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Research article

Long term operation of a phototrophic biological nutrient removal system: Impact of CO_2 concentration and light exposure on process performance

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ABSTRACT

The utilization of non-aerated microalgae-bacterial consortia for phototrophic biological nutrient removal (photo-BNR) has emerged as an alternative to conventional wastewater treatment. Photo-BNR systems are operated under transient illumination, with alternating dark-anaerobic, light-aerobic and dark-anoxic conditions. A deep understanding of the impact of operational parameters on the microbial consortium and respective nutrient removal efficiency in photo-BNR systems is required. The present study evaluates, for the first time, the long-term operation (260 days) of a photo-BNR system, fed with a COD:N:P mass ratio of 7.5:1:1, to understand its operational limitations. In particular, different CO_2 concentrations in the feed (between 22 and 60 mg C/L of Na_2CO_3) and variations of light exposure (from 2.75 h to 5.25 h per 8 h cycle) were studied to determine their impact on key parameters, like oxygen production and availability of polyhydroxyalkanoates (PHA), on the performance of anoxic denitrification by polyphosphate accumulating organisms. Results indicate that oxygen production was more dependent on the light availability than on the CO_2 concentration. Also, under operational conditions with a COD:Na_2CO_3 ratio of 8.3 mg COD/mg C and an average light availability of 5.4 ± 1.3 W h/g TSS, no internal PHA limitation was observed, and 95 ± 7%, 92 ± 5% and 86 ± 5% of removal efficiency could be achieved for phosphorus, ammonia and total nitrogen, respectively. 81 ± 1.7% of the ammonia was assimilated into the microbial biomass and 19 ± 1.7% was nitrified, showing that biomass assimilation was the main N removal mechanism taking place in the bioreactor. Overall, the photo-BNR system presented a good settling capacity (SVI ~60 mL/g TSS) and was able to remove 38 ± 3.3 mg P/L and 33 ± 1.7 mg N/L, highlighting its potential for achieving wastewater treatment without the need of aeration.

1. Introduction

High concentrations of nutrients, mainly in the form of ammonia and phosphorus, are directly responsible for eutrophication of rivers, lakes and seas, representing a challenge for wastewater treatment (WWT). However, this high nutrient load can also be a chance for simultaneous nutrient recovery and the production of added-value bioproducts, such as biofertilizers (Mulbry et al., 2005, 2006; Ramanan et al., 2016; Torres-Franco et al., 2021). Presently, the WWT industry uses several physical/chemical or biological methods to remove nutrients. Conventional technology, such as biological nutrient removal (BNR) and phosphate/ammonia precipitation, are well established and present high nutrient removal efficiencies (Crini and Lichtfouse, 2019; Winkler and Straka, 2019). Indeed, the latest years have witnessed a great development of BNR systems like the Integrated Fixed-Film Activated Sludge (IFAS) (Naghipour et al., 2022) and the Moving Bed Biofilm Reactor (MBBR) (Zekker et al., 2011), which combine suspended and attached activated sludge, and allow a decrease of areal and operational costs in relation to conventional activated sludge technologies. However, these processes are highly dependent on energy (due to the intensive aeration of BNR systems) and nutrient precipitation requires the use of chemicals, which presses the need for more environmentally sustainable technologies (Rosso et al., 2008; Torres-Franco et al., 2021)). Nutrient removal in conventional BNR systems is normally performed by polyphosphate accumulating organisms (PAOs), ammonia oxidizing bacteria (AOBs) and nitrite oxidizing bacteria (NOBs).

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Conventional BNR is favored by higher Chemical Oxygen Demand (COD):P ratios (Kuba et al., 1996; Wang et al., 2009), which normally requires extra COD addition, creating additional WWT expenses.

Algal based technology has been investigated for WWT, using single microalgae technology (De-Bashan and Bashan, 2004) or through consortia of microalgae and bacteria (Carvalho et al., 2018; Alcántara et al., 2015, de Godos et al., 2014; Torres-Franco et al., 2021).

Microalgae – bacterial consortia for wastewater treatment have a great potential for biosorption and nutrient removal, being considered an environmentally friendly technology (Oruganti et al., 2022; Chan et al., 2022; Toledo-Cervantes et al., 2019; Young et al., 2017). These consortia require less COD when compared with conventional BNR systems, since CO_2 is used by microalgae and nitrifiers as carbon source, thereby lowering the COD:Nutrient ratio needed for an efficient nutrient removal (Fatemeh et al., 2021; Judd et al., 2015; Wang et al., 2018). For phototrophic growth, microalgae use nutrients and CO_2 as their main source of inorganic carbon (Fatemeh et al., 2021) and produce high amounts of photosynthetic O_2 through solar energy capture. Heterotrophic bacteria consume O_2 and remove organic carbon and nutrients, producing CO_2 that is used for autotrophic growth (Boelee et al., 2012; Zhang et al., 2018). Also, the consortium of microalgae and bacteria can form granular biomass (Zhang et al., 2018), improving settling and overcoming the problem of solids separation from the treated water that occurs in WWT with single microalgae systems.

A further advantage of microalgae - bacterial technology is the reduction of greenhouse gas emissions (de Godos et al., 2014). Besides CO_2 fixation by microalgae that reduce CO_2 emissions, photosynthetic oxygenation also reduces the need of mechanical aeration, decreasing the energy demands of the process (Fatemeh et al., 2021). Higher ammonia assimilation into the microalgal biomass, instead of the dominance of nitrification-denitrification processes, may also decrease the emissions of N_2O, a gas which presents a greenhouse potential 300 times stronger than CO_2 (Alcántara et al., 2015; Plouviez and Guieysse, 2020).

Microalgae-bacterial photobioreactor performance, when fully optimized, is expected to be comparable with the classical BNR process in terms of nutrient removal, but with less energy requirements (de Godos et al., 2014). Whilst parameters such as C:N:P ratios, dissolved oxygen, pH and temperature have been widely studied for the conventional BNR process, the impact of key parameters in phototrophic biological nutrient removal (photo-BNR) systems, like CO_2 concentration and light exposure period, still requires further evaluation.

Previous studies indicated that the availability of CO_2 is one of the principal limiting factors for microalgae growth (Arias et al., 2017) and for microalgae-bacterial consortia nutrient removal capacity, since nitrifying bacteria also compete with microalgae for the CO_2 (Choi et al., 2010, 2010de Godos et al., 2014; García et al., 2017). CO_2 sequestration is influenced by light intensity, pH, temperature, CO_2 loading and biomass concentration (Judd et al., 2015). Also, algal activity increases with light intensity, until a threshold value where the photosynthetic apparatus is saturated. Indeed, light availability will not only influence the nutrient removal capacity of microalgae and other phototrophic organisms, but will also impact the photosynthetic oxygen production. In turn, this oxygen production will influence the growth and nutrient removal capacity of chemotrophic bacteria (PAOs, AOBs, NOBs and others) present in the microalgae-bacterial consortia (Carvalho et al., 2021). The photo-BNR process developed by Carvalho et al. (2021) displayed the potential to achieve higher levels of P removal as compared to other microalgal-bacterial processes. However, there is a knowledge gap on how CO_2 concentrations and light availability should be adjusted to assure high removal of both N and P. On one hand, sufficient oxygen should be photosynthetically produced to allow PAO respiration and nitrification. On the other, oxygen concentration should not compromise the achievement of anoxic phases for efficient denitrification.

Therefore, the objective of the present study is to clarify what is the impact of CO_2 concentration and light exposure on the nutrient removal capacity of Photo-BNR systems. Thus, a non-aerated photo-BNR system operated under dark (anaerobic)/light (aerobic)/dark (anoxic) conditions, selected for a consortium of microalgae, PAOs, AOB and NOBs that was studied to elucidate how the adjustment of key operational parameters can influence process stability and removal efficiency. The goal is to understand the impact of CO_2 concentration and the length of light/dark phases on the long-term operation of the photo-BNR system, particularly on O_2 production, nitrification, denitrification and phosphorus removal.

2. Materials and methods

2.1. Photo-BNR reactor

A sequencing batch reactor (SBR), with a working volume of 3.8 L was inoculated with sludge from a photo-BNR reactor already enriched in PAOs (*Accumulibacter* phosphatis), microalgae, cyanobacteria and *Thiocapsa,* amongst other microorganisms (Carvalho et al., 2021). The reactor was continuously operated for 260 days and subjected to transient illumination provided by an internal halogen lamp (200 W), with a light intensity of 532 W/m^2 (similar to the average light intensity in Portugal during summer days) (Gschwind et al., 2006), which corresponds to a volumetric light intensity of 4.6 W/L. The sludge retention time (SRT) was 19 ± 1 day to guarantee proliferation of nitrifiers, and the HRT was 16 h. The photo-BNR reactor was operated in 8 h cycles with sequential dark (anaerobic), light (aerobic) and dark (anoxic) periods, and continuously mixed through magnetic stirring (~700 rpm). In the end of the anoxic phase, there was a 15 min settling period, followed by a 30 min idle period. The idle period included the removal of supernatant and the beginning of argon sparging before the beginning of the next cycle. Argon was continuously sparged during the dark (anaerobic) phase to assure anaerobic conditions. The reactor was fed in the beginning of the dark (anaerobic) phase with 1.9 L of synthetic medium, where the carbon source was a mixture of two Volatile Fatty Acids (VFAs), acetate and propionate (75%/25% of COD), to guarantee the proliferation of PAOs over GAOs (Lopez-Vazquez et al., 2009). The synthetic medium fed to the reactor was composed of 75% (v/v) of a phosphate solution (168 mg/L of K_2HPO_4 and 103 mg/L of KH_2PO_4) and 25% (v/v) of carbon medium, which after the reactor start-up stage, presented a concentration per liter of: 1.92 g $C_2H_3O_2Na.3H_2O$; 204 μL $C_3H_6O_2$ (99.5%); 0.59 g NH_4Cl; 0.95 g $MgSO_4.7H_2O$; 0.44 g $CaCl_2.2H_2O$; 31.7 mg EDTA and 3.17 mL of a micronutrients solution, with a concentration per liter of: 1.5 g $FeCl_3.6H_2O$; 0.15 g H_3BO_3; 0.03 g $CuSO_4.5H_2O$; 0.18 g KI; 0.12 g $MnCl_2.4H_2O$; 0.06 g $Na_2MoO.2H_2O$; 0.12 g $ZnSO_4.7H_2O$ and 0.15 g $CoCl_2.6H_2O$. These concentrations corresponded to a COD, phosphorus and ammonia concentration in the feed of 300 mg COD/L, 40 mg P/L and 40 mg N/L, respectively. Sodium carbonate (Na_2CO_3) was fed at the end of the dark anaerobic period as a supplemental inorganic carbon (IC) source for autotrophic microorganisms. Carbonate was used instead of a CO_2 rich stream gas stream for simplification purposes and for a better control of CO_2 concentrations in the reactor. The temperature of the reactor was set to 20 ± 4 °C, using a thermostat bath, while pH was controlled at 7.5 through the addition of 0.1 M HCl.

2.2. Evolution of operational conditions

Throughout the photo-BNR operation, light intensity, phosphorus and ammonia concentration in the feed remained unchanged. To improve the reactor nutrient removal efficiency and evaluate the impact of CO_2 concentration and illumination periods on the culture's performance, the length of the different phases was adjusted during the photo-BNR operation, as well as the Na_2CO_3 concentration in the feed (Fig. 1).

The length of the dark (anaerobic) phase was adjusted to guarantee total COD consumption in the dark phase, preventing the growth of

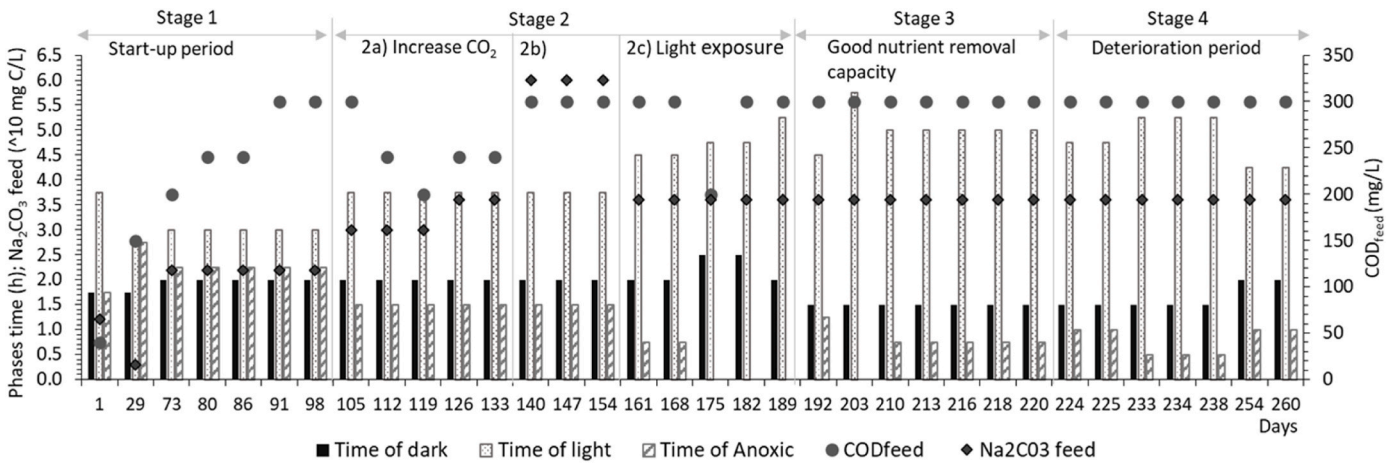

Fig. 1. Operational adaptations of the photo-BNR operation. Stage 1: start-up period; Stage 2: increase auto-oxygenation of photo-BNR (2a: increase of CO_2 and temporary adjustment of COD concentration; 2 b: increase of CO_2 and reestablishment of COD concentration; 2c: increase of the length of the light phase); Stage 3: Photo-BNR operation with good nutrient removal capacity; Stage 4: deterioration of photo-BNR performance. Settling and idle period was 15 and 30 min, respectively, during all the period of the photo-BNR operation.

heterotrophic organisms during the subsequent light aerobic phase that do not contribute for nutrient removal. The length of the light phase and concentration of Na_2CO_3 was adjusted to guarantee sufficient oxygen production for system oxygenation and allow oxygen detection by the sensor (>1 mg O_2/L). The length of the dark (anoxic) phase was modified to guarantee that oxygen was depleted after the light was turned off, achieving anoxic conditions for denitrification (Fig. 1). The reactor operation was divided into 4 stages: Stage 1 (104 days) was the start-up period, where COD was increased from 40 to 300 mg COD/L; Stage 2 (86 days) corresponds to the period that the CO_2 concentration, fed to the reactor as Na_2CO_3, was increased from 22 to 36 mg IC/L (2a) and to 60 mg IC/L (2 b) at constant values of light exposure time (3.75 h per cycle), followed by a period that the light exposure time was increased from 3.75 h to 5.25 h per cycle (2c) at constant CO_2 concentration of 36 mg IC/L; Stage 3 (30 days) corresponds to the period with good capacity of nutrients removal, with a COD:IC:N:P: ratio of 7.5:0.9:1:1 (mg basis) and 5.00 h of light exposure time; Stage 4 (39 days) corresponds to the period where NO_3 leaked to the dark-anaerobic phase of the following cycle (Fig. 1).

2.3. Analytical methods

Acetate and propionate were determined by high-performance liquid chromatography (HPLC), using a VWR Hitachi Chromaster with a Bio-rad Aminex HPX-87H 300 × 7.8 MM column and a DAD detector. 0.01 N sulfuric acid was used as eluent, with an elution rate of 0.5 mL/min and an operating temperature of 30 °C.

Phosphate, ammonia, nitrate and nitrite concentrations were determined by colorimetric methods implemented in a flow segmented analyzer (Skalar 5100, Skalar Analytical, The Netherlands). For the total phosphorus content, an acidic digestion of a mixed liquor sample was performed with 0.3 M H_2SO_4 and 400 mg of $K_2S_2O_8$ and analyzed using the flow segmented analyzer. PHAs were determined by gas chromatography (GC) using the method described by Lanham et al. (2013), using a Bruker 430-GC gas chromatograph equipped with a FID detector and a Restek column (60 m, 0.53 mm internal diameter, 1 μM df, crossbond). For carbohydrates determination, an acidic digestion, with 0.9 M HCl was made during 3 h and the supernatant was analyzed by HPLC as described by Lanham et al. (2012). Total suspended solids (TSS) and volatile suspended solids (VSS) were calculated according to the standard methods (APHA/AWWA/1995).

The light intensity provided by the halogen lamp was measured using a LI-COR light meter (LI-250 A), equipped with a pyranometer sensor LI-200 SA.

Phylogenetic analysis of the bacterial community was done through Fluorescence *in situ* hybridization (FISH), as previously described by Amann (1995), on fixed samples with 4% paraformaldehyde or ethanol, according to Nielson et al., 2009). The oligonucleotide probes used were the fluorescein isothiocyanate (FITC)-labelled EUBmix (EUB338, EUB338II, EUB338III) for all bacteria, applied with the cyanine 3 (Cy3)-labelled probes: PAOmix (PAO651, PAO462, PAO846) for *Candidatus Accumulibacter* phosphatis; Acc-I-444 which targets type I *Accumulibacter* PAOs and Acc–II–444 for *Accumulibacter* PAOs type II; CPB_654 for *Candidatus Competibacter* phosphatis; Nso1225 for ammonia oxidizing bacteria (AOBs) (*Nitrosomonadaceae*; *Nitrosomonadales*); and NIT3 (*Nitrobacter* spp.) and Ntspa662 (*Nitrospira* spp.) for nitrite oxidizing bacteria (NOBs). More details are available at probeBase 2016. The biomass samples were visualized using a Zeiss Imager D2 epifluorescence microscope (Germany), at 1000 X amplification. Sequencing analysis was performed at DNASense ApS Inin Aalborg, Denmark.

2.4. Calculations

Ammonia, phosphorous, and total nitrogen removal efficiency was calculated as the difference between the concentrations in the influent and in the effluent, divided by the concentration in the influent. Nitrate removal efficiency was calculated as the difference between the concentration in the end of the light period and in the effluent, divided by the concentration in the end of the light period. Nitrification rate was calculated by dividing the nitrate concentration in the end of the light period by the total amount of ammonia consumption during the light period. To determine the phosphorus content in the biomass (%), the supernatant phosphate concentration was subtracted from the total phosphate concentration obtained by sample digestion.

N and P removal rates (mg/L.h) in the light phase and anoxic phase were calculated as the difference between the concentrations in the beginning and end of each phase, divided by the time length of each phase.

The chlorophyll (mg Chlo/L) concentration was calculated according to Ritchie (2018) using the equations for pigments extraction with 100% ethanol.

Aqueous carbon dioxide was measured with a CO_2 Mettler Toledo sensor and then the concentrations were readjusted considering the pH of the reactor, taking into account the equations of CO_2 equilibrium in water and their respective constants according to Henry's Law, using the

following equations (K = 0.0017 M; Ka_1 = 4.47 E^{-7}M; Ka_2 = 4.69 E^{-11}M):

$$H^+ = 10^{pH} \quad \text{eq. 1A}$$

$$\alpha_{H_2CO_3} = \frac{[H^+]^2}{[H^+]^2 + [H^+]K_{a1} + K_{a1}K_{a2}} \quad \text{eq. 1B}$$

$$\alpha_{HCO_3^-} = \frac{[H^+]K_{a1}}{[H^+]^2 + [H^+]K_{a1} + K_{a1}K_{a2}} \quad \text{eq. 1C}$$

$$[CO_2]_{total} = [CO_2]_{aq} + \frac{\propto HCO_3^-[CO_2]_{aq} + \propto HCO_3^- K[CO_2]_{aq}}{\propto H_2CO_3} \quad \text{eq. 1D}$$

Sludge volume index (SVI) was measured inside the reactor in the end of the cycle, after 15 min of settling, and was calculated according to the following equation (Pierce et al., 1998).

$$SVI\left(\frac{mL}{g\ TSS}\right) = \frac{\frac{Volume\ of\ sedimented\ biomass\ (mL)}{Volume\ of\ reactor\ liquor\ (L)}}{TSS\ \left(\frac{g}{L}\right)} \quad \text{eq. 2}$$

3. Results and discussion

3.1. Photo-BNR start-up and operation

The photo-BNR reactor cyclic operation with sequential dark (anaerobic)/light (aerobic)/dark (anoxic) phases, targeted the selection of a microalgae-bacterial consortium capable of consuming the totality of the COD during the dark (anaerobic) phase, and removing nutrients (N and P) during the light and anoxic phases. During the start-up period (Stage 1), COD and Na_2CO_3 in the feed were increased until 300 mg COD/L and 22 mg IC/L, respectively, which corresponds to a COD:IC:N:P ratio of 15:1.1:2:2 on a mass basis (Fig. 1). The increase of COD concentration resulted in higher biomass concentration, consequently, resulting in higher oxygen necessities for heterotrophic biomass respiration and growth (Fig. 2A, Stage 1). The oxygen that was being photosynthetically produced could be consumed by other microorganisms in the photo-BNR process, including autotrophic aerobes. As PAOs were oxygen limited during the light phase, they had reduced energy for P uptake and polyphosphate formation, showing low phosphorus content in the biomass (Fig. 2B, Stage 1). This gradually decreased PAOs' capability to perform COD consumption and P release during the dark anaerobic phase. Since low nutrient removal was obtained (<70% for phosphorus and ammonia) and O_2 was barely detected during the start-up period, it was concluded that nutrient removal was limited by oxygen availability. For this reason, the aim of Stage 2 was to increase oxygen production by microalgae/cyanobacteria by means of increasing the CO_2 concentration and the illumination length (Fig. 1). In Stage 2a, oxygen was still limited, and the culture was not able to fully consume the 300 mg COD/L fed to the reactor during the dark phase. Therefore, COD was temporarily decreased in Stage 2a to guarantee full COD consumption during the dark period and allow system stability until the adjustments of CO_2 concentration and illumination length became effective.

Indeed, an increase of chlorophyll concentration was observed in Stage 2 b (Fig. 2A), which suggested an increase of photosynthetic microorganisms and a consequent likely increase of photosynthetic oxygen production. As such, COD concentration in the feed was restored in Stage 2 b–300 mg COD/L. Along Stage 2a and 2 b, the oxygen concentration was routinely limited, prompting the increase of the light period in stage 2c from 3.75 h to 5.25 h. After around 30 days with higher light exposition time, oxygen production increased (Fig. 2A), allowing full removal of phosphorus and around 90% of ammonia removal in Stage 3. When oxygen inside the reactor exceeded a concentration of ~2 mg O_2/

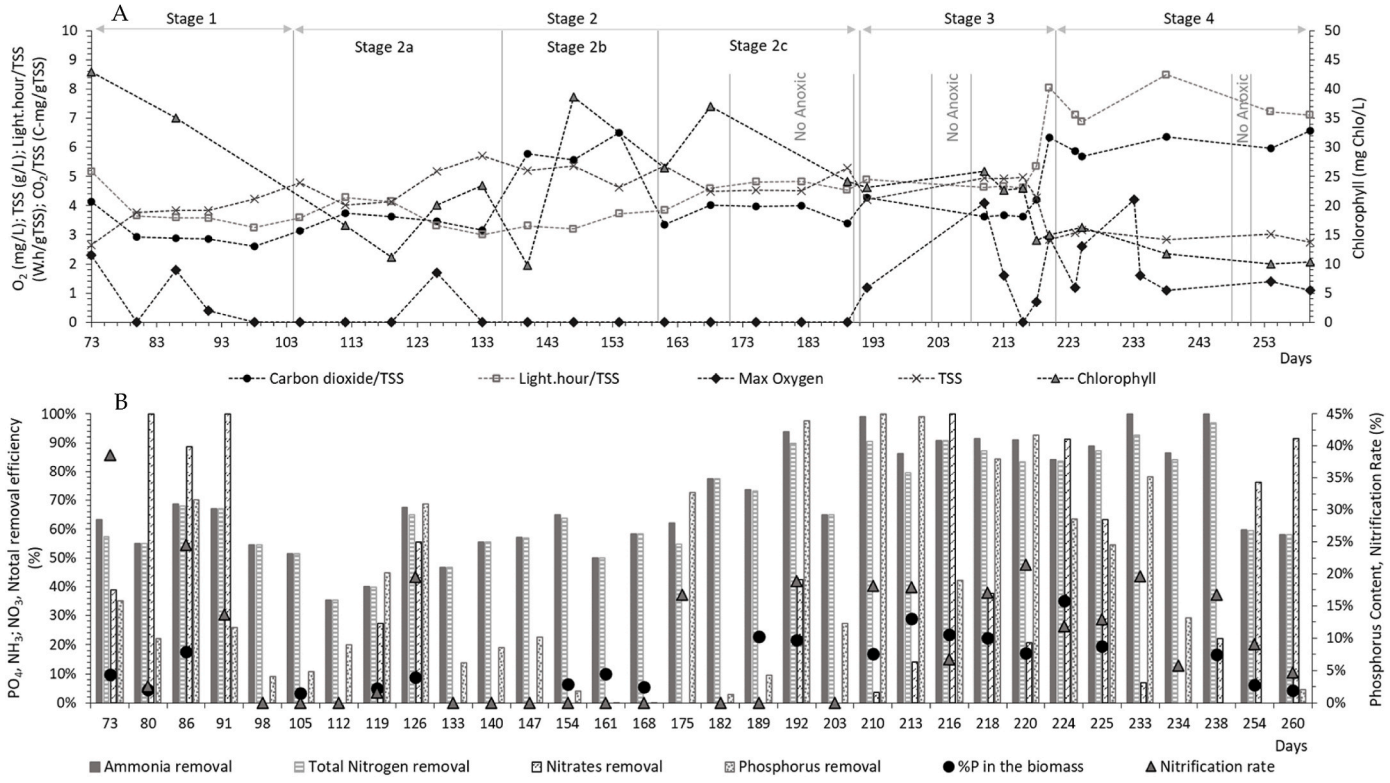

Fig. 2. Photo-BNR performance during the 260 days of operation. TSS values correspond to the average value of TSS during a daily cycle and Max O_2 corresponds to the oxygen concentration reached in the end of light phase. Stage 1 (until day 104): start-up period; Stage 2: (from day 105–191) increase auto oxygenation of photo-BNR; Stage 3 (from day 192–221): good capacity of nutrient removal; Stage 4 (from day 222–260): deterioration of photo-BNR performance. No anoxic phase corresponds to the period when the anoxic phase was removed to allow more time of light exposure.

L at the end of the illuminated phase, subsequent anoxic conditions were hard to achieve and, consequently, denitrification during the anoxic period was reduced, which decreased total N removal to around 80% (Fig. 2B). Overall, these results show that oxygen concentration is a key parameter to be controlled. O_2 production by microalgae/cyanobacteria is dependent on CO_2 availability and light intensity and exposition period (Fatemeh et al., 2021; Judd et al., 2015; Luo et al., 2017; Muñoz and Guieysse, 2006; Razzak et al., 2017). As already indicated, illumination period and intensity have a strong influence on algal-bacteria consortia dynamics and, thus, influence nutrient removal efficiency (Fatemeh et al., 2021). For this reason, the CO_2 concentration and the length of the light phase were the two key parameters explored in the present work, to understand its impact on photo-BNR performance when operated in dark (anaerobic)/light (aerobic)/dark (anoxic) phases.

3.2. Impact of CO_2 concentration

Changes in Na_2CO_3 concentration were made to adapt the photosynthetic oxygen production to the photo-BNR necessities. In stage 2a, O_2 was still not detected in the reactor (Fig. 2A) even with 30 mg IC/L of Na_2CO_3 concentration fed to the reactor. The increase of the TSS concentration was not accompanied by an increase of chlorophyll concentration. In fact, chlorophyll decreased in Stage 2a when compared with Stage 1, indicating the growth of other autotrophic microorganisms, rather than microalgae. The increase of Na_2CO_3 in the feed from 30 mg IC/L to 36 mg IC/L (during stage 2a) and more notably to 60 mg IC/L in stage 2 b, led to an increase of chlorophyll concentration (Fig. 2A), indicating higher microalgae growth and thus, higher O_2 production and better nutrient removal were expected. In reality, neither higher oxygen concentrations inside the reactor were achieved, nor was a higher P removal obtained, despite Stage 2 b being operated with the highest CO_2 concentration tested in this work. Only ammonia removal slightly increased from 48% ± 12–59% ± 5 in stage 2 b (Fig. 2B). The fact that dissolved oxygen could not be detected in the reactor bulk does not necessarily mean that O_2 production was unaffected by the CO_2, since the oxygen could have been produced and consumed at similar rates. With more CO_2 being consumed for autotrophic microorganisms' growth (bacteria or microalgae), higher oxygen is required to support this growth. Nitrification is dependent on both CO_2 and O_2 availability and during stage 2 b, even with the increase in the Na_2CO_3 concentration fed, no nitrification was observed (Fig. 2B). These results indicate that photosynthetic O_2 production was not enough to fulfil all the microorganisms' requirements during this stage of reactor operation.

Fatemeh et al. (2021) and Singh and Singh (2014) reported that low availability of inorganic carbon can limit the microalgae growth and consequently oxygen production, directly affecting the amount of nitrogen and phosphorus assimilated into the biomass. On the other hand, Razzak et al. (2017) and Singh and Singh (2014) also observed that increasing the CO_2 concentration in microalgae systems increased the amount of biomass. However, the optimal CO_2 concentration for microalgae growth was not clear from those studies.

A CO_2/TSS ratio of 3.4 ± 0.3 mg IC/g TSS in Stage 2a and 6.0 ± 0.5 mg IC/g TSS in Stage 2 b did not result in oxygen detection. The higher length of the light phase in Stages 3 and 4 resulted in ratios of 4.3 ± 1.0 mg IC/g TSS (Stage 3) and 6.1 ± 0.4 mg IC/g TSS (Stage 4), which was sufficient to allow higher oxygen production, even more than the oxygen demand, since O_2 increased above negligible levels (Fig. 2A). Excess O_2 production was also observed in Carvalho et al. (2021), in a microalgae-bacterial system operated with a ratio of 3.1 ± 0.8 mg IC/g TSS. However, in Stage 2a and Stage 2 b, there was no excess of oxygen production, probably due to the shadowing effect caused by the higher TSS concentration (4.8 ± 0.7 g TSS/L on Stage 2a and 5.1 ± 0.4 g TSS/L on Stage 2 b), or because the period of illumination was not enough, limiting oxygen production. Anbalagan et al. (2017) found that for higher TSS concentrations, higher light intensities are needed to reach higher oxygen production, since in microalgae-bacterial consortia, an excessive solids concentration may reduce light penetration, causing self-shading and resulting in oxygen consumption by microalgae dark respiration, with subsequently lower oxygen availability for bacteria respiration. Literature is unanimous about recommending to maintain the biomass concentration at relatively lower levels to avoid self-shading problems (Luo et al., 2017; Muñoz and Guieysse, 2006). Foladori et al. (2020) found that the best TSS values for maximum ammonia removal in microalgae-bacterial reactors (light intensity = 45 μmol/m^2s^{-1}) without external aeration was between 0.7 and 2.6 g TSS/L, depending on the floccular structure. Most of the published studies, either from microalgae or microalgae-bacterial consortia, used biomass concentrations between 0.35 and 2.8 g TSS/L (García et al., 2017; Judd et al., 2015). The results of stage 4 (2.7 g TSS/L) are more consistent with this range, and is much lower than the biomass concentration obtained in stage 2a (5.7 g TSS/L) (Fig. 2A). A possible way to decrease the high TSS values observed in the present work, and thus attain higher light availability, could be by decreasing the SRT of the reactor below 19 days. Future studies are needed to evaluate this hypothesis and clarify its impact on slow growing populations (e.g. nitrifiers) and the respective nitrification performance.

Overall, the obtained results indicate that increases of the Na_2CO_3 concentration will not increase photosynthetic oxygen production per se, since the biomass concentration inside the reactor will affect light penetration and microalgae growth. Higher biomass concentration and associated self-shading appears to be limiting the photosynthetic O_2 production. These results suggest that O_2 production could be more dependent on the light availability than on the CO_2 concentration.

3.3. Impact of light phase length

Since Stage 2 b results indicated that operation at high CO_2 concentrations (60 mg IC/L) did not result in oxygen detection in the reactor and improved nutrient removal, for subsequent stages, CO_2 concentration was restored to 36 mg IC/L and the light availability was increased. Given that the light intensity to which the culture was exposed (532 W/m^2) was already high, to achieve higher light availability, and consequently allow more oxygen production, the period of light exposure was increased in Stage 2c and in Stage 3 (Fig. 1). To attain this in the 8 h cycle, the anoxic phase length was reduced or even removed. In fact, when oxygen was limited, the culture did not produce nitrate and, consequently, no anoxic phase was needed.

The results obtained during the reactor operation support the importance of the length of the light phase, since its increase allowed oxygen detection in Stage 3 (Fig. 2A), without necessarily implying an increase in photosynthetic microorganisms. Considering that algal organic matter contains on average 1.5% of chlorophyll *a* (Kang et al., 2018), the algal biomass concentration during stage 2 b was 1.9 ± 0.5 g/L (67 ± 22% of the VSS) and in stage 3 was 1.3 ± 0.4 g/L, (42 ± 7% of the VSS). These results indicate that the photosynthetic efficiency was limited by the lower time of light exposition in stage 2 b and not by the amount of CO_2. The increased photosynthetic efficiency in Stage 3 promoted higher phosphorus and ammonia removal efficiency (Fig. 2B). Phosphorus content in the biomass ranged between 8 and 13%, values that are comparable to conventional EBPR processes (Carvalheira et al., 2014a). Also, the higher oxygen concentrations available enabled nitrification (Fig. 2B). It should be mentioned that nitrite was never detected along the system operation, possibly due to its immediate oxidation into nitrate by NOBs. Nevertheless, ≤21% of the ammonia that was consumed was nitrified during Stages 2, 3 and 4, which was lower than the 33 ± 5% reported by Carvalho et al. (2021). Lower nitrate concentrations measured during the light phase could occur for at least 2 reasons: 1) the lower O_2 concentrations achieved promote simultaneous nitrification and denitrification and leads to underestimated nitrate production (Foladori et al., 2020); 2) lower AOB activity due to the lower oxygen production caused by the shadow effect and/or reduced light penetration (Mohd Udaiyappan et al., 2017). Due

to the diversity of the photo-BNR sludge, further research is needed to understand which microorganisms are responsible for nitrification (see section 3.4).

Overall, in addition to the full COD removal that occurred during the dark-anaerobic phase, the higher oxygen availability at Stage 3 allowed good nutrient removal, with an average removal of 95 ± 7%, 92 ± 5% and 86 ± 5% for PO_4, NH_4 and total N, leading to an effluent with 2.1 ± 2.7 mg P/L and 5.3 ± 2.0 mg N/L (Fig. 3, Table 1, Fig. S1).

Nearly full ammonia removal together with high concentrations of P removal (>10 mg P/L) was not previously found in other microalgae-bacteria consortia (Guo et al., 2021; Ji et al., 2020; Toledo-Cervantes et al., 2019; Posadas et al., 2015). However, high concentrations of P removal, up to 40 mg P/L, were obtained in the present work, as a result of the consortia enrichment in PAOs (which are capable of significant accumulation of P as poly-P) combined with the adjustments made to the light availability. Furthermore, this high enrichment in PAOs also contributed to the high COD removal efficiency of the system, a feature that must be accomplished in wastewater treatment, alongside with nutrient removal.

Along Stage 3, from day 191 to day 220, the implemented period of illumination (5.0 h) resulted in a light availability per TSS of 5.4 ± 1.3 W h/g TSS and allowed the production of oxygen in excess of the culture needs (Fig. 4, Fig. S2).

P removal during Stage 3 (38 ± 3.3 mg P/L) was 1.5 times higher than in Carvalho et al. (2021) (25 ± 9.2 mg P/L), while ammonia removal was similar (33 ± 1.7 mg N/L in the present work, and 38 ± 0.9 mg N/L in Carvalho et al. (2021)). PHA was always available during the light phase, contrary to observations of Carvalho et al. (2021), showing that phosphorus removal by *Accumulibacter* is dependent on the PHA availability, leading to nearly complete P removal (Figs. 3 and 4). Higher PHA availability during the photo-BNR cycles in the present study could potentially be explained by the higher ammonia assimilation for biomass growth in this study (81 ± 1.7%, in contrast to 67 ± 5% in Carvalho et al. (2021)) and, consequently, higher P would also be assimilated to sustain biomass growth, necessitating less PHA consumption. The latter factor indicates that an increase of N:P ratio (1:1 in the present study in comparison to 1:1.5 of Carvalho et al. (2021)) favors nutrient assimilation into microalgae biomass and prevents the complete exhaustion of PHA reserves during the light, which are necessary for dark denitrifying P removal by PAOs.

The loss of nutrient removal efficiency in stage 4 was likely due to a drop in the TSS concentration at the end of Stage 3, which resulted in a quick increase of both light and CO_2 availability (Fig. 2A). No adjustments were made to the length of illumination nor to the CO_2 concentration in the feed, in order to evaluate the impact of this boundary condition on the system. The consortia responded with a population change (see section 3.4) and high oxygen concentrations could be detected in the end of the light period (>2 mg/L). This hindered the subsequent anoxic phase and denitrification efficiency became generally poor. As a result, nitrate was frequently present in the next cycle during COD feeding, resulting in reactor destabilization. The proliferation of heterotrophic denitrifiers is known for leading to the failure of conventional EBPR systems (Izadi et al., 2020; Valverde-Pérez, 2015). Similarly, nitrate leakage to the anaerobic phase can lead to the failure of the photo-BNR system, since ordinary heterotrophic denitrifiers compete with PAOs for the organic carbon. Indeed, phosphorous removal became minimal by the end of Stage 4 (Fig. 2B). This result shows that there is an optimal range of light availability for stable operation of photo-BNR systems (in the present work of 5.4 ± 1.3 W h/g TSS) and that corrective measures should be implemented when deviations occur (e.g. temporary SRT adjustments).

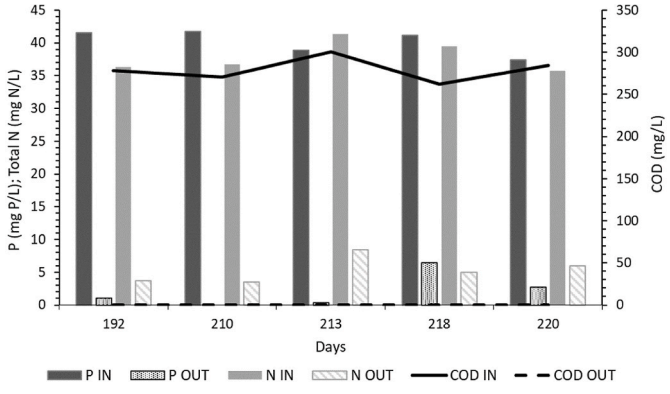

Fig. 3. Value of total nitrogen, phosphorus and COD in the influent and effluent during Stage 3 of photo-BNR operation.

3.4. Characterization of the microbial consortium

The light availability is a key parameter in the photo-BNR process, not only because of oxygen production, but also because the consortium responds to different photoperiod conditions with changes in the microalgae and bacterial populations. Whilst FISH results showed *Accumulibacter* as the most abundant microorganism present in photo-BNR between days 192 and 210 (Table 2), this genus was not detected by sequencing analysis. This inconsistency between FISH and sequencing data has already been mentioned in several previous works (Carvalho et al., 2021; Albertsen et al., 2016; Rubio-Rincón et al., 2019; Valverde-Pérez et al., 2016), and is due to differing biases within each analytical procedure. During stage 3, the abundance of *Accumulibacter* detected by FISH increased in relation to the previous operational stages. At this same time, Type II *Accumulibacter* became the more abundant group as compared to Type I (Table 2), though it is unclear if this had any direct impact on the level of P removal.

Sequencing results (Table 3) indicate that cyanobacteria species (Chloroplast_OTU_4) were present in high abundance in the photo-BNR. Cyanobacteria from the genus *Calothrix*, which was not present in Carvalho et al. (2021), appears in this work as a microorganism with high relative abundance. Its presence increased towards the end of Stage 3 and became dominant in Stage 4, likely due to the increase of light time exposure. *Calothrix* is also known to form microalgae blooms (Bischoff et al., 2019). *Candidatus* Chloroploca, an anoxygenic phototrophic bacteria capable of storing polyphosphate and PHB (Gorlenko et al., 2014; Grouzdev et al., 2018), appears in the 10 most abundant species in the photo-BNR process, and was found in higher amounts between days 154 and 210 (Table 3). This suggests that *Candidatus* Chloroploca contributed to P removal in the photo BNR process.

Both FISH (Table 2) and sequencing results (Table 3) indicate that AOBs were not abundant in the photo-BNR, thus, it can be hypothesized that nitrification from NH_3 to nitrite could be performed by a side-population, as for example, *Limnohabitans* species (Baskaran et al., 2020). Although these microorganisms were not amongst the 10 most abundant species in the reactor, they were present in the photo-BNR community (Table S1). Nitrification from nitrite to nitrate could be performed by phototrophic bacteria, such as *Thiocapsa* and *Rhodobacter* (Table 3) (Levy-Booth et al., 2014).

Since no carbon source was fed during the anoxic period, denitrification could be mainly attributed to dPAOs (*Accumulibacter*) and dGAOs (*Competibacter*) (Table 3). Nevertheless, the possibility that starch fermentation by microalgae during the dark anoxic phase contributed towards denitrification cannot be excluded. However, PHA consumption and P removal also accompanied denitrification, supporting the hypothesis of denitrifying PAO activity.

In terms of microalgae composition (Table 4), the organisms with highest relative abundance were from the class Chlorophyceae, followed by the class Trebouxiophyceae, both from the phylum *Chlorophyta*. Similar results were observed by Carvalho et al. (2021), Zhang et al. (2018) and Jiménez-Bambague et al. (2020), although in the last 2 works the microalgae-bacterial reactor was operated with light and

Table 1
Nutrient removal and production rates during Stage 3 of the Photo-BNR operation.

Rate	Parameter	Day						
		192	210	213	218	220	Average	Std
Light Uptake	PO_4 (mg-P/L.h)	15	16	13	14	13	14	1.1
	NH_4 (mg-N/L.h)	3.0	3.2	3.1	3.1	3.3	3.2	0.11
Light Production	NO_3 (mg-N/L.h)	1.9	1.4	1.3	1.4	1.2	1.5	0.27
Anoxic Uptake	PO_4 (mg-P/L.h)	0.43	0.00	0.03	4.4	3.8	1.7	2.2
	NH_4 (mg-N/L.h)	0.35	0.12	0.63	0.54	0.47	0.42	0.20
	NO_3 (mg-N/L.h)	0.87	0.21	0.95	1.4	0.70	0.84	0.45
	TSS[a]	4.2	5.0	4.9	4.3	2.8	4.2	0.85
	VSS[a]	3.0	3.8	3.7	3.1	2.1	3.1	0.69

[a] Average TSS and VSS values over one cycle.

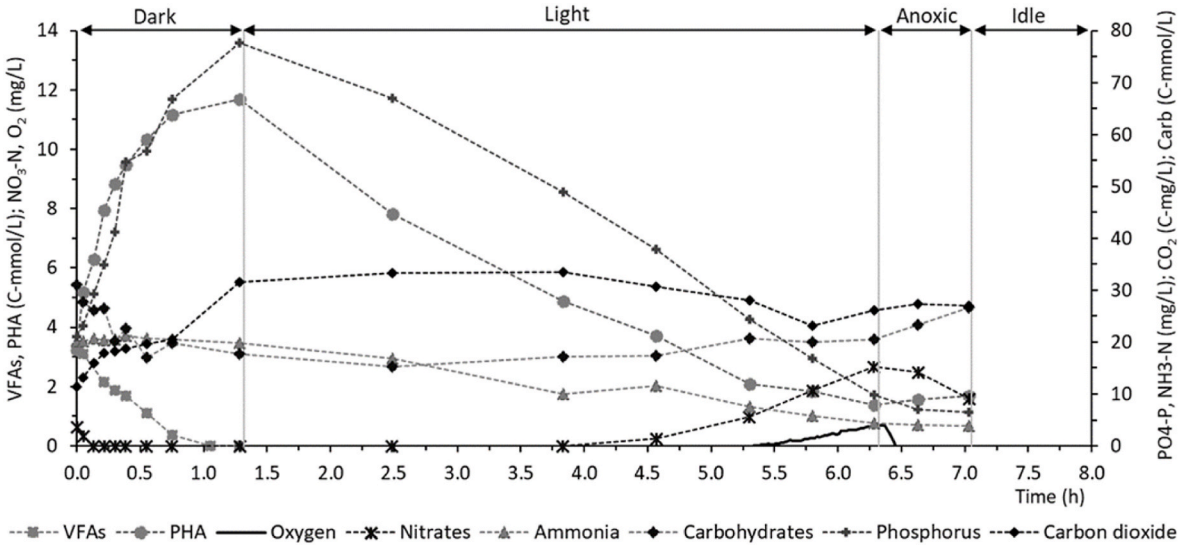

Fig. 4. Representative cycle of photo-BNR performance over Stage 3. Cycle performed at day 218.

Table 2
FISH results during the photo-BNR operation.

Stage	FISH PROBE	PAOS			GAOS	AOBs	NOBs	
		PAOMIX	ACC-I-444	ACC–II–444	CPB_654	NSO 1225	NIT 3	NTSPA 662
1	Day 73	++	++	+-	++	–	–	–
2	Day 154	++	++	++	++	+-	–	–
3	Day 192	+++	+	+++	++	–	–	–
	Day 210	+++	+	+++	++	+-	–	–
	Day 216	++	++	+++	++	–	–	–
	Day 220	++	++	+++	+	–	–	–

(−) non-existent; (+-) almost non-existent; (+) present; (++) abundant; (+++) dominant. Probes: PAOmix for *Candidatus* Accumulibacter phosphatis; Acc-I-444 which targets Type I *Accumulibacter* PAOs and Acc–II–444 for *Accumulibacter* PAOs Type II; CPB_654 for *Candidatus* Competibacter phosphatis; Nso1225 for AOBs; NIT3 and Ntspa662 for NOBs.

aeration. Members of the *Chlorophyta* are reported in various studies as organisms with good capacity for nutrient removal (Abinandan et al., 2018; Cai et al., 2013; Ji et al., 2020; Toledo-Cervantes et al., 2019). Indeed, although PAOs are likely to be mainly responsible for phosphorus removal, the role of microalgae in phosphorus removal through assimilation for biomass growth cannot be ignored (Bunce et al., 2018; Powell et al., 2011; Shilton et al., 2012). Also, as microalgae biomass accounts for almost 50% of the VSS concentration in the photo-BNR system, and since >80% of the ammonia was assimilated into the microbial biomass, microalgae were likely the main contributors to ammonia assimilation. These results are in accordance with studies that described biomass assimilation as the main mechanism of nitrogen removal in algae-bacterial consortia (Carvalho et al., 2021; Su et al., 2011; Wágner et al., 2021).

A further aspect of the present photo-BNR system is that it showed a good settling capacity during the entire operational period (Fig. S3), with SVI values ranging from 42 mL/g TSS (day 86) to 61 mL/g TSS (day 213) and increasing to 123 mL/g TSS on day 260, which coincided with the increase of *Chalotrix,* a filamentous cyanobacteria. It is important to note that settling time was short, only 15 min, indicating fast and good settling properties (Pierce et al., 1998) of the microalgae-bacterial flocs both on day 86 and 213. This is an extremely important aspect for cost-efficient microalgae biomass separation by gravity sedimentation (Foladori et al., 2020), since no extra separation steps and, thus, no more

Table 3
10 most abundant prokaryotic species, obtained from DNA sequencing, in the photo-BNR process.

Stage 1	Stage 2b	Stage 3				Stage 4	
Day 73	Day 154	Day 192	Day 210	Day 216	Day 220	Day 260	
37.4	28.3	21.8	30.8	23.2	16.7	2.4	(C) Gammaproteobacteria; (G) Thiocapsa
19.0	24.5	20.9	17.2	15.9	17.3	0.0	(P) Cyanobacteria; (C) Chloroplast_OTU_4
6.3	14.5	25.6	19.6	22.1	21.3	0.7	(C) Gammaproteobacteria; (F) Competibacteraceae; (G) CPB_S18
0.5	2.4	6.8	7.8	13.9	23.3	56.1	(C) Cyanobacteria; (G) Calothrix
0.1	1.9	3.3	3.1	2.2	2.0	3.4	(C) Gammaproteobacteria; (F) Competibacteraceae; (G) CPB_C22&F32
2.4	1.4	1.6	1.7	1.7	1.7	0.0	(P) Cyanobacteria; (C) Chloroplast_OTU_19
1.0	2.7	2.2	2.2	1.3	1.0	1.5	(C) Chloroflexia (F) Chloroflexaceae; (G) Candidatus Chloroploca
0.4	4.4	1.0	1.3	1.3	1.4	0.8	(C) Gammaproteobacteria; (F) Competibacteraceae; (G) CPB_P15
1.7	4.5	2.0	0.6	0.5	0.2	0.0	(C) Chloroflexia (F) Chloroflexaceae; (G)Chloronema
2.8	0.8	0.9	1.0	1.2	1.2	0.5	(C) Alphaproteobacteria; (F) Rhodobacteraceae; (G)Rhodobacter

(P – Phylum; C – Class; O – Order; F – Family; G- Genus).

Table 4
9 most abundant Archaea species, obtained from DNA sequencing, in the photo-BNR process.

Stage 2	Stage 3				
Day 154	Day 192	Day 210	Day 216	Day 220	
93.2	93.8	88.0	90.4	89.9	(P) Chlorophyta; (C) Chlorophyceae_OTU_1
4.7	3.1	6.4	5.1	6.7	(P) Chlorophyta; (C) Trebouxiophyceae_OTU2
0.1	1.1	3.4	2.3	1.4	(P) Chlorophyta; (C) Chlorophyceae; (G) Uronema
1.3	0.8	1.0	0.8	0.8	(P) Chlorophyta; (C) Chlorophyceae_OTU_4
0.2	0.8	0.3	0.8	0.6	(P) Cercozoa; (C) Thecofilosea; (F) Rhizaspididae
0.2	0.2	0.5	0.3	0.3	(P) Unassigned
0.2	0.2	0.3	0.2	0.2	(K) Fungi; (P) Ascomycota_Saccharomycotina

(K – Kingdom; P – Phylum; C – Class; O – Order; F – Family; G- Genus)

energy consumption is necessary, to efficiently separate the biomass from the treated liquid before discharge.

3.5. Prospecting photo-BNR for outdoor deployment

In this study, the photo-BNR process was operated with a SRT of 19 ± 1 days and an HRT of 16 h. Near full nutrient removal was obtained with a COD:IC:N:P mass ratio of 7.5:0.9:1:1, when the culture was exposed to 5.4 ± 1.3 W h/g TSS.

The results obtained from the present work highlight the importance of light availability for efficient nutrient removal, a parameter directly dependent on the length of the photoperiod, but also, on the biomass concentration and its self-shading effect at high concentration values. Future process optimization should evaluate the impact of biomass concentration on microalgae light absorbance capacity, since adequate photosynthetic oxygen production is critical for an efficient photo-BNR operation. As previously mentioned, biomass concentration could be controlled by decreasing the SRT, however, more investigation is needed to understand the impact of reducing this parameter on the microbial consortium composition and thus, on nutrient removal efficiency.

Regarding the length of the illuminated and anoxic periods, its adjustment was constrained by the reactor laboratorial operation under 8 h cycles. However, the photo-BNR operation in a daily cycle of 12 h light/12 dark, could overcome these constraints, since more time of light exposure, and consequently higher oxygen production by microalgae, and a longer anoxic phase for denitrification, could be obtained. On the other hand, the increase of cycle time would also increase the HRT and, thus, decrease the amount of wastewater treated per day. Still, if HRT is maintained at 2 days (simulating the present work with half the reactor volume discharged per cycle), it is still much lower than the 10 days HRT used, for example, by Torres-Franco et al. (2021) or the 4 and 4.5 days HRT of Anbalagan et al. (2017) and de Godos et al. (2014), respectively, for nutrient removal with microalgae-bacterial consortia. The proposed HRT of 2 days for outdoor operation of the photo-EBPR is in-line with observations of Judd et al. (2015) that HRT between 2 and 5 days are needed to obtain up to 80% nutrient removal in HRAPs.

The Photo-BNR technology presented here is dependent on illumination, and therefore, it must be robust to address the challenges of outdoor implementation. As this study found, the removal efficiency of the Photo-BNR is dependent on the light availability per biomass (W.h/g TSS) and CO_2 supply, which may be tuned to overcome winter seasons (short daylight) or operational disturbances, like the excess of oxygen production that hinders the denitrification in the anoxic stage. In the case of winter, the longer nighttime favors the non-illuminated anaerobic and anoxic phases, thus benefitting COD removal and denitrification, respectively. Also, the oxygen production by phototrophs during the short daytime may be improved through a control of the biomass concentration (e.g. SRT control). This can prevent the self-shading of light and consequently, increase the light availability per biomass and lead to higher oxygen production. In the case of periods where operational disturbances lead to an excess of oxygen production (like the TSS drop observed in the present study, which resulted in the simultaneous high presence of CO_2 and light exposure) corrective measures can be implemented, like a temporary increase of the SRT to recover biomass concentration and/or dosing the CO_2 supply. This Photo-BNR flexibility in combining different operational adjustments to respond to practical adversities, prospects its robustness in future outdoor applications.

4. Conclusions

The results obtained during the 260 days of photo-BNR operation indicate that the proposed photo-BNR system does not require mechanical aeration, since the oxygen necessary could be photosynthetically produced. Higher photosynthetic oxygen production was obtained

when the photo-BNR culture was exposed to longer periods of light, rather than to higher concentrations of CO_2. Best results for nutrient removal were obtained with a COD:IC:N:P mass ratio of 7.5:0.9:1:1, and a CO_2/TSS ratio of 4.3 ± 1.04 mg C/g TSS, when the culture was illuminated at 5.4 ± 1.3 W h/g TSS. In this case, it was possible to achieve removal efficiencies higher than 90% for phosphorus and ammonia and higher than 85% for total nitrogen. The stability of the photo-BNR system with good nutrient removal efficiency is closely related with photosynthetic oxygen production, which needs to be strictly controlled to values < 2 mg O_2/L, allowing PAO respiration and nitrification, but without compromising the development of an anoxic phase for efficient denitrification. The present study provides insight on the key parameters that must be tuned and controlled for stable photo-BNR operation and future technology deployment into outdoor applications.

Credit author statement

VCF Carvalho: Conceptualization, Investigation, Formal analysis, Writing-Original Draft. **JC Fradinho**: Conceptualization, Writing-Reviewing and Editing, Visualization, Supervision. **A Oehmen**: Conceptualization, Writing-Reviewing and Editing, Supervision. **MAM Reis**: Conceptualization, Resources, Writing-Reviewing and Editing, Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2023.117490.

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