Chapter

Performance of Anoxic-Oxic Sequencing Batch Reactor for Nitrification and Aerobic Denitrification

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Abstract

The biological nitrogen removal (BNR) involves two processes: nitrification and denitrification. Denitrification occurs almost exclusively under facultative anaerobic or microaerophilic conditions; however, aerobic denitrification can occur in aerated reactors. In this chapter, the feasibility of achieving nitrogen removal using a lab-scale biological sequencing batch reactor (SBR) exposed to anoxic/oxic (AN/OX) phases is described in order to attain aerobic denitrification. The SBR was fed with acetate and ammonium sulfate. Nitrite generation was controlled in order to avoid the N_2O production by nitrifier denitrification. Experiments under four different operating conditions were carried out: low and high aeration, each one with low and high organic loads. For all the tested conditions, a complete COD removal was achieved. The highest inorganic N removal close to 80% was obtained at pH = 7.5, high organic load (880 mg COD/(L day)) and high aeration given by 12 h cycle, AN/OX ratio = 0.5:1.0, and dissolved oxygen concentration higher than 4.0 mg O_2/L. Nitrification followed by high-rate aerobic denitrification took place during the aerobic phase. Denitrification took place mainly from the intracellular reserves of polyhydroxyalkanoates (PHA) during the aerobic phase. The proposed AN/OX system constitutes a simple and potentially eco-friendly process for biological nitrogen removal, providing N_2 as the end product and decreasing the formation of N_2O, a powerful greenhouse gas.

Keywords: nitrogen removal, sequencing batch reactor, nitrification, aerobic denitrification, polyhydroxyalkanoates, glycogen

1. Introduction

The biological removal of nitrogen (N) comprises two processes: nitrification and denitrification. The nitrification is a strict aerobic process that involves the oxidation of ammonia (NH_3) to nitrate (NO_3^-) by autotrophic bacteria. Firstly,

ammonia is oxidized to nitrite (NO_2^-), by means of ammonia-oxidizing bacteria (AOB), and then nitrite is oxidized to nitrate by the nitrite-oxidizing bacteria (NOB) [1]. In the second step, named denitrification, nitrate is converted into a gaseous product, nitrous oxide (N_2O) or molecular nitrogen (N_2), which is finally eliminated into the atmosphere. Denitrification is an anoxic process performed by heterotrophic bacteria using nitrite and/or nitrate as the electron acceptor. In full denitrification, NO_3^- is reduced to NO_2^- and then to nitric oxide (NO), N_2O, and finally to N_2 [2].

Nitrosomonas is the most common genus of autotrophic bacteria capable of oxidizing ammonium to nitrite; however, *Nitrosococcus*, *Nitrosospira*, *Nitrosovibrio*, and *Nitrosolobus* also have that ability. These ammonium oxidizers belong to the beta subdivision of the *Proteobacteria* [3]. *Nitrobacter*, *Nitrospira*, *Nitrospina*, *Nitrococcus*, and *Nitrocystis* are known to be involved in the nitrite oxidation [3]. Nitrite-oxidizing genera belong to the alpha, gamma, and delta subdivisions of the *Proteobacteria* [4]. Denitrification is carried out by several bacterial genera such as *Achromobacter*, *Aerobacter*, *Alcaligenes*, *Bacillus*, *Brevibacterium*, *Lactobacillus*, *Micrococcus*, *Proteus*, *Pseudomonas*, and *Spirillum* [5].

Carbon is not a difficult compound to eliminate by biological processes; on the contrary, one of the most common problems in wastewater treatment plants is the lack of organic carbon to carry out the denitrification process. Particularly, treatment plants with low chemical oxygen demand/nitrogen (COD/N) ratios exhibit difficulties for nitrogen removal due to a shortage of organic substrate [6, 7].

Several biological processes have been proposed for nitrogen removal. The modified Ludzack-Ettinger process is a widespread conventional technology for nitrogen biological removal. This process is a modification of a conventional activated sludge process where an anoxic reactor is located upstream of the aerobic reactor. This process with pre-anoxic configuration is commonly named anoxic/oxic (AN/OX) process. In the first reactor, denitrification is carried out using organic carbon from wastewater. For this, the process requires an internal recycle that carries nitrate, generated from ammonia by the nitrification process (aerobic reactor), to the anoxic reactor. The amount of nitrate removed in the anoxic reactor depends on both the recycle flow and availability of influent organic carbon. Several disadvantages are associated with this process: (a) high costs involved in the recirculation; (b) production of nitrogen oxides as end products, instead of N_2, which is caused by microaerophilic conditions, generated by recirculation [8]; and (c) limitation of the carbon source in the anoxic tank, caused by the recirculation of the nitrate-rich mixed liquor, resulting in accumulation of intermediate products such as nitrites and nitrogen oxides [9].

Systems based on postanoxic denitrification have the anoxic tank located downstream of the aerobic tank. Nitrification and consumption of the organic carbon take place in the first reactor. Denitrification is carried out in the anoxic stage. Thus, mixed liquor recycle from the aerobic to the anoxic stage is not required. However, this oxic/anoxic (OX/AN) system leads usually to a total consumption of the organic carbon. This configuration was firstly proposed by Wuhrmann [10], where organic substrates required for denitrification were probably supplied from endogenous death and lysis of active biomass [11]. Then, Wuhrmann process was modified to improve denitrification by carbon addition [11]. However, additional operational costs are caused by the addition of exogenous carbon such as methanol or acetate [12]. Another disadvantage is attributed to the postanoxic denitrification process. Microaerophilic conditions generated from the transfer of oxygen by mixing in the anoxic reactor can exert an inhibitory effect on the denitrification rate [13]. This phenomenon can finally trigger the production of nitrogen oxides due to incomplete denitrification.

Three main routes for biological production of N_2O have been proposed: hydroxylamine oxidation and nitrifier denitrification, both processes by AOB, and heterotrophic denitrification by heterotrophic denitrifiers [14]. N_2O emissions from heterotrophic denitrification can occur under microaerophilic conditions, because oxygen could inhibit the activity of nitrous oxide reductase [15]. At low DOC, N removal takes place via partial nitrification, and formed nitrite is denitrified to N_2/N_2O by AOB [16].

Simultaneous nitrification and denitrification (SND) are an alternative process to the conventional configurations previously described. The SND process is carried out in a single reactor where partial nitrification, from ammonia to nitrite, coupled to denitrification, takes place. SND process is based on gradients of dissolved oxygen (DO) within the flocs. The nitrifying autotrophic bacteria are distributed on the periphery of the floc, where the dissolved oxygen concentration (DOC) is above 2 mg O_2/L, while the denitrifying bacteria are located inside the floc, where the concentration of oxygen is very low [17, 18]. Large flocs (>125 μm) allow generating an oxygen gradient with anoxic conditions in the center of the floc [19, 20]. SND can be accomplished at low DOC [21]. However, at concentrations of about 0.4 mg O_2/L, N_2O instead of N_2 may be the final product of denitrification [22]. In addition, nitrite accumulation above 1 mg/L triggers the production of N_2O, and at higher nitrite levels, the denitrification process could be inhibited [21].

Another alternative process to the conventional nitrification-denitrification is based on shortcut nitrification (nitritation) followed by denitritation. In this process, AOBs oxidize NH_4^+ to NO_2^-, and then, the formed NO_2^- is denitrified [23]. Nitrogen elimination via nitrite requires high ammonia concentration and low DOC (<0.4 mg O_2/L) in order to prevent NOB growth [24]. In this process, oxygen consumption (aerobic phase) and organic carbon demand (anoxic stage) are reduced 25 and 40%, respectively, in comparison to the conventional nitrification-denitrification [25]. However, NO_2^- accumulated after nitritation is considered a key factor that triggers the N_2O generation by means of the nitrifier denitrification in a low DO environment [26]. Partial nitritation/anammox was proposed 20 years ago as key strategy for achieving a more sustainable treatment of municipal wastewater. Partial nitritation/anammox is an autotrophic nitrogen removal process based on two successive processes: partial oxidation of ammonium to nitrite by AOBs followed by oxidation of the residual ammonium with the formed nitrite to nitrogen gas [27]. The last process named anammox is carried out by a group of *Planctomycete* bacteria, which grow with CO_2 as the sole carbon source and use nitrite as the electron donor [3]. Partial nitrification, which occurs usually at low DO conditions (involving lower energy demands), can lead to NO_2^- accumulation. Nitrifier denitrification, in the presence of NO_2^- and low DO, has been considered the most likely pathway of production of N_2O in both nitritation reactor and anammox reactor [23].

Advanced N-removal processes such as partial nitrification-denitrification (shortcut nitrification, nitritation, followed by denitritation), SND, or partial nitritation-anammox are applied with the view to reducing the energy demands. However, N_2O emissions still occur and can even be higher than the ones observed during conventional nitrification-denitrification [23].

Aerobic denitrification is an alternative process to conventional anoxic denitrification, which can achieve complete denitrification at high oxygen concentrations. This process constitutes a good strategy to diminish N_2O emissions [28]. A total of 37 species (14 genera) has been reported as potential aerobic denitrifiers, which belong mainly to α, β, and γ *Proteobacteria* [29]. *Citrobacter diversus* [30], *Alcaligenes faecalis* [31], *Pseudomonas aeruginosa* [32], *Microvirgula*

aerodenitrificans [33], *Paracoccus denitrificans* [32], and *Bacillus licheniformis* [34], among others, have been reported to be able to carry out aerobic denitrification. Ji et al. [29] have proposed that nitrate and oxygen co-respiration is a microbial adaption that allows the degradation of toxic nitrate in an aerobic environment. Aerobic denitrification can be an auxiliary pathway next to aerobic respiration [35]. It has been suggested that the enzymatic system for aerobic and anaerobic denitrification is probably the same. Anaerobic denitrification is negatively affected by aerobic conditions, being widely accepted that nitrous oxide reductase is inhibited by oxygen. However, N_2 generation as final product under high oxygen concentrations suggests the probable existence of different nitrous oxide reductases, which are insensitive to oxygen [35]. Denitrification via nitric oxide dismutation has been also proposed. In this process, denitrification of nitrate and nitrite to nitric oxide is followed by dismutation of nitric oxide into oxygen and N_2, which did not require nitrous oxide reductase. However, it still needs to be investigated if nitric oxide dismutation is a common and widespread process between bacteria [35].

The organic carbon required for denitrification has been considered the critical element in conventional nitrogen removal processes [36]. Therefore, it is crucial to achieve a nitrogen removal process using completely the organic carbon from wastewaters. Intracellular carbon such as PHA (polyhydroxyalkanoates) and/or glycogen is commonly stored in wastewater treatment systems. These carbon reserves could drive denitrification. Anaerobic/oxic (ANA/OX) configuration can enrich two kinds of organisms: polyphosphate-accumulating organisms (PAOs) and glycogen-accumulating organisms (GAOs) [37]. PAOs and GAOs are able to store PHA and glycogen. Denitrifying PAOs and denitrifying GAOs are able to denitrify using PHA and/or glycogen as carbon source.

The sequential batch reactor (SBR) is one of the main technologies for the biological treatment of wastewaters, being successfully used in urban wastewater [38, 39], as in industrial wastewaters [40, 41]. A SBR with anaerobic/oxic/anoxic configuration (ANA/OX/AN SBR) has been used for the removal of carbon and nitrogen. Efficient nitrogen removal via nitrification followed by post-denitrification, without the addition of external organic carbon, was reported. For this, PHA and glycogen stored during the anaerobic phase were later used as electron donors during post-anoxic denitrification. Denitrification was attributed to denitrifying glycogen-accumulating organisms [36].

In this chapter, a nitrogen removal process based on nitrification-aerobic denitrification was proposed. An anoxic/oxic (AN/OX) SBR with DOC higher than 1.5 mg O_2/L during the aerobic period was utilized. In this system, two requirements must be met: (a) growth of denitrifying bacteria able to store internally sufficient carbon reserves (PHA and/or glycogen) in the anoxic phase and (b) ability of the denitrifying bacteria to denitrify during the aerobic phase by using the intracellular carbon reserves. The AN/OX SBR would avoid both mixed liquor recirculation and exogenous carbon addition, and additionally potential emissions of N_2O could be minimized. Thus, the proposed system offers important advantages with respect to both conventional nitrification-denitrification and advanced N-removal processes.

2. Activated sludge reactor

A lab-scale SBR (1.2 L working volume) was operated for 10 months. The SBR was inoculated with sludge from a lab-scale activated sludge plant in Center of Research and Development in Food Cryotechnology (CIDCA,

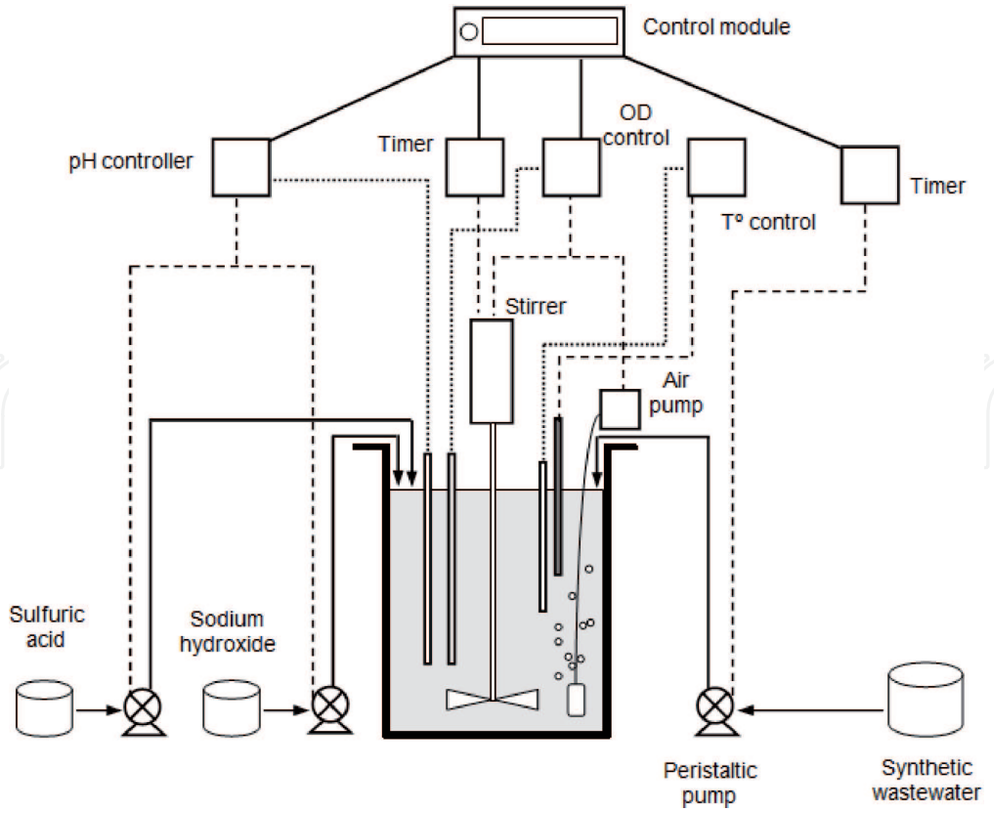

Figure 1.
Scheme of the lab-scale sequencing batch reactor (from Alzate Marin et al. [42]).

UNLP-CONICET-CIC, Argentina). The SBR was operated with cycles comprising the following phases: reaction (with anoxic and aerobic stages), biomass settling, and supernatant draw. The reactor was completely mixed at a stirring rate of 100 rpm, except during the settle and draw periods. The reactor was automatically controlled by a data acquisition and control system (DACS) developed in the electronic laboratory of CIDCA; pH was measured by a pH probe (Phoenix, Houston, TX, USA). Air was introduced 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 **Figure 1**.

3. Volumetric oxygen transfer coefficient

Oxygen is known to increase the oxidative state of biological systems, which could negatively affect anaerobic and anoxic processes. Microaerophilic conditions can be caused by stirring. The volumetric oxygen transfer coefficient (k_La, h^{-1}) is an important parameter in the aerobic wastewater treatment, particularly when anaerobic or anoxic conditions are required. In the present study, k_La was determined in order to evaluate the oxygen amount supplied to the reactor by agitation during the anoxic phase. k_La was measured by the clean water non-steady-state method [43] at 20°C, agitation rate of 100 rpm, and different aeration rates (vvm = 12–137 L/(L h)). Firstly, the SBR (1.2 L) was continuously aerated until the saturation concentration of oxygen (DOC*, mg O_2/L) in water was reached. Then, DO is completely removed by the addition of sodium sulfite. Finally, the aeration was turned on to the oxygen saturation level. DOC was measured at several points during the aeration period. k_La in the reactor was measured by integration of the following equation:

$$\frac{dDOC}{dt} = k_L a \ (DOC^* - DOC) \quad (1)$$

where DOC* is the saturation concentration of oxygen in water (mg O_2/L) at the working temperature and DOC is the dissolved oxygen concentration (mg O_2/L) at time (t). The driving force of the oxygen transfer process is given for the difference between DOC* and DOC.

A linear relationship between $k_L a$ and the aeration rate has been proposed by the following expression:

$$k_L a = m\,AER + n \quad (2)$$

where AER is the aeration rate (L/(L h)), m is the slope (L/L), and n (h^{-1}) corresponds to the $k_L a$ produced by stirring without aeration (AER = 0). The parameters m and n were determined through linear regression analysis (Sigma Plot 10.0) resulting in 0.10 L/L and 2.34 h^{-1}, respectively.

For clean water, at working conditions of the SBR, 25°C, stirring rate of 100 rpm, and without aeration, a $k_L a$ value of 2.63 h^{-1} was estimated by using the following expression [43]:

$$k_L a_{(25^oC)} = k_L a_{(20^oC)}\ 1.024^{(25-20)} \quad (3)$$

Based on this estimation, it was assumed that only stirring will cause oxygen penetration through liquid surface during the anoxic stage of the SBR operation.

4. Synthetic wastewater and operating conditions

Synthetic wastewater (SWW) contained sodium acetate (carbon and energy source), ammonium sulfate (nitrogen source), and potassium phosphate (phosphorus source). A micronutrient solution (1 ml) was added to the SWW (1 L) [44]; influent COD/N/P ratio was 100:10:5. SWW 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, leading to a cellular residence time (CRT) of 10 days. Treated wastewater was removed from the SBR after settling period. A volumetric exchange ratio of about 27% was set. The effects of different operating parameters, such as DOC, organic load, cycle duration, and AN/OX ratio on the ability of nitrification and denitrification were studied.

5. Analytical methods

The SBR was monitored by determination of the following physical–chemical parameters: oxidation-reduction potential (ORP, mV), orthophosphate (PO_4^{3-}-P, mg/L), ammonia nitrogen (NH_3-N, mg/L), nitrate nitrogen (NO_3^--N, mg/L), nitrite nitrogen (NO_2^--N, mg/L), soluble COD (COD_S, mg/L), and total COD (COD_T, mg/L). The oxidation-reduction potential is a measure of the oxidative state in an aqueous system. ORP reflects the concentration of DO, organic substrate, activity of organisms, and some toxic compounds in the system, the DOC being the most important factor [45]. The ORP of the SBR was measured off-line using an ORP probe (Phoenix, Houston, TX, USA). The other physical-chemical parameters were determined by spectrophotometric methods using commercial reagents (Hach Company, Loveland, CO). COD_S corresponded to the organic substrate. Biomass concentration was determined as COD (COD_B, mg/L) from the difference between COD_T and COD_S. COD_B was correlated with volatile suspended solids (VSS, mg/L).

Intracellular poly-P and PHA granules were detected by Neisser and Sudan Black staining, respectively [46]. Total carbohydrate (TC) content was determined following a modified version of the anthrone method proposed by Jenkins et al. [47].

6. Inorganic nitrogen removal

Inorganic nitrogen (Ni) corresponded to the sum of ammonia, nitrite, and nitrate concentrations. The inorganic nitrogen removal (NiR) was measured throughout the operational cycle as follows:

$$\% \text{ NiR} = \left(1 - \frac{Ni_t}{Ni_O}\right) 100 \quad (4)$$

where Ni_O is the Ni concentration at the start of the anoxic phase (mg/L) given by the NH_3-N from the wastewater and Ni_T corresponds to the Ni concentration (mg/L) at time t of the SBR operational cycle. Residual nitrate and nitrite (from of the previous cycle) were not considered in the determination of Ni_O.

7. Simultaneous nitrification-denitrification (SND) followed by denitrification (DN)

Simultaneous nitrification and denitrification (SND) took place from the beginning of the aerobic phase until the moment when the ammonium was exhausted. Later, subsequent nitrogen removal occurred by denitrification (DN).

Nitrogen removed via SND was determined in the aerobic phase 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). For SND determination, NH_3-$N_{oxidized}$ was calculated from the difference between the total NH_3-N consumption and NH_3-N assimilated into heterotrophic biomass (NH_3-$N_{assimilated}$). Nitrogen assimilated by nitrifying bacteria was assumed to be negligible [48]. The total consumption of NH_3-N was determined by spectrophotometry. NH_3-N assimilated into heterotrophic biomass was estimated for the aerobic period in the presence of ammonium. For this, theoretical mass balances of carbon and nitrogen were carried out using typical values for stoichiometric coefficients of the studied biological process. In SBR with feast-famine regime, PHB (polyhydroxybutyrate) is synthetized from acetate under anaerobic or anoxic phase, and then biomass is produced during the aerobic phase from stored PHB. In our system, PHB production was estimated using a yield $Y_{PHB/Acetate}$ of 0.52 C-mol PHB/C-mol Ac for anoxic condition [49]. Available acetate for PHB synthesis was estimated from difference between initial COD and COD required for anoxic denitrification using a stoichiometric coefficient of 3.8 mg COD_{Ac}/mg NO_3^--N. Biomass production from PHB was estimated assuming a heterotrophic biomass yield $Y_{X/PHB}$ of 0.5 C-mol X/C-mol PHB. Finally, NH_3-$N_{assimilated}$ by heterotrophs was determined assuming a biomass molecular formula of $CH_{1.8}O_{0.5}N_{0.2}$, which is equivalent to 24.6 g VSS/C-mol X [48].

SND was calculated from the following equation [48]:

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

where NO_x^--N is the sum of the oxidized nitrogen species (nitrite and nitrate) at the moment when ammonia was exhausted and NH_3-$N_{oxidized}$ corresponds to the ammonia nitrogen oxidized during the aerobic period.

Nitrogen removed via denitrification (DN) was calculated from the difference between oxidized nitrogen at the end of nitrification (NO_x^--N_{FN}) and oxidized nitrogen at the end of the aerobic phase (NO_x^--N_{FA}) as follows:

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

8. Experimental design

Experiments were carried out at low and high dissolved oxygen concentrations (oxygen saturation levels, OSL, of 20 and 60%, respectively) using in each case low and high organic loads (440 and 880 mg COD/L day). The following notation was used to describe and report the results of the experiments: low oxygen concentration and low organic load (LOLC), low oxygen concentration and high organic load (LOHC), high oxygen concentration and low organic load (HOLC), and high oxygen concentration and high organic load (HOHC).

9. Experiments at low dissolved oxygen concentrations

In these experiments, the effect of organic load on the nitrification process was evaluated at low DOC. An oxygen saturation level (OSL) of 20%, equivalent to a DOC of 1.6 mg O_2/L, was set for the aerobic phase (**Table 1**). Experiments were carried out at two different organic volumetric loads. In experiment low oxygen concentration and low organic load (LOLC), 440 mg COD/(L day) was used, and in experiment low oxygen concentration and high organic load (LOHC), the value was 880 mg COD/(L day).

In the experiments LOLC, the SBR showed at steady state a good performance with a biomass concentration of 1220 ± 215 mg COD_B/L. For organic carbon, a removal higher than 99% was reached in anoxic phase. Ammoniacal nitrogen removal was about 99%, mainly in the aerobic phase (**Figure 2**). In this phase, about 70% of the ammonium was nitrified up to nitrate as was determined by mass balance. According to these results, a redox potential of about +295 mV was measured during the aerobic phase, which involves a suitable oxidizing environment for autotrophic nitrification. It must be considered that ORP values between +100 and +350 mV are necessary for the nitrification process to take place [50]. A relatively low concentration of oxygen (<2.0 mg O_2/L) was enough to achieve a good nitrifying activity without accumulation of nitrite. Volumetric and specific nitrification rates are shown in **Table 2**.

PHA accumulation followed by degradation of the polymer took place in the anoxic and aerobic phases, respectively, as was detected by Sudan Black staining. Cocci-shaped cells arranged in tetrads (tetrad-forming organisms, TFOs) displayed that metabolic ability (**Figure 3a** and **b**). Some subgroups of *Alphaproteobacteria* and *Gammaproteobacteria* exhibit TFO morphology with GAO phenotype. These microorganisms are commonly associated with enhanced biological phosphorus removal (EBPR) deterioration [51]. In the present study, TFOs corresponded likely to some group of GAO commonly found in systems without EBPR.

PHA could be used as intracellular carbon source for denitrification. However, poor denitrification took place since at the end of the operational cycle, the final effluent exhibited a nitrate concentration of 4.75 ± 0.25 mg NO_3^--N/L, equivalent to about 70–80% of the nitrified ammoniacal nitrogen. According to these results,

Parameters	Experiment LOLC	Experiment LOHC
Anoxic phase (min)	150	150
Aerobic phase (min)	150	150
Settling phase (min)	50	50
Draw phase (min)	10	10
Total cycle length (h)	6	6
Anoxic/aerobic ratio	1.0:1.0	1.0:1.0
Temperature (°C)	25 ± 0.5	25 ± 0.5
pH (anoxic and aerobic phases)	7.0 ± 0.1	7.0 ± 0.1
Oxygen saturation level (%)	20	20
Organic volumetric load (mg COD/(L day))	440	880
Nitrogen volumetric load (mg NH_3-N/(L day))	44	88
Phosphorus volumetric load (mg P/(L day))	22	44

Adapted from Alzate Marin et al. [42].

Table 1.
Operating conditions for experiments at low oxygen concentration.

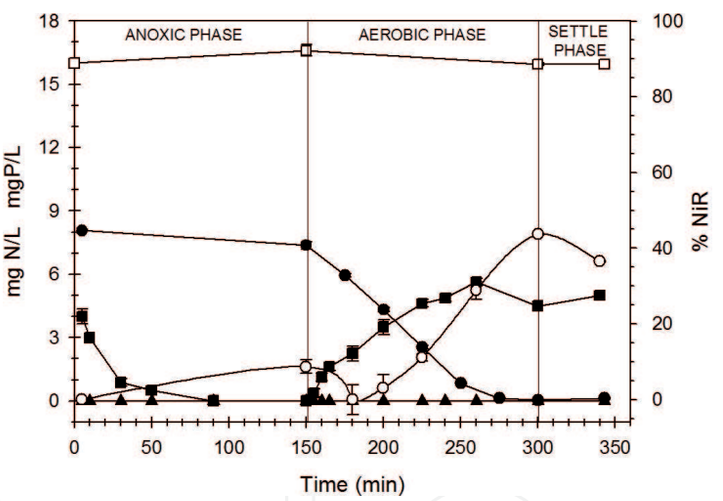

Figure 2.
Changes of phosphorus and nitrogen concentrations during operational cycles of the steady-state SBR. Experiment with low oxygen concentration and low organic load (LOLC). (□) Orthophosphate (PO_4^{3-}-P, mg P/L), (●) ammonia (NH_3-N, mg N/L), (■) nitrate (NO_3^--N, mg N/L), (▲) nitrite (NO_2^--N, mg N/L), and (○) % inorganic nitrogen removal (% NiR).

nitrogen removal through the SND and DN processes represented 11 ± 10% and 5 ± 5%, respectively. The final effluent exhibited an inorganic nitrogen concentration of 4.84 ± 0.40 mg N/L, which resulted in a mean discharge of 5.80 mg N/day. These results involved an inorganic nitrogen removal of 45 ± 2% (**Table 2**). This poor nitrogen removal was associated with the low denitrification ability of the system. It must be considered that the residual nitrate, after the discharge of the final effluent, was completely removed by denitrification in the first 90 min of the following cycle (**Figure 2**).

Poly-P staining by Neisser method resulted negative (**Figure 3c**), and soluble phosphorus (orthophosphate) concentration did not show important changes (**Figure 2**). These results involve that PAO activity and hence the EBPR process did not take place. According to these findings, positive ORP values (+286 ± 8 mV) were

Parameters	Experiment LOLC	Experiment HOLC	Experiment HOHC
VNR (mg NH_3-N/(L h))	3.96 ± 0.10	3.71 ± 0.45	4.09 ± 0.08
SNR (mg NH_3-N/(g VSS h))	4.22 ± 0.10	4.14 ± 0.48	1.33 ± 0.00
VDNR (mg NO_3^--N/(L h))	ND	2.53 ± 0.96	2.57 ± 0.36
SDNR (mg NO_3^--N/(g VSS h))	ND	2.94 ± 1.10	0.83 ± 0.10
% NAS	—	10.0 ± 1.0	28.7 ± 0.5
% SND	11 ± 10	0 ± 0	9 ± 2
% DN	5 ± 5	55 ± 3	57 ± 2
% AR	99 ± 1	99 ± 1	99 ± 1
% NiR	45 ± 2	67 ± 2	78 ± 1

Adapted from Alzate Marin et al. [42].
ND, not determined.

Table 2.
Biological parameters of the SBR for the different experiments.

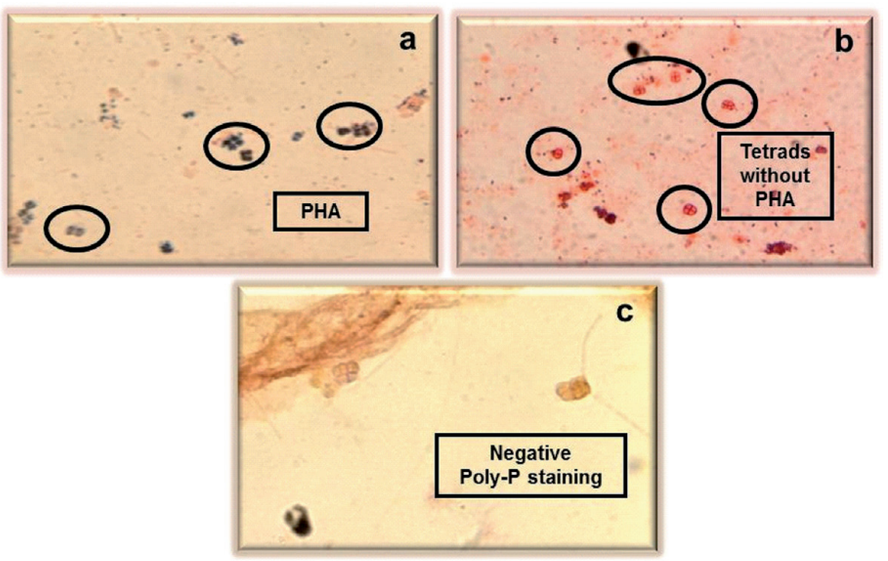

Figure 3.
Micrographs of activated sludge stained with Sudan black (a and b) and Neisser (c). (a) Tetrad-forming organisms (TFOs) showing positive PHA staining (final anoxic phase), (b) TFOs with negative PHA staining (final aerobic phase), and (c) negative Neisser staining.

recorded throughout the anoxic phase, which are not suitable for anaerobic PHA metabolism. It is well known that negative ORP values between −50 and − 200 mV are usually required for anaerobic polyphosphate breakdown [52]. In the anoxic phase, zero DOC was registered, and a k_La value of 2.63 h^{-1} was estimated by using Eq. (3). For these conditions, an oxygen transfer rate of 21.3 mg O_2/(L h) was estimated at 25°C by using Eq. (1). The oxygen transfer by stirring increased the oxidative state (positive ORP) during the anoxic phase. It can be assumed that this phenomenon would lead to unfavorable ecological conditions for anaerobic metabolism of PAOs.

In the experiments with low oxygen concentration and high organic load (LOHC), the organic volumetric load was increased from 440 to 880 mg COD/(L day) under identical operational conditions to those of the experiment LOLC (**Figure 4**). The nitrogen and phosphorus volumetric load were 88 mg

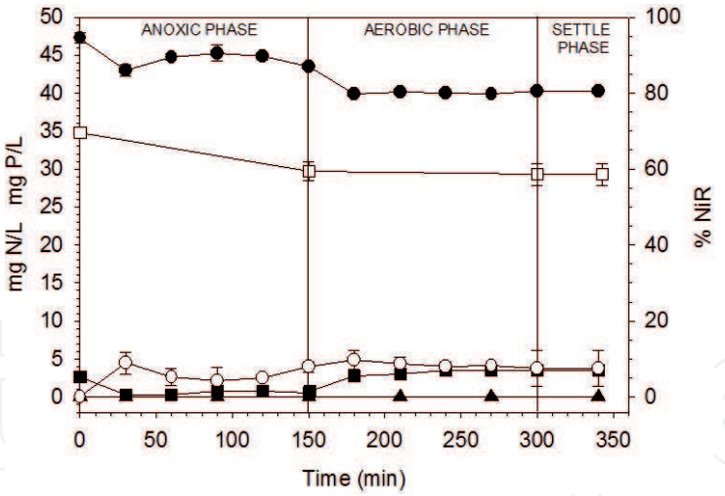

Figure 4.
Changes of phosphorus and nitrogen concentrations during operational cycles of the steady-state SBR. Experiment with low oxygen concentration and high organic load (LOHC). (□) Orthophosphate (PO_4^{3-}-P, mg P/L), (●) ammonia (NH_3-N, mg N/L), (■) nitrate (NO_3^--N, mg N/L), (▲) nitrite (NO_2^--N, mg N/L), and (○) % inorganic nitrogen removal (% NiR) (adapted from Alzate Marin et al. [42]).

NH3-N/(L day) and 44 mg P/(L day), respectively, in order to maintain the same COD/N/P ratio (100:10:5) (**Table 1**). The steady-state SBR reached a biomass concentration of 1850 ± 120 mg COD_B/L. Ammoniacal nitrogen was removed only 15% throughout the operational cycle. Poor nitrification was observed as only 7% of ammonia from anoxic phase was nitrified, even though adequate oxidizing conditions were registered during the aerobic phase (ORP > +100 mV). Low nitrate concentrations were generated, and hence the denitrification process did not take place; nitrite was not accumulated. The final effluent showed a high inorganic nitrogen concentration (43.5 ± 0.20 mg N/L), resulting in a mean discharge of 57.42 mg N/day. Thus, a poor Ni removal of only 8% was achieved (**Figure 4**). It is important to highlight that even though the influent nitrogen load was only two times higher to that of the experiment LOLC, the daily nitrogen discharge was about ten times greater than that corresponding to the previous assay. EBPR activity was not observed; as was previously discussed for experiment LOLC, oxidizing conditions during the anoxic phase (positive ORP) were unfavorable for PAO growth.

In the tested system, a COD/N/P ratio of 100:10:5 was utilized in experiments LOLC and LOHC in order to ensure excess conditions of nitrogen and phosphorus. Nevertheless, a relatively low DO concentration was used, which can lead to competition between heterotrophic and nitrifying bacteria. In the experiment LOHC, the higher organic load led to a greater intracellular PHA production, in anoxic phase, in comparison to LOLC. Thus, a higher growth of heterotrophic bacteria from PHA took place in the aerobic phase, which would involve a greater oxygen uptake rate by heterotrophs. This observation was reported by Third et al. [48] working with an aerobic SBR fed with acetate. Nitrifying bacteria, with very low growth rate, were likely outcompeted by heterotroph overgrowth under low oxygen availability. This phenomenon could explain the poor nitrifying activity in experiment LOHC. In conclusion, the organic load stimulated strongly the competition by oxygen between heterotrophic and nitrifying bacteria at low DO concentrations.

10. Experiments at high dissolved oxygen concentration

In these assays, at high dissolved oxygen concentration, a value of OSL (60%), equivalent to a DOC of 4.8 mg O_2/L, was set for the aerobic phase (**Table 3**). As in

Parameters	Experiment HOLC	Experiment HOHC
Anoxic phase (min)	220	220
Aerobic phase (min)	440	440
Settling phase (min)	51	51
Draw phase (min)	9	9
Total cycle length (h)	12	12
Anoxic/aerobic ratio	0.5:1.0	0.5:1.0
Temperature (°C)	25 ± 0.5	25 ± 0.5
pH (anoxic and aerobic phases)	7.5 ± 0.1	7.5 ± 0.1
Oxygen saturation level (%)	60	60
Organic volumetric load (mg COD/(L day))	440	880
Nitrogen volumetric load (mg NH_3-N/(L day))	44	44
Phosphorous volumetric load (mg P/(L day))	22	44

Table 3.
Operational conditions for experiments at high dissolved oxygen concentration with different organic loads.

the previous experiments, two organic volumetric loads were evaluated: 440 and 880 (mg COD/(L day)) (**Table 3**). The effects of cycle duration, anoxic/aerobic ratio, and organic load on the denitrification process were evaluated. The purpose of these experiments was to determine optimal experimental conditions to attain a good denitrifying activity and hence an acceptable process of nitrogen removal. Therefore, in addition to achieving efficient nitrification, sufficient organic carbon must be supplied for the denitrification process to take place. High oxygen availability permitted to minimize competition by oxygen between heterotrophic and nitrifying bacteria. In these experiments, the extension of the operating cycle was increased from 6 h to 12 h, and the anoxic/aerobic ratio was decreased from 1.0:1.0 to 0.5:1.0. These conditions were set in order to provide a longer aerobic period to favor the denitrification process.

In the experiment HOLC, the volumetric loads of organic carbon, nitrogen, and phosphorus were the same as those used in the experiment LOLC. All the operating conditions are shown in **Table 3**.

The COD/N/P ratio (100:10:5) and oxygen saturation level (60%) used in this assay would minimize competition between heterotrophs and nitrifiers. Oxidizing conditions were registered in the anoxic phase (ORP = +187 ± 13), being unfavorable for the EBPR process to occur. Ammonium was almost completely removed (99%). About 80% was eliminated in the aerobic phase. Nitrification produced nitrate concentrations of about 10–12 mg NO_3^--N/L in the first 2 h of the aerobic period. ORP values higher than +190 mV favored the nitrifying activity. Then, the nitrate concentration gradually decreased, which was attributed to the activity of denitrifying bacteria (**Figure 5**). The mean discharge of nitrate was 3.2 mg N/day. This concentration was about 32% lower than the one obtained in experiment LOLC for a same nitrogen volumetric load.

Residual nitrate was denitrified at the beginning of the following cycle (anoxic phase). Nitrite was not accumulated in the SBR, as was also observed in the previous experiments. The mean discharge of inorganic nitrogen was 3.2 mg N/day (corresponding totally to nitrate), being about 45% lower than the results obtained in experiment LOLC. According to the nitrogen mass balance, about 85% of the incoming ammonia in aerobic period was nitrified; nitrogen assimilation by heterotrophic bacteria corresponded to 15%. Nitrogen assimilated by heterotrophs represented

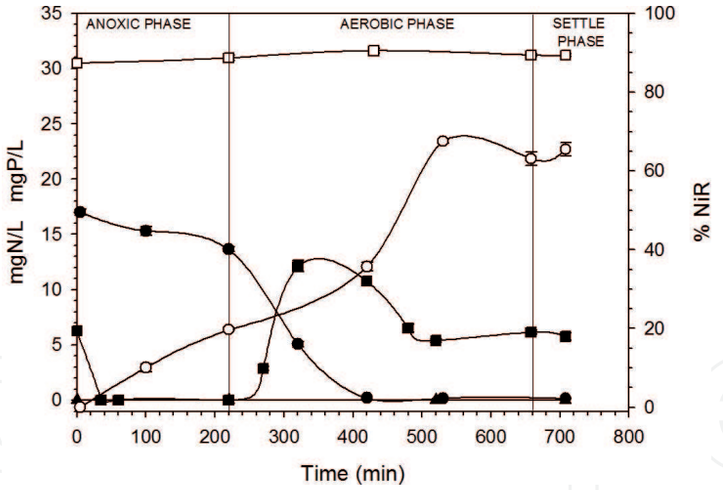

Figure 5.
Changes of the phosphorus and nitrogen concentrations during an operational cycle of the steady-state SBR (experiment HOLC). (□) orthophosphate (PO_4^{3-}-P, mg P/L), (●) ammonia (NH_3-N, mg N/L), (■) nitrate (NO_3^--N, mg N/L), (▲) nitrite (NO_2^--N, mg N/L), and (○) % inorganic nitrogen removal (% NiR) (adapted from Alzate Marin et al. [42]).

10% of the total ammonia load applied to the SBR. Volumetric and specific nitrification rates were not significantly different to those determined in the experiment LOLC. SND did not take place; denitrification began once the nitrification process was completed; 55 ± 3% of the generated nitrate was removed (**Table 2**).

Nitrification followed by denitrification was the most important process for nitrogen removal. The elimination of Ni was about 50% higher than that achieved in experiment LOLC (**Table 2**). The greater efficiency for nitrogen removal was attributed to a higher denitrifying activity in the experiment HOLC. In addition, the improved denitrification process of this assay can be attributed to a greater extension of the aerobic phase. However, the denitrification was probably limited by a low availability of intracellular organic carbon during the aerobic phase. In the experiment HOHC, the organic volumetric load was increased from 440 to 880 mg COD/(L day), while the ammoniacal nitrogen load was the same as that corresponding to the HOLC (44 mg NH_3-N/(L day)). This led to an increase in the COD/N ratio from 100:10 to 100:5. The volumetric load of phosphorus was 29 mg P/(L day). The other operating conditions were identical to those used in the experiment HOLC (**Table 3**).

Organic substrate was completely removed in anoxic phase. Ammonium was almost depleted during the process; about 80–85% was eliminated in the aerobic phase (**Figure 6**). Nitrogen assimilated by heterotrophs represented almost 30% of the incoming ammonia to the SBR (**Table 2**). Oxidizing conditions were similar to those corresponding to previous assays, with positive ORP values. The specific nitrification rate was significantly lower than that corresponding to the assay HOLC (**Table 2**). This result was attributed to the enrichment of the biomass in heterotrophic bacteria because of the higher organic load applied in experiment HOHC. Biomass concentration was twice the value reached in the HOLC assay.

The SND process showed little improvement. The denitrification was similar to that obtained in experiment HOLC, and the specific denitrification rate was significantly lower than that observed in the previous experiment. The mean discharge of inorganic nitrogen was 2.2 mg N/day. The inorganic nitrogen removal was 78 ± 1%, being significantly higher than that observed in the previous assay (**Table 2**). In the experiments HOHC, the higher organic load generated a greater PHA production, as was estimated by material balance, in comparison with HOLC assay. Thus, a higher content of endogenous carbon and energy reserve for the denitrification

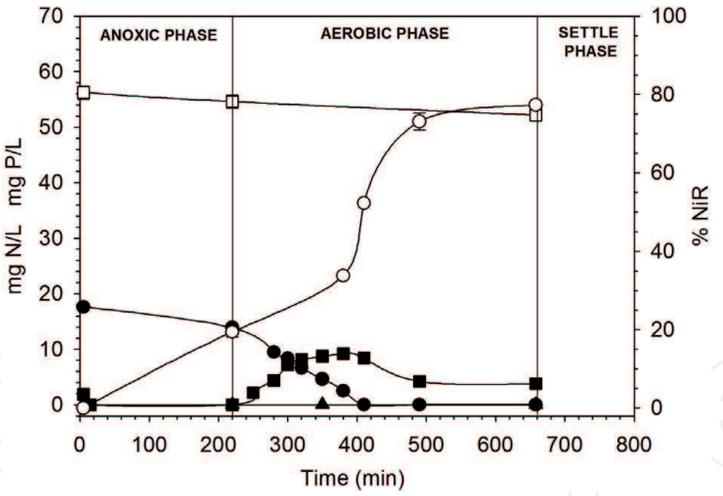

Figure 6.
Changes of the phosphorus and nitrogen concentrations during an operational cycle of the steady-state SBR (experiment HOHC). (□) orthophosphate (PO_4^{3-}-P, mg P/L), (●) ammonia (NH_3-N, mg N/L), (■) nitrate (NO_3^--N, mg N/L), (▲) nitrite (NO_2^--N, mg N/L), and (○) % inorganic nitrogen removal (% NiR).

process was available. However, the higher efficiency of inorganic nitrogen removal attained in experiment HOHC was attributed mainly to a greater assimilation of nitrogen by heterotrophic bacteria, which was about three times larger than that observed at low organic load (**Table 2**).

As was mentioned, the highest inorganic nitrogen removal was attained in the experiments HOHC; however, the specific denitrification rate was significantly lower than that corresponding to the assay HOLC. It must be considered that a high organic load led to an excessive growth of heterotrophs, which probably involved an intense competition by different growth factors among heterotrophic bacteria. Under these conditions, it can be inferred that denitrifying bacteria would preferably use oxygen as the final acceptor of electrons instead of nitrate, which represents a competitive advantage in terms of energy efficiency. This would explain the low specific denitrification rate obtained in the HOHC experiment.

11. Endogenous carbon sources as affecting microbial consortia in denitrification process

In all the experiments, the denitrification process at aerobic phase took place without external organic carbon. Denitrification occurred from intracellular carbon and energy reserves; the specific denitrification rates obtained were higher than those corresponding to endogenous decay (0.2–0.6 mg NO_3^--N/(g VSS h) [53]). Under steady-state conditions, the total carbohydrate (TC) concentration of the biomass was determined by the anthrone method throughout the operational cycle of the reactor. TC increased slightly during the anoxic phase and initial period of the aerobic phase, and then it decreased slightly at the end of the aerobic phase. These TC changes could not be attributed to cyclic changes of intracellular glycogen, which are typical of reactors with anaerobic/aerobic regime. In these systems, the microbial community is commonly enriched with GAOs and/or PAOs, which are responsible for the degradation and synthesis of glycogen during the anaerobic and aerobic stages, respectively. In the case of GAOs, glycogen constitutes the primary source of energy for both uptake of exogenous organic carbon and PHA storage during the initial anaerobic stage [51, 54]. Then, glycogen is replenished aerobically from PHA. In the anoxic/oxic SBR of the present study, GAOs as tetrad-arranged cocci and positive PHA staining were microscopically detected. However, typical

GAO metabolism regarding glycogen cycling was not observed. TC increase was mainly attributed to microbial growth instead of glycogen accumulation, even though a light glycogen increase during the anoxic phase of the operational cycle cannot be discarded. Slight decay of TC at final aerobic phase could be attributed to the glycogen component. Anyway, GAO was not a representative microbial phenotype in the anoxic-oxic SBR. This result could be explained considering that oxidative conditions were prevalent in the anoxic period generated by the high oxygen transfer during the agitation.

Based on this analysis, it can be argued that the denitrification achieved in the SBR took place from the intracellular reserves of PHA during the aerobic phase. Denitrification process could also be driven from intracellular glycogen but to a lesser extent. PAOs and GAOs are able to denitrify using intracellular carbon source. In the present study, PAO activity was not observed. The absence of EBPR activity was associated to high oxidative conditions not favorable to PAOs during anoxic phase more than to the GAO-PAO competition. GAOs with tetrad-type morphology were probably responsible of the denitrification process; however, the denitrifying activity of other microbial groups should not be discarded.

The specific denitrification rates obtained in the present study were similar (experiment HOHC) or higher (experiment HOLC) than those reported in literature for anoxic denitrification carried out by PAOs; intracellular glycogen was the carbon source used for anoxic denitrification [9, 55]. Vocks et al. [56] reported a similar SDNR to that obtained in the experiment HOLC, using a membrane bioreactor (ANA/OX/AN); denitrifying GAOs were considered as responsible for the denitrification using stored glycogen as internal carbon source [56]. Li et al. [36] reported SDNRs of 0.5 and 1.24 mg NO_3^--N/(g VSS h) using glycogen and PHA, respectively, at anoxic conditions. These SDNRs were similar to that obtained in the experiment HOHC and 2–6 times lower than that corresponding to experiment HOLC.

Anoxic denitrification rates are commonly higher than those obtained under aerobic conditions [57]. In contrast, the specific denitrification rates (SDNR) obtained in the present study, at bulk DO concentration higher than 4.0 mg O_2/L, were similar or higher to those reported for anoxic conditions.

12. Conclusions

A lab-scale sequencing batch reactor (SBR) operated with two phases, anoxic and aerobic, achieved complete COD removal. At low DO concentration, the nitrification process depended on the organic load. Low DO concentration and relatively high organic load (LOHC) led to significant growth of heterotrophic bacteria and poor nitrification. At low DO concentration and low organic load (LOLC), a good nitrifying activity led to an inorganic nitrogen removal of about 45%. It is known that in activated sludge systems, competition by growth factors (macro- and micronutrients and DO) between heterotrophic and nitrifying bacteria can occur. In both experiments, LOLC and LOHC, a COD/N/P ratio of 100:10:5 assured excess conditions of nitrogen and phosphorus. Nevertheless, competition by oxygen between both groups of microorganisms took place at high organic load.

With reference to the experiments carried out at high oxygen concentration (HOLC and HOHC), a high DOC minimized competition by oxygen between heterotrophs and nitrifiers. Higher inorganic nitrogen removal (67–78%) was achieved at the following conditions: pH = 7.5, higher dissolved oxygen concentration, and prolonged aerobic phase. Nitrification followed by denitrification during the aerobic phase was the most important process for nitrogen removal. The

elimination of Ni was 50–70% higher than that achieved in experiment LOLC. The greater efficiency for nitrogen removal was attributed to a higher denitrifying activity, due to a greater extension of the aerobic phase. From the results obtained using high dissolved oxygen concentrations (HOLC and HOHC), it can be concluded that there was no shortage of intracellular carbon and energy reserve. Thus, organic carbon was not the limiting substrate for the denitrification process under aerobic conditions. Denitrification took place mainly from the intracellular reserves of PHA during the aerobic phase. Aerobic denitrification could be attributed to glycogen-accumulating organism (GAOs) with tetrad-type morphology; activity of polyphosphate-accumulating organisms (PAOs) was not observed. Other microbial groups have probably contributed to the denitrifying activity. The nitrification followed by denitrification, under aerobic conditions, analyzed in the present chapter, is an alternative process to the conventional configurations. The specific denitrification rates, at bulk DO concentration higher than 4.0 mg O_2/L, were similar or higher to those reported for anoxic conditions. It is widely accepted that in an aerobic environment, denitrifying bacteria can survive in the anaerobic/anoxic center of the microbial flocs. If not, denitrifiers could tolerate oxygen so that the denitrification process is not affected. Aerobic denitrifiers can use alternatively nitrate or oxygen as final electron acceptor. In the present study, denitrifying activity was attributed to the aerobic denitrification process.

The proposed AN/OX system constitutes a simple and potentially eco-friendly process for biological nitrogen removal, providing N_2 as the end product and decreasing the formation of N_2O, a greenhouse gas that has an important influence on atmosphere warming.

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Nomenclature

AER	aeration rate (L/(L h)
AN	anoxic
AR	ammonia removal
ANA	anaerobic
AOB	ammonia-oxidizing bacteria
COD	chemical oxygen demand
COD_B	biomass concentration as COD (mg COD_B/L)
COD_S	soluble COD (mg/L)
COD_T	total COD (mg/L)
CRT	cellular residence time (days)
DACS	data acquisition and control system
DN	denitrification
DOC	dissolved oxygen concentration (mg O_2/L)
DOC*	saturation concentration of oxygen (mg O_2/L)
GAOs	glycogen-accumulating organisms
k_La	volumetric oxygen transfer coefficient (h^{-1})
LOHC	low oxygen concentration and high organic load

LOLC	low oxygen concentration and low organic load
HOHC	high oxygen concentration and high organic load
HOLC	high oxygen concentration and low organic load
N_2	molecular nitrogen
N_2O	nitrous oxide
NAS	nitrogen assimilated by heterotrophic bacteria
NH_3	ammonia
NH_3-N	ammonia nitrogen (mg/L)
NH_3-$N_{assimilated}$	ammonia nitrogen assimilated by heterotrophs (mg/L)
NH_3-$N_{oxidized}$	oxidized ammonia nitrogen (mg/L)
Ni	inorganic nitrogen (mg/L)
Ni_O	Ni concentration at the start of the anoxic phase (mg/L)
Ni_T	Ni concentration at time t (mg/L)
NiR	inorganic nitrogen removal
NO	nitric oxide
NO_2^-	nitrite
NO_2^--N	nitrite nitrogen (mg/L)
NO_3^-	nitrate
NO_3^--N	nitrate nitrogen (mg/L)
NO_x^--N	oxidized nitrogen (mg/L)
NO_x^--N_{FA}	oxidized nitrogen at the end of the aerobic phase (mg/L)
NO_x^--N_{FN}	oxidized nitrogen at the end of nitrification (mg/L)
NOB	nitrite-oxidizing bacteria
OSL	oxygen saturation level
OX	oxic
PHA	polyhydroxyalkanoates
PAOs	polyphosphate-accumulating organisms
PO_4^{3-}-P	orthophosphate (mg/L)
SBR	sequencing batch reactor
SDNR	specific denitrification rate (mg NO_3^--N/(g VSS h)
SND	simultaneous nitrification and denitrification
SNR	specific nitrification rate (mg NH_3-N/(g VSS h)
SWW	synthetic wastewater
TFOs	tetrad-forming organisms
TC	total carbohydrates
VDNR	volumetric denitrification rate (mg NO_3^--N/(L h))
VNR	volumetric nitrification rate (mg NH_3-N/(L h))
VSS	volatile suspended solids (mg VSS/L)
$Y_{PHB/Acetate}$	yield coefficient for PHB from acetate (C-mol PHB/C-mol Ac)
$Y_{X/PHB}$	yield coefficient for heterotrophic biomass from PHB (C-mol X/C-mol PHB)

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