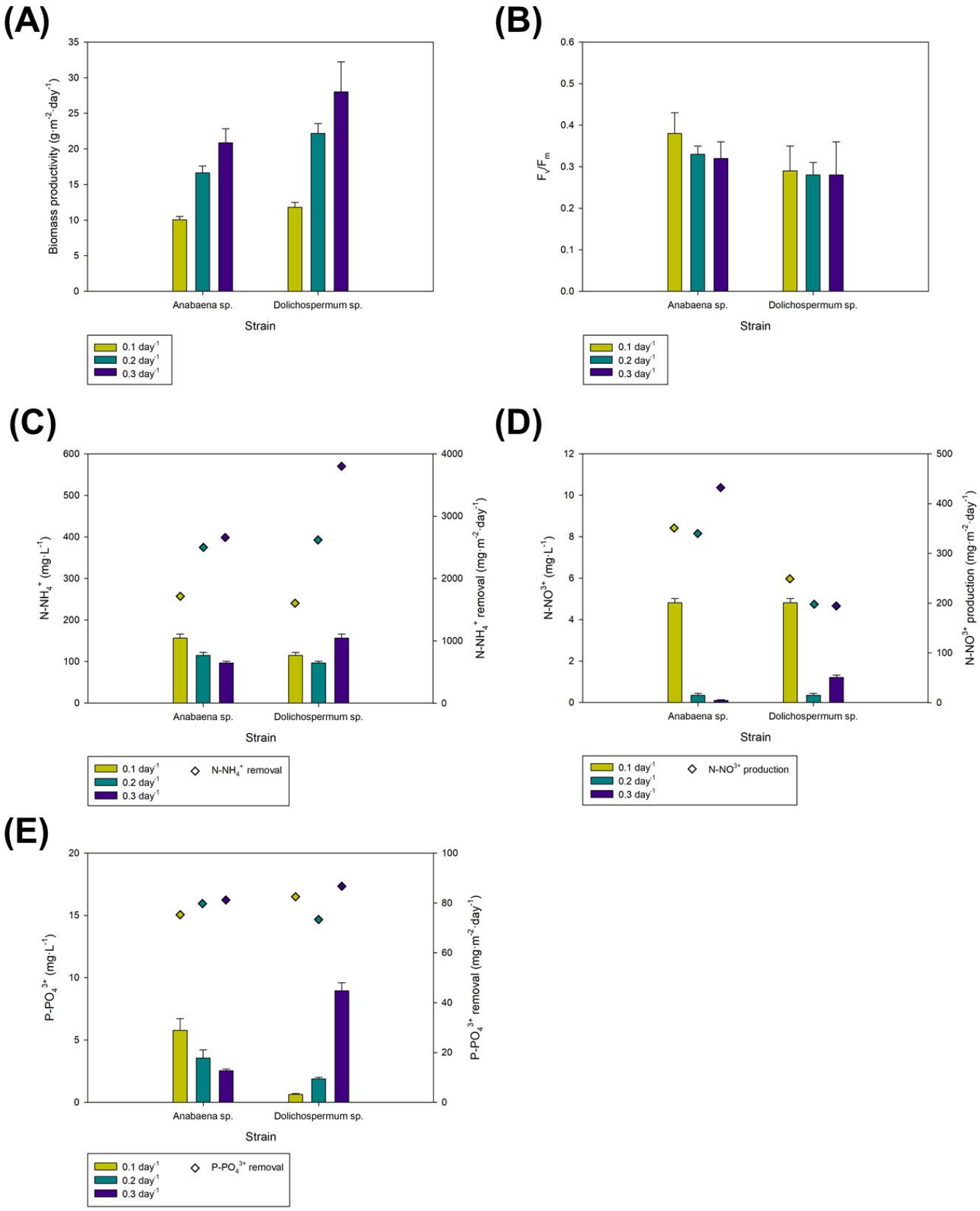
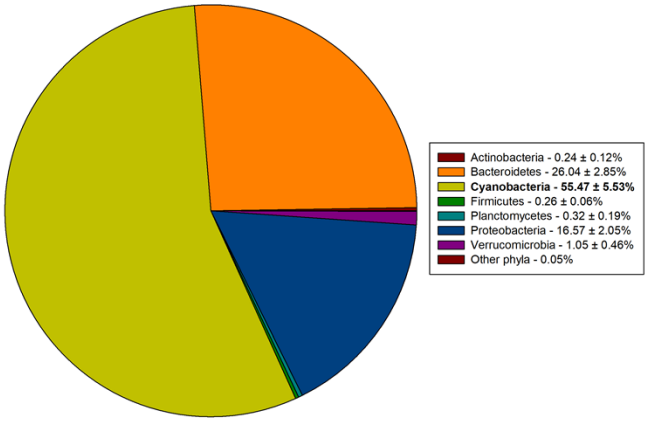

(A)
Biomass productivity (g·m-2·day-1)
Anabaena sp.
Dolichospermum sp.
Strain
0.1 day-1
0.2 day-1
0.3 day-1
(B)
FV/Fm
Anabaena sp.
Dolichospermum sp.
Strain
0.1 day-1
0.2 day-1
0.3 day-1
(C)
N-NH4+ (mg·L-1)
N-NH4+ removal (mg·m-2·day-1)
Anabaena sp.
Dolichospermum sp.
Strain
0.1 day-1
0.2 day-1
0.3 day-1
N-NH4+ removal
(D)
N-NO3+ (mg·L-1)
N-NO3+ production (mg·m-2·day-1)
Anabaena sp.
Dolichospermum sp.
Strain
0.1 day-1
0.2 day-1
0.3 day-1
N-NO3+ production
(E)
P-PO43+ (mg·L-1)
P-PO43+ removal (mg·m-2·day-1)
Anabaena sp.
Dolichospermum sp.
Strain
0.1 day-1
0.2 day-1
0.3 day-1
P-PO43+ removal

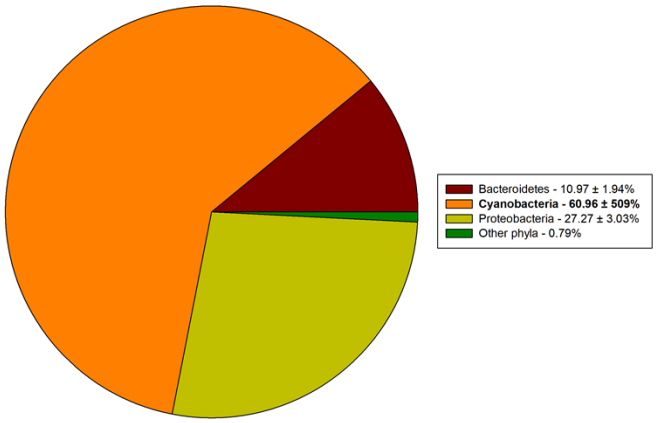
Figure 5

(A)

Anabaena sp.

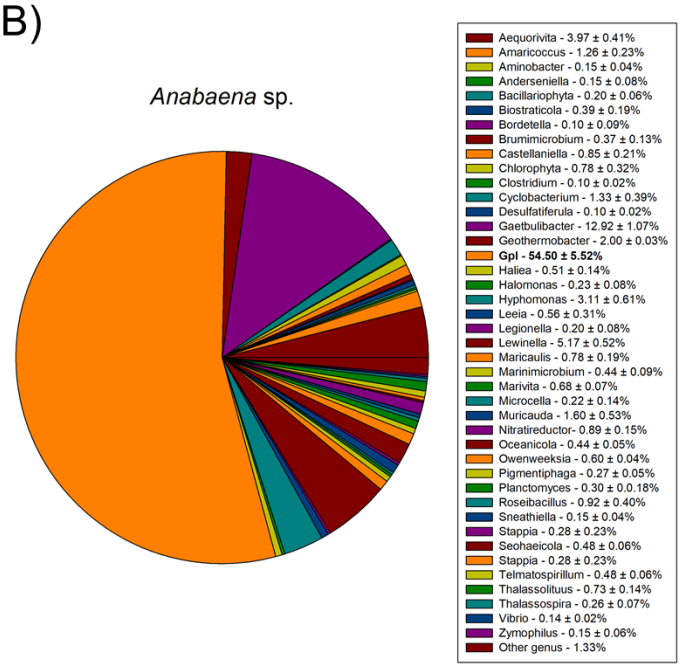


Dolichospermum sp.

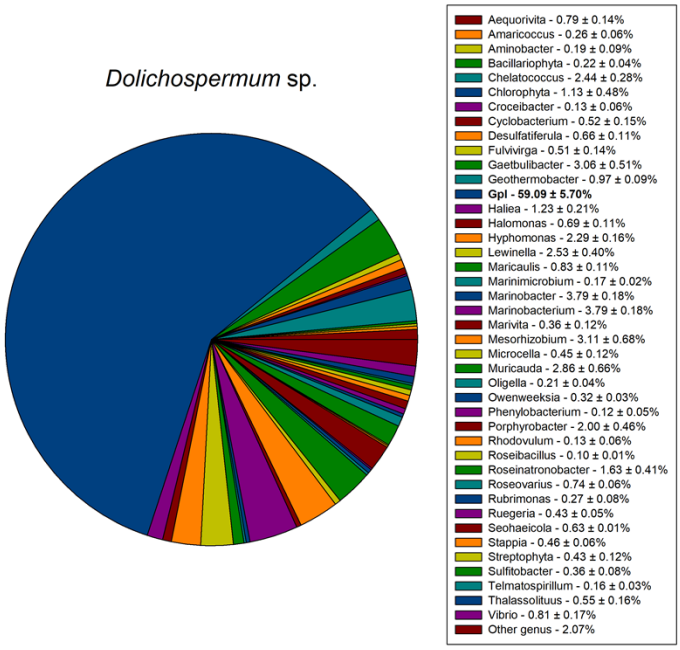


(B)

Anabaena sp.



Dolichospermum sp.



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