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Research article

Assessment of microalgae and nitrifiers activity in a consortium in a continuous operation and the effect of oxygen depletion

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ABSTRACT

Background: Industrial wastewaters with a high content of nitrogen are a relevant environmental problem. Currently, treatments to remove nitrogen are not efficient, so is necessary to develop alternative methods. The objective of this study is to investigate a consortium of microalgae — nitrifying, that due to the symbiosis between them could be an interesting alternative.

Results: In this study, it was possible to obtain a consortium of nitrifying bacteria (NB) and microalgae (MA) capable of operating with low requirements of dissolved oxygen, using aerobic sludge from wastewater treatment plants. During the operation, this consortium presents removal percentages above 98% of ammonia, even at concentrations of DO of 0.5 mg O_2 L^{-1}. It is estimated that the removal was caused both by the action of nitrifying bacteria and microalgae. It was determined that approximately 60% of the ammonia feed was oxidized to nitrate by nitrifying bacteria, while the algae assimilated 40% of the nitrogen feed at steady state. A methodology for measuring the specific activities of nitrifying bacteria and microalgae by comparing the rates in the variation inorganic nitrogen compounds was established with satisfactory results. An average specific activity of 0.05 and 0.02 g NH_4^+ $gVSS^{-1}$ d^{-1} for nitrifying bacteria and microalgae was determined, respectively.

Conclusions: The consortium it can be obtained in a single continuous operation, and has a high capacity for nitrogen removal with low oxygen content. The consortium could prove to be a more economical method compared to traditional.

1. Introduction

Nitrogen is found in most of industrial wastewaters such as in pharmaceutical, chemical, petrochemical and food wastewaters [1]. Nitrogen can be found in the form of ammonium which can result in eutrophication when disposed in a body of water. The stripping of ammonium can release soluble ammonia to the environment which is toxic [2]. Ammonium is usually removed from wastewater by the nitrification–denitrification process which is quite efficient but requires oxygen and a source of organic matter, *i.e.*, extra costs. An interesting microbial symbiosis for ammonium removal is the use of both nitrifying bacteria (NB) and microalgae (MA), where the NB oxidizes ammonia to nitrite (AOB) and then to nitrate (NOB) and the MA would produce O_2 through photosynthesis [3,4]. The contribution of oxygen by the MA could reduce the requirement for aeration, which is a costly process in wastewater treatment plants [5]. In addition, it has been reported that MA are capable of removing inorganic nitrogen compounds, hydrocarbons, organic matter and coliforms [6]. However, in the treatment of effluents with a high nitrogen content and low chemical oxygen demand (COD), the addition of an external electron donor is required to complete the removal of nitrogen, as are the NB [7].

The joint activity of NB and MA has been observed in studies at laboratory scale [7,8,9]. However, the specific activity of each microorganism in this consortium has not been evaluated. In fact, the effect of environmental variables on the consortium of NB and MA is unknown [10]; however, these have been studied extensively on NB and MA, individually [11]. For instance, proper culture ranges that have been reported in literature for cultures of MA have a temperature range of 16–27°C, a pH between 4 and 11 and intensities of light of 18.5–185 μmol m^{-2} s^{-1} [12]. For NB, the optimum temperature range is 30–40°C, pH optimum working for AOB and NOB is 7.2–7.6 and 7.9–8.2, respectively and an oxygen concentration of

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Nomenclature

Act_{NB} specific activity of nitrifying bacteria (gNH_4^+ $gVSS^{-1}$ d^{-1})
Act_{MA} specific activity of microalgae (gNH_4^+ $gVSS^{-1}$ d^{-1})
Δm_{NB} respiration of nitrifying bacteria (gO_2 L^{-1} d^{-1})
Δm_{MA} oxygenation by microalgae (gO_2 L^{-1} d^{-1})
$m_{respiration}$ oxygen consumption slope (gO_2 L^{-1} d^{-1})
$m_{respiration\ endogenous}$ endogenous oxygen consumption slope (gO_2 L^{-1} d^{-1})
$m_{oxygenation}$ oxygen production slope (gO_2 L^{-1} d^{-1})
$m_{oxygenation\ basal}$ basal oxygen production slope (gO_2 L^{-1} d^{-1})
$Y_{NH_4^+/O_2}$ theoretical yield of ammonium (gNH_4^+ gO_2^{-1}).
f_d dilution factor of the sample.
X_{Sample} biomass sample ($gVSS$ L^{-1})
$r_{NH_4^+}$ rate of nitrification (gNH_4^+ $gVSS^{-1}$ d^{-1})
$R_{NH_4^+}$ rate of nitrogen assimilation (gNH_4^+ $gVSS^{-1}$ d^{-1})
HRT hydraulic retention time (d)
$[N\text{-}NO_3^-{}_{effluent}]$ concentration of nitrate-nitrogen in the effluent ($gN\text{-}NO_3^-$ L^{-1})
$[N\text{-}NH_4^+{}_{feeding}]$ concentration of ammonium-nitrogen in the inlet ($gN\text{-}NH_4^+$ L^{-1})
$[N\text{-}total_{Effluent}]$ concentration of total nitrogen in effluent of the reactor (sum of $N\text{-}NH_4^+$, $N\text{-}NO_2^-$ and $N\text{-}NO_3^-$ effluent; gN L^{-1})
$MW_{NH_4^+}$ molecular weight of ammonium (18 gNH_4^+ mol^{-1})
AW_N atomic weight nitrogen (14 gN mol^{-1})

> 2 mg O_2 L^{-1} [11]. Therefore, pH and temperature ranges appropriate for the growth of both microorganisms are overlapped. Also, NB and MA have been found in wastewater treatment plants [13,14], making them an attractive source of inoculum for the cultivation of both microorganisms. On the other hand, it is known that light is essential for the growth of MA [6,15], and it has been reported that NB may be inhibited by the light as well [16,17]; therefore, the light could be a negative factor in a mixed culture of both microorganisms.

The aim of this paper is to develop a strategy to obtain a NB and MA consortium in a bioreactor that is capable to remove ammonia from wastewater. To do this, a novel methodology, adapted from conventional respirometry, for determining the specific activity of NB and MA in a consortium is developed and employed. Furthermore, the effect of the diminishing external oxygen supply on the consortium performance is also assessed.

2. Material and methods

2.1. Lab-scale bioreactors

A CSTR-type reactor, made of plexiglass, was used in order to allow the light to penetrate and reach the biomass. The reactor receives a light intensity of 67.5 μmol m^{-2} s^{-1} (along the reactor walls), which was measured using a luxometer (Extech model 403,125, 0.0135 μmol m^{-2} s^{-1} for lux), The reactors consist of a cylindrical tank of 1.5 L equipped with a pH control (Hanna Instruments, model BL 931700) connected to a diaphragm pump (STROKE, BL1.5 model) that injects a solution of HCO_3^- (49 g L^{-1}, Merck, when the pH was below 7.0). An automatic feeding system (peristaltic pump, Masterflex L/S), aeration by an air compressor (ABAC model B 2800-100, 5 L min^{-1}) and agitation by a magnetic stirrer (Stuart, model SB161) were also implemented. The culture medium composition was the same as used in previous works [18].

2.2. Consortium generation

The reactor was operated continuously with a constant flow of 0.15 L d^{-1} (HTR 10 d), which was inoculated with aerobic sludge (1 L, 3.4 gVSS L^{-1}) from a WWTP (La Farfana, Santiago, Chile). At the reactor's start-up, the ammonium concentration was increased, stepwise until it reached a value of 1400 mg $N\text{-}NH_4^+$ L^{-1}. The values of pH, temperature and dissolved oxygen (DO) were stable throughout the operation (80 d) and values of 7.8 ± 0.1, 27.2 ± 0.2°C and 7.0 ± 0.5 mg O_2 L^{-1} were obtained respectively.

2.3. Oxygen concentration effect in the consortium

Once the NB-MA consortium was obtained, in a second experiment the reactor was operated at a constant flow rate of 0.30 L d^{-1} (HRT 5 d). The aeration flow was diminished in stages; the operation lasted 50 d. The pH of 7.2 and the temperature of 25°C were stable throughout the operation.

2.4. Determining the activity of nitrifying bacteria and microalgae

Determining the specific activity of NB and MA in a consortium requires the measurement of the oxygen consumption during the oxidation of ammonium to nitrate by NB and the ability to produce O_2 by MA during photosynthesis. An acrylic respirometer of 0.14 L, an oxygen probe (Design Instruments, DO2-WWT) and a reader (Design Instruments, model series modular control FMC) were used to monitor the variation of DO. The respirometer was exposed to artificial light with and intensity of 67.5 μmol m^{-2} s^{-1} along the reactor walls. Air was injected with a Fishbowl pump (Sea Star, model HX-108 A) and the mixing was performed using a magnetic stirrer during the determination. The following procedure was used:

- Aerate for 12 h (to consume the residual ammonium), stop aeration and monitor consumption oxygen for 20–30 min ($m_{respiration\ endogenous}$)
- Turn lighting on and monitor production of oxygen for 20–30 min, turning off lighting ($m_{oxygenation\ basal}$).
- Add 50 μL of medium culture, monitor oxygen consumption for 30–45 min ($m_{respiration}$).
- Turn lighting on and monitor variation in dissolved oxygen ($m_{oxygenation}$).

The $m_{respiration\ endogenous}$ represents the sum of the endogenous respiration of the NB and MA (dark respiration); while the $m_{oxygenation\ basal}$ is the resulting factor between the endogenous respiration of NB and endogenous oxygenation of MA. The $m_{respiration}$ is the respiration of the NB by the oxidation of ammonium to nitrate, together with the dark respiration of MA. Finally, the relationship $m_{oxygenation}$ represents the activity of MA to, and the NB activity the presence of a nitrogen source. By plotting the dissolved oxygen (DO) *versus* time, the slope or rates through linear regression for the different stages above mentioned, are determined.

Due to the fact that, Act_{BN} [Equation 1] and Act_{MA} [Equation 3] are determined in a respirometer (off-line) and not in the reactor, a dilution factor (f_d) must be determined. The specific activity of NB and MA of the consortium samples were determined using the following equations:

$$Act_{NB} = \frac{\Delta m_{BN} * Y_{NH_4^+/O_2} * f_d}{X_{Sample}} \quad \text{[Equation 1]}$$

$$\Delta m_{NB} = \left(\left[m_{respiration}\right] - \left[m_{respieraction\ endogenous}\right]\right) \quad \text{[Equation 2]}$$

$$Act_{MA} = \frac{\Delta m_{MA} * Y_{NH_4^+/O_2} * f_d}{X_{Sample}} \quad \text{[Equation 3]}$$

$$\Delta m_{MA} = \left(\left[m_{oxygenation}\right] - \left[m_{oxygenation\ basal}\right]\right) + \Delta m_{NB} \quad \text{[Equation 4]}$$

The theoretical yield ($Y_{NH4+/O2}$) was assumed to be 0.281 gNH_4^+ gO_2^{-1} for the NB [8] and 0.055 gNH_4^+ g O_2^{-1} for MA. In the case of MA, to obtain an estimation of the yield, a stoichiometric equation is proposed [Equation 5] based on a general biomass formula of MA [19] and the substrates used in the experiments.

$$CO_2 + 0.11NH^+{}_4 + 0.01PO_4^{=} + 0.695\ H_2O + h\nu \rightarrow CH_{1.83}O_{0.48}N_{0.11}P_{0.01} + 1.1295O_2 \quad \text{[Equation 5]}$$

In order to compare the specific activities determined externally, two parameters are defined using variations of the inorganic nitrogen compounds. These new parameters were named as the rate of nitrification and nitrogen assimilation, which were calculated using the following equations:

$$r_{NH_4^+} = \frac{\left[N-NO_{Effluent}^-\right] * \left(\frac{MW_{NH_4^+}}{AW_N}\right)}{HRT * X_{sample}} \quad \text{[Equation 6]}$$

$$R_{NH_4^+} = \frac{\left(\left[N-NH_{4\ feeding}^+\right] - \left[N-total_{Effluent}\right]\right) * \left(\frac{MW_{NH_4^+}}{AW_N}\right)}{HRT * X_{sample}} \quad \text{[Equation 7]}$$

The rates of nitrification and assimilation are parameters determined from variables of the reactor by calculating the balance of inorganic nitrogen compounds, thus they are considered in-line parameters.

2.5. Analytical methodology

The concentration of nitrogen as ammonium (N-NH_4^+), nitrite (N-NO_2^-) and nitrate (N-NO_3^-) was measured daily by using a spectrophotometric method [20]; the dissolved oxygen (DO) and the temperature were measured through an oxygen probe (DO2-WWT connected to a FMC series modular control). The pH was measured with an electrode (OAKTON, WD 35802-00). The inlet flow and HCO_3^- solution injection were measured by volume displacement. Biomass was determined through the determination of volatile suspended solids (VSS) by using a gravimetric (dry weight) method. The qualitative biomass analysis was performed with a fluorescence microscope (Nikon, model 50I). The content of chlorophyll a was determined by using a handheld fluorimeter (Aquaflor, Design Turner) and the relative percentages of NB and MA were measured with a Flow Cytometer (FACs Canto II).

3. Results and discussions

3.1. Consortium development

Fig. 1 shows the nitrogen compounds evolution during the operation of the reactor. The presence of nitrogen as nitrate (N-NO_3^-) was observed in the reactor's effluent throughout the operation, which indicates the presence of nitrifying microorganisms. In addition, at the beginning of the operation, the concentration of nitrite increased (N-NO_2^-) in the reactor's effluent, which is an intermediate product of nitrification, as the concentration of nitrogen as ammonium (N-NH_4^+) at the inlet increased. After 20 d of operation, the concentration of N-NO_2^- dropped and increased in the form of N-NO_3^- in the effluent from the reactor, maintaining concentrations between 600 and 800 mg N-NO_3^- L^{-1}, which stabilized after 60 d of operation at an average value of 770 ± 35 mg N-NO_3^- L^{-1}. The presence of nitrite has been reported in the effluent of experiments with NB, which is attributed to a momentary and reversible inhibition of NOB by increasing concentrations of ammonium [21]. This would explain the accumulation of N-NO_2^- and the increase in the concentration of N-NO_3^- when the concentration of N-NH_4^+ was kept constant in the inlet.

After 30 d of operation, the average inlet concentration of N-NH_4^+ was 1214 ± 40 mg N-NH_4^+ L^{-1} which is significantly different from the N-NO_3^- concentration at the outlet which was 770 ± 35 mg N-NO_3^- L^{-1}. From the N-NO_3^-outlet/N-NH_4^+ inlet ratio, it was possible to estimate the amount of ammonia that was nitrified or oxidized to nitrate by the NB. Nevertheless, the removal percentage of ammonia was always kept above 99% despite that only a fraction of the removal was due to nitrification. This difference is explained by the microalgal growth, together with the uptake of nitrogen for assimilation by NB, which mainly consumes ammonia as a nitrogen source [22,23]. This was confirmed by the increasing presence of microorganisms with substances capable of fluoresce as well as the variation of chlorophyll a during operation. The images for microscope fluorescence (Fig. 2) showed the presence (from 7 d on) of MA containing chlorophyll a, which is capable of fluoresce.

The chlorophyll a content in the effluent samples is shown in Fig. 3a. The major peak of chlorophyll a is observed between d 21 and 28 (25 mg chlorophyll a L^{-1}, approximately), a concentration of around 11 mg chlorophyll a L^{-1} was achieved on the other days. Similar profiles of chlorophyll a was demonstrated by Chen et al. [15] in the

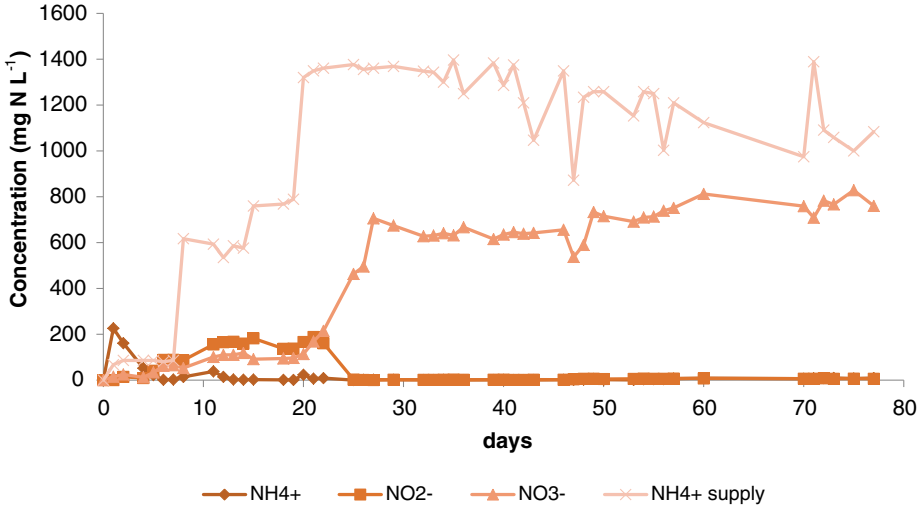

Fig. 1. Variations in the concentration of inorganic nitrogen compounds in the reactor. N-NH_4^+, N-NO_2^- and N-NO_3^- output; N-NH_4^+ supply the reactor.

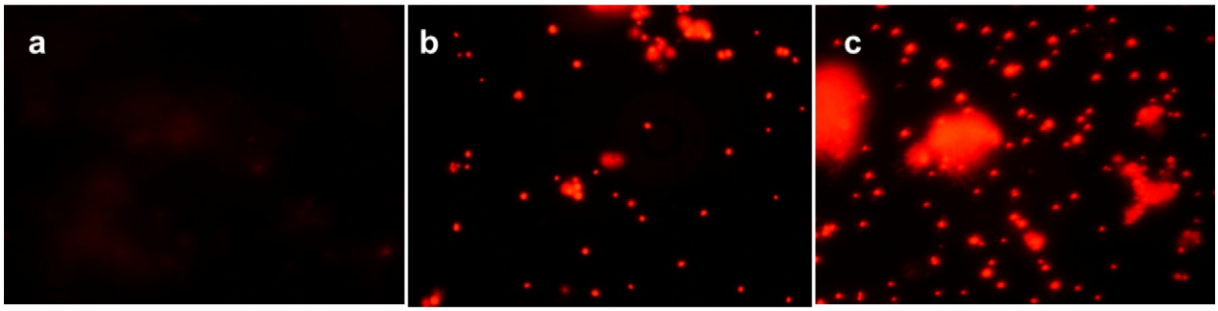

Fig. 2. Images of fluorescence microscopy (scale 10×). Samples taken on day 0 (a), 35 (b) and 70 (c) of the reactor operation. Microorganisms with fluorescent substance are observed in red.

culture of *Chlorella* sp. in a continuous tubular photobioreactor, where the peaks were attributed to a period of photoacclimation to avoid photoinhibition. Results show that both microorganisms obtained in the NB and MA consortium contributed to the removal of ammonia, since it has been reported that the culture of MA possesses great capability of nitrogen removal [24]. Assuming that the difference between the fed ammonia and total nitrogen effluent was assimilated by MA, it can be estimated that a 42 ± 5% on the average (from 80 d of operation) was assimilated by MA.

Fig. 3b shows the percentage of NB and MA, determined by flow cytometry. At the beginning of the experiment, a high content of bacteria and a negligible presence of MA in the inoculum (aerobic sludge) were found. However, during the course of operation, the percentage of bacteria decreased (up to 40 d) due to the washing of the NB. While the MA presented a pronounced increase (up to 28–35 d), followed by a slight decline and stabilization after 50 d of operation, which is quite similar to the variation pattern of chlorophyll a. The variation in the proportions of NB and MA coincides with what was observed with the nitrogen compounds after 50 d of operation, since the percentages of both microorganisms in the N-NO_3^- reactor effluent were stable. From this point on, the consortium presence is confirmed.

Similar trends for the specific activities and calculated rates for both the NB and MA were observed (Fig. 4). This demonstrates that the external determination of the specific activities can be used to monitor the behavior inside the reactor; which is monitored by calculating the rates of nitrification and nitrogen assimilation. In the case of the specific activities of NB, these are consistent with increasing nitrate concentrations and the difference between the rates and the specific activity showed a consistency of around 50%. In the case of MA, the differences between the specific activities and assimilation rates, decreased to 18% at steady state. Average specific activities of 0.05 and 0.02 gNH_4^+ $gVSS^{-1}$ d were determined for NB and MA respectively when the operation was stable.

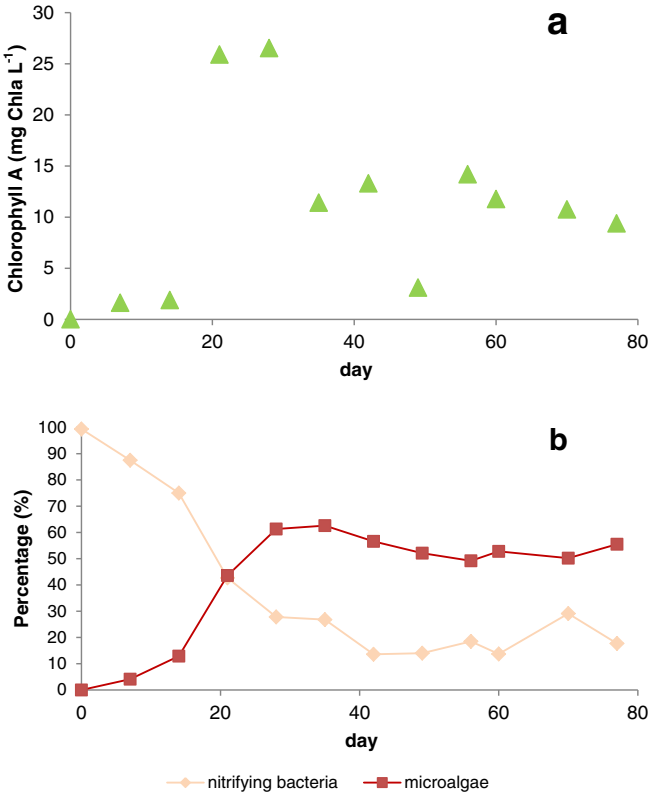

Fig. 3. (a) Variation in the concentration of chlorophyll (a) *in vivo* in the reactor; (b) Relative Percentages of microalgae and nitrifying bacteria determined by flow cytometry.

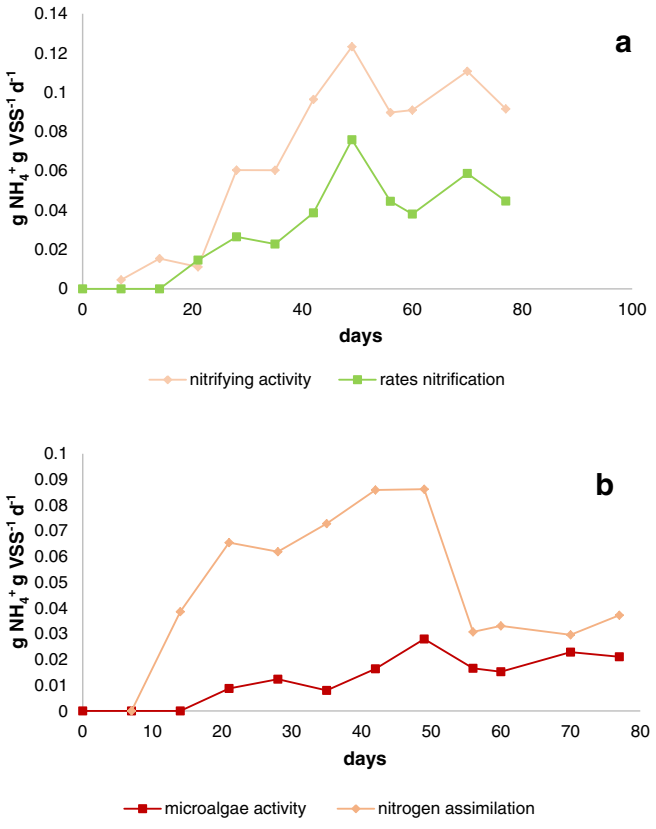

Fig. 4. (a) Evolution of the specific nitrifying activity and rates nitrification. (b) Microalgae activity and nitrogen assimilation in the obtaining of the consortium. The specific activity is determined for the methodology external (off-line), and the rates (in-line parameters) for the balance of nitrogen in the reactor.

3.2. Effect of dissolved oxygen in the consortium

The consortium development process was carried out with constant aeration with DO concentrations above 4 mg O_2 L^{-1}, since it has been reported that concentrations lower than 2 mg O_2 L^{-1} impairs NB activity [11]. The aeration flow was diminished stepwise to lower the DO content inside the reactor, and thus assessing the requirement of oxygen by NB in the consortium. In the first 30 d of operation, when the DO concentration was higher than 0.5 mg O_2 L^{-1}, 40% of N-NH_4^+ at the inlet was assimilated by MA and 60% was oxidized to nitrate by NB (Fig. 5). These percentages were similar to those determined for obtaining the consortium, demonstrating that the decrease in DO had no effect on the microorganisms activity. Subsequently, a decrease in the percentage of ammonium oxidized to nitrate, around a concentration of 0.5 mg O_2 L^{-1}, was observed. This value may represent the limit DO concentration that would negatively affect the NB activity. Throughout the operation, the ammonia removal was high, with percentages above 98%, even after the decrease of the NB activity, which is explained by the increase in nitrogen assimilation by MA.

In regard to MA, high concentrations of oxygen may impair the microalgal growth since it leads to photo-oxidation damage and to a decrease in the capability of removing pollutants [25,26]. However, the highest oxygen concentration attained was 7.0 ± 0.5 mg O_2 L^{-1}, which is below the DO concentration reported as inhibitory for MA of 20 mg O_2 L^{-1} [25]. The effect of the DO upon the NB has been widely studied and, unlike MA, low concentrations of DO exert a negative effect on the nitrification [11,18]. Before the 20th d of operation the DO concentrations were kept at values where no inhibition had been detected for NB; however, from d 20 onwards, the DO concentration dropped to values where inhibition of the process has been observed. Ruiz et al. [18] found that at a concentration of 0.7 mg O_2 L^{-1} an accumulation of 65% of the N-NO_2^- that comes from the oxidation of ammonia was attained, which showed a reduction in the NOB activity. Afterwards, at 0.5 mg O_2 L^{-1} a complete inhibition of NOB and AOB took place since the ammonia was not oxidized at all. In the present case, only a partial decrease of the nitrification activity occurred, which is observed in the slight reduction of ammonia that is oxidized to nitrate.

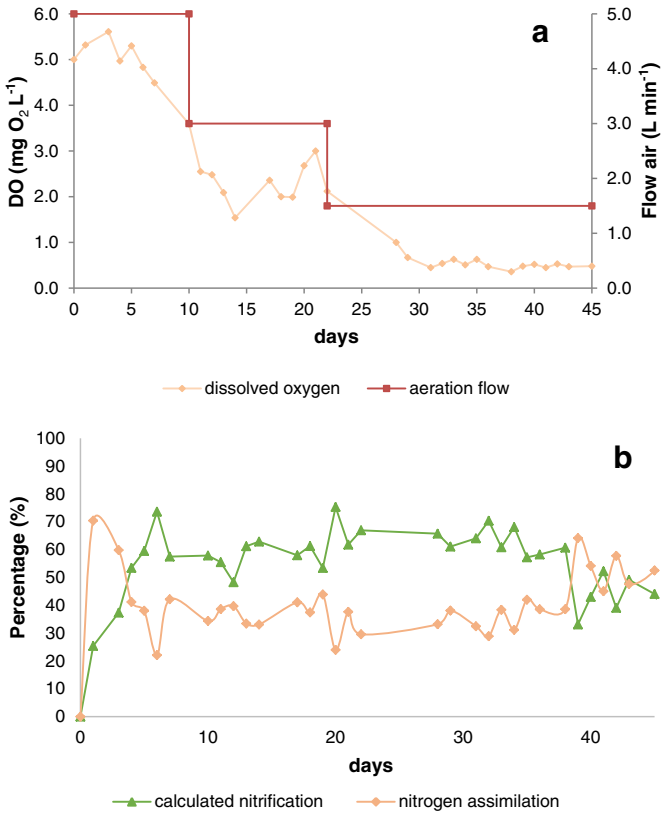

Fig. 5. (a) Variation in the aeration flow and the content of dissolved oxygen. (b) Percentage of calculated nitrification and nitrogen assimilation.

Same positive syntrophic effects have been reported for heterotrophic microorganisms and MA consortia [4]. On one hand, MA were able to compensate the loss of nitrification activity, and on the other hand the NB were able to nitrify at unexpectedly low DO concentration [11,18]. The persisting nitrifying activity at these DO concentrations, can be explained by the agglomeration of NB and MA, which can be observed in Fig. 2c. The MA have the ability to generate self-flocculation phenomenon in the presence of bacteria [27,28]. In these flocs, where NB and MA coexist, the DO produced by the MA would go directly to NB, instead of being diluted into the bulk solution. In other words, in the close proximity to the MA that are agglomerated with NB, there will be a higher DO content than the one in the solution.

4. Conclusions

It is possible to obtain a consortium of NB and MA from aerobic sludge, in a continuous system operation with a simple reactor design and with high percentages of removal of ammonia and nitrogen, even at low levels of dissolved oxygen. A positive effect of the presence of MA upon the NB activity, was observed when operating at low oxygen concentration. Further research is required in order to determine the exact need of oxygen supply for this type of consortia and to develop control strategies for maintaining a stable operation at minimum energy costs. Furthermore, the proposed adapted respirometry system allowed measuring both the activity NB and MA in the consortium.

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