Nitrogen removal in a shallow maturation pond with sludge accumulated during 10 years of operation in Brazil

V. A. J. Rodrigues, E. F. A. Mac Conell, D. F. C. Dias, M. von Sperling, J. C. de Araújo and J. L. Vasel

ABSTRACT

Accumulated sludge in polishing (maturation) ponds reduces the hydraulic retention time (smaller useful volume), and this could potentially lead to a decrease in performance. However, settled biomass, present in the sediments, can contribute to nitrogen removal by different mechanisms such as nitrification and denitrification. This study investigated the influence of the bottom sludge present in a shallow maturation pond treating the effluent from an anaerobic reactor on the nitrification and denitrification processes. Nitrification and denitrification rates were determined in sediment cores by applying ammonia pulses. Environmental conditions in the medium were measured and bacteria detected and quantified by real-time polymerase chain reaction (real-time PCR). The pond showed daily cycles of mixing and stratification and most of the bacteria involved in nitrogen removal decreased in concentration from the upper to the lower part of the sludge layer. The results indicate that denitrifiers, nitrifiers and anammox bacteria coexisted in the sludge, and thus different metabolic pathways were involved in ammonium removal in the system. Therefore, the sediment contributed to nitrogen removal, even with a decrease in the hydraulic retention time in the pond due to the volume occupied by the sludge.

Key words | bacterial activity, denitrification, nitrification, polishing ponds, sediment core, stratification, upflow anaerobic sludge blanket (UASB) effluent

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INTRODUCTION

Polishing ponds are used to improve the quality of urban wastewater after anaerobic treatment, like upflow anaerobic sludge blanket (UASB) reactors, so that the final effluent quality may be compatible with legal standards or desired targets (Cavalcanti et al. 2001). Polishing ponds are designed in a similar way to maturation ponds (that usually follow facultative ponds), and exert approximately the same treatment objectives. This type of wastewater treatment configuration performs well in the removal of organic matter, pathogenic organisms and nitrogen (Dias et al. 2014).

After years of operation, the accumulation of sludge at the bottom of the ponds deserves special attention. In principle, the volume occupied by the sludge could potentially influence treatment efficiency due to the reduction of the hydraulic retention time in the liquid column. However, Keffala et al. (2011) studied a system of ponds in Belgium (Bertrix) and observed that the sediment contributed to

the removal of nitrogen through nitrification and denitrification processes. In another study (Yamamoto et al. 2009), the role of the sediment for nitrogen removal in waste stabilization ponds (WSP) was investigated. The results demonstrated that the nitrification capacity was enhanced in the presence of the sediment. Zimmo et al. (2004) also reported higher nitrification rates in wastewater incubations containing sediment compared to those containing only wastewater.

Studies on complementary areas such as hydrodynamics of ponds and molecular biology are important to better understand the biological mechanisms involved in nitrogen removal in ponds. Thermal stratification is usually reported in ponds with depths greater than five metres (Addy & Green 1997). However, due to the turbidity that blocks part of the solar radiation that enters the pond, this phenomenon may occur in systems with shallower depths, especially

in the summer (Ukpong *et al.* 2006). Daily cycles of stratification and mixing can take place as a function of the differences in temperature and liquid density during the day.

The vertical mixing of the liquid transports nutrients and oxygen to the bottom, favoring bacterial growth responsible for nitrogen removal. As reported by Namèche *et al.* (1997), Chabir *et al.* (2000) and Keffala *et al.* (2011), the conditions for the development of nitrifying and denitrifying bacteria are present in the sludge due to the stratified environment in terms of dissolved oxygen (DO) and the hydrodynamic behavior of the ponds.

The objectives of this research were: (i) to determine the nitrogen removal rate by nitrification and denitrification processes measured in sediment cores, (ii) to quantify bacteria involved in the nitrogen cycle and (iii) to evaluate mixing and stratification conditions in a polishing pond with sludge accumulated over 10 years of operation. The major goal was the understanding of possible relevant mechanisms for nitrogen removal in maturation ponds in tropical climates, since there is substantial controversy in the ponds literature on this topic.

METHODS

Description of the pond system

The small full-scale wastewater treatment system investigated is located in the Center for Research and Training in Sanitation (CePTS UFMG/COPASA), which receives actual wastewater from the city of Belo Horizonte, Brazil. The system involved the following units in series: one UASB reactor and two shallow polishing (maturation) ponds (Figure 1). The UASB reactor was cylindrical, with the gas-solids-liquid separator in the central part, with gas collection. The ponds were rectangular with a length-to-width ratio around 5. The population equivalent of the wastewater treatment line was around 250 inhabitants. Belo Horizonte is located at a latitude of 19 °53′ S in Cfa or Cwa humid subtropical climate according to the Köppen classification, with a mean annual temperature of 22.1 °C.

The studies were concentrated on Pond 1, which had the following dimensions: length: 25.00 m; width: 5.25 m; depth: 0.80 m. During the experimental period, the median flow was 19.9 m^3/d and the median hydraulic retention time was 5.7 d for this pond. This unit was in operation for more than 10 years, and the bottom sludge occupied 40% of the useful volume of the pond (Possmoser-Nascimento *et al.* 2014). The description of the ponds system can be seen in Rodrigues *et al.* (2015).

This study investigated the influence of sediments in nitrogen removal (nitrification and denitrification processes). The nitrification and denitrification rates were determined in sediment cores taken from the pond by applying ammonia pulses. Environmental conditions of importance in the nitrogen cycle, represented by pH, DO, temperature (T) and oxidation-reduction potential (ORP), were measured at two different depths: the pond surface
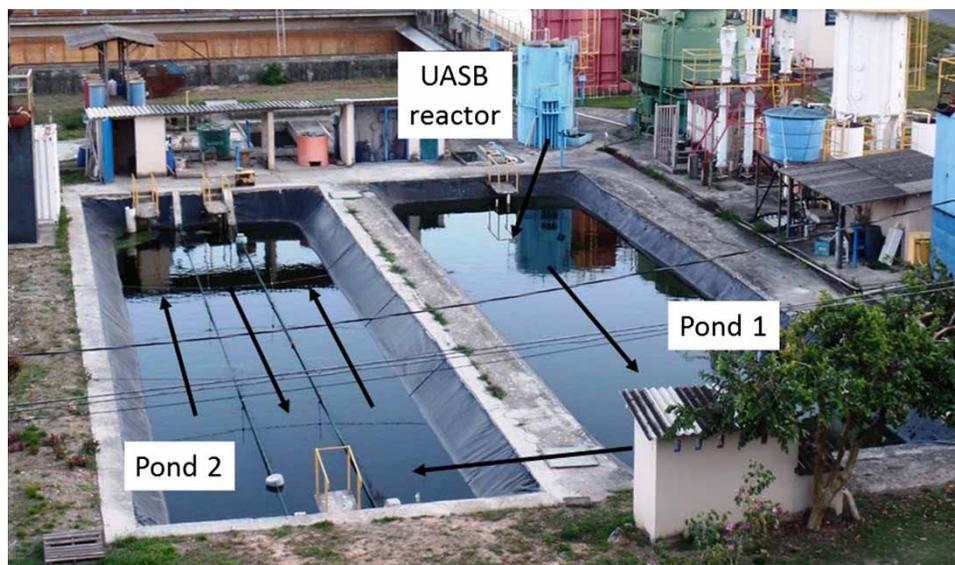

Figure 1 | View of the treatment system (UASB reactor and ponds).

and liquid-sediment interface in the pond. Analyses of total solids, total volatile solids (TVS) and total organic carbon (TOC) were performed in samples from the liquid layer and sludge layer (at different depths). TOC was measured in a Shimadzu model TOC-V analyzer. The different bacterial groups involved in the nitrogen cycle were detected by real-time polymerase chain reaction (real-time PCR). The following items show a brief description of the effluent and sludge sampling during the experimental phase.

Evaluation of system performance in terms of nitrogen removal

The monitoring results for the nitrogen fractions (organic, ammonium, nitrite and nitrate) in the treatment line (from the UASB reactor and Pond 1) in this specific study were obtained from May to December 2013. Sampling was done on a predominantly weekly basis. The nitrogen fractions were analyzed according to the methodology described in *Standard Methods for the Examination of Water and Wastewater* (APHA/AWWA/WEF 2005).

Core sampling

Sampling of the sludge was carried out with sediment cores (diameter $= 0.045$ m, cross-sectional area $= 0.0016$ m^2) to keep the real characteristics of the sludge in each layer and to preserve the spatial variability of biological activity in the sludge taken from ponds (Chabir *et al.* 2000; Keffala *et al.* 2011). This technique consists of sampling different points in

the pond (sediment core) by using a tube made from transparent acrylic material to encase the sediment core. A detailed description of the tube collector can be seen in Keffala *et al.* (2011). Eight sampling points were chosen based on a bathymetric survey of the pond (Possmoser-Nascimento *et al.* 2014) and samples were collected in June 2014 and January 2015. In the laboratory, each sample was separated into four layers with different volumes according to their oxygen concentration – liquid supernatant and sediment layers (aerobic, anoxic and anaerobic) (Figure 2). The volume of each layer was: 10 mL (0.63 cm) for the aerobic layer located at the sludge-liquid interface, 20 mL (1.25 cm) for the anoxic layer located in the middle of the sludge layer and 150 mL (9.37 cm) for the anaerobic layer located at the bottom of the sludge (Keffala *et al.* 2011). For the supernatant, the liquid from the pond was replaced by the UASB reactor effluent (to avoid interference from algae) plus an additional mass of ammonia nitrogen. In order to confirm the absence of nitrification in the liquid column, preliminary tests were carried out on the water column samples without sludge. Identical cores were reconstructed with the capacity of preserving the vertical layers, as shown in Figure 2.

DO, T, ORP and pH

In order to understand the pond's vertical dynamics, DO, T, ORP and pH values were monitored at two different depths, one at 2 cm below the pond surface and the other at the liquid-sediment interface (bottom), in the first polishing pond during a sequence of 7 days. Sampling intervals were
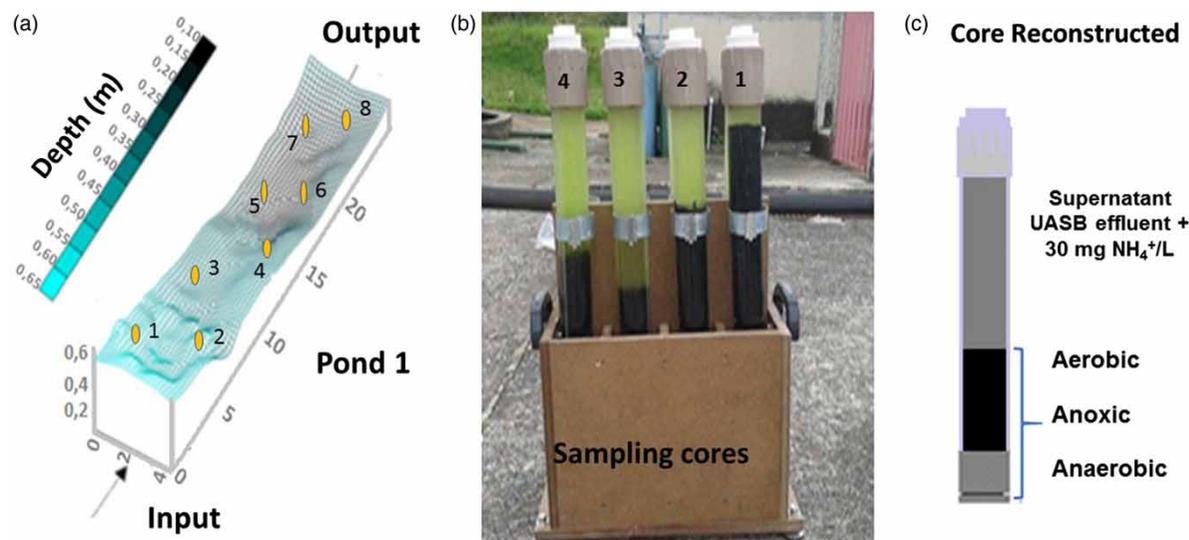

Figure 2 | (a) Bathymetric survey and different sampling points in Pond 1 from which the sediments were collected, (b) different samples of liquid and sediment core – 1 to 4, (c) four different layers in the vertical profile (reconstructed core).

30 minutes. Measurements were made using a multiparametric probe YSI XLM 600 with datalogger.

DNA extraction and bacteria quantification by real-time PCR

DNA was extracted from 0.5 g of frozen biomass samples, taken from the sediment core, according to the protocol described by Egli et al. (2003). The DNA samples were extracted from samples of the sediment core, which was constructed based on eight different samples collected from the pond that were pooled together in order to represent one sediment core. This sediment core was prepared in June 2014 (a mild winter in Brazil) and January 2015 (summer in Brazil). In total, four samples were used from each sediment core, from the liquid column, and sludge from the different layers (upper layer – aerobic, middle layer – anoxic and bottom layer – anaerobic). The extracted DNA was purified using the Wizard DNA Kit Clean-Up System (Promega, Madison, USA) and stored at $-20\,°C$. The abundance of total bacteria, ammonia oxidizing bacteria (AOB), nitrite oxidizing bacteria (NOB), anammox and denitrifiers was investigated by real-time quantitative PCR (qPCR) using SyBR green assays on biomass samples taken from the sediment core. qPCR assays were conducted on a real-time PCR thermal cycler (Applied Biosystems 7500 instrument). Each 20 µL reaction mixture contained 10 ng of template DNA, 375 nM of forward and reverse primers (each) and 10 µL of Maxima SYBR Green/ ROX q PCR Master Mix 2X (Thermo Scientific, USA). The total bacterial abundance was quantified using eubacterial 16S rRNA-targeted primers, anammox bacteria were quantified using primers Pla46F and Amx667R,

and the abundance of denitrifiers was quantified using primers targeting the nitrous oxide reductase gene nosZF and nosZ1622R (Table 1). PCR conditions were as follows: initial denaturation at $95\,°C$ for 10 min, followed by 40 cycles of $94\,°C$ for 1 min, 53 to $56\,°C$ for 1 min (annealing temperature varied depending on primer used – Table 1), and $72\,°C$ for 1 min. The program finished with the melting curve, which consisted of 15 s at $95\,°C$, 1 min at $60\,°C$, 30 s at $95\,°C$ and 15 s at $60\,°C$. The concentration of the different bacterial groups (as measured by qPCR) was expressed in the number of gene copies (Whelan et al. 2003) per gram of TVS.

Nitrogen mass balance in the cores

Ammonium was added to each core sample in order to make up a concentration of 30 mg of $N\text{-}NH_4^+/L$ in the supernatant in each of the reconstructed cores, which contained 600 mL of UASB effluent and the sludge layer. The cores were covered with aluminium paper to prevent the growth of algae due to solar radiation incidence. After 4 h of contact between the sediment and the solution (supernatant), samples in each core were taken for initial characterization (t_0). During 5 days, tests were carried out every 24 hours for nitrite ($N\text{-}NO_2^-$), nitrate ($N\text{-}NO_3^-$), ammonium ($N\text{-}NH_4^+$), chemical oxygen demand, DO, T and pH in the supernatant sample. Ammonium was measured with a photometer, Hach DR2800 model 10031 Hach (salicylate method). Nitrite and nitrate were measured with chromatography equipment, Dionex model ICS1000. Ammonium ($N\text{-}NH_4^+$) and oxidized nitrogen ($N\text{-}NO_x$ = nitrite + nitrate) concentrations ($mg\cdot L^{-1}$) measured in the supernatant during 5 days of testing were transformed into mass (mg) for calculating the mass balance.

Table 1 | Specific primers used in quantification of total bacteria and bacteria involved in the nitrogen cycle by real-time PCR

Target group	Primer set	Sequence (5′ to 3′)	Annealing temperature (°C)	Reference
Domain Bacteria	1055F 1392R	ATGGCTGTCGTCAGCT ACGGGCGGTGTGTAC	53	Ferris et al. (1996)
AOB	amoA-1F amoA-2R	GGGGTTTCTACTGGTGG CCCCTCKGSAAAGCCTTCTTC	57	Rotthauwe et al. (1997)
Nitrobacter sp.	Nitro-1198f Nitro-1423r	ACCCCTAGCAAATCTCAAAAAACCG CTTCACCCCAGTCGCTGACC	58	Graham et al. (2007)
Nitrospira sp.	NTSPAf NTSPAr	CGCAACCCCTGCTTTCAGT CGTTATCCTGGGCAGTCCTT	60	Kindaichi et al. (2006)
Anammox	Pla46F Amx667R	GGATTAGGCATGCAAGTC ACCAGAAGTTCCACTCTC	56	Neef et al. (1998), Van der Star et al. (2007)
Denitrifiers	NosZ F NosZ1622R	CGYTGTTCMTCGACAGCC AG CGSACCTTSTTGCCSTYGCG	53	Enwall et al. (2005)

The mass of ammonium (N-NH$_4^+$) consumption and the mass of oxidized nitrogen formed were calculated based on the mass balance proposed by Keffala et al. (2011).

Nitrification and denitrification rate in the sediment core

Based on temporal variations of ammonium and oxidized forms of nitrogen concentrations in the eight cores, a linear regression slope of decreasing N-NH$_4^+$ and increasing N-NO$_x$ was calculated, allowing for the calculation of the nitrification and denitrification rates. The ammonium consumption rate and nitrate formation rate during the experiments were calculated according to Equation (1), as reported by Keffala et al. (2011).

$$r(N\text{-}NH_4^+) \text{ or } r(N\text{-}NO_x) = \frac{a.V}{S} \qquad (1)$$

with: r(N-NH$_4^+$): kinetic rate of ammonium consumption (g/m^2 d); r(N-NO$_x$): kinetic rate of oxidized nitrogen formation (g/m^2 d); a: linear regression slope (mg·L^{-1}·d^{-1}); V: volume of supernatant at t$_0$ (L); S: core surface (m^2).

RESULTS AND DISCUSSION

Overall system performance

Nitrogen in raw sewage is present mainly in the forms of organic nitrogen and ammonium, with the organic form being converted to ammonium by the ammonification process. Ammonium concentration may decrease during treatment due to conversion to other forms (e.g. in the nitrification process), and total nitrogen concentration may decrease due to removal (e.g. volatilization and denitrification). The removal of ammonium by volatilization was not investigated here, because previous studies in the same system (Assunção & von Sperling 2012) indicated that it was very low, accounting for only around 3% of the applied load. Median values of total nitrogen decreased from 45.1 mg·L^{-1} to 34.4 mg·L^{-1} (23.7% removal) through the UASB reactor and Pond 1 (Figure 3). In the effluent from Pond 1, most of the nitrogen (30.4 mg·L^{-1}) was present in the form of ammonium. Nitrate and nitrite were detected in very low concentrations (<1.0 mg·L^{-1}). The different nitrogen fractions in the system are shown in Figure 3.

As expected, part of the organic nitrogen was converted to ammonium in the UASB reactor due to ammonification. Oxidized nitrogen concentrations in raw sewage were virtually insignificant, but increased slightly in the pond, indicating low nitrification or possibly denitrification. The oxidized forms of nitrogen remained low in the effluent from Pond 1. Regarding the conversion mechanisms, it appears that sediments can play an important role in the global nitrogen mass balance in WSP (Keffala et al. 2011). On the other hand, Camargo Valero & Mara (2007) found that the nitrification process was masked by simultaneous algal nitrate uptake during the peak of algal activity in a maturation pond in the UK. However, in situations in which water quality changes are small, any understanding of the fate of nitrogen is particularly difficult, due to the possibility of having simultaneous processes (e.g. nitrification-denitrification, nitrification-biological uptake).
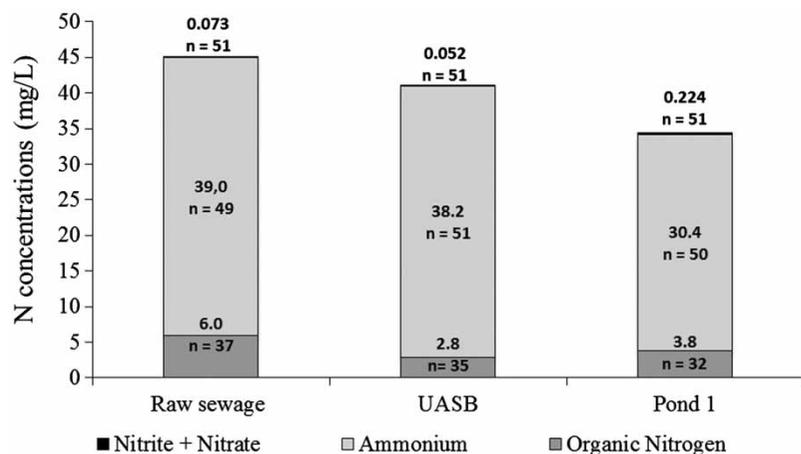

Figure 3 │ Median concentrations of nitrogen fractions along the system.

Mixing and stratification in Pond 1

The incidence of thermal radiation in stabilization ponds is not evenly distributed throughout the pond depth. This phenomenon causes higher temperatures at the surface during the day, creating a temperature gradient through the depth (Chowdhury *et al.* 2014) and stratification of the water masses due to convection currents as a function of density. During the night, the opposite occurs because solar radiation ceases, and destratification with vertical mixing can take place. Typical temperature, DO, ORP and pH variations versus time in Pond 1 are illustrated in Figure 4.

The pond remained stratified from 6:00 am until 10:00 pm. From 6:00 am, the increase in solar radiation at the surface stimulated algal growth and photosynthetic production, and consequently DO increased, reaching supersaturation at the top layer ($\sim$40 mg·L^{-1}) (Figure 4). The fluctuations in DO are also associated with the oxygen balance between production and consumption in the system (algae and bacteria), leading to the following values: liquid surface: 42.2 mg·L^{-1} (max), 15.7 mg·L^{-1} (average) and 0.06 mg·L^{-1}
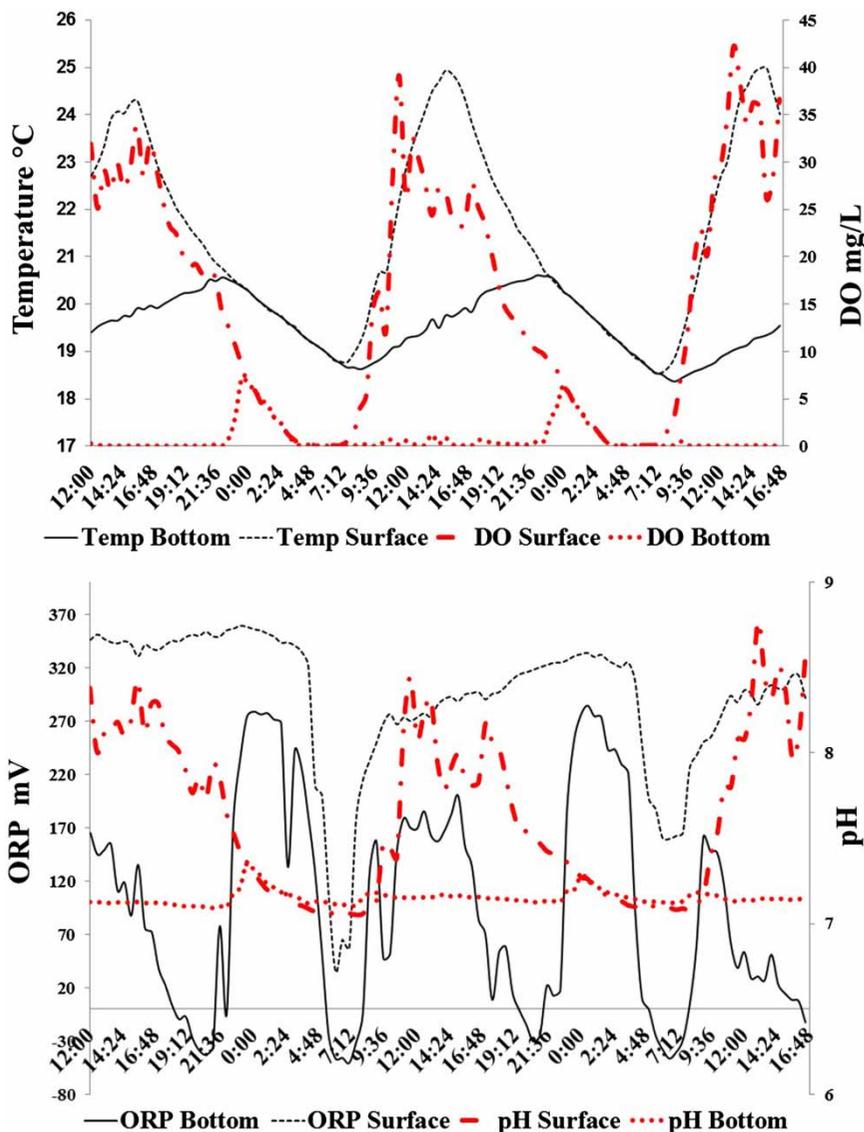

Figure 4 | Diurnal concentration profiles through 2 days at two different depths – liquid surface and liquid-sediment interface. Top graph: temperature and DO; bottom graph: ORP and pH.

(min); bottom: 7.10 mg·L^{-1} (max), 0.79 mg·L^{-1} (average) and 0.03 mg·L^{-1} (min). At night, the water temperature decreased, leading to mixing of the liquid masses and oxygenation at the bottom.

The pH and ORP values at the surface presented the same tendency, namely the increase in pH accompanied by the increase in ORP (Figure 4). This behavior was also related to photosynthetic activity of algae that consumed H$^+$ ions and increased the pH. The surface pH values ranged from 7.0 (min) to 9.0 (max), with an average of 7.6, while pH values at the bottom showed lower values, ranging from 7.1 (min) to 7.4 (max) and averaging 7.1, probably due to lower photosynthetic activity (Figure 4). pH values above 9.0 enhance free ammonia formation, which may inhibit the activity of nitrifying bacteria (*Nitrospira* and *Nitrobacter*) (Blackburne *et al.* 2007).

Ammonium conversion to nitrite and nitrate followed by their reduction to nitrogen gas depends on environmental conditions (aerobic and anoxic conditions), which also impact bacterial populations responsible for the nitrogen transformations in the ponds. At the surface of the pond, favorable conditions for nitrification could be found (ORP > 300 mV), while in the bottom the ORP values ($-50 < ORP < +250$ mV) suggest that nitrification and denitrification could take place (in the sludge layer). In addition, anaerobic conditions (with negative ORP values) were observed in the bottom of the pond, late at night and in the early morning, suggesting that nitrogen removal via anammox activity could occur.

Bacteria involved in the nitrogen cycle in the pond

The distribution and abundance of bacteria were related to their metabolic capabilities, and their success depends on several abiotic factors, such as temperature, which influences the growth rate of microorganisms. During the day, at the surface of the pond the DO concentration reached 42 mg·L^{-1} due to intense photosynthetic activity, while in the bottom of the pond, anoxic conditions prevailed over the whole period, with pH around 7.2 and an absence of light. At night, mixing of the water column occurred, and anoxic niches appeared at the surface, while the bottom was oxygenated (around 5 mg·L^{-1} of DO). Once the light intensity and DO concentrations were limited or practically absent in this region, the anoxic and anaerobic processes (such as fermentation, denitrification and anammox) were predominant.

The variation of environmental conditions between the top and bottom of the pond could explain the presence of different bacterial groups involved in the nitrogen cycle through the depth of the sediment core (Figure 5). The abundance of bacterial groups involved in the nitrogen cycle and total bacterial populations per gram of TVS in each layer of the sediment core, performed with samples taken in June 2014 and January 2015, are shown, respectively, in Figure 5(a) and 5(b). In general, the concentration of each bacterial group investigated in this study (total bacteria, denitrifiers, AOB, NOB and anammox) were in the same order of magnitude in both sediment cores, thus suggesting that there was no temporal variability between these samples.

The abundances of the different bacterial groups remained more or less uniform through the depth of the sediment core. However, the relative abundance of bacteria involved in the nitrogen cycle in relation to total bacteria increased through the depth (from 1.0% in the liquid layer to 7.0% in the anaerobic sludge layer). The concentration of denitrifying bacteria (as measured by *nosZ* gene abundance) and *Nitrobacter* (as measured by 16S rRNA gene abundance) were higher than the other groups investigated (AOB, *Nitrospira* and anammox bacteria).
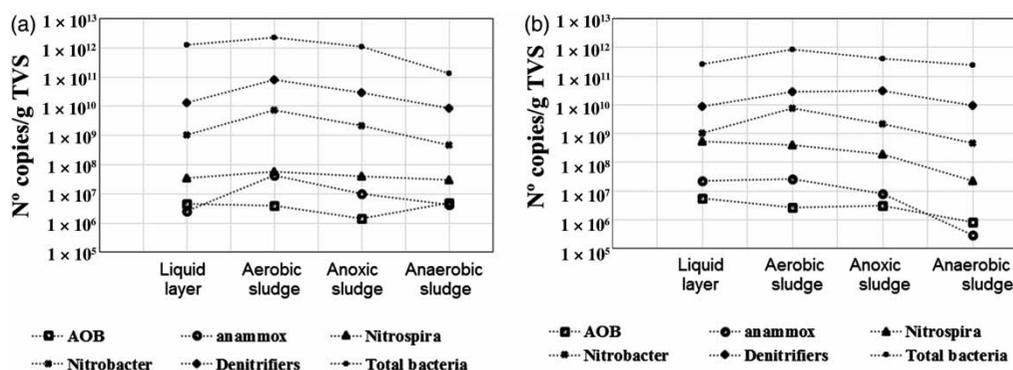

Figure 5 | Abundance of bacteria of the nitrogen cycle and total bacteria through the depth of the sediment core of Pond 1 in June 2014 (a) and in January 2015 (b).

The presence of denitrifiers through the pond depth was expected, since they can grow under aerobic and anoxic conditions and in the presence of organic matter. In fact, the concentration of denitrifying bacteria correlated well with TOC concentrations observed in each layer. The TOC concentrations were 250, 600, 450 and 500 mg·L^{-1}, respectively, for the liquid layer, aerobic sludge, anoxic sludge and anaerobic sludge. In this pond and in other biological nitrogen removal systems (Mac Conell *et al.* 2015), denitrifying bacteria were predominant among the nitrogen cycle bacteria, with relative abundances above 91%. The predominance of denitrifying bacteria over the other bacterial groups investigated can be explained by the fact that most of them are heterotrophic bacteria and, therefore, have higher growth rates than the autotrophic bacteria (such as AOB, NOB and anammox).

In relation to AOB, the relative abundance of this group (AOB and anammox) was much lower (ranging from 0.05% to 0.1% in relation to bacteria from the nitrogen cycle) than the abundance of NOB (abundance ranged from 5.5% to 8.5% in relation to bacteria from the nitrogen cycle).

Among the NOB, higher concentrations of *Nitrobacter* compared with *Nitrospira* were observed in all layers (Figure 5). Such behavior may be related to changes in nitrite concentration and oxygen availability in the deeper layers. The intense mixing of the water column that was observed might have also contributed to these findings. Several studies have shown that the NOB typically found in sewage treatment systems are *Nitrospira* and *Nitrobacter*. *Nitrospira* are k-strategists, well adapted to low nitrite and oxygen concentrations (half-saturation constant, Ks = 0.9 ± 0.07 mg NO$_2^-$N·L^{-1}; Blackburne *et al.* 2007), whereas Nitrobacter are r-strategists, and therefore proliferate better under high concentrations of nitrite and oxygen (Schramm *et al.* 1999). The predominance of *Nitrobacter* over *Nitrospira* observed in this study might also be related to the ammonium concentration found in this pond (around 30.4 mg·L^{-1}). According to Blackburne *et al.* (2007), *Nitrospira* is affected by free ammonia concentrations of 0.04 to 0.08 mg NH$_3$-N·L^{-1}, while *Nitrobacter* is affected by concentrations of 50 mg NH$_3$-N·L^{-1}.

Since the abundance of ammonia oxidizers was very low, particularly AOB, responsible for supplying nitrite, it is likely that other metabolic routes favored the growth of *Nitrobacter*. Firstly, nitrite may have been supplied by partial denitrification. Secondly, *Nitrobacter* may have grown mixotrophically using organic compounds. These two processes were observed and discussed by Winkler *et al.* (2015) in studies with aerobic granular sludge.

In a previous study, Mac Conell *et al.* (2015), investigated the microbial community (by 454 pyrosequencing of the 16S rRNA genes) in a trickling filter, also used as post-treatment of anaerobic effluent, receiving wastewater with the same origin as the one in the current study. The authors observed that the relative abundance of denitrifiers ranged from 6% to 16% of total reads; anammox bacteria ranged from 0.5% to 1.0% of the total sequences; and *Nitrospira* abundance ranged from 0.3% to 1.0% of total reads. The authors concluded that the bacterial community involved in the nitrogen cycle changed with filter depth and during different operational periods. Nitrification occurred in all compartments of the filter, whereas the increase in relative abundance of anammox bacteria occurred when the organic loading rate was lower. The differences observed between these two studies are obviously related to the differences in the treatment processes, but are worth mentioning here because of the similarities in terms of influent sewage and anaerobic pretreatment.

Nitrification and denitrification rates in the sludge

Nitrogen removal via assimilation by cyanobacteria and microalgae can occur intensely in the liquid column of ponds up to a depth where sunlight can reach and while sludge allows for other mechanisms, e.g. via anammox bacteria. Nitrogen removal rate via nitrification and denitrification was tested in the first sample of the prepared solution (UASB reactor effluent spiked with ammonium) and after that in samples comprising stratified sludge with the prepared solution. In the test carried out with only the solution, despite the availability of substrate (ammonium) and oxygen, the results showed that the nitrification rate was zero. After applying 30 mg of N-NH$_4^+$·L^{-1} in the eight cores containing the samples of sediment and solution, ammonium decreased and was associated with the increase of nitrate and nitrite over time (Figure 6). Examples showing the temporal variations of ammonium and oxidized nitrogen concentrations in core number 8 are illustrated in Figure 6, representing a typical behavior.

Under the test conditions, part of the removed ammonia was not fully recovered in the form of oxidized nitrogen, suggesting that nitrification was not the only process occurring in the system. This difference in the mass balance could be explained by an alternative process such as denitrification. The loss of ammonia was calculated by mass balance in the system. It was considered that the only nitrogen input was in the form of ammonium and the loss was associated with denitrification. These considerations were made
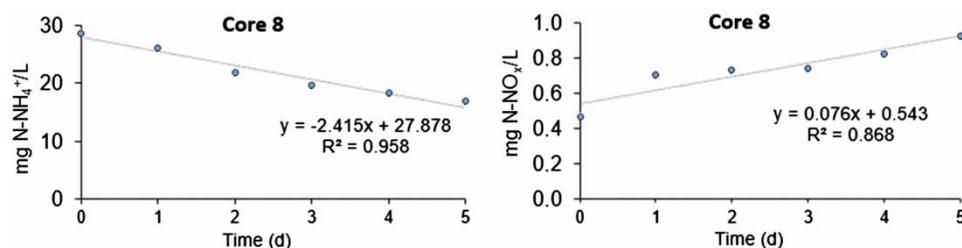
Figure 6 | Temporal variation of ammonium and oxidized nitrogen forms in core 8 with a sample of sludge and a solution made of UASB effluent spiked with ammonium.

Table 2 | Mass balance (ammonium and oxidized nitrogen) in each core containing the synthetic solution and the sludge

Mass	Cores							
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**
$M_{(NT)\ initial}$ (mg)	17.8	17.5	18.5	18.1	18.5	18.4	17.6	18.1
$M_{(N-NH4+)\ consumed}$ (mg)	3.37	1.91	2.87	3.44	3.52	3.61	2.64	4.06
$M_{(N-NOx)\ formed}$ (mg)	−0.28	−0.32	−0.34	−0.34	−0.33	−0.36	−0.33	−0.27
$M_{(NT)\ lost}$ (mg)	3.09	1.59	2.53	3.10	3.18	3.25	2.31	3.79
$M_{(NT)\ calculated}$ (mg)	14.5	15.9	16.0	15.0	15.3	15.2	15.3	14.3
$M_{(NT)\ final}$ (mg)	9.86	10.3	10.2	9.63	9.38	9.27	9.35	9.33

Note: see equations in Table 3 (supplementary material, available with the online version of this paper).

once the algal influence was attenuated (core covered with aluminium paper) and the biological uptake was considered low. The mass balance of the system can be seen in Table 2. The related equations are presented as supplementary material (available with the online version of this paper).

Total nitrogen here represents the sum of ammonium and oxidized nitrogen. The concentrations obtained through mass balance and the final mass measured in each core were not equal. The calculated mass was always greater than the measured mass in practically all cores. It seems that other processes in the system occurred, such as exchange of material between the liquid-sediment interfaces. Moreover, part of the ammonia present in the pond may have come from organic nitrogen degradation in the sediment, deposited in the form of ammonium and not recorded in the mass balance. Unlike the test with samples from sludge and effluent, nitrification and denitrification were close to zero in the test containing only supernatant (the control sample). This result confirmed that nitrogen removal occurred in the sludge layers. Average values of kinetic rates of ammonium consumption were $0.88\,\mathrm{g\,N\text{-}NH_4^+/m^2\,d}$ and for formation of oxidized nitrogen were $0.025\,\mathrm{g\ N\text{-}NO_x/m^2\,d}$.

CONCLUSIONS

Aerobic and anaerobic conditions were observed in the first polishing pond during 24-hour periods due to mixing and thermal stratification of the liquid column. The bacteria involved in the nitrogen cycle were found in all layers of the sediment core and the concentrations of each bacterial group (AOB, NOB, anammox and denitrifiers) were similar in the liquid layer and in the sludge, which could be explained by the intense mixing of the water column carrying oxygen to the sediment. Denitrifying bacteria were predominant among the nitrogen cycle bacteria, while AOB and anammox were detected in very low concentrations. The availability of oxygen and nitrite might have favored the development of *Nitrobacter*, since the abundance of this group was much higher than *Nitrospira*. The mass balance showed that the concentration of oxidized forms of nitrogen was lower than the concentration of the ammonium removed, suggesting the occurrence of denitrification or anammox removal. The results indicate that denitrifiers, nitrifiers and anammox bacteria coexisted in the sludge of this pond, and thus different metabolic pathways were involved in ammonium removal in this wastewater treatment system.

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