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Nitrogen and phosphate removal from wastewater with a mixed microalgae and bacteria culture

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ABSTRACT

Microalgae are able to convert nutrients (nitrogen and phosphorus) from wastewater into biomass and bio-products, thus improving the sustainability of wastewater treatment. In High Rate Algal Ponds (HRAP), biomass productivity and water treatment efficiency are highly dependent on environmental parameters such as temperature, light intensity and photoperiod. The influence of temperature and photoperiod on biomass productivity and the removal of dissolved nitrogen and phosphorus from municipal wastewater by a native microalgae-bacteria consortium was assessed in batch cultures in view of the development of an HRAP at a larger scale. Temperature affected the growth rate and microalgae biomass production as well as ammonium and phosphate removal rates. At the temperatures 15 and 25 °C, the average total nitrogen and phosphorus removal extents ranged from 72 to 83% and 100% respectively. Additionally 33.0 ± 0.1% of the total nitrogen was eliminated by stripping at 25 °C, and 50 ± 2% was assimilated by the microorganisms under all conditions tested.

1. Introduction

Microalgae have been at the focus of attention in recent years as an alternative system for biological wastewater treatment with several applications in wastewater treatment [1–5]. Microalgae are photosynthetic microorganisms that can grow rapidly and live in harsh conditions due to their unicellular or simple multicellular structure [6]. They provide a way for contaminants-removal (nitrogen, phosphorus and carbon) from wastewater while producing biomass that could find use for the production of high-value chemicals (algal metabolites) and/or biogas through anaerobic digestion [7]. Additionally, microalgae can diminish the harmful effects of sewage effluent and reduce eutrophication in aquatic environments [8]. Wang et al. [9] reported a decrease in nitrogen (83% N as NH_4^+) and phosphorus (90% P as PO_4^{3-}) in municipal wastewater by *Chlorella* sp. They suggested that the nutrient removal rates were independent of the optimal N/P ratio but that the concentrations of these nutrients were important for the algal growth systems. In another study, Samori et al. [10] clearly demonstrated the high potential of using an isolated algal strain from an artificial freshwater pond, *Desmodesmus communis*, to remove contaminants from primary wastewater. These photosynthetic microorganisms can potentially provide oxygen for the heterotrophic aerobic bacteria that could in turn biodegrade organic pollutants from municipal wastewater and release carbon dioxide to be used by the microalgae in the presence of light. It must be born in mind that microalgae can exhibit many types of metabolism (e.g. autotrophic, heterotrophic, mixotrophic, photo-heterotophic) and are capable of metabolic shift in response to changes in environmental conditions [6], hence they may compete with heterotrophic bacteria [11] under certain conditions. The net pollutant removal in these systems is basically the additive effect of their assimilation by the algal [5,12], biological processes (nitrification/denitrification) and stripping phenomena such as ammonia volatilization and phosphorus precipitation. The latter is potentially brought about by the high pH levels induced through photosynthetic microalgal growth [7,9,13]. The efficiency of the algae-bacteria consortium for nutrient uptake is not only affected by the bioavailability of nutrients but also depends on the complex interactions between physico-chemical factors such as pH, light intensity, photoperiod, temperature and biological factors. Examples of the latter are the presence of pathogen, viral attack, protozoa predation and competition with bacteria over the available nutrients [14–16].

Mixed bacteria-microalgae cultures are often grown in high rate algal ponds (HRAPs) to treat municipal, industrial and/or

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agricultural wastewaters. HRAP technology essentially consists of a shallow race-track reactor of 0.3–0.4 m in depth, with mechanical mixing, in which algae and bacteria grow. The wastewater, usually primary or secondary, is fed into the HRAP and mixed with the algal and bacterial culture inside the bioreactor. Stirring promotes algal growth and prevents biomass settling [17]. Studies have been carried out to understand the influence of the various parameters in HRAP and to explain how these parameters can improve the microalgal performance at larger scales. Hidiyanto et al. [18] developed a hydrodynamic model of HRAP and showed that a ratio L/W (length/width) higher than 10 allowed better performance with respect to velocity uniformity and reduced shear stresses; Sutherland et al. [19] indicated that the overall areal productivity significantly increased with increasing depth (200, 300 and 400 mm). Kim et al. [20] demonstrated high removal efficiencies for COD (86%), total nitrogen (93%) and total phosphorus (83%) from untreated municipal wastewater in an algae dominated consortium in a HRAP.

Critical parameters, such as light intensity, photoperiod, temperature and pH affect HRAP wastewater treatment and biomass production. For instance, the photoperiod and the temperature are subject to large seasonal variations with a consequent effect on wastewater treatment efficiency and biomass productivity that fluctuate all year round. Recently, Lee et al. [21] showed that the photoperiod impacts greatly on nutrient removal, biomass production and alters algal-bacterial population dynamics in a photo-bioreactor used for municipal wastewater treatment. Understanding the effects of environmental parameters (temperature, light . . .) on biotic and abiotic phenomena is a major way to improve and optimize HRAPs performance.

In this preliminary work, the effect of both temperature and photoperiod on microalgae biomass production and pollutant removal (soluble nitrogen and phosphorus) from municipal wastewater was investigated. A native microalgae-bacteria consortium originating from a pilot HRAP was assessed with a view to improve the HRAP pilot plant's performance in pollutant removal.

2. Material and methods

2.1. Preparation of an active microalgal-bacteria inoculum

The microbial biomass used in all batch experiments was obtained from a HRAP pilot plant in Pau (France). The microbial consortium (microalgae, bacteria and other microscopic organisms) collected was firstly allowed to settle for 2 h and the settled solids were used as an "enriched microbial biomass inoculum". The wastewater collected from another similar pilot plant (Ouistreham – France) was clarified through centrifugation (2000*g*; 10 min) and used as the nutrient medium for batch experiments.

The enriched microbial biomass was used to inoculate (10% v/v) the clarified nutrient medium (2900 mL). This culture was continuously stirred (35 × 6 mm magnetic stirrer; 100 rpm) in an Erlenmeyer flask under continuous illumination (45 $\mu mol\, s^{-1}\, m^{-2}$ at the culture surface with fluorescent lamps, Biolux OSRAMJ 30W/965) and was maintained at room temperature (approx. 20 °C). The constant mixing served to avoid sedimentation.

2.2. Wastewater characteristics

The municipal wastewater (primary effluent) used in the batch experiments was obtained from the inlet into the HRAP at Ouistreham (France). The typical composition of this water is described in Table 1.

2.3. Batch experiments

Batch experiments lasted 8 days and were performed in open Duran (Schott) bottles (250 mL; 200 mL working volume; 7 cm culture depth). The system consisted of 15 stirred flasks, immersed in a water-bath attemperated by means of water recirculation through a chiller-heater (Frigomix U-1/Thermomix BU). Illumination was provided from above the flasks using fluorescent lamps (4× Mazdafluor 18 W) at 200–250 $\mu mol\, m^{-2}\, s^{-1}$ measured at the surface of the cultures (Licor LI250A, LI-COR, USA).

The reproducibility of the replicates was estimated by starting 15 identical cultures of *Chlorella vulgaris* in modified Bristol medium (20 °C; photoperiod 12 h) and estimating the microalgal growth (as indicated in Section 2.4, by the total concentration of chlorophyll *a* and *b*) in all the bottles. In our conditions, chlorophyll concentration was revealed as an indirect measure of the algal biomass in the wastewater (see Section 2.4). A standard deviation of 7.60% of the values of the apparent specific growth rate, based on chlorophyll measurements, for the 15 cultures was determined indicating a good reproducibility between the cultures in the incubation system. In other words, there was no influence of the position of the bottles in the water bath on the results.

After confirming the positional uniformity of the incubator, five experiments were performed at different temperatures and photoperiods. Each experiment contained duplicate cultures randomly placed in the incubator. The temperatures (5, 15 and 25 °C) and photoperiods (6, 12 and 18 h of light) were chosen to mimic average winter and summer conditions in the open-pond of Ouistreham (Table 2). The "photoperiod" refers to the length of continuous illumination applied to the cultures during any 24 h period. In the following, when referring to cultures conditions (Bi–ii), i refers to the temperature and ii the photoperiod.

In addition two control experiments were performed: (a) Batch cultures in obscurity and (b) Abiotic cultures without inoculation. These experiments were performed to assess the impacts of microbial respiration, nitrification and abiotic losses (nutrient stripping and precipitation). Batch cultures were performed in obscurity at 5, 15 and 25 °C (referred to as B5, B15 and B25). In order to measure ammonia volatilization and phosphorus precipitation the abiotic experiments were performed at 15 °C and a photoperiod of 18 h. These cultures were prepared with successive filtrations (1.2 μm and 0.2 μm) of the nutrient medium to remove the biomass. This experiment was repeated at two different pH values; unmodified (pH ≈ 8) and strongly alkaline (pH = 10.0 with addition of NaOH 2 mol L^{-1}). The latter pH corresponded to the value routinely encountered at the end of the experiments.

2.4. Analytical methods

Daily samples were collected at the same time of the day (at the beginning of the light cycle) and were analysed according to the Standards Methods for Examination of Water and Wastewater [22].

Table 1
Average composition of the wastewater (average of five samples).

Parameter	Unit	Value
Total Chemical Oxygen Demand (tCOD)	$mgO_2\ L^{-1}$	278 ± 124
Soluble Chemical Oxygen Demand (sCOD)	$mgO_2\ L^{-1}$	193 ± 71
Ammonium (NH_4^+)	$mgN\ L^{-1}$	41.6 ± 17.1
Nitrite (NO_2^-)	$mgN\ L^{-1}$	6.4 ± 3.7
Nitrate (NO_3^-)	$mgN\ L^{-1}$	1.4 ± 0.6
Phosphorus (PO_4^{3+})	$mgP\ L^{-1}$	3.1 ± 1.3
Nitrogen particulate (N_p)	$mgN\ L^{-1}$	3.1 ± 2.9
Total suspended solid (TSS)	$mg\ L^{-1}$	88.9 ± 27.5
Volatile suspended solid (VSS)	$mg\ L^{-1}$	61.2 ± 22.8
pH		7.8 ± 0.4

Table 2
Environmental conditions of the batch experiments and apparent specific growth rates (μ), duplication time (t_d), biomass productivity (P_B), ammonium and phosphate removal (R_{NH4},R_{PO4}).

Temperature (°C)	Photoperiod (light/dark cycle)	μ (h^{-1})	t_d (h)	P_B (mg Chl L^{-1} h^{-1})	R_{NH4} (mgN L^{-1} h^{-1})	R_{PO4} (mgP L^{-1} h^{-1})
5	6 h/18h	0	0	0	0	0
5	12 h/12h	0	0	0	0	0
15	12 h/12h	0.020 ± 0.001	34.7 ± 1.7	0.011 ± 0.001	0.12 ± 0.03	0.016 ± 0.001
15	18 h/6h	0.020 ± 0.002	34.7 ± 3.8	0.016 ± 0.001	0.19 ± 0.01	0.024 ± 0.002
25	12 h/12h	0.025 ± 0.002	27.7 ± 2.1	0.018 ± 0.003	0.32 ± 0.03	0.024 ± 0.001

The following parameters were measured: pH, dissolved oxygen (DO), temperature, concentrations of chlorophyll (*a* and *b*), concentrations of ammonium (NH_4^+), nitrite (NO_2^-), nitrate (NO_3^-) and phosphate (PO_4^{3-}), particulate nitrogen (N_p) content of the biomass, total suspended solids (TSS) and volatile suspended solids (VSS). The pH measurements were carried out twice a day. Data on pH represent the highest value of the two measurements of the day day (à supprimer).

The temperature, DO and pH were measured with the multiparameter probe ODEON (SN—ODOEA-0175), an oxygen sensor (SN-PODOA-0180) and a pH sensor (SN-PPHRA-0144) respectively. The concentrations of the dissolved nitrogen species (NH_4^+, NO_2^-, NO_3^-) and PO_4^{3-} were determined using an Ion Chromatograph (DIONEX LC25-LC20). The anions were separated using an Ion Pac AS11-HC analytical column and NaOH (30 mM – Dionex LC25) as eluent. The cations were separated using an IonPac CS12A analytical column and methanesulfonic acid (20 mN – Dionex LC20) as eluent. The particulate nitrogen content (N_p) of the biomass was determined by elemental analysis (Organic Elemental Analyzer FLASH 2000CHNS/O, base unit operated with helium). TSS and VSS were determined by filtration of the suspension and heating the solid at 105 °C and at 550 °C, respectively.

Microalgal growth was monitored by measuring the total chlorophyll concentration (Chl; mg L^{-1}) during the experiment. The chlorophyll concentration was determined by a modified method proposed by Porra [23] with extraction of chlorophyll *a* and *b* with a 85% methanol aqueous solution containing 1.5 mM of sodium dithionite at 40 °C. The chlorophyll concentration was calculated from the absorbance of the extracted solution at 650 and 664 nm (Spectrophotometer Cary 50 Scan UV–vis). Under our conditions, the chlorophyll and biomass (VSS) concentrations were well correlated ($R^2 = 0.99$) over the range observed in our experiments (50–400 mg/L).

The apparent specific growth rate (μ) and the algal biomass productivity (P_B) were calculated in the exponential phase of growth, according to Eqs. (1) and (2).

$$\mu = \frac{\ln(Chl_t/Chl_0)}{t_t - t_0} \quad (1)$$

$$P_B = \frac{Chl_t - Chl_0}{t_t - t_0} \quad (2)$$

where Chl_{t0} and Chl_{0t} are the chlorophyll concentrations at the times t_0 and t_t, corresponding to the beginning and end of the exponential growth phase, respectively.

The time required to double the population, doubling time (t_d), was calculated from the value of the specific growth rate ($t_d = \ln(2)/\mu$).

The removal rates (R_i) were calculated according to Eq. (3).

$$R_i = \frac{S_0 - S_f}{t_f} \quad (3)$$

where R_i represents the nutrient removal rate of the substrate i (NH_4^+ or PO_4^{3-}), S_0 its initial concentration at $t = 0$. S_f is its concentration at t_f (either just as the concentration of the nutrient falls below its detection level or at the end of the experiment, ~190 h).

2.5. Statistical analysis

To evaluate differences between mean values of the apparent specific growth rate (μ), the microalgal biomass productivity (P_B), doubling time (t_d) and nutrient (pollutant) removal rates (R_i), Student's *T*-tests were carried out with a confidence level of 95%. The null hypothesis stated that the means were equal. A value $p \leq 0.05$ indicate that there is a significant difference between tested means.

3. Results and discussion

3.1. Microalgal growth and nutrients removal at 25 °C

Only the results from the cultures at 25 °C are presented here but similar trends were observed for the cultures developed under the other two conditions tested: B15–12, B15–18 (data not shown).

For the batch experiment at 25 °C and a photoperiod of 12 h, the chlorophyll concentration profile followed the same trend as those of VSS and TSS (Fig. 1a). Additional results showed a linear correlation ($R^2 = 0.95$, data not shown) between the chlorophyll concentration and the volatile solids (VSS) under our experimental conditions. This suggests that the chlorophyll concentration could be used as an indicator of microalgal growth or at least photosynthetic activity under our conditions.

The TSS/VSS ratio increased (Fig. 1a) over the last 80 h of the experiment. This could be due to an increase in the TSS brought about by mineral precipitation as the pH increased towards the end of the experiment. We propose phosphate as the likely candidate (see Section 3.4). The control experiments in which the pH was constant at around 8 exhibited no significant precipitation or increase in the VSS/TSS ratio.

The dissolved oxygen concentration had a tendency to increase during the experiment under the different conditions where there was algal growth, indicating a prevalence of photosynthetic activity over heterotrophic carbon-oxidation and nitrification.

Nitrification was observed when the cultures were grown under obscurity (Figs. 1 b and 4 c); the decrease in ammonium concentration was accompanied by nitrite formation that peaked at 70 h and then decreased, presumably due to further nitrite assimilation by the microalgae. Comparing the nitrite concentration profile for the illuminated cultures at 25 °C (Fig. 1b) and the cultures in obscurity (Fig. 4c), one can expect nitrite uptake by microalgae to occur after ammonium exhaustion under illuminated conditions. The nitrate concentration remained approximately constant and low (about 2 mgN L^{-1}) over the entire experiment (data not shown). Its consumption due to denitrification was unlikely to occur under aerobic conditions and increasing dissolved oxygen concentration in the culture. On the other hand, data obtained for the cultures in obscurity (Fig. 4c) suggest no nitrification by conversion of nitrite to nitrate after ammonium exhaustion.

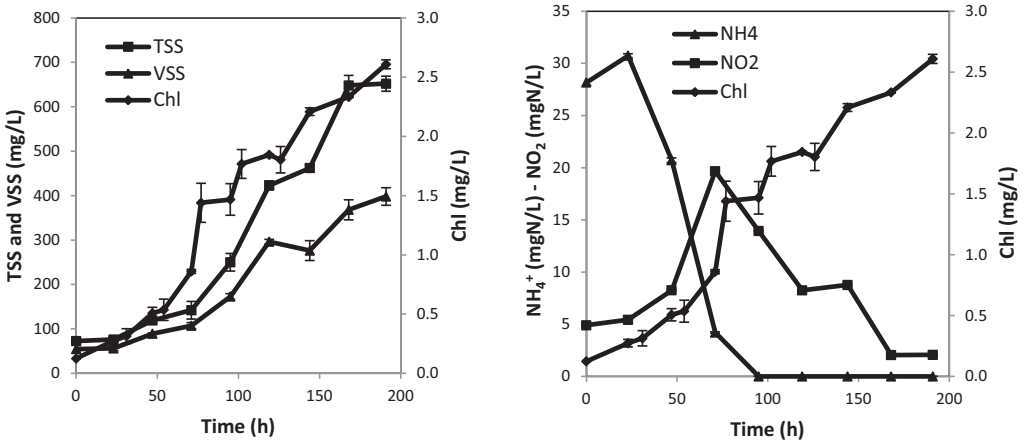

Fig. 1. Batch Culture growth and nitrogen evolution with time for the assay developed at (25 °C, 12 h/12 h light/dark cycle, B25-12) (a) Chlorophyll, TSS and VSS concentrations (b) NH_4^+, NO_2^- and chlorophyll concentrations with time. The error bars represent the standard deviation around each point.

In general, microalgae are able to assimilate nitrogen from a variety of nitrogen sources including ammonium, nitrate, nitrite and urea [9,12] although ammonium is the preferred nitrogen source. Ammonia is the most energetically efficient nitrogen source, since less energy is required for its uptake. Ruiz-Marin et al. [24] reported that the microalgae *C. vulgaris* and *S. obliquus* showed preferences for ammonium to any other form of nitrogen present in wastewater. Under both autotrophic and heterotrophic conditions, ammonium is transported across the membrane by a group of proteins belonging to the ammonium transporter family, a group of evolutionarily related proteins commonly found in bacteria, yeasts, algae and higher plants [25]. At 25 °C as well as in all other conditions (except at 5 °C where no biological activity occurred, see Section 3.2), the order of priority for the consumption of nitrogen sources was as expected ammonium (NH_4^+) first and then (NO_2^-). Under our conditions nitrate did not seem to be assimilated by the microalgae (similar nitrate profiles were measured in light and darkness conditions, data not shown).

The pH increased during the experiments from 7.8 to 10.0 (Fig. 2). Several factors might explain the pH variation, such as microalgal growth (pH increase as a result of CO_2 uptake from the medium by autotrophic and/or mixotrophic microalgae) and/or excretion of basic metabolites from biodegradation of organic matter [5].

During the culture, a decrease in the phosphate concentration over time was also detected (Fig. 2, discussed later).

3.2. Effect of temperature and photoperiod on microalgal growth and biomass productivity

The total chlorophyll ($a+b$) concentration in terms of absolute values, or normalized by its initial concentration, were followed through the batch experiments at different temperatures and photoperiods (Figs. 2 and 3).

At the lowest temperature tested (5 °C), no net microalgal growth (increase in chlorophyll concentration) was observed whatever the photoperiod duration (cf. B5–6, B5–12, Fig. 3). Roleda et al. [26] also reported low to negative growth rates for microalgal cultures at low temperatures (10 °C). They reported that microalgae were just able to sustain metabolic activity, without cell division; in some cases cell death was observed resulting in a negative growth rate. Reduced microbial activity and the absence of microalgal growth were expected at 5 °C. Furthermore, the inoculum for our experiments had come from a culture at room

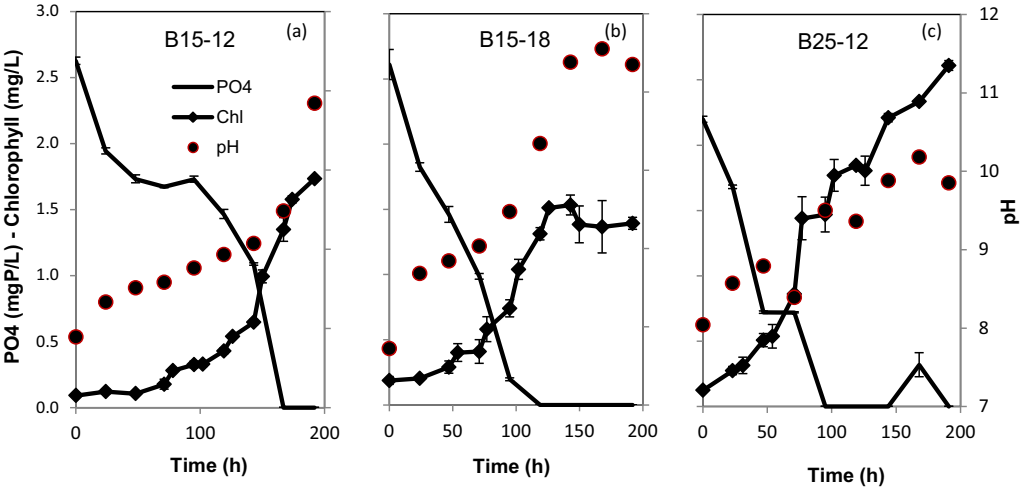

Fig. 2. PO_4^{3-}, pH and Chlorophyll profiles during batches batch cultures under photosynthetic conditions. According to our nomenclature (Bi-ii), i refers to the experiment temperature and ii stands for the length of the continuous light exposure within a 24 h period. Hence in the experiment B15-12, the cultures were incubated at 15 °C and received 12 h of light and 12 h of darkness. The error bars represent the standard deviation around each point.

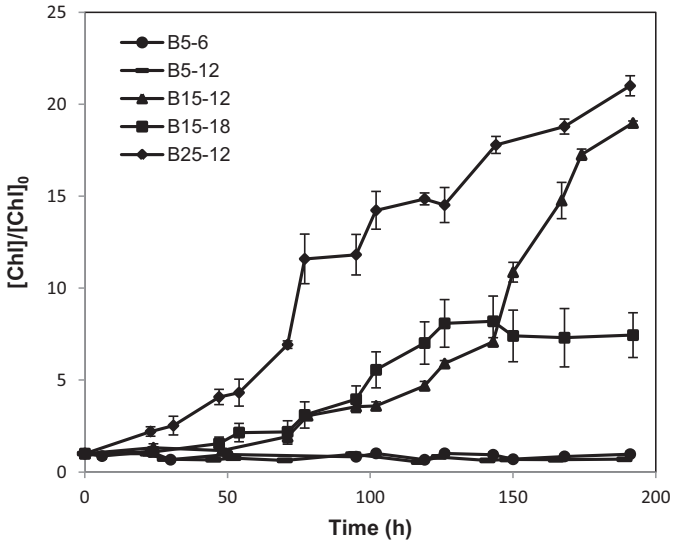

Fig. 3. Chlorophyll concentration over time in batch experiments. According to our nomenclature (Bi-ii), i refers to the experiment temperature and ii stands for the length of the continuous light exposure within a 24 h period. Hence in the experiment B5-6, the cultures were incubated at 5 °C and received 6 h of light and 18 h of darkness. Y-axis, [Chl]/[Chl_0], is the chlorophyll concentration normalized in relation to its initial concentration. The error bars represent the standard deviation around each point.

temperature, so inadequate acclimatisation to the lower temperature cannot be ruled out as a possible explanation for our results.

The apparent specific growth rate (μ) at 15 °C was determined to be 0.020 h^{-1} for the two photoperiods tested (Table 2) suggesting that the photoperiod did not affect the μ value at this temperature. At 25 °C, μ increased slightly to 0.025 h^{-1} ($p < 0.05$). The duration of the exponential phase also depended on the conditions: 126 h for B15–12 (from 48 to 174 h); 79 h for B15–18 (from 47 to 126 h) and 79 h for B25–12 (from 23 to 102 h).

Biomass productivity was positively affected by temperature (Table 2). The highest value was observed at the highest temperature (25 °C) ($p < 0.05$). Similar results have been reported by Martinez et al. [27] where the optimal temperature seemed to be 25 °C for algal biomass production in stirred cultures of the microalgae *Scenedesmus obliquus* in urban wastewater.

The final chlorophyll concentration, measured at the end of the experiment, strongly depended on the temperature (Fig. 2: about 1.5 mg/L at 15 °C and 2.6 mg/L at 25 °C). The photoperiod had little influence on this parameter, although, higher biomass values were determined for the culture B15-18 compared with those of B15–12 after approximately 70 h and until 170 h. This could be attirbuted to the lower chlorophyll concentration at time zero for the culture B15–12.

The main differences between the experiments at the two photoperiods are the shorter exponential growth phase and the presence of a stationary phase for B15–18. This may be explained by the pH profile of the cultures (Fig. 2) over time. After approximately 70 h, a significantly higher pH of the culture B15–18 is observed compared with B15–12. This is expected to be linked to a higher CO_2 uptake rate, related to increased microalgae concentration in the culture B15–18 (Fig. 2b). Since pH has a strong impact in microbial growth and activity, this increase might have contributed to the halt of the exponential growth phase for B15–18 sooner than that for B15–12.

In the experiment B25–12 (Fig. 1), the exponential growth phase (until approximately 100 h) was followed by a phase of linear growth. A possibility is that this linear phase resulted from a light and/or nitrogen limitation [27]; at 100 h, ammonium depletion in the medium was observed, as previously-stated in Section 3.1.

In order to quantify the relative significance of non-phototrophic microbial metabolism, experiments were also performed in obscurity (Fig. 4). In darkness the pH of the cultures remained approximately constant over time at 8.5 for all the temperatures tested (data not shown). No increase in the chlorophyll concentration (neither in the TSS or VSS concentrations, data not shown) was observed, suggesting that there was no microbial growth and, in particular, no algal biomass production. This suggested that the microalgae in our cultures were obligate phototrophs unable to grow or perform heterotrophic metabolism with the available organic carbon in the medium. This may be also due to the presence of refractory organic carbon that are not biodegraded by heterotrophic bacteria or microalgae. Thus photosynthesis seems to be the predominant biological phenomenon in our cultures.

3.3. Nitrogen removal

The rate of NH_4^+ removal in all batch cultures increased at higher temperatures and longer photoperiods ($p < 0.05$) (Table 2). NH_4^+ and PO_4^{3-} were not removed in batch cultures at 5 °C (B5–6 and B5–12). This is in agreement with the absence of

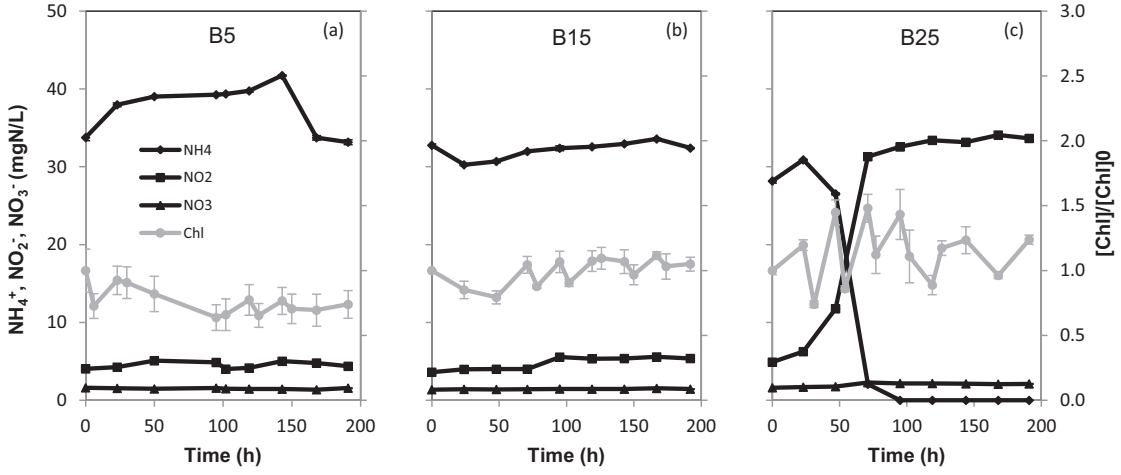

Fig. 4. Nitrogen species and chlorophyll concentrations over time for control batch in darkness at different temperatures (5, 15 and 25 °C). The error bars represent the standard deviation around each point.

biological activity in those cultures. Neither biomass production nor nitrification was observed in these conditions (Fig. 4).

A linear correlation between chlorophyll and ammonium concentration in the culture was observed for the experiments at 15 °C and 25 °C (Fig. 5a). The pattern suggests that the consumption of this compound was associated with microalgal growth, nevertheless, ammonium may have been also depleted by abiotic phenomena such as stripping related to the high pH levels induced by the photosynthetic activity (see later) and respired by nitrifying bacteria.

The slopes of the graphs were dependent on the conditions tested (Fig. 5a). The maximal slope was found for the B25–12 test (25 °C and 12 h photoperiod) which suggested that microalgae-related ammonium depletion is temperature dependent. The slope of ammonium depletion at 25 °C seems to be additionally influenced by bacterial nitrification as demonstrated by our experiments performed in the dark (Fig. 4c).

At 15 °C, ammonium depletion was expected to be only related to algal activity as no ammonium depletion occurred in the darkness at this temperature (Fig. 4b). Similarly, a very low nitrification activity has been detected for cultures of *Nitrosomonas* and *Nitrobacter* at 15 °C [28]. Likewise, larger ammonium removal was determined for the culture B15-18 compared with the culture B15-12 (Fig. 5 and Table 2, Eq. (3)). Certainly, ammonium was first depleted by the culture developed at 18 h/6 h (approximately at 143 h) whereas the presence of NH_4^+ was still available at the end of the experiment for the culture B15–12 (data not shown). This could have been due to the higher biomass concentration produced over time in the former culture as previously stated.

In order to get a better understanding of the mechanisms of nitrogen removal, the nitrogen balance at time zero and at the end of each experiment (the molar ratio in% between each nitrogen species and the total nitrogen at times t_0 and t_f) was examined (Fig. 6). The incubation time for the experiment was 192 h. At the beginning of the experiment, nitrogen was mainly in the form of ammonium (72–81%) and at the end of the experiments, a high percentage was in the particulate form (54–63%).

In our experiments the difference between the particulate nitrogen concentrations (N_p) at the beginning and the end of the experiment corresponded to the nitrogen assimilated by the microorganisms (algae and bacteria). For all experiments, the nitrogen assimilation into the biomass accounted for $50 \pm 2\%$ of the total nitrogen at the beginning of the assays. Su et al. [5] reported similar results on nitrogen assimilation in filamentous blue-green algae; the nitrogen assimilation into biomass was between $40.7 \pm 0.4\%$ and $52.9 \pm 0.3\%$ in four batches with a photoperiod of 12 h. Similar nitrogen contents were measured in biomass obtained in our different tested conditions (between 0.035 and 0.051 gN g^{-1} DW), suggesting identical nitrogen assimilation abilities. These low nitrogen content values may be due to the low nitrogen concentration in the medium.

In the presence of light (Fig. 6), 83% of the ammonium was removed from the medium at 15 °C and 12 h photoperiod and 100% in the other two conditions. This suggests that both temperature and photoperiod have an impact on ammonium depletion. Similar results were obtained by Martinez et al. [27], in a batch culture at 25 °C (t_f = 188 h) with 100% removal of NH_4^+. Wang et al. [9] reported 83% of ammonium removal when treating raw wastewater with *Chlorella* sp. These authors also confirmed that ammonium or NO_x were used as nitrogen source by the microalgae. In semi-continuous cultures with immobilized green algae, the ammonium depletion depended strongly on the algal species tested [24]. Aslan and Kapdan [29] showed that the medium composition and the environmental conditions such as the initial nutrient concentration, the light intensity, the nitrogen/phosphorus ratio, the photoperiod duration as well as the algae species highly affected nitrogen and phosphorus removal.

At the end of the experiments, between 25 and 33% of the total nitrogen could not be accounted for: 25% at 15 °C and 12 h photoperiod, 26% at 15 °C and 18 h photoperiod and 33% at 25 °C and 12 h photoperiod. As previously mentioned, denitrification requires anoxic conditions and is unlikely to have occurred in the stirred open bottles. Thus, stripping must be the major phenomenon leading to nitrogen loss. Ammonia volatilization is favoured by pH values higher than 10, as observed at the end of the cultures (Fig. 2). These pH values are greater than the pKa of the NH_4^+/NH_3 system, which ranges from 9.24 (at 25 °C) to 9.90 (at 5 °C). However, stripping also depends on the gas-liquid equilibrium of ammonia; Henry's coefficients increase with temperature, from 5×10^{-6} at 5 °C, 9.1×10^{-6} at 15 °C and 1.6×10^{-5} atm m^3 mol^{-1} at 25 °C, favouring greater stripping at higher temperatures. The more extensive N loss observed at the higher temperatures could be explained by ammonium stripping.

The results from the abiotic experiments (15 °C and 18 h photoperiod) confirmed that under unmodified pH (pH ≈ 8), the ammonium concentration remained constant over time (192 h), while in the abiotic assay at pH 10, $17 \pm 0.8\%$ of ammonium was lost. No ammonium stripping was measured in cultures in the

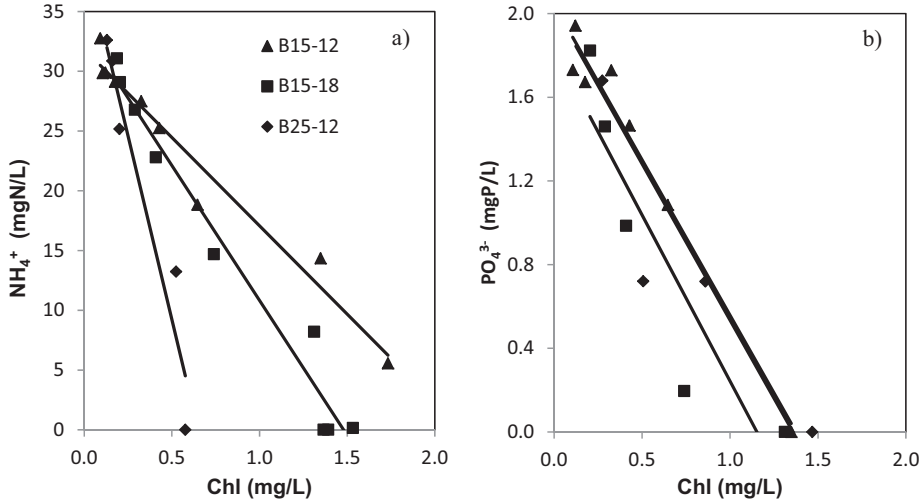

Fig. 5. Correlation between chlorophyll and depleted nutrients concentrations in the medium for all conditions tested (a) NH_4^+ and (b) PO_4^{3-} concentrations. The correlation coefficients for the removal of nutrients were between 0.85 and 0.97.

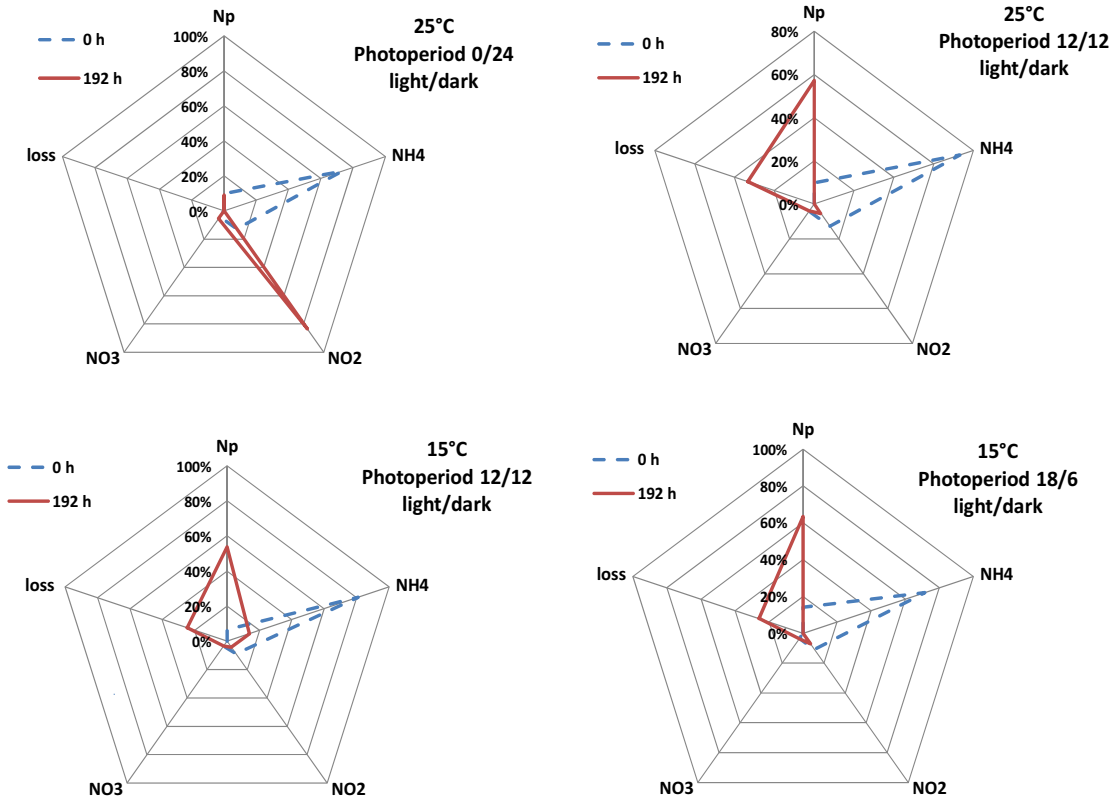

Fig. 6. Nitrogen balance for batch cultures between the start and the end of the experiment (192 h). "Loss" indicates the amount of N, that could not be accounted for at the end of the experiment (ammonia stripping).

absence of light for all tested temperatures (results presented in Fig. 6 for 25 °C; data are not shown for 5 °C and 15 °C). Under these conditions, no microalgal growth was observed and consequently the pH remained constant at around 8.5 (data not shown). Martinez et al. [27] also reported the strong influence of pH on ammonium removal by ammonia desorption in anoxic batch cultures for the treatment of urban wastewater at different temperatures. In our experiments the loss of nitrogen was mainly attributed to this stripping phenomenon. In conclusion, high nitrogen removals portions (72–83%), linked to biomass assimilation and stripping, are observed for the different temperatures and photoperiods tested.

3.4. Phosphorus removal

When correlating the concentration of the residual phosphate in the medium with the chlorophyll concentration of the culture (algal biomass), the correlation coefficients were low (Fig. 5b). This suggested that factors other than assimilation could be at play.

Differences in the depletion rate of phosphate (R_{PO4}) were observed for different temperatures and photoperiods (Table 2). However, the experiments B15–18 (15 °C and 18 h photoperiod) and B25–12 (25 °C and 12 h photoperiod) displayed the same phosphorus removal rates (0.024 mgP L^{-1} h^{-1}). This could have been due to a trade-off between the temperature and the photoperiod duration. Moreover, a higher phosphate removal rate was determined for the culture B15–18 compared with the one at 12 h photoperiod (Table 2). This was due to the fact that in the former culture, phosphate was depleted earlier than in the culture B15–12 (Eq. (3)). In all the experiments phosphate was totally removed from the medium after 100–150 h (Fig. 2). In continuous cultures, phosphorus removal efficiencies have been reported to be between 70 and 95% in summer conditions and 25% in winter conditions [30].

Su et al. [5] observed phosphate removal efficacies ranging from 56 to 73% in batch cultures (8 days of incubation) with a low nitrogen/phosphorus ratio (N/P = 3:1). This low level of phosphate depletion was attributed to nitrogen limitation encountered by the cultures. Similar results were shown by Aslan and Kapdan [29] using cultures of *C. vulgaris* in synthetic wastewater (10 days of treatment) where 78% of phosphorus was removed by keeping N/P = 2:1. In contrast Wang et al. [9] showed that up to 90% of phosphorus was removed from wastewater (9 days of incubation) by *Chlorella* sp. (N/P = 6:1). Previous studies have shown that the optimal N/P ratio for maximum nitrogen and phosphorus uptake by microalgae-bacteria cultures is N/P = 3:1 [5]. In our study the initial N/P ratio was 17:1. The phosphate removal measured in our work did not seem to have been affected by the N/P ratio in the medium, indeed this high ratio should have favoured phosphate removal. Phenomena other than phosphorus uptake by microalgae could be responsible for phosphorus removal.

The variation in chlorophyll and phosphate concentrations over time were tested at two temperatures and photoperiods tested (Fig. 2). During the first 50 h of the experiment the phosphate concentration fell significantly. At 15 °C, the algae could have accumulated phosphate even though during this period the algal biomass remained approximately constant (Fig. 2a and b). The fall in the phosphate concentration may be due to adsorption on the cell surface, which has been previously reported to contribute significantly to phosphorus removal from wastewater [27]. At the beginning of the experiment, the magnitude of phosphorus removal was not affected by temperature or photoperiod. This observation could also be explained by adsorption.

Phosphate removal and biomass production correlated well from 50 h incubation onwards (Fig. 2). From approximately 100 h (168 h for the culture B15–12) onwards the pH of the cultures rose above 9.5. At this elevated pH, the chemical precipitation of

phosphorus is possible and in every graph it can be seen that complete phosphate removal occurs at this pH. No phosphate removal was detected for cultures in darkness where pH values were between 7.8 and 8.3 all along the incubation period.

It can be seen that microalgal growth slowed down and stopped when phosphate was no longer detected in the medium at 15 °C (Fig. 2a and b), suggesting that the exhaustion of this nutrient may limit growth. For the cultures at 25 °C, the growth did not stop after phosphate exhaustion (Fig. 2c). This may be explained by the fact that microalgae are known to uncouple nutrient uptake such as phosphorus from growth. They can continue to grow after nutrient exhaustion. This has been previously demonstrated and modelled [31–33]. Additionally, the phosphate concentration increased in the medium after 150 h. This could have been as a result of cell rupture, releasing the intercellular phosphate content into the medium, a phenomenon that has been reported by Martinez et al. [27]; in this work the cell rupture was confirmed by microscopic examination showing the progressive whitening of the cells.

In conclusion, phosphate seems to be depleted from the medium through different mechanisms: starting with adsorption onto the cells surface, followed by assimilation by the biomass for a part of the phosphate ions and chemical precipitation for the other part. Phosphate has been widely reported to be eliminated through biotic processes such as assimilation [5,25] into biomass (bacteria and microalgae) and abiotic processes such as adsorption [27] and chemical precipitation [30].

4. Conclusions

In this work, the effects of temperature and photoperiod on dissolved nitrogen and phosphate removal, microalgal growth and productivity were studied. A clear understanding of the abiotic and biotic mechanisms involved is required in order to improve HRAP performances in terms of biomass productivity and wastewater treatment. An increase in temperature from 15 to 25 °C led to a slightly higher apparent specific growth rate, an increase in the microalgae concentration and the biomass productivity. Neither growth, nor metabolic activity was detected for cultures at 5 °C. The apparent specific growth rate was mainly influenced by temperature and not by photoperiod duration.

High nitrogen depletion levels (between 72 and 83%) were measured in our study. The majority (72–81%) of the nitrogen in the wastewater was found in the form of ammonium. The highest ammonium removal rate was determined at 25 °C. The biomass nitrogen content obtained under different conditions (temperature and photoperiod) was similar, suggesting identical nitrogen assimilation abilities. Nitrogen assimilation by the biomass contributed to nitrogen removal from the wastewater ($50 \pm 2\%$ of the total nitrogen). Ammonia stripping seems one of the mechanisms responsible of nitrogen elimination, contributing to at least 17% of N-removal. Nitrification was also a pathway for ammonium transformation in our experimental system.

All phosphate ions were removed from the wastewater after approximately 100–150 h. The highest rate of phosphate removal was measured in cultures at 25 °C and 12 h photoperiod together with 15 °C and 18 h photoperiod. The phosphate concentration profile suggests a peculiar trend: a theoretical adsorption of phosphate on the cell surface, followed by consumption associated with microalgal growth and finally chemical precipitation due to the high pH values.

Under our conditions the nitrogen and phosphate depletion rates were high and that the system did not seem to require organic carbon supply for this process. This could be attractive for the treatment of secondary effluents where there is little organic carbon available. Nevertheless, further work must be carried out to confirm these preliminary results. Studies should assess the effect of daily and seasonal temperature and light intensity changes on nutrient removal and microalgal growth. The use of continuous HRAP and finally an LCA (Life Cycle Assessment) and cost analysis must be performed to fully confirm this system's applicability at wastewater treatment with an indigenous microalgae-bacteria consortium. Moreover, mechanisms of adsorption of phosphate on the cell surface and its assimilation by microalgae should be further studied. In order to improve the understanding of this complex microbial system, research is required to characterize the microbial consortium in batch systems.

As a conclusion, microalgal treatment of wastewater, through biological and physico-chemical mechanisms, could represent an attractive addition to existing biological treatments used to purify wastewaters. The advantages of using microalgae for this purpose are: an in situ production of oxygen by microalgae for bacterial use and the possibility of recycling assimilated nitrogen and phosphorus as a fertilizer.

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