Optimisation of *Scenedesmus almeriensis* production using pig slurry as the sole nutrient source

Martina Ciardi [1,2], Cintia Gómez-Serrano [1,2], María del Mar Morales-Amaral [1], Gabriel Acién [1,2], Tomas Lafarga [1,2*], & José María Fernández-Sevilla [1,2].

[1] Department of Chemical Engineering, University of Almería, 04120, Almería, Spain

[2] CIESOL Solar Energy Research Centre, Joint Centre University of Almería-CIEMAT, 04120, Almería, Spain

Corresponding author: lpt365@ual.es

Abstract

Pig slurry was used as the sole nutrient source to produce biomass of the microalga *Scenedesmus almeriensis* using laboratory-scale bubble column photobioreactors. No differences in terms of biomass productivity were observed between fresh and digested pig slurry. The optimum dilution rate to enable the processing of the largest amount of pig slurry per litre was 5%, which led to a biomass productivity rate of 0.68 $g \cdot L^{-1} \cdot day^{-1}$, comparable to that of the standard growth medium formulated using pure chemicals (0.70 $g \cdot L^{-1} \cdot day^{-1}$). The inlet $N\text{-}NH_4^+$ concentration was approximately 180 $mg \cdot L^{-1}$, with inhibitory effects being observed at concentrations higher than 200 $mg \cdot L^{-1}$. The $N\text{-}NH_4^+$, $N\text{-}NO_3^-$, $P\text{-}PO_4^{3-}$, and chemical oxygen demand removal rates were 34.1, 0.5, 2.1, and 519.3 $mg \cdot L^{-1} \cdot day^{-1}$. The observed turbidity of the media did not affect microalgal growth at the studied dilutions. Ozonation effectively reduced the chemical oxygen demand concentration of the media, but no effect on microalgal growth was observed. Overall, pig slurry was an effective nutrient source for the mass production of *S. almeriensis*, and this strain was shown to be robust with a $N\text{-}NH_4^+$ tolerance up to 200 $mg \cdot L^{-1}$ and the potential use in the bioremediation of agro-industrial wastes.

Keywords: bioremediation, microalgae, nutrient removal, nitrogen, phosphorus, biomass

1. Introduction

Eutrophication is a serious pollution problem mainly caused by large amounts of nutrients, principally nitrogen and phosphorus, emanating from agricultural sources. Pig slurry management is of great importance and a key challenge in intensive livestock production areas. The current system consists of using slurry directly on agricultural land. However, current legislation limits its direct use to 170 $kg_N \cdot ha^{-1} \cdot year^{-1}$ in the EU [1]. In many regions, slurry production often exceeds the capacity of the land to assimilate it [2]. Its storage involves significant amounts of greenhouse gas emission and utilising it in agriculture contributes to nitrogen and phosphorus accumulation in groundwater [3].

Pig slurry is rich in nitrogen (essentially $N\text{-}NH_4^+$) and phosphorus (mainly $P\text{-}PO_4^{3-}$) as well as organic matter and minerals such as calcium and magnesium [3]. These nutrients, along with carbon, are the main nutrients needed for microalgae production; hence utilising pig slurry as the nutrient source for microalgal biomass production is currently being investigated. Microalgae biotechnology is a relatively new research area that, despite having increased exponentially over recent years, is still limited by high production costs [4]. Nutrients contribute significantly to the total production costs, so using wastewater or other agricultural wastes as nutrient sources has been suggested as a feasible way to render the process viable [5]. The microalgal biomass produced can be used for high-value applications such as the production of agricultural products (biostimulants and biofertilisers) or animal feeds (mainly aquafeeds). This strategy has another added benefit in reducing the amount of nutrients that end up in the receiving water bodies, thus making the process more sustainable.

The mass production of microalgae in controlled facilities using pig slurry as the nutrient source has been shown to be an excellent strategy for reducing pig slurry ecotoxicity and minimising biomass production costs. Previous studies have reported total inorganic nitrogen and phosphorus removal rates of 14.4-59.5 and 1.4-5.2 $mg \cdot L^{-1} \cdot day^{-1}$, respectively [6]. One of the main pig slurry limitations is that it contains high $N\text{-}NH_4^+$

concentrations. Although $N\text{-}NH_4^+$ is the preferred nitrogen source for most microalgae and cyanobacteria [7], concentrations higher than 160 $mg \cdot L^{-1}$ have been shown to inhibit microalgal growth [8]. Therefore, pig slurry must be diluted prior to microalgae production, according to the initial $N\text{-}NH_4^+$ content – this reduces the amount of waste that can be processed per surface area of land. The optimum dilution rate will depend on the initial $N\text{-}NH_4^+$ content and on the strain used, since some strains are more tolerant to $N\text{-}NH_4^+$ [9]. Moreover, digestate and animal manure may also contain heavy metals, several different organic pollutants, and antibiotic residues that can negatively affect microalgal growth or the composition of the microalgae-bacteria consortia [10]. This highlights the need for selecting the optimum dilution rate to avoid toxic effects on microalgal growth. Another challenge of using pig slurry as the nutrient source for microalgae production is its turbidity [11], which varies greatly depending on the source of the slurry and the dilution used.

The present study assessed the potential utilisation of pig slurry as the sole nutrient source in the production of *Scenedesmus almeriensis* (CCAP 276/24), a robust and fast-growing microalga that has the potential use in bioremediation processes [12–14] and in agriculture [15]. Preliminary trials were conducted to identify whether there is a need for an anaerobic digestion step prior to microalgae production. One of the main goals of this study was to determine the slurry dilution that allowed maximal biomass productivity. Moreover, the effects of the $N\text{-}NH_4^+$ concentration and turbidity on microalgal growth were also assessed.

2. Materials and methods

2.1. Microalgal strain used

The microalgal used was *S. almeriensis* (CCAP 276/24), a robust and fast-growing strain that is particularly adapted to stressful conditions. It was initially isolated in a

photobioreactor located inside a greenhouse in Almería (Spain), where it was exposed to high-temperature (45 °C) and irradiance (2,000 µmol photons·m^{-2}·s^{-1}) conditions [16]. This strain can adapt to pH, temperature, and salinity values ranging from 7-10, 26-40 °C, and 0-5 g NaCl·L^{-1}, respectively and can be produced outdoors throughout the year [14,17], thus making it a potential candidate for wastewater or pig slurry treatment processes. The strain was obtained from the culture collection of the Department of Chemical Engineering at the University of Almería. The inocula were maintained at 23 ± 2 °C, pH 8.0 ± 0.1, and 150 µmol photons·m^{-2}·s^{-1} in batch mode using a modified Arnon medium, as described previously [18].

2.2. Microalgae production

The *S. almeriensis* biomass was produced indoors using 300 mL bubble-column photobioreactors with spherical bases (3 cm in diameter and 45 cm in height) filled with 250 mL of culture [19]. The reactors were inoculated with 10% of the culture volume using a standard inoculum at a concentration of 0.4 g·L^{-1}, maintained as described above. Cultures were operated in a batch mode for one week and then in semi-continuous mode using a dilution rate of 0.3 day^{-1} until the total volume of the photobioreactor was replaced at least twice (7-8 days of semi-continuous operation). The temperature, pH, and light levels were monitored and controlled online. The temperature and pH were kept constant at 25.0 °C and 8.0 respectively. The pH was controlled by on-demand injection of carbon dioxide. Illumination was performed using 28 W Philips Daylight T5 fluorescence tubes (Madrid, Spain) placed horizontally, 1 cm away from each other and 4 cm from the culture. The light was programmed to mimic outdoor conditions, progressively increasing in intensity from 08:00 to 15:30 and then progressively decrease from 15:30 to 20:00 h. In the absence of cells, the maximum irradiance inside the photobioreactors was 293, 809, 1419, 1650, 1419, 809, and 293 µmol photons·m^{-2}·s^{-1} in the time frames 08:00-09:30, 09:30-11:00, 11:00-12:30, 12:30-15:30, 15:30-17:00, 17:00-18:30, and 18:30-20:00 respectively. The irradiance inside the photobioreactors in

the absence of cells was measured using an SQS-100 spherical quantum sensor (Walz GmbH, Effeltrich, Germany). Microalgae production was conducted in triplicate using three independent photobioreactors and considering each photobioreactor as a natural replicate unit. The microalgae production and the location of the photobioreactors inside the illuminated chamber were conducted in a random order. Analytical determinations were conducted in triplicate at the end of the experiment, also randomly.

2.3. Pig slurry and composition of the standard medium

Fresh pig slurry was collected from a local farm in Níjar (Almería, Spain). The composition of the fresh samples was 203.8 ± 9.5 $mg \cdot L^{-1}$ $N\text{-}NO_3^-$; 2,741.7 ± 18.2 $mg \cdot L^{-1}$ $N\text{-}NH_4^+$; and 64.7 ± 2.1 $mg \cdot L^{-1}$ $P\text{-}PO_4^{3-}$. The chemical oxygen demand (COD) of the fresh slurry was 20,200.0 ± 65.2 $mg \cdot L^{-1}$. Analytical determinations were carried out in triplicate, as described in the subsections below.

A modified Arnon medium [18] was used as the control standard medium (SM) and was prepared using 0.24 $mg \cdot L^{-1}$ $NaVO_3$, 1.26 $mg \cdot L^{-1}$ $Na_2MoO_4 \cdot 2H_2O$, 2.86 $mg \cdot L^{-1}$ H_3BO_4, 1.81 $mg \cdot L^{-1}$ $MnCl_2 \cdot 4 \cdot H_2O$, 0.22 $mg \cdot L^{-1}$ $ZnSO_4 \cdot 7H_2O$, 0.08 $mg \cdot L^{-1}$ $CuSO_4 \cdot 5H_2O$, 0.04 $mg \cdot L^{-1}$ $CoCl_2 \cdot 6H_2O$, 124.0 $mg \cdot L^{-1}$ $MgSO_4 \cdot 7H_2O$, 15.0 $mg \cdot L^{-1}$ $CaCl_2 \cdot 2H_2O$, 117 $mg \cdot L^{-1}$ NaCl, 19 $mg \cdot L^{-1}$ KOH, 24.9 $mg \cdot L^{-1}$ $FeSO_4 \cdot 7H_2O$, 174.0 $mg \cdot L^{-1}$ $K_2HPO_4 \cdot 7H_2O$, and 850.0 $mg \cdot L^{-1}$ $NaNO_3$. The $N\text{-}NO_3^-$ and $P\text{-}PO_4^{3-}$ contents of the SM were 140.0 and 26.1 $mg \cdot L^{-1}$, respectively, and the N/P ratio was 5.4.

2.4. Culture media opimisation

2.4.1. Preliminary trials: Anaerobic digestion

Preliminary trials were conducted to assess whether an anaerobic digestion step was needed before use in microalgae production. Briefly, fresh and digested pig slurries were diluted using tap water until an $N\text{-}NH_4^+$ concentration of 150 $mg \cdot L^{-1}$ was achieved (10% v/v). The diluted slurries were used as the culture media for microalgae cultivation, as described in the previous subsections.

2.4.2. Identification of the optimal $N\text{-}NH_4^+$ inlet concentration

Fresh pig slurry was used to prepare different culture media by dilution at concentrations of 10.0, 6.6, 5.0, 2.3, and 1.2% using distilled water. The media formulated using these dilutions were labelled as PS-10%, PS-6%, PS-5%, PS-2%, and PS-1%, respectively (where PS stands for pig slurry). These media were used to produce *S. almeriensis* following the procedure and conditions described in the previous subsection. All the media used for microalgae production were filtered using 1 µm Macherey-Nagel filters. The cultures were produced in triplicate as described above using three independent photobioreactors; all the replicates, including those of the SM, were produced under the same conditions and at the same time.

2.4.3. Supplementation of pig slurry using commercial fertilisers

PS-1%, PS-2%, and PS-5% were supplemented using analytical grade chemicals until $N\text{-}NO_3^-$ and $P\text{-}PO_4^{3-}$ concentrations of 140.0 and 40.0 $mg \cdot L^{-1}$, respectively were achieved. The supplemented media were labelled as S-PS1%, S-PS2%, and S-PS5%, where S-PS stands for supplemented pig slurry. Again, these media were used to produce *S. almeriensis* as described above. The cultures were produced in triplicate using three independent photobioreactors; all the replicates, including those of the SM, were produced under the same conditions and at the same time.

2.4.4. Ozonation

The PS-5% culture medium was ozonised over either 30, 60, or 100 min and the resulting media were labelled as PS-5%-I, PS-5%-II, and PS-5%-III respectively. Ozonation was performed using a portable 3060 ozone generator (E. Ecology SL, Madrid, Spain) that produces ozone at a flux of 950 $mg \cdot h^{-1}$. Ozone was injected at the bottom of the reactor via a porous diffuser while a magnetic stirrer ensured good contact between the liquid and gas phases. Experiments were conducted at 23 ± 3 °C in 2 L reactors covered with aluminium foil to avoid radiation losses. The ozonised media were used to produce *S.*

almeriensis as described above. For these trials, a different batch of pig slurry (collected from the same location) was used. The composition of the diluted slurry used for these trials was: 7.7 ± 0.3 mg·L^{-1} N-NO_3^-, 186.2 ± 6.1 mg·L^{-1} N-NH_4^+, 11.4 ± 2.3 mg·L^{-1} P-PO_4^{3-}, and 1,990 ± 16 mg·L^{-1} COD.

The reactors were washed and autoclaved between the different experiments. Biomass was harvested by centrifugation using a Sigma 3-18 KS centrifuge (Sigma Laborzentrifugen GmbH, Osterode am Harz, Germany) operating at 8,000 × *g* for 10 min. The harvested biomass was freeze-dried and stored in a sealed container at -20 ºC until further analysis. Both the biomass and the culture media were analysed as described in the following section.

2.5. Analytical determinations

2.5.1. Biomass concentration

The biomass concentration was calculated by dry weight filtering 25 mL aliquots of the culture through Macherey-Nagel MN 85/90 glass fibre and drying it in an oven at 80 ºC for 24 h. The biomass productivity was calculated as the product of the biomass concentration and the dilution rate (0.3 day^{-1}).

2.5.2. Chlorophyll fluorescence ratio

The chlorophyll fluorescence ratio or maximum potential quantum yield efficiency of photosystem II was determined using an AquaPen AP 100 fluorometer (Photon System Instruments, The Czech Republic).

2.5.3. Average irradiance inside the culture

Absorbance at 400-700 nm was daily measured using a Genesys 10S UV-Vis spectrophotometer (Thermo Fisher Scientific, Spain). The extinction coefficient (k_a) was calculated using the equation:

$$k_a = \frac{Abs}{C_b \cdot p}$$

where Abs is the above-mentioned absorbance, C_b is the biomass concentration, and p is the cuvettes' light path (1 cm). The extinction coefficient was used to calculate the average irradiance inside the culture (I_{av}), and this was calculated as a function of the irradiance on the culture surface (I_0), k_a, C_b, and the light path inside the reactor (p) using the equation:

$$I_{av} = \frac{I_0}{k_a \cdot C_b \cdot p} \cdot (1 - e^{-k_a \cdot c_b \cdot p})$$

2.5.4. Concentration of nutrients

The concentration of ammonium, nitrates, and phosphates at the inlets and outlets of the photobioreactors was measured using standard official methods approved by the Spanish Ministry of Agriculture [20]. Briefly, phosphorus and nitrates were measured spectrophotometrically through the phospho-vanado-molybdate complex and by measuring the absorbance at 220-275 nm using a Genesys 10S UV-Vis spectrophotometer (Thermo Fisher Scientific, Spain). The ammonium concentration was determined using the Nessler reactive method. In addition, the COD was determined spectrophotometrically using HachLange kits (LCK-555). Turbidity was determined according to the ISO 7027 International Standard using an HI 93703 portable turbidity meter (Hanna Instruments, RI, USA). Determinations were conducted in triplicate and expressed as NTU (nephelometric turbidity unit).

2.5.5. Biomass composition

Crude protein was determined by the Kjeldahl method following acid digestion using a nitrogen-to-protein conversion factor of 5.95 [21]. Lipids were extracted following the Folch method using chloroform:methanol (2:1 v/v) as the solvent and the total lipid content was calculated gravimetrically [22]. The ash content was determined by

calcination of the biomass to constant weight in a muffle oven at 600 ºC for 12 h [23]. The carbohydrate content was determined by difference.

The pigment contents, namely chlorophyll-a (Ch_a), chlorophyll-b (Ch_b), and total carotenoids (TC) were estimated by means of the equations proposed by Wellburn [24] using a Genesys 10S UV-Vis spectrophotometer (Thermo Fisher Scientific, Spain). Briefly, 100 mg of freeze-dried biomass were suspended in 40 mL of acetone and sonicated for 10 s using a UP400S ultrasonic processor (Hielscher Ultrasonics GmbH, Germany) operating at 400 W and 24 kHz. The extract was stirred at 150 rpm for 5 min and centrifuged. The supernatant was evaporated under a nitrogen stream, resuspended in methanol and then the pigment concentration was calculated as follows:

$$Ch_a(mg \cdot L^{-1}) = 16.72 \cdot A_{665} - 9.16 \cdot A_{652}$$

$$Ch_b(mg \cdot L^{-1}) = 34.09 \cdot A_{652} - 15.28 \cdot A_{665}$$

$$TC(mg \cdot L^{-1}) = \frac{1000 \cdot A_{470} - 1.63 \cdot Ch_a - 104.96 \cdot Ch_b}{221}$$

where A_{665}, A_{652}, and A_{470} are the optical density of the extract at 665, 652, and 470 nm, respectively. Two technical replicates were conducted per natural replicate and results were expressed on a dry weight basis. The dry matter content was determined by drying in an oven at 105 ºC for 24 h.

2.6. Statistical analysis

The temperature, pH, and illumination levels were controlled, and all the treatments were subjected to the same experimental conditions, except for the culture media (the fixed factor). The normality and homoscedasticity of the variables within each group were initially checked and then the data 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. A bivariate

Pearson's' correlation analysis was conducted to identify relationships between different variables.

3. Results and discussion

3.1. Preliminary trials

Preliminary trials revealed that pig slurry could be used as the sole nutrient source to produce *S. almeriensis* without any pre-treatment (besides filtering through 1 µm filters). These trials were conducted to assess whether an anaerobic digestion step was required before introducing the nutrients into the reactor. Overall, the $N\text{-}NH_4^+$ and $P\text{-}PO_4^{3-}$ contents at the inlets were not affected by the anaerobic digestion process, with average values of 148.8 ± 11.7 and 18.2 ± 2.6 $mg \cdot L^{-1}$, respectively. Anaerobic digestion led to a slight reduction in the $N\text{-}NO_3^-$ content at the inlets from 12.2 ± 0.3 to 10.5 ± 1.1 $mg \cdot L^{-1}$ and to a 2-fold reduction in COD values ($p<0.05$). The observed reduction in the $N\text{-}NO_3^-$ content was not a problem given that when both $N\text{-}NH_4^+$ and $N\text{-}NO_3^-$ are present in the media, microalgae generally prefer the former [7]. No differences were observed in biomass productivity when the microalgae were produced using digested or fresh pig slurry, with biomass productivity values of 0.54 ± 0.04 and 0.50 ± 0.04 $g \cdot L^{-1} \cdot day^{-1}$, respectively. Consequently, the results described from now on were obtained using fresh pig slurry as the main nutrient source. This strategy has the added advantages of simplifying the process and increasing sustainability by reducing the emission of greenhouse gases during digestion.

3.2. Optimum dilution: Effect of $N\text{-}NH_4^+$ on biomass productivity

Fresh pig slurry was diluted at different levels (1.2-10.0%) to identify the maximum amount of slurry that could be processed without negatively affecting the biomass productivity. It is important to highlight that the biomass produced includes both, microalgae and bacteria. It is not possible to produce 100% microalgal biomass as

bacteria and other microorganisms are always present in the medium, especially when using waste streams. The composition of the consortia depends on environmental and operational conditions [12,25] but, if managed properly, over 95% of the biomass produced will be microalgae [26]. The results, shown in Figure 1A, suggest that the pig slurry negatively affected biomass productivity when compared to the SM (p<0.05). The decrease observed in biomass productivity was especially high when it was produced in PS-1%, PS-6%, or PS-10%, meaning pig slurry diluted at 1.2, 6.6, or 10.0%, respectively (p<0.05). The PS-2% and PS-5% media resulted in a lower (but significant) 10-11% decrease in biomass productivity, with productivity values of 0.71 ± 0.02 and 0.70 ± 0.04 $g \cdot L^{-1} \cdot day^{-1}$, respectively. The quantum yield of the cultures was monitored daily. The quantum yield values, shown in Figure 1B, represent a non-invasive measurement of photosystem PSII activity [27]. A decrease in quantum yield indicates that the culture is subjected to stress conditions such as excess light [28], lack of nutrients [29], or the presence of toxins or heavy metals [30]. The quantum yield values were lower for the media formulated using pig slurry than for the SM medium, and the results were in line with the biomass productivities. Given that the cultures' illumination, temperature, and pH monitored and controlled online, low values must have been caused either by a lack of nutrients or by the presence of a toxic or inhibitory compound.

The main nutrients needed for microalgal grown are nitrogen and phosphorus (along with carbon). Figure 2 shows the $N\text{-}NH_4^+$, $N\text{-}NO_3^-$, and $P\text{-}PO_4^{3-}$ concentrations at the reactors' inlets and outlets. In the present study, the molar N/P ratio of the diluted slurries was 27-32, higher than that of the Redfield ratio (16), which is considered to be the optimal [31]. The Redfield ratio is not a universal biochemical optimum but represents an average of species-specific N/P ratios [32]. The N/P ratios calculated here suggest that PS-1% and PS-2% could be phosphorus-limited. The calculated N/P ratios were also higher than those recently reported in Spanish farms (6-9) [33]. It is important to point out that, in that study, the growth phases of the animals (gestating and lactating sows, nursery piglets,

and growing pigs) significantly affected the N/P ratios; however, in the present study only the $N\text{-}NH_4^+$, $N\text{-}NO_3^-$, and $P\text{-}PO_4^{3-}$ concentrations were considered, not the total nitrogen and phosphorus. Although microalgae can use ammonia to produce biomass, high ammonia concentrations lead to toxic effects on them. In our study, the $N\text{-}NH_4^+$ content of PS-6% and PS-10% was 223.1 and 375.4 $mg \cdot L^{-1}$, respectively (Figure 2A). $N\text{-}NH_4^+$ toxicity is common when producing microalgae using wastewater or agricultural wastes. *Muriellopsis* sp. and *Pseudokirchneriella subcapitata* were produced in media containing $N\text{-}NH_4^+$ concentrations up to 200 $mg \cdot L^{-1}$ with no inhibitory effects being observed [9]. Nonetheless, growth inhibition is generally seen at concentrations above 160 $mg \cdot L^{-1}$ [8]. The high $N\text{-}NH_4^+$ concentration in PS-6% and PS-10% was probably the reason for the lower productivity when compared to the SM. Surprisingly, *S. almeriensis* presented a relatively high biomass productivity when produced using an $N\text{-}NH_4^+$ concentration of 183.6 $mg \cdot L^{-1}$ (PS-5%), which is slightly higher than the above-mentioned 100-160 $mg \cdot L^{-1}$ range. In the present study, $N\text{-}NH_4^+$ removal rates varied from 12.2 to 65.5 $mg \cdot L^{-1} \cdot day^{-1}$, being higher in PS-10% because of the higher initial $N\text{-}NH_4^+$ content. Not all the $N\text{-}NH_4^+$ was used for microalgal growth - a mass balance of the system revealed that approximately 46.5, 35.0, and 33.8% of the nitrogen present in PS-10%, PS-6%, and PS-5%, respectively was “lost” into the atmosphere (stripping). Some of the $N\text{-}NH_4^+$ might also have been nitrified by the action of nitrifying bacteria, although nitrifying bacteria are also very sensitive to high $N\text{-}NH_4^+$ concentrations [34].

The second most abundant source of nitrogen present in fresh pig slurry is $N\text{-}NO_3^-$. The $N\text{-}NO_3^-$ concentrations at the inlets and outlets of the reactors are shown in Figure 2B. The $N\text{-}NO_3^-$ inlet concentrations varied from 3.70 $mg \cdot L^{-1}$ in PS-1% to 15.9 $mg \cdot L^{-1}$ in PS-10%. The $N\text{-}NO_3^-$ removal rates ranged from 1.0 to 3.4 $mg \cdot L^{-1} \cdot day^{-1}$, representing 94.3% removal in PS-1%, which had the lowest initial $N\text{-}NO_3^-$ concentration. In terms of $P\text{-}PO_4^{3-}$, the inlet concentrations varied from 3.9 $mg \cdot L^{-1}$ in PS-1% to 22.2 $mg \cdot L^{-1}$ in PS-10% (Figure 2C).

Phosphorus is a critical component in the physiological ecology of eukaryotic microalgae and prokaryotic cyanobacteria. It plays a key role in most cellular processes that involve energy transfer, signal transduction, biosynthesis of macromolecules, photosynthesis, and respiration [35]. In the present study, $P\text{-}PO_4^{3-}$ removal rates varied from 1.2 to 2.2 $mg \cdot L^{-1} \cdot day^{-1}$ and represented 100% removal in PS-1%. This means that when the microalgae were grown in PS-1%, they consumed all the $P\text{-}PO_4^{3-}$ that entered the system. Phosphorus deficiency can cause a marked reduction in membrane phospholipids [35] and this might be the cause of the observed decrease in biomass productivity when the microalgae were produced in PS-1%, which was not inhibited by high $N\text{-}NH_4^+$ concentrations. Regarding COD removal, values ranged from 25.7 $mg \cdot L^{-1} \cdot day^{-1}$ in PS-1% to 420.6 $mg \cdot L^{-1} \cdot day^{-1}$ in PS-10%. The higher COD removal in PS-10% can be attributed to a higher COD concentration at the inlet and to a higher bacterial content in the microalgae-bacteria consortia due to the inhibitory effect of high $N\text{-}NH_4$+ concentrations on microalgal growth. COD removal was higher than 60% in all the cultures.

3.3. Supplementation of fresh pig slurry

The results shown in Figure 2 demonstrate that the biomass productivity achieved with the most concentrated culture media (PS-6% and PS-10%) was lower than that of the control; this was because the $N\text{-}NH_4^+$ content exceeded 200 $mg \cdot L^{-1}$. Therefore, these two culture media were not assessed further. Moreover, the results showed that the most diluted medium (PS-1%) led to lower biomass productivities, when compared to the control; this was because the culture was phosphorus-limited. To assess the reasons for the lower biomass productivities achieved when the microalgae were produced using PS-2% and PS-5% (with potentially inhibitory effects from the compounds/microorganisms present in the pig slurry) and to avoid the phosphorus limitation observed in PS-1%, these media were supplemented with nitrogen and phosphorus. As one can observe in Figure 3A, the results show that when *S. almeriensis*

is not nitrogen- or phosphorus-limited, the biomass productivities achieved using fresh pig slurry (diluted at a concentration of 1.2 or 2.5%) are comparable to those achieved using the SM. In this case, the averaged N/P ratios were 12.5, 13.5, and 16.2 for S-PS1%, S-PS2%, and S-PS5%, respectively; this highlights the importance of the N/P ratio in microalgal culture media. Previous studies also noted the importance of the initial N/P ratio on biomass productivity and wastewater treatment, suggesting that the optimal ratio can vary from 5 to 30, depending on the ecological conditions in the wastewater [32]. In addition, the quantum yield values (shown in Figure 3B) indicate that the cultures were not subjected to any stress conditions, given that the values were comparable to those of the SM.

Figure 4 shows that the cultures were not limited in terms of nitrogen or phosphorus. The $N\text{-}NO_3^-$ concentration at the outlets ranged from 74.4 $mg \cdot L^{-1}$ in S-PS1% to 79.5 $mg \cdot L^{-1}$ in S-PS5%, representing $N\text{-}NO_3^-$ removal rates of 16.7, 21.7, and 21.5 $mg \cdot L^{-1} \cdot day^{-1}$ in S-PS1%, S-PS2%, and S-PS5% respectively. Similar results were observed for $P\text{-}PO_4^{3-}$. The concentration at the outlets ranged from 26.5 to 39.9 $mg \cdot L^{-1}$, demonstrating that the cultures were not phosphorus-limited. The removal rates varied from 1.9 $mg \cdot L^{-1} \cdot day^{-1}$ in S-PS5% to 2.4 $mg \cdot L^{-1} \cdot day^{-1}$ in S-PS1%, which is comparable to results reported in previous research [36]. The lower $P\text{-}PO_4^{3-}$ removal rate in S-PS5% was caused by this cultures' low biomass productivity. Indeed, a positive correlation was determined between the biomass productivity and $P\text{-}PO_4^{3-}$ removal (R^2=0.885; 0.05). The COD removal rate in SPS-5% was 287.1 $mg \cdot L^{-1} \cdot day^{-1}$, slightly higher than that for PS-5% because of the higher COD inlet concentration. In these trials, COD removal ranged from 70-80%, demonstrating the potential of the microalgae-bacteria consortia to degrade organic matter.

Overall, despite the fresh slurrys' high microbial load, which can contain pathogens [37] and/or heavy metals [38], the results suggested that it has no toxic effect on *S. almeriensis* when diluted at 1.2 or 2.5%. However, when the biomass was produced

using fresh pig slurry diluted at a higher concentration (5.0%) and supplemented with nutrients, its productivity decreased from 0.74 ± 0.02 $g \cdot L^{-1} \cdot day^{-1}$ in the SM to 0.68 ± 0.03 $g \cdot L^{-1} \cdot day^{-1}$ in S-PS5% (p<0.05). Figure 4A shows the $N\text{-}NH_4^+$ concentration at the reactor inlets. The results indicate that the lower biomass productivity might be partially attributable to a slight increase in the $N\text{-}NH_4^+$ content at the inlets (205.4 $mg \cdot L^{-1}$), although the same strain did manage to grow at a similar $N\text{-}NH_4^+$ concentration (183.6 $mg \cdot L^{-}1$; PS-5%; Figure 2). Not only high $N\text{-}NH_4^+$ concentrations but also high turbidity in the pig slurry were suggested as potential factors inhibiting microalgal growth [11]. Another hypothesis is that light availability was lower in the PS-5% and S-PS5% media because of the increased turbidity. Light availability is the most important factor when producing any photosynthetic organism [39]. For this reason, we calculated the average irradiance inside the culture, finding a decrease from 303.9 ± 10.2 to 239.5 ± 6.9 µmol photons $\cdot m^{-2} \cdot s^{-1}$ when comparing the SM and the S-PS5% medium (p<0.05). This parameter refers to the average irradiance to which the cells are exposed inside the culture; hence this decrease indicated that the turbidity observed in the latter might be the reason for the lower biomass productivity. The average irradiance inside the culture is dependent on the photobioreactor design and, therefore, the decrease we observed might be even higher in larger reactors, which are subjected to solar radiation rather than the artificial illumination of a controlled environment.

3.4. Effect of ozonation on turbidity and biomass productivity

Previous reports have suggested that the turbidity of wastewater or pig slurry might limit microalgal growth [11]. In the present study, lower average irradiance inside the culture was observed when the microalgae were produced using PS-5% rather than the SM (p<0.05). Different strategies have been proposed for decreasing the turbidity of pig slurry and wastewater. Ozone is a strong antioxidant that can effectively degrade organic compounds, especially those with high electronic moieties [40]. Its high oxidation potential makes ozonation an effective strategy for processing waste streams [41]. As

an example, this strategy led to a 55.6% turbidity removal in petrochemical wastewater [42] and similar results were reported for tannery effluents [43]. In the present study, pig slurry diluted at a concentration of 5% was ozonised for either 30, 60, or 100 min (PS-5%-I, PS-5%-II, and PS-5%-III, respectively). The turbidity of the media decreased from 10.8 ± 0.6 NTU (before ozonation) to 9.2 ± 0.3, 6.9 ± 0.2, and 4.6 ± 0.3 NTU after processing for 30, 60, or 100 min respectively. By decreasing the turbidity, ozonation led to an increase in the average irradiance inside the culture (Table 1; $p<0.05$). The effect of ozonation on biomass productivity is shown in Figure 5A. Surprisingly, despite the lower light availability observed, the ozonation of PS-5% for either 30, 60, or 100 min did not affect biomass productivity which was 0.68 ± 0.01 $g \cdot L^{-1} \cdot day^{-1}$ when produced using pig slurry (the average of the processed and unprocessed pig slurries). This means that the turbidity of fresh diluted pig slurry did not negatively affect microalgae production, as was expected. It is important to note that the scale of the reactors used, as well as the use or artificial illumination in a controlled environment, increases light availability; thus, turbidity might limit microalgal growth when pig slurry is processed using large-scale open photobioreactors located outdoors. Raceway reactors, which operate at a culture depth of 0.15-0.30 m (or even deeper) are inefficient in terms of light availability even when using freshwater – this is because of the self-shading effect of the microalgae. In turn, thin-layer cascade reactors operate using a shallow water column, generally 0.01-0.02 m, which allows one to maximise the light availability and, therefore, the biomass productivity. These photobioreactors have been used to produce *S. almeriensis* using different types of water [14,17] and show potential for use in the large-scale processing of pig slurry.

No differences were observed between the biomass produced in the SM and PS-5% media. This was surprising as S-PS-5% led to a lower biomass productivity than SM ($p<0.05$) and both media consisted of pig slurry diluted at a 5% concentration. The reason for this was the small but significant increase in the $N\text{-}NH_4^+$ concentration from

186.2 mg·L^{-1} in PS-5% to 205.4 mg·L^{-1} in S-PS5% (p<0.05). These results demonstrate the importance of diluting fresh pig slurry down to N-NH_4^+ concentrations that do not inhibit microalgal growth, which in our case was below 190-200 mg·L^{-1}. This threshold is relatively high as most studies reported growth inhibition at approximately 160 mg·L^{-1} [8]. The results also emphasise the importance of selecting a robust microalgae strain for wastewater or pig slurry bioremediation, and they demonstrate the robustness of the *S. almeriensis* strain selected. The N-NH_4^+ removal rates varied from 27.3 to 30.4 mg·L^{-1}·day^{-1} and were comparable to those achieved when processing anaerobically digested pig slurry in 3 L open ponds [44]. In that study, the authors concluded that nitrogen recovery by microalgae biomass is frequently overestimated because of denitrification or stripping. In the present paper, when microalgae were produced in PS-5%, 47.1 mg·L^{-1}·day^{-1} of N-NH_4^+ was stripped away, representing 32.0% of the total N-NH_4^+ removal rate.

The N-NO_3^- removal rates were low (0.3-0.9 mg·L^{-1}·day^{-1}), probably caused by the high N-NH_4^+ concentration in the inlet and because N-NH_4^+ is generally the preferred nitrogen source for microalgae. For example, ammonium carbonate led to higher *Scenedemus obliquus* productivities than sodium nitrate in a recent study [45]. It has been suggested the reason for this is that a redox reaction is not involved in its assimilation, and therefore N-NH_4^+ assimilation requires less energy [46]. The P-PO_4^{3-} removal rates (1.9-2.2 mg·L^{-1}·day^{-1}) were comparable to those reported for pig manure bioremediation using *S. almeriensis*, with maximum P-PO_4^{3-} removal rates of 1.9 mg·L^{-1}·day^{-1} [36]. The results were also similar to those obtained when processing undiluted pig slurry using *Chlorella vulgaris*, with the total nitrogen and phosphorus contents dropping from 362.5 and 18.3 mg·L^{-1} to 155.9 and 10.2 mg·L^{-1}, respectively [47]. When processing diluted pig slurry using the same microalgae, the total nitrogen and phosphate removal rates were 33.7 and 2.4 mg·L^{-1}·day^{-1}, respectively [6].

In terms of COD removal, ozonation led to a significant reduction in the COD concentration from 1990.0 $mg \cdot L^{-1}$ in PS-5% to 1410.0 $mg \cdot L^{-1}$ in PS-5%-III ($p<0.05$). These results were in line with previous reports that achieved a decrease in COD concentration from 2752 $mg \cdot L^{-1}$ to 1168 $mg \cdot L^{-1}$ following ozonation for 110 min [43]. Moreover, significant differences were observed between the inlet and outlet of all the reactors, suggesting that microalgae assimilated part of the removed COD. Several microalgae, including species of the *Scenedesmus* genus, are mixotrophic and can simultaneously assimilate carbon dioxide and organic carbon [47]. In the present study, COD removal rates varied between 357.3 and 519.3 $mg \cdot L^{-1} \cdot day^{-1}$, being higher in PS-5% because of the higher initial content (Figure 6D). Similarly, the COD removal was higher than in the previous trials because of the higher COD concentration at the inlets. Slurry composition values vary significantly according to the animals´ age and physiological state [33] and its analysis is mandatory prior to developing depuration processes or even before being used as fertilisers in agriculture.

The COD concentration at the outlets was high because of the very high nutrient content of the pig slurry, and this does not comply with the maximum discharge limits of most Wester countries. In Spain, this limit is set at 125 $mg \cdot L^{-1}$ [48]. Similarly, the concentration at the reactor outlets did not meet the maximum discharge limits in terms of total nitrogen and phosphorus, set at 10-15 and 1-2 $mg \cdot L^{-1}$, respectively [48]. It is important to highlight that the slurry was processed using laboratory-scale photobioreactors. When produced outdoors, the culture (which is still rich in nutrients, mainly $N\text{-}NO_3^-$, $P\text{-}PO_4^{3-}$, and COD) could be recirculated to improve nutrient recoveries. Further studies on this topic using pilot-scale outdoor reactors are needed (and ongoing). Moreover, the outlet effluents have a nutrient content that is comparable to that of urban wastewater, meaning they could be further processed in a treatment plant as urban wastewater, prior to utilisation – for example, in irrigation.

3.5. Composition of the microalgal biomass

Microalgae are especially interesting not only because of their potential for recovering nutrients from waste streams but also because of their composition, which makes microalgal biomass a valuable product. Microalgae are rich sources of high-value proteins, pigments, lipids, and bioactive molecules [49]. The composition and bioactivity of microalgal biomass depends not only on the selected strain but also on the cultures' environmental and operational conditions. For example, nitrogen limitation can lead to increased lipid and carotenoid production and accumulation, as well as reduced protein production. However, the response to nitrogen limitation is strain-dependent [50]. Table 2 shows the effect of diluted pig slurry on the macromolecular composition of the produced biomass. Overall, producing *S. almeriensis* using pig slurry led to significant differences in the protein, lipid, carbohydrate, ash, and pigment contents ($p<0.05$). The protein content decreased from 34.5% when the biomass was produced using freshwater to 29.3% when using pig slurry as the nutrient source. Depending on the end use of the biomass, this might represent a problem, for example, some agricultural products, such as biofertilisers and biostimulants require a high amino acid content. The biomass produced using pig slurry had a higher lipid content. In *S. almeriensis,* the high lutein content is especially interesting because it is an antioxidant carotenoid that is important for maintaining eye health as well as being highly valued for use in the feed industry [51]. The total carotenoid content of the biomass produced using pig slurry was lower when compared to that of the control. Further studies are needed to fully characterise the effect of pig slurry on the composition of the produced biomass. The observed differences can be attributed to the different nitrogen and phosphorus contents of both media and to the presence of compounds in the pig slurry that can affect microalgal growth. Moreover, the biomass produced contains microalgae and bacteria (and other microorganisms) at different concentrations, which could partially explain the observed differences. As mentioned before, the composition of the microalgae-bacteria consortia depends on environmental and operational conditions. However, phototropism is promoted in the presence of light and inorganic nutrients, with the potential for over 95% of the produced

biomass being microalgae [26]. To precisely determine this, *in vitro* assessment would be required.

Conclusions

Fresh pig slurry has shown itself to be a promising source of nutrients for the large-scale production of *S. almeriensis.* The results reported in this study were obtained using laboratory-scale controlled photobioreactors. The process needs to be further validated and scaled-up using pilot-scale photobioreactors located outdoors. Anaerobically digesting the pig slurry prior to microalgae production did not improve the biomass productivity values, thus simplifying the process and facilitating its implementation in rural areas. However, the pig slurrys' nutrient load varies significantly and it is important to select the most suitable dilution rate for each process independently. If utilised for *S. almeriensis* production, it is advisable to use dilution rates that lead to $N\text{-}NH_4^+$ concentrations below 190-200 $mg \cdot L^{-1}$, so avoiding inhibitory effects and achieving productivity values comparable to those obtained when using a standard medium. This threshold is slightly higher than that reported for other microalgae, demonstrating the robustness of the strain used here. Moreover, if the pig slurry is too diluted, cultures growth could be phosphorus-limited, which would have a marked effect on biomass productivity. When diluted at a concentration of 5%, the turbidity of pig slurry led to a decrease in the average irradiance inside the culture, although this did not affect biomass productivity. However, this might be due to the controlled environment of the laboratory-scale reactors; hence, further studies on scaling–up the process are needed. Because of the higher light availability in thin-layer cascade reactors (when compared to conventional raceways), these designs show potential for use in the processing of slightly turbid waste streams. Finally, given the large amounts if water required to dilute pig slurry and to avoid $N\text{-}NH_4^+$ toxicity or high turbidity values that limit microalgal growth, recirculating the outlet effluents back into the system would be inevitable at the industrial

scale. Further studies are therefore needed to assess the effect of recirculation on biomass productivity and nutrient recoveries.

Acknowledgements

This work is part of the SABANA Project funded by the H2020 Research and Innovation Framework Programme (727874) and the PURASOL Project, funded by the Spanish Ministry of Science, Innovation, and Universities (CTQ-2017-84006-C3-3-R). Tomás 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. Authors thank the Institute for Agricultural and Fisheries Research and Training (IFAPA).

CRediT author statement

M. Ciardi: Investigation, Formal analysis, and Writing – Original draft; **C. Gómez:** Investigation and Supervision; **M.M. Morales-Amaral:** Investigation, Formal analysis; **G. Acién:** Supervision, Funding acquisition; **T. Lafarga:** Formal analysis, visualization and Writing – Original draft; and **J.M. Fernández-Sevilla:** Supervision, Funding acquisition.

Declaration of competing interest

Authors declare no conflict of interests.

Statement of informed consent, human/animal rights

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

Table 1. Effect of ozonation on the average irradiance inside the culture and turbidity. Different letters in the same column indicate significant differences (*p*<0.05). Data is represented as mean of three independent determinations ± SD.

	Turbidity (NTU)	I_{av} (µmol·m^{-2}·s^{-1})
PS-5%	10.8 ± 0.6 ^{A}	239.5 ± 6.9 ^{A}
PS-5%-I	9.2 ± 0.3 ^{B}	249.2 ± 3.6 ^{B}
PS-5%-II	6.9 ± 0.2 ^{C}	269.2 ± 9.1 ^{C}
PS-5%-III	4.6 ± 0.3 ^{D}	283 ± 1.0 ^{D}

Table 2. Composition of microalgal biomass produced using the standard medium (SM) and diluted pig slurry (PS-5%). Different letters in the same row indicate significant differences (p<0.05). Data is represented as mean of three independent determinations ± SD.

	SM	PS-5%
Protein ($g \cdot 100g^{-1}$)	34.5 ± 0.2 ^{A}	29.3 ± 0.4 ^{B}
Lipid ($g \cdot 100g^{-1}$)	4.3 ± 0.2 ^{B}	6.4 ± 0.1 ^{A}
Carbohydrate ($g \cdot 100g^{-1}$)	47.2 ± 1.8 ^{B}	59.3 ± 5 ^{A}
Ash ($g \cdot 100g^{-1}$)	8.3 ± 1.8 ^{A}	5.6 ± 4.1 ^{B}
Chlorophyll-*a* ($g \cdot 100g^{-1}$)	0.62 ± 0.09 ^{A}	0.57 ± 0.02 ^{A}
Chlorophyll-*b* ($g \cdot 100g^{-1}$)	0.48 ± 0.13 ^{A}	0.27 ± 0.03 ^{B}
Carotenoids ($g \cdot 100g^{-1}$)	0.33 ± 0.01 ^{A}	0.23 ± 0.03 ^{B}

Results are expressed on a dry weight basis.

Figure legends

Figure 1. (A) Biomass productivity and (B) maximum potential quantum yield efficiency of photosystem II of *S. almeriensis* cultures produced in diluted fresh pig slurry. Data is represented as mean of three independent determinations ± SD. Different letters indicate significant differences (p<0.05)

Figure 2. Inlet and outlet concentration and removal rates of (A) N-NH_4^+, (B) N-NO_3^-, (C) P-PO_4^{3-}, and (D) COD of *S. almeriensis* cultures produced in diluted fresh pig slurry. Data is represented as mean of three independent determinations ± SD.

Figure 3. (A) Biomass productivity and (B) maximum potential quantum yield efficiency of photosystem II of *S. almeriensis* cultures produced in supplemented diluted fresh pig slurry. Data is represented as mean of three independent determinations ± SD. Different letters indicate significant differences (p<0.05)

Figure 4. Inlet and outlet concentration and removal rates of (A) N-NH_4^+, (B) N-NO_3^-, (C) P-PO_4^{3-}, and (D) COD of *S. almeriensis* cultures produced in supplemented diluted fresh pig slurry. Data is represented as mean of three independent determinations ± SD.

Figure 5. (A) Biomass productivity and (B) maximum potential quantum yield efficiency of photosystem II of *S. almeriensis* cultures produced in ozonised pig slurry diluted at a concentration of 5%. Data is represented as mean of three independent determinations ± SD. Different letters indicate significant differences (p<0.05)

Figure 6. Inlet and outlet concentration and removal rates of (A) N-NH_4^+, (B) N-NO_3^-, (C) P-PO_4^{3-}, and (D) COD of *S. almeriensis* cultures produced in ozonised pig slurry diluted at a concentration of 5%. Data is represented as mean of three independent determinations ± SD.

Figure 1

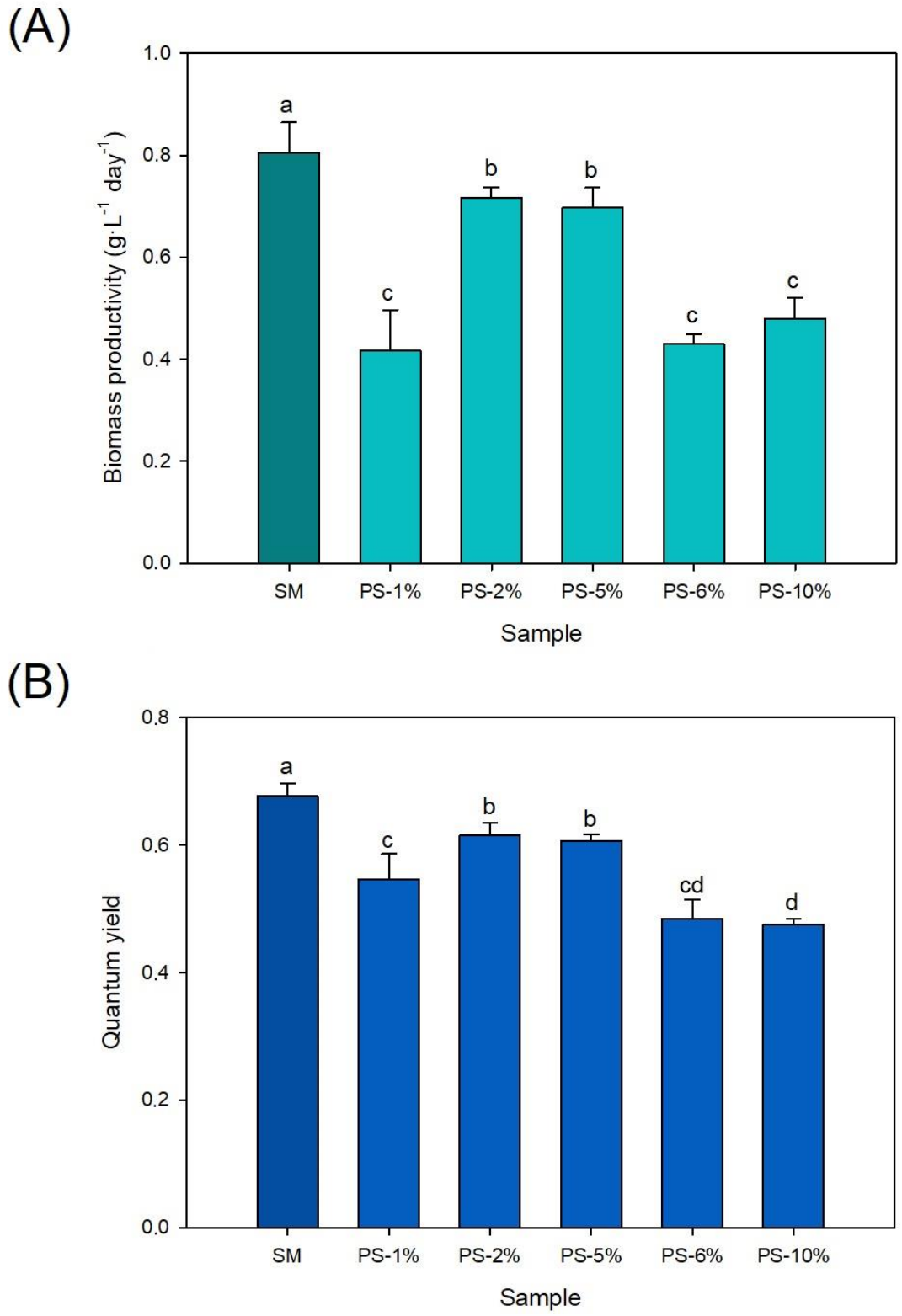

(A)
Biomass productivity (g·L-1 day-1)
1.0
0.8
0.6
0.4
0.2
0.0
a
c
b
b
c
c
SM
PS-1%
PS-2%
PS-5%
PS-6%
PS-10%
Sample
(B)
Quantum yield
0.8
0.6
0.4
0.2
0.0
a
c
b
b
cd
d
SM
PS-1%
PS-2%
PS-5%
PS-6%
PS-10%
Sample

Figure 2

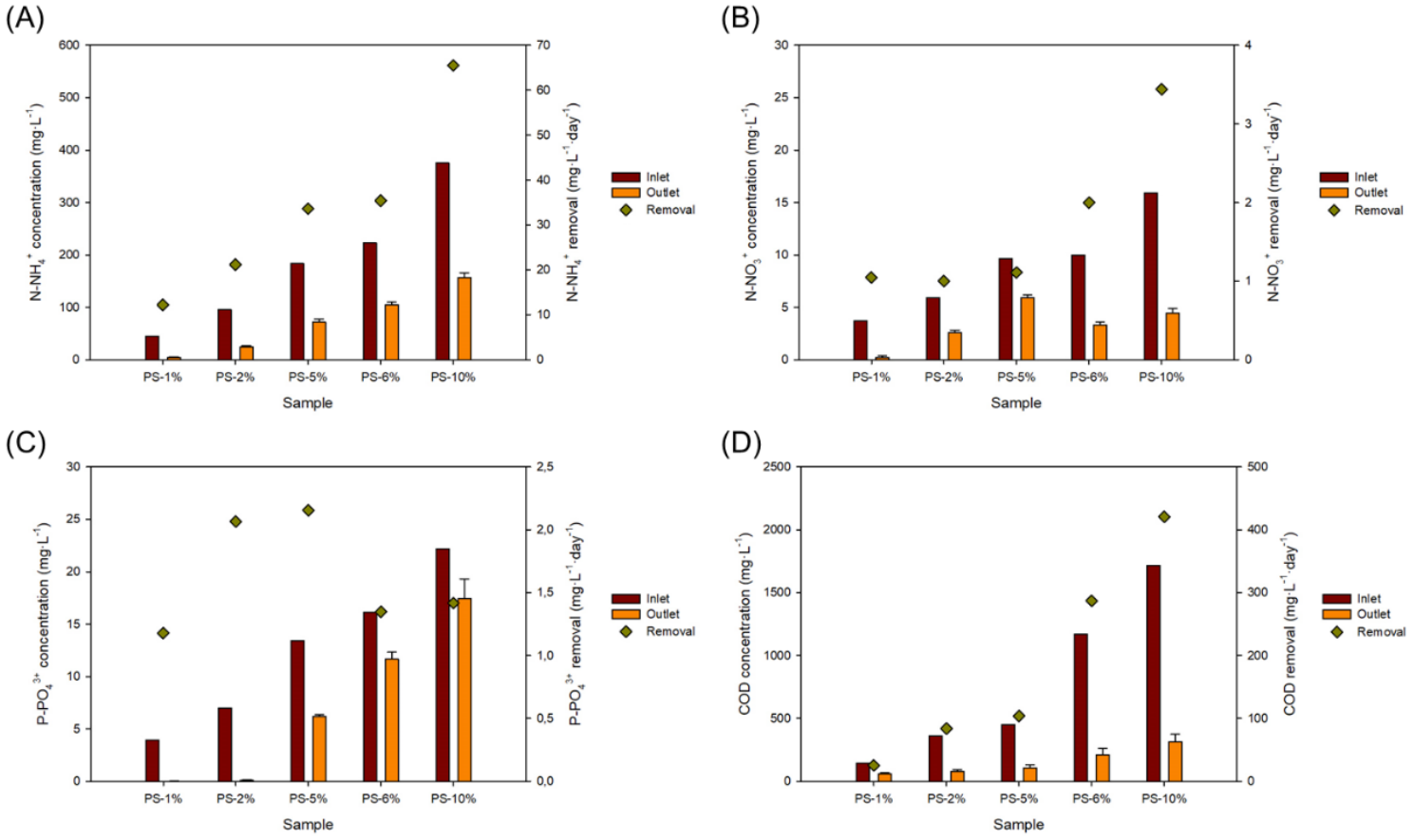

(A)
N-NH4+ concentration (mg·L-1)
N-NH4+ removal (mg·L-1·day-1)
Inlet
Outlet
Removal
PS-1%
PS-2%
PS-5%
PS-6%
PS-10%
Sample
(B)
N-NO3+ concentration (mg·L-1)
N-NO3+ removal (mg·L-1·day-1)
Inlet
Outlet
Removal
PS-1%
PS-2%
PS-5%
PS-6%
PS-10%
Sample
(C)
P-PO43+ concentration (mg·L-1)
P-PO43+ removal (mg·L-1·day-1)
Inlet
Outlet
Removal
PS-1%
PS-2%
PS-5%
PS-6%
PS-10%
Sample
(D)
COD concentration (mg·L-1)
COD removal (mg·L-1·day-1)
Inlet
Outlet
Removal
PS-1%
PS-2%
PS-5%
PS-6%
PS-10%
Sample

Figure 3

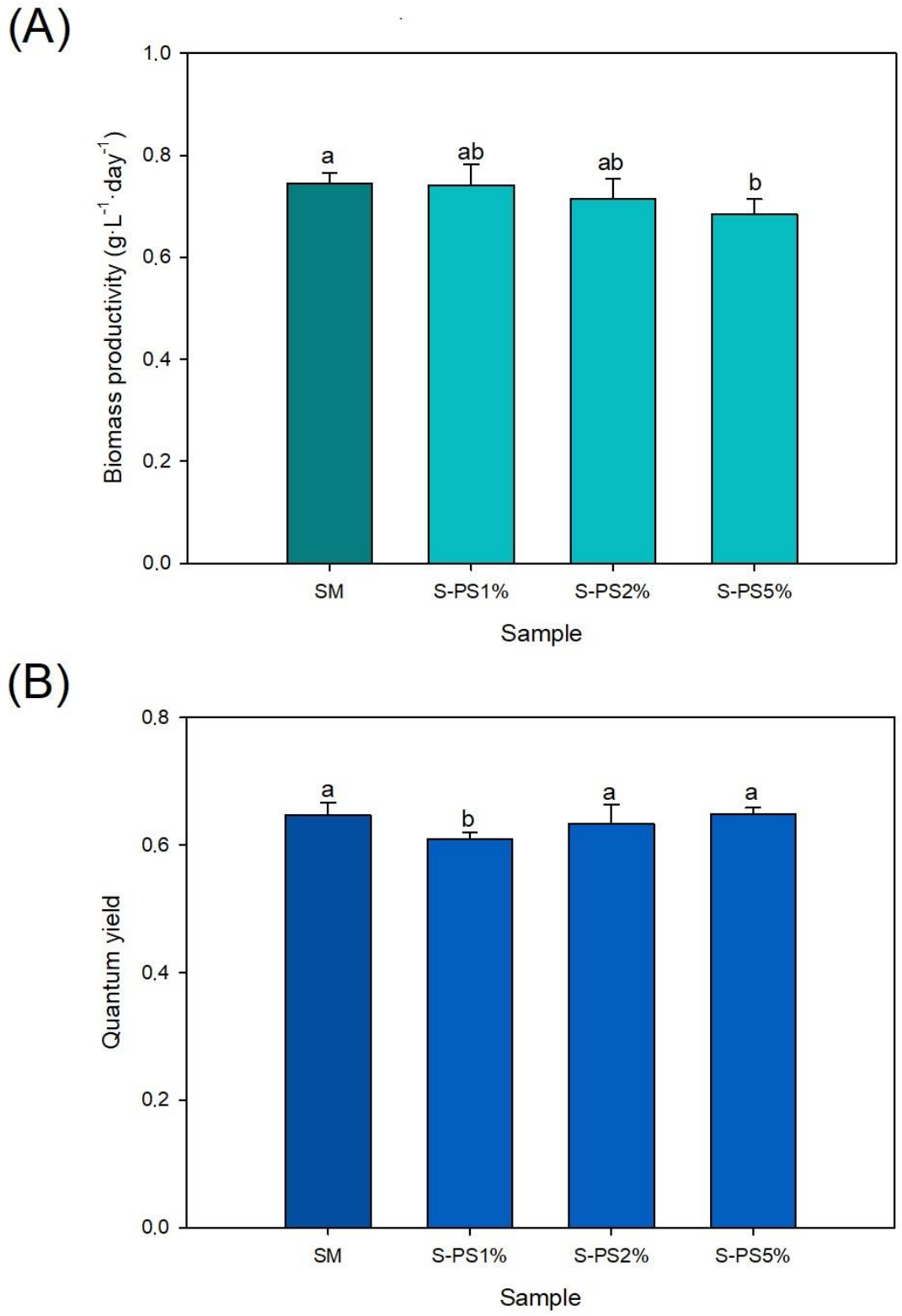

(A)
Biomass productivity (g·L-1·day-1)
1.0
0.8
0.6
0.4
0.2
0.0
a
ab
ab
b
SM
S-PS1%
S-PS2%
S-PS5%
Sample
(B)
Quantum yield
0.8
0.6
0.4
0.2
0.0
a
b
a
a
SM
S-PS1%
S-PS2%
S-PS5%
Sample

Figure 4

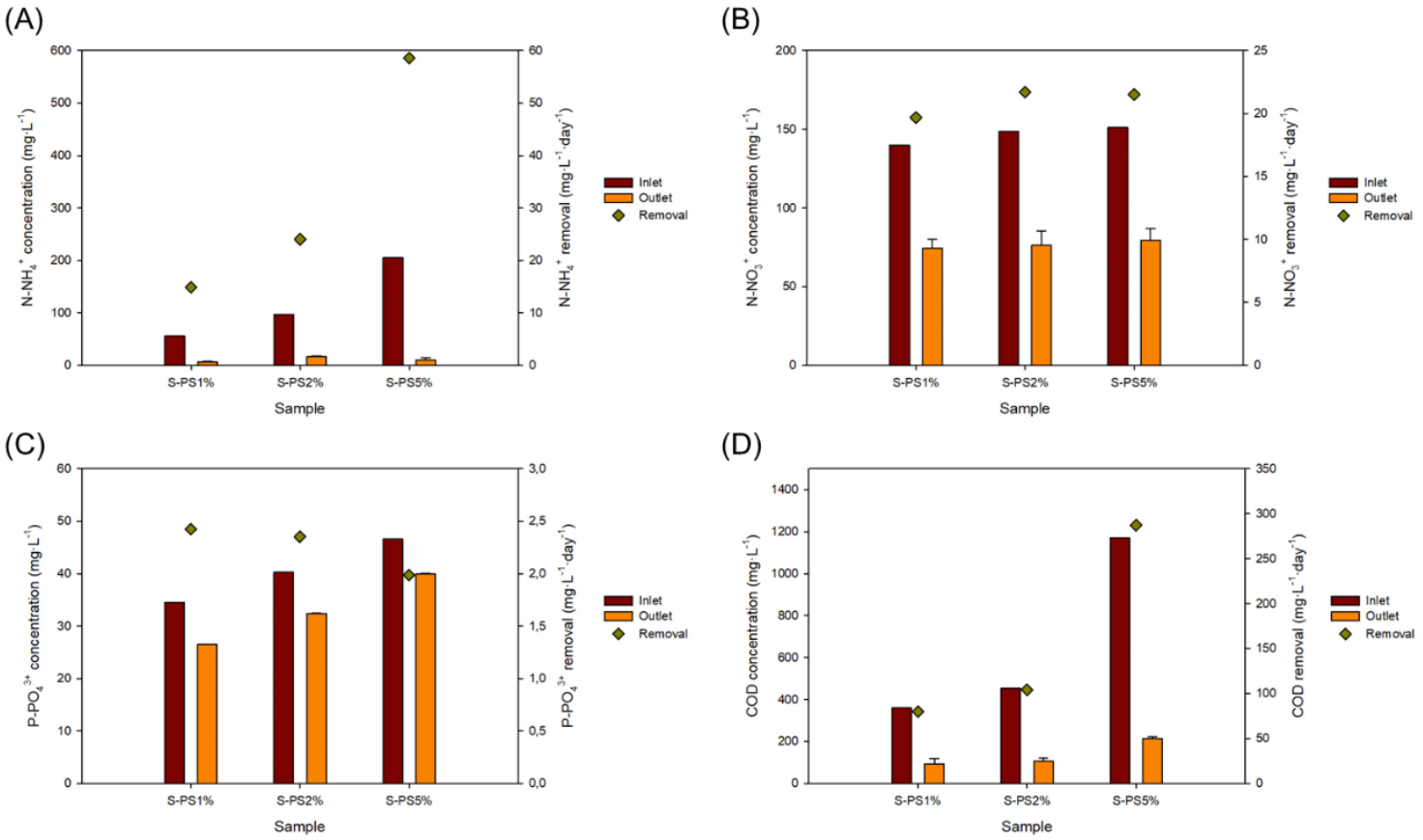

(A)
N-NH4+ concentration (mg·L-1)
N-NH4+ removal (mg·L-1·day-1)
Inlet
Outlet
Removal
S-PS1%
S-PS2%
S-PS5%
Sample
(B)
N-NO3+ concentration (mg·L-1)
N-NO3+ removal (mg·L-1·day-1)
Inlet
Outlet
Removal
S-PS1%
S-PS2%
S-PS5%
Sample
(C)
P-PO43+ concentration (mg·L-1)
P-PO43+ removal (mg·L-1·day-1)
Inlet
Outlet
Removal
S-PS1%
S-PS2%
S-PS5%
Sample
(D)
COD concentration (mg·L-1)
COD removal (mg·L-1·day-1)
Inlet
Outlet
Removal
S-PS1%
S-PS2%
S-PS5%
Sample

Figure 5

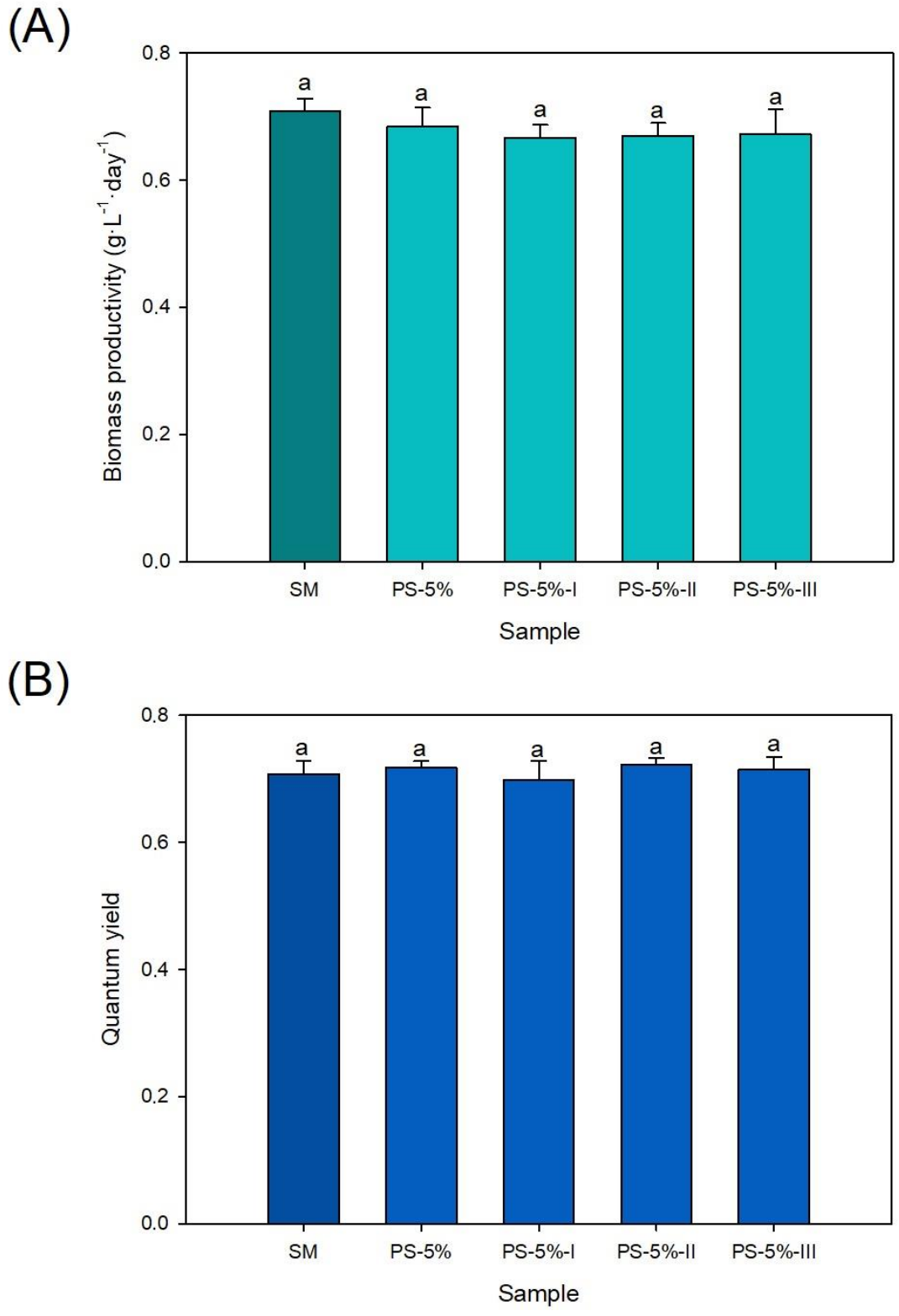
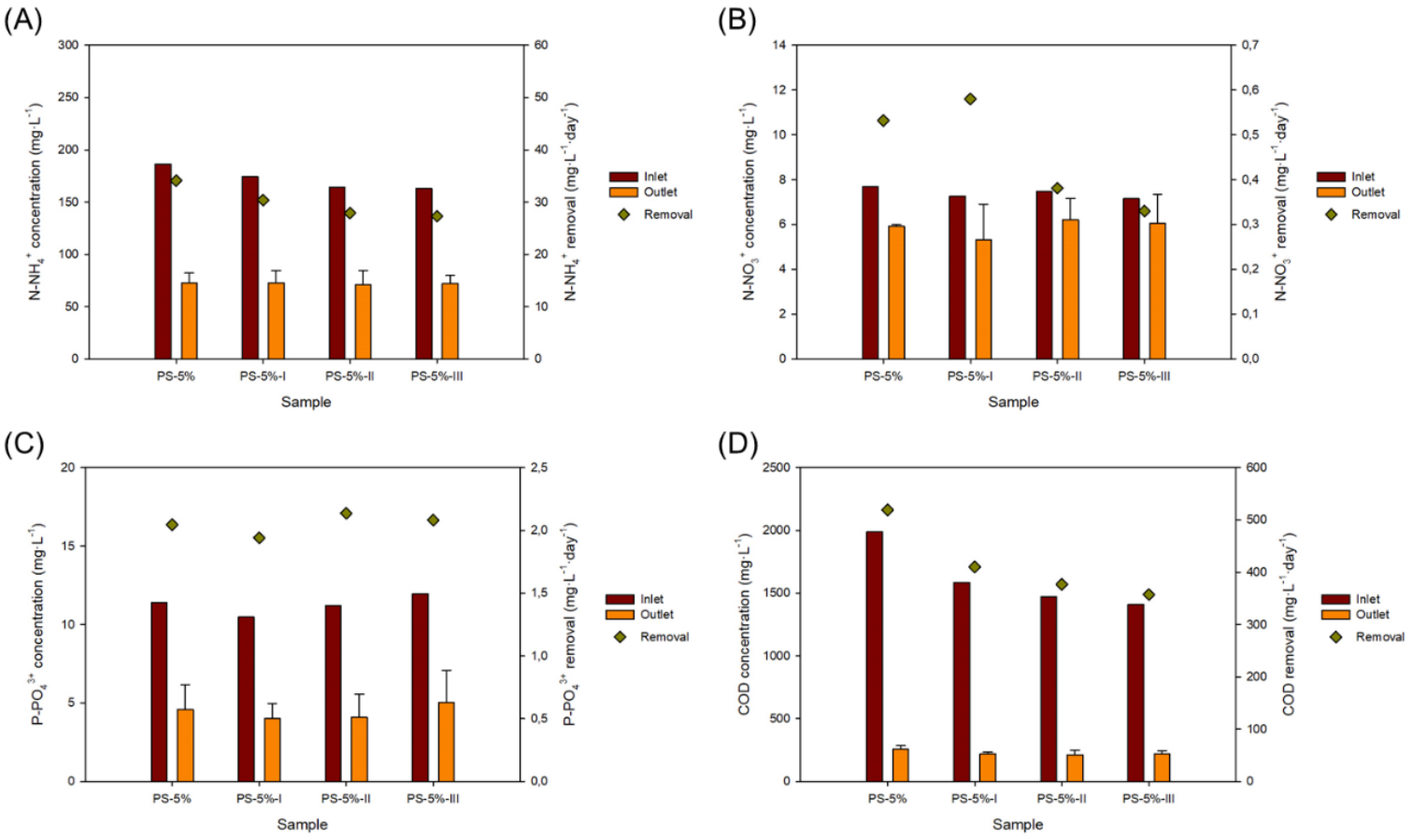

(A)
Biomass productivity (g·L-1·day-1)
0.0
0.2
0.4
0.6
0.8
a
a
a
a
a
SM
PS-5%
PS-5%-I
PS-5%-II
PS-5%-III
Sample
(B)
Quantum yield
0.0
0.2
0.4
0.6
0.8
a
a
a
a
a
SM
PS-5%
PS-5%-I
PS-5%-II
PS-5%-III
Sample

Figure 6


(A)
N-NH4+ concentration (mg·L-1)
N-NH4+ removal (mg·L-1·day-1)
Inlet
Outlet
Removal
PS-5%
PS-5%-I
PS-5%-II
PS-5%-III
Sample
(B)
N-NO3+ concentration (mg·L-1)
N-NO3+ removal (mg·L-1·day-1)
(C)
P-PO43+ concentration (mg·L-1)
P-PO43+ removal (mg·L-1·day-1)
(D)
COD concentration (mg·L-1)
COD removal (mg·L-1·day-1)

References

[1] EC, Council directive of 12 December 1991 concerning the protection of waters against pollution caused by nitrates from agricultural sources (91/676/EEC), Off. J. Eur. Communities. L375 (1991) 1–8.

[2] D. Biagini, C. Lazzaroni, Eutrophication risk arising from intensive dairy cattle rearing systems and assessment of the potential effect of mitigation strategies, Agric. Ecosyst. Environ. 266 (2018) 76–83. https://doi.org/10.1016/j.agee.2018.07.026.

[3] M.J. Luján-Facundo, M.I. Iborra-Clar, J.A. Mendoza-Roca, M. Also-Jesús, Alternatives for the management of pig slurry: Phosphorous recovery and biogas generation, J. Water Process Eng. 30 (2019) 100473. https://doi.org/10.1016/j.jwpe.2017.08.011.

[4] J.A. Garrido-Cardenas, F. Manzano-Agugliaro, F.G. Acien-Fernandez, E. Molina-Grima, Microalgae research worldwide, Algal Res. 35 (2018) 50–60. https://doi.org/10.1016/j.algal.2018.08.005.

[5] F.G. Acién, E. Molina, J.M. Fernández-Sevilla, M. Barbosa, L. Gouveia, C. Sepúlveda, J. Bazaes, Z. Arbib, Economics of microalgae production, in: Microalgae-Based Biofuels Bioprod. From Feed. Cultiv. to End-Products, Elsevier Inc., 2017: pp. 485–503. https://doi.org/10.1016/B978-0-08-101023-5.00020-0.

[6] M. Franchino, V. Tigini, G.C. Varese, R. Mussat Sartor, F. Bona, Microalgae treatment removes nutrients and reduces ecotoxicity of diluted piggery digestate, Sci. Total Environ. 569–570 (2016) 40–45. https://doi.org/10.1016/j.scitotenv.2016.06.100.

[7] A. Solimeno, R. Samsó, E. Uggetti, B. Sialve, J.-P. Steyer, A. Gabarró, J.

García, New mechanistic model to simulate microalgae growth, Algal Res. 12 (2015) 350–358. https://doi.org/10.1016/j.algal.2015.09.008.

[8] P. Scarponi, A.M. Volpi Ghirardini, M. Bravi, C. Cavinato, Evaluation of Chlorella vulgaris and Scenedesmus obliquus growth on pretreated organic solid waste digestate, Waste Manag. 119 (2021) 235–241. https://doi.org/10.1016/j.wasman.2020.09.047.

[9] M. del M. Morales-Amaral, C. Gómez-Serrano, F.G. Acién, J.M. Fernández-Sevilla, E. Molina-Grima, Production of microalgae using centrate from anaerobic digestion as the nutrient source, Algal Res. 9 (2015) 297–305. https://doi.org/10.1016/j.algal.2015.03.018.

[10] K. Risberg, H. Cederlund, M. Pell, V. Arthurson, A. Schnürer, Comparative characterization of digestate versus pig slurry and cow manure – Chemical composition and effects on soil microbial activity, Waste Manag. 61 (2017) 529–538. https://doi.org/10.1016/j.wasman.2016.12.016.

[11] E. Uggetti, B. Sialve, E. Latrille, J.P. Steyer, Anaerobic digestate as substrate for microalgae culture: The role of ammonium concentration on the microalgae productivity, Bioresour. Technol. 152 (2014) 437–443. https://doi.org/10.1016/j.biortech.2013.11.036.

[12] A. Sánchez-Zurano, T. Lafarga, M. del M. Morales-Amaral, C. Gómez-Serrano, J.M. Fernández-Sevilla, F.G. Acién-Fernández, E. Molina-Grima, Wastewater treatment using Scenedesmus almeriensis: effect of operational conditions on the composition of the microalgae-bacteria consortia, J. Appl. Phycol. 2021. 1 (2021) 1–13. https://doi.org/10.1007/S10811-021-02600-2.

[13] A. Morillas-España, T. Lafarga, A. Sánchez-Zurano, F.G. Acién-Fernández, E. Rodríguez-Miranda, C. Gómez-Serrano, C.V. González-López, Year-long evaluation of microalgae production in wastewater using pilot-scale raceway

photobioreactors: Assessment of biomass productivity and nutrient recovery capacity, Algal Res. 60 (2021) 102500. https://doi.org/10.1016/J.ALGAL.2021.102500.

[14] A. Morillas-España, T. Lafarga, F.G. Acién-Fernández, C. Gómez-Serrano, C.V. González-López, Annual production of microalgae in wastewater using pilot-scale thin-layer cascade photobioreactors, J. Appl. Phycol. 2021. 1 (2021) 1–11. https://doi.org/10.1007/S10811-021-02565-2.

[15] E. Navarro-López, M. del C. Cerón-García, M. López-Rodríguez, F.G. Acién-Fernández, E. Molina-Grima, Biostimulants obtained after pilot-scale high-pressure homogenization of Scenedesmus sp. grown in pig manure, Algal Res. 52 (2020) 102123. https://doi.org/10.1016/j.algal.2020.102123.

[16] J.F. Sánchez, J.M. Fernández, F.G. Acién, A. Rueda, J. Pérez-Parra, E. Molina, Influence of culture conditions on the productivity and lutein content of the new strain Scenedesmus almeriensis, Process Biochem. (2008). https://doi.org/10.1016/j.procbio.2008.01.004.

[17] A. Morillas-España, T. Lafarga, C. Gómez-Serrano, F.G. Acién-Fernández, C.V. González-López, Year-long production of Scenedesmus almeriensis in pilot-scale raceway and thin-layer cascade photobioreactors, Algal Res. 51 (2020) 102069. https://doi.org/10.1016/j.algal.2020.102069.

[18] M. del M. Morales-Amaral, C. Gómez-Serrano, F.G. Acién, J.M. Fernández-Sevilla, E. Molina-Grima, Outdoor production of Scenedesmus sp. in thin-layer and raceway reactors using centrate from anaerobic digestion as the sole nutrient source, Algal Res. 12 (2015) 99–108. https://doi.org/10.1016/j.algal.2015.08.020.

[19] C. Gómez, A. Guzmán-Carrasco, T. Lafarga, F.G. Acién-Fernández, OPTIMIZATION OF A NEW CULTURE MEDIUM FOR THE LARGE-SCALE

PRODUCTION OF PROTEIN-RICH ARTHROSPIRA PLATENSIS (OSCILLATORIALES, CYANOPHYCEAE), J. Phycol. 27 (2020) 636–644. https://doi.org/10.1111/jpy.13111.

[20] BOE, Métodos Oficiales de Análisis: Suelos y Aguas, (1982).

[21] C.V.G. López, M. del Carmen Cerón García, F.G.A. Fernández, C.S. Bustos, Y. Chisti, J.M.F. Sevilla, Protein measurements of microalgal and cyanobacterial biomass, Bioresour. Technol. 101 (2010) 7587–7591. https://doi.org/10.1016/j.biortech.2010.04.077.

[22] E. Perera, D. Sánchez-Ruiz, M.I. Sáez, A. Galafat, A. Barany, M. Fernández-Castro, A.J. Vizcaíno, J. Fuentes, T.F. Martínez, J.M. Mancera, F.J. Alarcón, J.A. Martos-Sitcha, Low dietary inclusion of nutraceuticals from microalgae improves feed efficiency and modifies intermediary metabolisms in gilthead sea bream (Sparus aurata), Sci. Reports 2020 101. 10 (2020) 1–14. https://doi.org/10.1038/s41598-020-75693-3.

[23] J. García-Márquez, R.M. Rico, M. del P. Sánchez-Saavedra, J.L. Gómez-Pinchetti, F.G. Acién, F.L. Figueroa, F.J. Alarcón, M.Á. Moriñigo, R.T. Abdala-Díaz, A short pulse of dietary algae boosts immune response and modulates fatty acid composition in juvenile Oreochromis niloticus, Aquac. Res. (2020). https://doi.org/10.1111/are.14781.

[24] A.R. Wellburn, The Spectral Determination of Chlorophylls a and b, as well as Total Carotenoids, Using Various Solvents with Spectrophotometers of Different Resolution, J. Plant Physiol. 144 (1994) 307–313. https://doi.org/10.1016/S0176-1617(11)81192-2.

[25] J. Collao, M. del M. Morales-Amaral, F.G. Acién-Fernández, S. Bolado-Rodríguez, N. Fernandez-Gonzalez, Effect of operational parameters, environmental conditions, and biotic interactions on bacterial communities

present in urban wastewater treatment photobioreactors, Chemosphere. 284 (2021) 131271. https://doi.org/10.1016/j.chemosphere.2021.131271.

[26] A. Herrera, G. D'Imporzano, F.G. Acién Fernandez, F. Adani, Sustainable production of microalgae in raceways: Nutrients and water management as key factors influencing environmental impacts, J. Clean. Prod. (2020). https://doi.org/10.1016/j.jclepro.2020.125005.

[27] E.H. Murchie, T. Lawson, Chlorophyll fluorescence analysis: A guide to good practice and understanding some new applications, J. Exp. Bot. (2013). https://doi.org/10.1093/jxb/ert208.

[28] Q. He, H. Yang, L. Wu, C. Hu, Effect of light intensity on physiological changes, carbon allocation and neutral lipid accumulation in oleaginous microalgae, Bioresour. Technol. 191 (2015) 219–228. https://doi.org/10.1016/j.biortech.2015.05.021.

[29] L. Tan, W. Xu, X. He, J. Wang, The feasibility of Fv/Fm on judging nutrient limitation of marine algae through indoor simulation and in situ experiment, Estuar. Coast. Shelf Sci. 229 (2019) 106411. https://doi.org/10.1016/J.ECSS.2019.106411.

[30] T. Gan, N. Zhao, G. Yin, M. Chen, X. Wang, J. Liu, W. Liu, Optimal chlorophyll fluorescence parameter selection for rapid and sensitive detection of lead toxicity to marine microalgae Nitzschia closterium based on chlorophyll fluorescence technology, J. Photochem. Photobiol. B Biol. 197 (2019) 111551. https://doi.org/10.1016/J.JPHOTOBIOL.2019.111551.

[31] R.J. GEIDER, J. LA ROCHE, Redfield revisited: variability of C[ratio]N[ratio]P in marine microalgae and its biochemical basis, Eur. J. Phycol. 37 (2002) 1–17. https://doi.org/10.1017/S0967026201003456.

[32] H.J. Choi, S.M. Lee, Effect of the N/P ratio on biomass productivity and nutrient removal from municipal wastewater, Bioprocess Biosyst. Eng. 2014 384. 38 (2014) 761–766. https://doi.org/10.1007/S00449-014-1317-Z.

[33] W.C. Antezana De Blas P García-Rebollar C Rodríguez A Beccaccia P Ferrer A Cerisuelo V Moset F Estellés M Cambra-López S Calvet, W. Antezana, Á.P. Ferrer Á F Estellés Á M Cambra-López Á S Calvet, S. Calvet, C.P. De Blas Á García-Rebollar Á C Rodríguez Á A Beccaccia, A. Cerisuelo, V. Moset, Composition, potential emissions and agricultural value of pig slurry from Spanish commercial farms, Nutr Cycl Agroecosyst. 104 (2016) 159–173. https://doi.org/10.1007/s10705-016-9764-3.

[34] J. Coppens, R. Lindeboom, M. Muys, W. Coessens, A. Alloul, K. Meerbergen, B. Lievens, P. Clauwaert, N. Boon, S.E. Vlaeminck, Nitrification and microalgae cultivation for two-stage biological nutrient valorization from source separated urine, Bioresour. Technol. 211 (2016) 41–50. https://doi.org/10.1016/j.biortech.2016.03.001.

[35] K. Liang, Q. Zhang, M. Gu, W. Cong, Effect of phosphorus on lipid accumulation in freshwater microalga Chlorella sp., J. Appl. Phycol. 25 (2013) 311–318. https://doi.org/10.1007/s10811-012-9865-6.

[36] A. Sánchez-Zurano, M. Ciardi, T. Lafarga, J.M. Fernández-Sevilla, R. Bermejo, E. Molina-Grima, Role of Microalgae in the Recovery of Nutrients from Pig Manure, Processes. 9 (2021) 203. https://doi.org/10.3390/pr9020203.

[37] J. Venglovsky, N. Sasakova, G. Gregova, I. Papajova, F. Toth, T. Szaboova, Devitalisation of pathogens in stored pig slurry and potential risk related to its application to agricultural soil, Environ. Sci. Pollut. Res. 25 (2018) 21412–21419. https://doi.org/10.1007/s11356-017-0557-2.

[38] X. Qian, Z. Wang, G. Shen, X. Chen, Z. Tang, C. Guo, H. Gu, K. Fu, Heavy

metals accumulation in soil after 4 years of continuous land application of swine manure: A field-scale monitoring and modeling estimation, Chemosphere. 210 (2018) 1029–1034. https://doi.org/10.1016/j.chemosphere.2018.07.107.

[39] T. Lafarga, Cultured Microalgae and Compounds Derived Thereof for Food Applications: Strain Selection and Cultivation, Drying, and Processing Strategies, Food Rev. Int. 36 (2020) 559–583. https://doi.org/10.1080/87559129.2019.1655572.

[40] K. Zhang, Z. hua Zhang, H. Wang, X. mao Wang, X. hui Zhang, Y.F. Xie, Synergistic effects of combining ozonation, ceramic membrane filtration and biologically active carbon filtration for wastewater reclamation, J. Hazard. Mater. 382 (2020) 121091. https://doi.org/10.1016/j.jhazmat.2019.121091.

[41] D. Bertagna Silva, A. Cruz-Alcalde, C. Sans, J. Giménez, S. Esplugas, Performance and kinetic modelling of photolytic and photocatalytic ozonation for enhanced micropollutants removal in municipal wastewaters, Appl. Catal. B Environ. 249 (2019) 211–217. https://doi.org/10.1016/j.apcatb.2019.02.072.

[42] P. Ding, L. Chu, J. Wang, Advanced treatment of petrochemical wastewater by combined ozonation and biological aerated filter, Environ. Sci. Pollut. Res. 25 (2018) 9673–9682. https://doi.org/10.1007/s11356-018-1272-3.

[43] D. Saranya, S. Shanthakumar, An integrated approach for tannery effluent treatment with ozonation and phycoremediation: A feasibility study, Environ. Res. 183 (2020) 109163. https://doi.org/10.1016/j.envres.2020.109163.

[44] C. González-Fernández, B. Molinuevo-Salces, M.C. García-González, Nitrogen transformations under different conditions in open ponds by means of microalgae-bacteria consortium treating pig slurry, Bioresour. Technol. 102 (2011) 960–966. https://doi.org/10.1016/j.biortech.2010.09.052.

[45] O.M. Darwesh, I.A. Matter, M.F. Eida, H. Moawad, Y.-K. Oh, Influence of Nitrogen Source and Growth Phase on Extracellular Biosynthesis of Silver Nanoparticles Using Cultural Filtrates of Scenedesmus obliquus, Appl. Sci. 2019, Vol. 9, Page 1465. 9 (2019) 1465. https://doi.org/10.3390/APP9071465.

[46] T. Cai, S.Y. Park, Y. Li, Nutrient recovery from wastewater streams by microalgae: Status and prospects, Renew. Sustain. Energy Rev. 19 (2013) 360–369. https://doi.org/10.1016/j.rser.2012.11.030.

[47] M. Wang, Y. Yang, Z. Chen, Y. Chen, Y. Wen, B. Chen, Removal of nutrients from undiluted anaerobically treated piggery wastewater by improved microalgae, Bioresour. Technol. 222 (2016) 130–138. https://doi.org/10.1016/j.biortech.2016.09.128.

[48] BOE, Real Decreto 509/1996, de 15 de marzo, de desarrollo del Real Decreto-ley 11/1995, de 28 de diciembre, por el que se establecen las normas aplicables al tratamiento de las aguas residuales urbanas., Bol. Of. Del Estado. 77 (1996) 12038–12041.

[49] T. Lafarga, Effect of microalgal biomass incorporation into foods: Nutritional and sensorial attributes of the end products, Algal Res. 41 (2019) 101566. https://doi.org/10.1016/j.algal.2019.101566.

[50] S.I. Mustapha, I. Rawat, F. Bux, Y.M. Isa, Catalytic pyrolysis of nutrient-stressed Scenedesmus obliquus microalgae for high-quality bio-oil production, Renew. Energy. 179 (2021) 2036–2047. https://doi.org/10.1016/J.RENENE.2021.08.043.

[51] J.F.F. Sánchez, J.M.M. Fernández-Sevilla, F.G.G. Acién, M.C.C. Cerón, J. Pérez-Parra, E. Molina-Grima, Biomass and lutein productivity of Scenedesmus almeriensis: Influence of irradiance, dilution rate and temperature, Appl. Microbiol. Biotechnol. 79 (2008) 719–729. https://doi.org/10.1007/s00253-008-1494-2.