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Nitrification and aerobic denitrification in anoxic–aerobic sequencing batch reactor

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HIGHLIGHTS

- An anoxic–aerobic sequencing batch reactor was proposed for nitrogen removal.
- Nitrification and aerobic denitrification could be considered an ecofriendly process.
- High oxygen concentration and prolonged aerobic phase favored nitrogen removal.
- Anoxic–aerobic regime favored the growth of glycogen accumulating organisms.
- Aerobic denitrification was attributed to glycogen accumulating organisms.

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ABSTRACT

The aim of this study was to evaluate the feasibility of achieving nitrogen (N) removal using a lab-scale sequencing batch reactor (SBR) exposed to anoxic/aerobic (AN/OX) phases, focusing to achieve aerobic denitrification. This process will minimize emissions of N_2O greenhouse gas. The effects of different operating parameters on the reactor performance were studied: cycle duration, AN/OX ratio, pH, dissolved oxygen concentration (DOC), and organic load. The highest inorganic N removal (NiR), close to 70%, was obtained at pH = 7.5, low organic load (440 mgCOD/(L day)) and high aeration given by 12 h cycle, AN/OX ratio = 0.5:1.0 and DOC higher than 4.0 mgO_2/L. Nitrification followed by high-rate aerobic denitrification took place during the aerobic phase. Aerobic denitrification could be attributed to Tetrad-forming organisms (TFOs) with phenotype of glycogen accumulating organisms using polyhydroxyalkanoate and/or glycogen storage. The proposed AN/OX system constitutes an eco-friendly N removal process providing N_2 as the end product.

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1. Introduction

Biological nutrient removal (BNR) constitutes the most economical and sustainable technique to meet rigorous discharge requirements (Xu et al., 2013). The biological removal of nitrogen (N) involves two processes: nitrification and denitrification. Nitrification is an aerobic process performed by autotrophic bacteria, in which ammonia (NH_3) is oxidized to nitrite (NO_2^-), by means of ammonia-oxidizing bacteria (AOB), then nitrite is oxidized to nitrate (NO_3^-) by the nitrite-oxidizing bacteria (NOB). Denitrification is an anoxic process performed by heterotrophic bacteria using nitrite and/or nitrate as the electron acceptor. In this process, NO_3^- is reduced to NO_2^- and then to nitric oxide (NO), nitrous oxide (N_2O) and finally to N_2. Denitrification occurs almost exclusively under facultative anaerobic or microaerophilic conditions; however, complete denitrification can be achieved under high dissolved oxygen concentration (DOC). Aerobic denitrification (ADN) can offer several advantages as it occurs in aerated reactors (Ahn, 2006).

In most BNR systems, an anoxic stage is located upstream of the aerobic zone, named pre-anoxic configuration, e.g. anoxic/oxic (AN/OX) process. High mixed liquor recycle rates are required to bring nitrate and/or nitrite to the anoxic zone. Systems based on post-anoxic denitrification have the anoxic tank located downstream of the aerobic tank, thus mixed liquor recycle from the aerobic to the anoxic stage is not required. However, the oxic/anoxic (OX/AN) system leads usually to a total consumption of the chemical oxygen demand (COD); therefore an exogenous carbon source should be supplied to carry out the post-anoxic denitrification. Post-anoxic denitrification could be also driven by carbon reserves such as polyhydroxyalkanoates (PHAs) and glycogen (Coats et al.,

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2011). Simultaneous nitrification and denitrification (SND) can be accomplished at low DOC (Zeng et al., 2003).

OX/AN, AN/OX and SND process show disadvantages. In OX/AN system, microaerophilic conditions generated from oxygen transfer by mixing in open anoxic basins can exert an inhibitory effect on the denitrification rate (Plósz et al., 2003). In AN/OX system, DO return from the aerobic zone to the anoxic basin significantly increases this problem. In addition, at low O_2 concentrations, N_2O can be the final product of denitrification instead of N_2. This phenomenon occurs mainly in sequencing batch reactor (SBR) with SND process, where nitrite accumulation (>1 mg/L) seems to trigger N_2O production, and at higher levels could also inhibit the denitrification rate (Zeng et al., 2003).

N_2O is a greenhouse gas that traps heat in the atmosphere. Under complete anoxic conditions, N_2O emissions can take place from heterotrophic denitrification (Tallec et al., 2008). In the presence of oxygen, higher N_2O production is caused mainly for two reasons: first, oxygen could inhibit the activity of nitrous oxide reductase, causing N_2O accumulation (von Schulthess et al., 1994); second, at low DOC, N removal takes place via nitrite, i.e. ammonium is oxidized to nitrite, which is denitrified to N_2/N_2O by AOB (Kampschreur et al., 2009). The nitrifier denitrification is mainly responsible for the increased N_2O emission due to O_2 limitation (Kampschreur et al., 2009). The higher N_2O emissions have been reported at 0.4 mgO_2/L and lower rates were observed above this concentration (Tallec et al., 2008).

N removal by a coupled nitrification–denitrification process at high oxygen concentrations, for minimizing emissions of N_2O, has not been sufficiently studied. In this system, nitrifiers and aerobic denitrifying bacteria could be enriched. Anaerobic/oxic (ANA/OX) process can enrich two kinds of organisms: polyphosphate accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs) (Mino et al., 1998), which are able to store internally carbon as PHA and glycogen. Denitrifying PAO (DPAO) and denitrifying GAO (DGAO) are able to denitrify using PHA and/or glycogen as carbon source.

PAOs are responsible for enhanced biological phosphorus removal (EBPR). PAOs convert volatile fatty acids (VFA) in intracellular PHA under anaerobic conditions. For this, intracellular granules of both polyphosphate (poly-P) and glycogen are used as energy source and reducing power respectively. In the aerobic stage, PHA is used for maintaining, cell growth, and replenishment of glycogen and poly-P (Oehmen et al., 2007). GAOs are able of taking up VFA in the absence of oxygen and storing them as PHA, using glycogen as a source of energy instead of poly-P (Muszyński et al., 2013). As no Poly-P is synthesized, higher carbon and energy reserves as PHA and glycogen are expected for GAOs. Based on this analysis, an ANA/OX system enriched with nitrifiers and DGAO is postulated for N removal.

Oxidation–reduction potential (ORP) is a measurement of the ability of a solution to receive or donate electrons indicating an oxidative or reductive environment respectively. ORP values of −50 to −200 mV are typically accepted for anaerobic polyphosphate breakdown, whereas +50 to +150 mV is considered as the range for aerobic phosphorus uptake (WEF, 2013). An oxidative environment would be unfavorable for selecting PAOs, being GAOs the microorganisms probably enriched in the sludge.

The aim of the present study was to evaluate the feasibility of achieving nitrogen removal using a biological system exposed to anoxic/oxic phases, with DOC higher than 1.0 mg/L during the aerobic period. For this, a SBR fed with acetate and ammonium sulphate as the carbon and energy and N sources respectively is used focusing on attaining full nitrification followed by aerobic denitrification in the oxic stage. Nitrite generation was controlled in order to avoid the N_2O production by nitrifier denitrification. ORP was monitored for favoring the GAOs growth instead of PAOs, so that significant intracellular reserves of carbon and energy are available to carry out the denitrification process. Experiments under different operating conditions were carried out: (i) low aeration and low organic load, (ii) low aeration and high organic load, and (iii) high aeration and low organic load. The effects of the operational parameters, such as the cycle duration, AN/OX ratio, pH, DOC, and organic load, on the SBR performance were studied.

2. Methods

2.1. Activated sludge reactor and operating conditions

A lab-scale SBR, with a working volume of 1.2 L, was operated for 10 months. The SBR was seeded with non-EBPR sludge from a lab-scale activated sludge plant in CIDCA. The SBR was operated with successive cycles comprising the following phases: anoxic and aerobic as the reaction stages, biomass settling and finally supernatant draw. The reactor was completely mixed with an overhead mixer at a stirring rate of 100 rpm, except during the settle and draw periods.

The duration of phases, on/off control of mixer, air supply, influent and effluent peristaltic pumps and key parameters (pH, temperature and DO) were automatically controlled by a data acquisition and control system (DACS). This system was developed in the electronic laboratory of CIDCA. pH was measured by a pH probe (Phoenix, Houston, TX, USA). Air was introduced intermittently, through porous diffusers at the bottom of the reactor. Dissolved oxygen concentration was measured by a DO probe (Ingold Mettler Toledo, Urdorf, Switzerland) and expressed as percentage of the oxygen saturation level (OSL) by the DACS. The SBR scheme is shown in Fig. 1.

The volumetric oxygen transfer coefficient (k_La, h^{-1}) in the SBR was measured by the clean water non-steady state method (Al-Ahmady, 2006). k_La is an important parameter in the aerobic wastewater treatment as well as when anaerobic or anoxic conditions are required. The test involves the removal of dissolved oxygen from a volume of water by the addition of sodium sulfite. When a DOC of 0 mgO_2/L is reached in the reactor, the aeration is turned on to the saturation level. The DOC is measured at several points during the aeration period. k_La in the SBR was measured by integration of the following equation:

$$OTR = \frac{dDOC}{dt} = k_La\,(DOC^* - DOC) \quad (1)$$

where OTR is the oxygen transfer rate ($mgO_2/(L\,h)$), DOC^* is the saturation concentration of oxygen in water (mgO_2/L) at the working temperature and DOC is the dissolved oxygen concentration (mgO_2/L) at time (t). The driving force of the oxygen transfer process is given for the difference between DOC^* and DOC.

k_La was measured at a stirring rate of 100 rpm, standard temperature (20 °C), and different aeration rates (vvm, L/(L h)) ranging between 0.2 and 2.3 L/(L min).

At the operational temperature of the SBR (25 °C), the oxygen mass transfer rate can be estimated as follows (Al-Ahmady, 2006):

$$k_La_{\,(25\,^\circ C)} = k_La_{\,(20\,^\circ C)}\,1.024^{(25-20)} \quad (2)$$

In presence of microorganisms, the oxygen uptake rate (OUR, $mgO_2/(L\,h)$) is determined by the following expression:

$$OUR = q_{O_2}\,X \quad (3)$$

where q_{O_2} is the specific oxygen uptake rate ($mgO_2/(mgCOD_B\,h)$) and X is the biomass concentration ($mgCOD_B/L$).

The mass balance for the DO in presence of biomass can be expressed as follows:

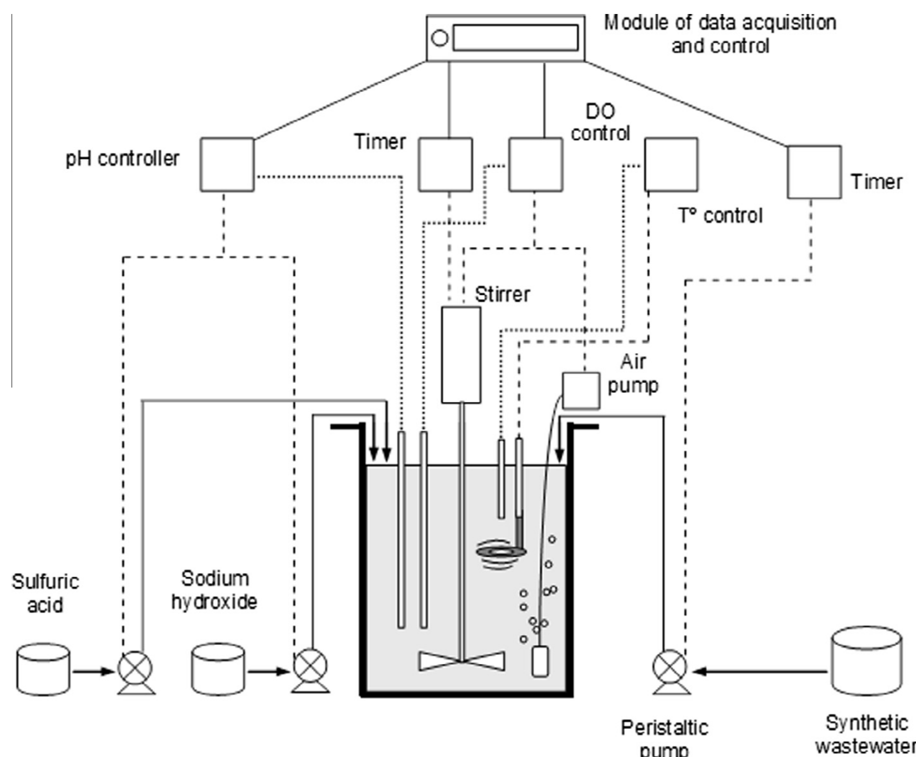

Fig. 1. Schematic diagram of the lab-scale sequencing batch reactor (SBR).

$$\frac{dDOC}{dt} = OTR - OUR \quad (4)$$

where OTR is determined by Eq. (1).

Three experiments were conducted under different operating conditions: (i) low aeration and low organic load (Experiment A), (ii) low aeration and high organic load (Experiment B), and (iii) high aeration and low organic load (Experiment C).

Table 1 shows the SBR operational conditions for all the experiments. In each case, they were maintained for three months. Aeration level was based on the following operating parameters: cycle length, anoxic/aerobic ratio and DOC values.

Synthetic wastewater (SWW, 330 mL) was fed to the reactor in the first 2 min of the anoxic period. Mixed liquor was withdrawn at the end of the aerobic phase (Experiment A and B: 30 mL, Experiment C: 60 mL). After the settling period, treated wastewater was removed from the SBR (Experiments A and B: 300 mL, Experiment C: 270 mL). These operational conditions led to a cellular residence time (CRT) of 10 days and a volumetric exchange ratio (VER) of about 27% for all experiments.

2.2. Synthetic wastewater

Synthetic wastewater containing sodium acetate as carbon and energy source and ammonium sulfate as nitrogen source was used. A 1 mL volume of a micronutrient solution (Lobo et al., 2013) was added to 1 L of synthetic wastewater. Two synthetic wastewaters named SWW1 and SWW2 were utilized. SWW1 with COD, N and P concentrations of 400 mg/L, 40 mg/L and 20 mg/L respectively was used in the Experiment A. SWW2 with COD, N and P concentrations of 800 mg/L, 80 mg/L and 40 mg/L respectively was utilized in the Experiments B and C (Table 2). These wastewaters allowed obtaining the organic, N and P loads utilized in each experiment (Table 1) without changing the VER, which was 27% as was previously indicated. For SWW1 and SWW2, the influent COD:N:P ratio was 100:10:5.

Table 1
Operational conditions during the experiments.

Parameters	Experiment A	Experiment B	Experiment C
Anoxic phase (min)	150	150	220
Aerobic phase (min)	150	150	440
Settling phase (min)	50	50	51
Draw phase (min)	10	10	9
Total cycle length (h)	6	6	12
Anoxic/aerobic ratio	1.0:1.0	1.0:1.0	0.5:1.0
Temperature (°C)	25 ± 0.5	25 ± 0.5	25 ± 0.5
pH (anoxic and aerobic phases)	7.0 ± 0.1	7.0 ± 0.1	7.5 ± 0.1
Oxygen saturation level (%)	20	20	60
Organic volumetric load (mgCOD/(L day))	440	880	440
Nitrogen volumetric load (mgNH_3-N/(L day))	44	88	44
Phosphorous volumetric load (mgP/(L day))	22	44	22

2.3. Analytical methods

The operation of the SBR was monitored by the following physical–chemical parameters: oxidation–reduction potential (ORP, mV), phosphate phosphorus (PO_4^{3-}-P, mg/L), ammonia nitrogen (NH_3-N, mg/L), nitrite nitrogen (NO_2^--N, mg/L), nitrate nitrogen (NO_3^--N, mg/L), soluble COD (COD_S, mg/L) as organic substrate, and total COD (COD_T, mg/L).

The oxidation–reduction potential of the biological system was measured off-line using an ORP probe (Phoenix, Houston, TX, USA). The ORP is the tendency of a solution to either gain or lose electrons. This parameter constitutes a measure of the oxidative state in an aqueous system, reflects the concentration of DO, organic substrate, activity of organisms and some toxic compounds in the system, among which the DO concentration is the most important factor (Cui et al., 2009).

Table 2
Synthetic wastewater and micronutrient solution.

Synthetic wastewater (SWW)	Concentration (g/L)	
	SWW1 (Experiment A)	SWW2 (Experiments B and C)
CH_3COONa	0.586	1.172
$(NH_4)_2SO_4$	0.188	0.376
KH_2PO_4	0.0543	0.1086
K_2HPO_4	0.0428	0.0856
$MgSO_4 \cdot 7H_2O$	0.18	0.18
$CaCl_2 \cdot 6H_2O$	0.0285	0.0285
Micronutrient solution		
$FeSO_4 \cdot 7H_2O$	15	15
$ZnSO_4 \cdot 7H_2O$	5	5
$MnSO_4 \cdot H_2O$	3	3
$CuSO_4 \cdot 5H_2O$	0.75	0.75
$CoCl_2 \cdot 6H_2O$	0.15	0.15
Citric acid	6	6
$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$	0.5	0.5
H_3BO_3	0.1	0.1
KI	0.1	0.1

The other physical–chemical parameters were determined by spectrophotometric methods using commercial reagents (Hach Company, Loveland, CO). Samples were taken from the reactor to measure COD_T (Hach Method No. 8000). Another aliquot was utilized for determination of orthophosphate, ammonia, nitrite, nitrate, and COD_S. For this, a volume of 35 mL was centrifuged for 10 min at 13,000 rpm (Eppendorf centrifuge model 5415C); then, the supernatant was filtered through 0.45 μm cellulose acetate membranes (GE Osmonics). Orthophosphate concentration in the filtrate was determined after reacting with vanadate-molybdate reagent in acid medium (Hach Method No. 8114, adapted from Standard Methods). Ammonia nitrogen in the filtrate was measured by the Nessler method (Hach Method No. 8038). Nitrite and nitrate were measured by the HACH methods No. 8153 and 8039 respectively. COD_S was measured using the Hach Method No. 8000. Biomass concentration was determined as COD (COD_B, mg/L), being calculated as the difference between COD_T and COD_S (Contreras et al., 2002). COD_B was transformed into volatile suspended solids (VSS, mg/L) using a calibration curve previously determined.

Inorganic nitrogen (Ni) corresponded to the sum of ammonia, nitrite and nitrate concentrations. Detection of intracellular poly-P and PHA granules was performed by specific staining methods and microscopic observation.

2.4. Microscopic detection of granules of poly-P and PHA

Neisser and Sudan Black stains were used for detection of poly-P and PHA granules respectively (Serafim et al., 2002). Samples of 30 μL of the mixed liquor from the reactor were spread on glass microscopic slides and dried at room temperature. Fixed smears were observed using a light microscope Leica DMLB (Germany) coupled with a photographic camera. Microscopic images were taken under phase contrast illumination at 1000X magnification.

2.5. Quantification of the SND and denitrification processes

The amount of nitrogen removed via simultaneous nitrification and denitrification (SND) was calculated from the difference between the amounts of oxidized ammonia nitrogen (NH_3-$N_{oxidized}$) and oxidized nitrogen (NO_x^--N: NO_3^--N + NO_2^--N). Oxidized ammonia nitrogen was calculated from the difference between the total consumption of ammonia nitrogen and NH_3-N assimilated into heterotrophic biomass. Nitrogen assimilated into nitrifying biomass was assumed to be negligible (Third et al., 2003).

The ammonia total consumption was measured by a spectrophotometric method as was previously explained in analytical methods Section. NH_3-N assimilated into heterotrophic biomass, for the period when ammonia was present, was estimated from theoretical mass balances of carbon and nitrogen using typical values for stoichiometric coefficients of the studied biological system. For this, amounts of PHB and biomass produced in the anoxic and aerobic phases respectively were estimated. PHB is the main polymer formed when acetate is used as carbon source (Dias et al., 2006). For the estimation of the PHB produced from acetate, a PHB yield $Y_{PHB/Ac}$ of 0.52 C-mol PHB/C-mol Ac (0.38 gPHB/gAc) for anoxic condition was utilized (Beun et al., 2002). Available acetate for PHB production was determined from difference between COD at the start of the anoxic phase and COD required for anoxic denitrification with a theoretical acetate demand of 3.8 mgCOD/mgNO_3^--N (3.65 mgAc/mgNO_3^--N). Biomass produced from PHB was estimated assuming a heterotrophic biomass yield $Y_{X/PHB}$ of 0.5 C-mol biomass/C-mol PHB (0.57 gVSS/gPHB). Finally, NH_3-N assimilated into heterotrophic biomass was determined assuming a molecular formula for biomass $CH_{1.8}O_{0.5}N_{0.2}$ equivalent to 24.6 gVSS/C-mol biomass (Third et al. 2003).

SND was calculated for the period when ammonia was present from the following equation (Third et al. 2003):

$$\%\,SND = \left(1 - \frac{NO_X^- - N}{NH_3 - N_{oxidized}}\right) x\,100 \tag{5}$$

where NO_x^--N is the sum of nitrite and nitrate at the moment when ammonia was depleted and NH_3-$N_{oxidized}$ is the amount of ammonia nitrogen oxidized during the aerobic period.

The amount of nitrogen removed via denitrification (DN), after the stage of SND, was calculated from the difference between the amounts of oxidized nitrogen (NO_x^--N) at the end of nitrification and final period of the aerobic phase through the following equation:

$$\%\,DN = \left(1 - \frac{NO_x^- - N_{FA}}{NO_x^- - N_{FN}}\right) x\,100 \tag{6}$$

where NO_x^--N_{FA} is the amount of oxidized nitrogen at the final period of aerobic phase and NO_x^--N_{FN} is the amount of oxidized nitrogen at the end of nitrification.

2.6. Calculation of Ni and P removal and rates of nitrification and denitrification

The inorganic nitrogen removal (NiR) was measured as follows:

$$\%\,NiR = \left(1 - \frac{Ni_F}{Ni_O}\right) x\,100 \tag{7}$$

where Ni_O is the Ni concentration (from the wastewater) at the start of the anoxic phase (mg/L) given only by the NH_3-N concentration and Ni_F is the Ni concentration (mg/L) from the SBR effluent. Ni_F corresponds to the sum of NH_3-N, NO_2^--N, and NO_3^--N concentrations. The nitrate and/or nitrite concentrations at the start of the cycle (from the residual supernatant of the previous cycle) were not considered in the determination of the Ni_O concentration.

An equation similar to Eq. (7) was applied for P removal. The volumetric nitrification rate (VNR, mgNH_3-N/(L h)) was determined from the ammonia decay curves after subtracting the NH_3 assimilated into heterotrophic biomass in the time interval. The specific nitrification rate (SNR, mgNH_3-N/(gVSS h)) was calculated from the ratio between VNR and the biomass concentration expressed as VSS. The volumetric denitrification rate (VDNR) and specific denitrification rate (SDNR) were calculated from the

nitrate decay curves for the period after the SND phase, and expressed as $mgNO_3^-$-N/(L h) and $mgNO_3^-$-N/(gVSS h) respectively.

2.7. Statistical analysis

For each experiment, three operational cycles were analyzed. All results correspond to average values. Analysis of variance was done using the Systat 12 software. A significance level of 0.05 was utilized.

3. Results and discussion

3.1. Oxygen mass transfer rate in the SBR

k_La was measured for clean water at 20 °C, stirring rate of 100 rpm and different aeration rates (12, 37, and 137 L/(L h)). A linear relationship between k_La and the aeration rate was determined as follows:

$$k_La = m \ \ AER + n \quad (8)$$

where AER is the aeration rate (L/(L h)), m is the slope (L/L) and n (h^{-1}) corresponds to the k_La produced by stirring without aeration (AER = 0).

The parameters *m* and *n* resulted 0.10 L/L and 2.34 h^{-1} respectively. Similarly, k_La produced only by stirring was determined by Third et al. (2003).

For clean water, at working conditions of the reactor (25 °C, stirring rate of 100 rpm) and zero aeration, a k_La value of 2.63 h^{-1} was estimated by using Eq. (2). Based on this estimation, it can be assumed that for a mixed culture only stirring will cause oxygen penetration through liquid surface during the anoxic stage of the SBR operation. Oxygen is known to increase the oxidative state of biological systems, which could negatively affect anaerobic and anoxic processes. Plósz et al. (2003) based on a mathematical model and simulation quantified the effect of the oxygen, entering to the anoxic reactor through the surface, on the denitrification. In the present study, ORP measurements were carried out in order to evaluate the oxidizing conditions of the anoxic and aerobic phases of each experiment and its relationship with the microbial composition of the sludge.

3.2. Experiment A

This experiment was conducted under low aeration and low organic load (Table 1). PHA accumulation occurred in the anoxic phase and degradation of this polymer took place in the subsequent aerobic phase, as was detected by microscopic observation of stained sludge samples. The biomass concentration reached 1220 ± 215 $mgCOD_B$/L. The organic substrate was almost completely removed in the anoxic phase (>99%). The orthophosphate concentration did not show significant changes, and the intracellular poly-P staining was negative, indicating that the EBPR process did not take place. Fig. 2(a) shows a typical operational cycle for the Experiment A.

DOC of zero and positive ORP values were measured throughout the anoxic phase (Table 3). It must be pointed out that ORP values lower than −50 mV are required for anaerobic polyphosphate breakdown (See Introduction section). In the anoxic phase, zero DOC did not change and OTR was the same to the OUR value according to Eq. (4). An OTR value of 21.3 mgO_2/(L h) was estimated by using a k_La value of 2.63 h^{-1} and the maximum driving force of oxygen transfer at 25 °C (DOC* − DOC = 8.11 mgO_2/L). Based on this analysis, it can be inferred that the O_2 transfer produced by stirring could increase the oxidative state of the system during the anoxic phase negatively affecting the P anaerobic meta-

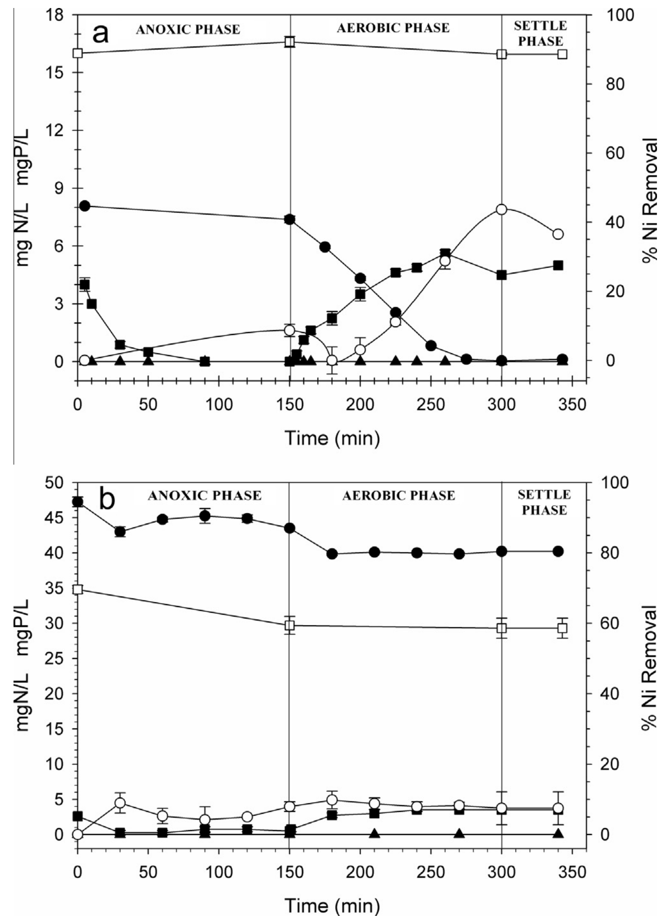

Fig. 2. Removal of phosphorus and nitrogen species during typical operational cycles of the SBR. (a) Experiment A. (b) Experiment B. (□) Orthophosphate (PO_4^--P, mgP/L); (●) Ammonia (NH_3-N, mgN/L); (■) Nitrate (NO_3^--N, mgN/L); (▲) Nitrite (NO_2^--N, mgN/L); (○)% inorganic nitrogen removal (% NiR).

Table 3
Physical–chemical and biological parameters of the SBR for the different experiments.

	Parameters	Experiment A	Experiment C
Anoxic phase	DOC[a] (mg/L)	0	0
	ORP[b] (mV)	286 ± 8	187 ± 13
Aerobic phase	DOC (mg/L)	1.6 ± 0.3	5.5 ± 1.2
	ORP (mV)	295 ± 7	199 ± 9
	VNR[c] ($mgNH_3^-$-N/(L h))	3.96 ± 0.10	3.71 ± 0.45
	SNR[d] ($mgNH_3^-$-N/(gVSS h))	4.22 ± 0.10	4.14 ± 0.48
	VDNR[e] ($mgNO_3^-$-N/(L h))	ND[f]	2.53 ± 0.96
	SDNR[g] ($mgNO_3^-$-N/(gVSS h))	ND	2.94 ± 1.1
	% SND[h]	11 ± 10	0 ± 0
	% DN[i]	5 ± 5	55 ± 3
Total cycle	% AR[j]	99 ± 1	99 ± 1
	% NiR[k]	45 ± 2	67 ± 2

[a] DOC: dissolved oxygen concentration.
[b] ORP: oxidation–reduction potential.
[c] VNR: volumetric nitrification rate.
[d] SNR: specific nitrification rate.
[e] VDNR: volumetric denitrification rate.
[f] ND: not determined.
[g] SDNR: specific denitrification rate.
[h] % SND: simultaneous nitrification and denitrification.
[i] % DN: denitrification.
[j] % AR: ammonia removal.
[k] % NiR: inorganic nitrogen removal.

bolism of PAOs. Thus, oxidizing conditions prevalent in the anoxic phase were probably unfavorable for selecting PAOs and could be responsible for the failure of the EBPR.

ORP values higher than +285 mV were registered during the aerobic phase (Table 3) resulting adequate oxidizing conditions for achieving autotrophic nitrification, since ORP values between +100 and +350 mV are required for achieving nitrification (Gerardi, 2007). Ammonia was removed about 99%, almost exclusively in the aerobic phase. About 70% of influent ammonia in aerobic phase was nitrified, i.e. it was used as the energy source by nitrifying bacteria leading to the formation of nitrate, as was determined by N mass balance. Residual ammonia was utilized as the N source for the biomass synthesis by mainly heterotrophic bacteria in the aerobic phase. Nitrite was not accumulated in the system. At the end of the cycle, the final effluent showed a nitrate concentration of 4.75 ± 0.25 mgNO_3^--N/L, resulting in a mean discharge of 5.70 mgNO_3^--N/day. In the following cycle, residual nitrate was completely removed by denitrifying bacteria in the first minutes of the anoxic phase (Fig. 2(a)). The final effluent showed an inorganic nitrogen concentration of 4.84 ± 0.40 mgN/L, resulting in a mean discharge of 5.80 mgN/day.

It should be indicated that the nitrification process was not limited by the relatively low DOC (<2.0 mgO_2/L). Significant VNR and SNR were determined but a low SND was achieved (Table 3). After SND, the process of denitrification was not evident (Fig. 2(a)); therefore the denitrification rate could not be determined. Nitrogen removed via SND and DN was lower than 21 and 10% respectively, leading to a moderate Ni removal (Table 3).

3.3. Experiment B

This Experiment was carried out at low aeration and high organic load (Table 1). The organic substrate was completely removed in the anoxic phase. The biomass concentration in the reactor under steady-state conditions was 1850 ± 120 mgCOD_B/L. Ammonia was removed about 15% throughout the operational cycle. Oxidizing conditions were registered during the aerobic phase (ORP > + 100 mV); however, only 7% of ammonia from anoxic phase was nitrified in aerobic phase generating relatively low nitrate concentrations. This situation did not stimulate the growth of denitrifying bacteria, so that denitrification was not observed under aerobic conditions. Nitrite was not accumulated in the reactor (Fig. 2(b)). Since very low nitrifying activity was achieved, the nitrification rate could not be reliably determined. In activated sludge systems, nitrifiers grow much slower than heterotrophic bacteria, and there is a competition for oxygen between both groups of organisms (Wang, 2012). It can be argued that, in Experiment B, the nitrifying bacteria were overgrown by the heterotrophic bacteria under high organic load and low DOC.

The final effluent exhibited a high inorganic nitrogen concentration of 43.5 ± 0.20 mgN/L, resulting in a mean discharge of 52.2 mgN/day. Thus, the Ni removal resulted only 8% (Fig. 2(b)).

EBPR activity was not observed. Cocci-shaped cells arranged in tetrads, known as Tetrad-forming organisms (TFOs), were commonly observed showing this metabolic ability of PHA accumulation and degradation. It is well known that several subgroups of Alphaproteobacteria and Gammaprotebacteria display TFO morphotype and exhibit GAO phenotype, being TFOs often associated with EBPR deterioration (Oehmen et al., 2007; Muszyński et al., 2013). Based on the experimental results of the present study, it can be argued that TFOs corresponded to some group of GAO typical of malfunctioning EBPR processes. PAOs were out competed by GAOs under probably the oxidizing conditions prevailing in the anoxic phase (positive ORP, Table 3), which are unfavorable for PAOs as it is recognized in literature (WEF, 2013).

3.4. Experiment C

The operation of the SBR was modified to recover the nitrification process. Cycle length was extended to 12 h, the anoxic/aerobic ratio decreased from 1.0:1.0 to 0.5:1.0, pH was increased to 7.5 ± 0.1 and OL was increased from 20 to 60% (Table 1). These operational conditions, which involved both a high aeration and a low organic load, should favor the nitrification process. The organic, N and P volumetric loads were identical to those of Experiment 1 with good nitrification.

The organic substrate was removed in the anoxic phase. The biomass concentration reached 1125 ± 66 mgCOD_B/L under steady-state conditions. EBPR process did not take place due probably to oxidizing conditions in the anoxic phase (positive ORP, Table 3). Ammonia was removed about 99%, being mostly eliminated in the aerobic phase (80%). In this phase, oxidizing conditions favored the nitrification process (ORP > + 190 mV, Table 3). Nitrification took place producing relatively high nitrate concentrations after about 2 h of the start of the aerobic phase. After this, a gradual reduction of the nitrate concentration was observed; this phenomenon was attributed to the denitrification process. At the end of the cycle, a mean discharge of 3.2 mgN/day as nitrate was determined. At the start of the anoxic phase of the following cycle, the residual nitrate was rapidly denitrified. Nitrite was not accumulated in the reactor (Fig. 3). The final effluent exhibited a Ni concentration of 5.95 ± 0.28 mgN/L, resulting in a mean discharge of 3.2 mgN/day. VNR and SNR were not significantly different to those corresponding to the Experiment A (Table 3).

SND did not take place as determined from the nitrogen mass balance. About 85% of the ammonia that entered in aerobic phase was nitrified and 15% was assimilated by heterotrophic bacteria. After completion of the nitrification, about 55% of the generated nitrate was removed by denitrification. The DN process together with the heterotrophic nitrogen assimilation led to an inorganic nitrogen removal close to 70% (Table 3).

It must be pointed out that the denitrification observed in both anoxic and aerobic phases occurred at ORP values higher than +170 mV and +190 mV respectively (Table 3). These values are significantly higher to those found in anoxic reactors of typical BNR plants (−100 to +100 mV, Dabkowski, 2008). This result suggests that the denitrification process observed in the present study is different from the anoxic process exhibited by BNR systems.

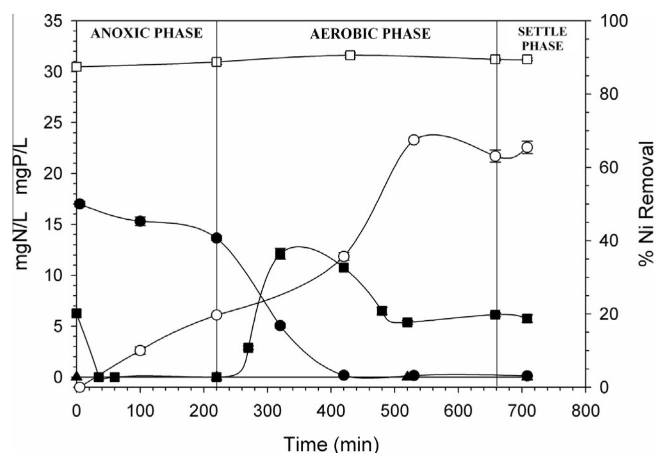

Fig. 3. Removal of phosphorus and nitrogen species during a typical operational cycle of the SBR (Experiment C). (□) Orthophosphate (PO_4^--P, mgP/L); (●) Ammonia (NH_3-N, mgN/L); (■) Nitrate (NO_3^--N, mgN/L); (▲) Nitrite (NO_2^--N, mgN/L); (○)% inorganic nitrogen removal (% NiR).

The Ni removal during the Experiment C was about 50% higher than that achieved in the Experiment A (Table 3). Increased inorganic nitrogen removal was attributed to a higher denitrifier activity in Experiment C.

The specific denitrification rate measured after completed nitrification (Table 3) was much higher than the typical values for endogenous decay, which have been shown to range from 0.2 to 0.6 mgNO_3^--N/(gVSS h) (Kujawa and Klapwijk, 1999). Since the external organic substrate was exhausted in the anoxic phase, the carbon used for the denitrification under aerobic conditions must originate from internal cell sources. As no PAO activity was observed, it can be argued that the denitrification during the aerobic phase was carried out by DGAO with TFO morphology. The observed SDNR was 3–9 times higher than those reported by Winkler et al. (2011), who achieved post-anoxic denitrification utilizing an ANA/OX/AN SBR. These authors suggested that the denitrification process was carried out by PAOs from its maintenance metabolism using glycogen. Similarly, Coats et al. (2011) reported a post-anoxic SDNR ranking between 0.53 and 1.36 mgNO_3^--N/(gVSS h), i.e. about 2–5 times lower than the obtained in the present study. In the work by Coats et al. (2011), PAOs were probably enriched in an A/O/A SBR, which showed post-anoxic denitrification mainly driven by glycogen. Vocks et al. (2005) using a bench-scale membrane bioreactor under ANA/OX/AN regime achieved a similar SDNR (2.2 mgNO_3-N/(gVSS h)) to that obtained in the present study. Post-anoxic denitrification took place from probably glycogen stored as internal carbon source. These authors proposed DGAOs as responsible for the post-denitrification, provided that denitrifying PAOs (DPAOs) are unable to denitrify without P removal. Recent studies attributed to DGAOs to play the major role in the process of post-anoxic denitrification. Li et al. (2014), working at an ANA/OX/AN SBR under operational conditions favorable for enrichment of GAOs, reported post-anoxic SDNRs of 1.24 and 0.5 mgNO_3-N/(gVSS h) for PHA and glycogen respectively. Post-anoxic SDNRs of 2.97 and 1.22 mgNO_3-N/(gVSS h) for the same carbon sources were informed by Zhu et al. (2013).

A priori it can be expected that anoxic denitrification rate is higher than that corresponding to aerobic conditions. Oh and Silverstein (1999) reported that the denitrification rate at a DOC of 0.4 mg/L represented about 50% of the anoxic rate, being only 4% at a DOC of 5.6 mg/L. However, in the present study, at a DOC of 5.5 mg/L, the specific denitrification rate was higher, in most cases, or similar to those found in literature for anoxic conditions as was previously discussed. This result can be explained by two reasons: (a) the denitrifying bacteria might grow in the anoxic inner zones of large flocs, (b) the dissolved oxygen did not affect the denitrification ability of the activated sludge, so that aerobic denitrifiers could be responsible for the achieved denitrification.

Aerobic denitrification can be considered as an acceptable environmentally process provided that N_2 is the end product as is the case of many aerobic denitrifiers mainly belonging to the Gammaprotebacteria. *Citrobacter diversus* (Huang and Tseng, 2001) and several strains of the genus Pseudomonas (Miyahara et al., 2010; Ji et al., 2015) among others has been reported as aerobic denitrifiers that produce mainly N_2 with low or undetectable levels of N_2O. *Microvirgula aerodenitrificans* is a denitrifying bacterium (Betaproteobacteria) able to produce N_2 from nitrous oxide or nitrate under several aeration conditions (Patureau et al., 1998).

In the present study, a low VER, high k_La for oxygen, and prolonged aerobic phase with high DOC led to highly oxidizing conditions. These conditions favored the aerobic denitrifying activity.

4. Conclusions

A lab-scale sequencing batch reactor (SBR) operated with two phases, anoxic and aerobic, achieved complete COD removal. High inorganic nitrogen removal was obtained at pH of 7.5, high dissolved oxygen concentration, prolonged aerobic phase and low organic load. Nitrification followed by aerobic denitrification took place during the aerobic phase. Aerobic denitrification could be attributed to Tetrad-forming organisms (TFOs) with glycogen accumulating organisms (GAOs) phenotype using polyhydroxyalkanoate and/or glycogen storage. The proposed AN/OX system constitutes a simple and potentially eco-friendly process for biological nitrogen removal, decreasing the formation of N_2O a greenhouse gas that has an important influence on atmosphere warming.

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