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RESEARCH ARTICLE

Nitrogen dynamic in vitro using sludge of a sewage stabilization pond from Patagonia (Argentina)

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Abstract

A relevant and current aspect of wastewater treatment systems is related to the processes of the nitrogen cycle that results in its elimination in gaseous forms. In the present study, we report the first measurements of nitrate-reducing rate (NRR) at lab-scale, using the flow-through reactor technique with sludge of a sewage stabilization pond system located in Patagonia (Argentina). Sludge was collected from Inlet and Outlet areas, in winter and summer. The sludge was characterized by having high moisture content (>94%) and organic matter concentration greater than 37%. The nitrate reduction experimental dates fitted significantly to the Michaelis-Menten model, allowing the estimation of the parameters that regulate the NR kinetics. The maximum potential nitrate reduction rate (R_{max}) showed great variability, registering a maximum of 131.6 μmol-N·gdw^{-1}·h^{-1} (Outlet-Summer) and a minimum of 4.1 μmol-N·gdw^{-1}·h^{-1} (Inlet-Winter). The lowest half saturation constant (K_m) was recorded in the Inlet sludge during the winter (6.1 mg N-NO_3^-·L^{-1}), which indicates a greater affinity for nitrate of this bacterial consortium. An unusually high activity of NR was registered, being higher with sludge from the Outlet zone and with summer temperature. In full-scale ponds, the NR activity could explain a relevant part of the nitrogen removal that involves the escape of gaseous forms.

Keywords Flow-through reactor · Nitrate reduction activity · Nitrogen removal · Patagonia · Sewage sludge · Wastewater treatment plant

Introduction

The nutrients discharge from sewage treatment systems into natural water bodies is a current environmental problem worldwide. Among the wastewater treatment systems, the stabilization ponds represent a simple and natural option, standing out for having comparative economic advantages with respect to construction, operation, and maintenance of conventional systems (Bouza-Deaño and Salas-Rodríguez 2013). In addition, a facultative stabilization pond working properly has the potential to develop a large number of the processes involved in the nitrogen cycle, and be efficient in its removal. However, the main mechanisms regarding nitrogen removal in stabilization pond systems have been a matter of great controversy in the technical literature (Lopes de Assunção and von Sperling 2012; Bastos et al. 2018).

In literature, the nitrification-denitrification and Anammox were described as the processes responsible for part of nitrogen removal in many full-scale wastewater treatment plants, both in conventional (Nourmohammadi et al. 2013; Tallec et al. 2008) and in natural systems (Saeed and Sun 2012; Rodrigues et al. 2017; Foladori et al. 2018). However, there are few studies focused to know the kinetics of the processes that occur in anoxic bottom environment in pond systems.

Nitrate is one of the main terminal electron acceptors in anaerobic respiration inside aquatic bottom environments. The nitrate-reducing activity is a metabolic process that can follow two ways: (1) Denitrification is the nitrate reduction in anoxic conditions resulting in the production of NO_2 with its subsequent reduction to NO, N_2O, and, finally, to N_2 or (2) dissimilatory nitrate reduction to ammonium (DNRA), the process which oxidizes organic matter using NO_3^- as an electron acceptor, reducing it to NO_2^-, and then to NH_4^+.

The nitrate reduction activity (NRR) has been extensively studied in sediments from natural aquatic systems (e.g., rivers,

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lakes, and coastal marine environments), allowing to quantify the reduction rates in these environments (Cook et al. 2006; Laverman et al. 2006; Jäntti et al. 2011; Peyton et al. 2001). It has been possible to infer different aspects that contribute to the metabolism involved, such as temperature, salinity, organic matter concentration, redox potential, and presence of microorganisms (JØrgensen and SØrensen 1985; Laverman et al. 2006). NRR has also been studied in batch reactors inoculated with activated sludge cultures, with a previous acclimation procedure (Albina et al. 2019). Moreover, many methods have been used to estimate the nitrogen dynamic in sediments: benthic chamber (Torres et al. 2016; Faleschini and Esteves 2013), experiments using labeled nitrogen (Enrich-Prast et al. 2016), in vitro incubation corer with sediment (Gardner et al. 2006), the acetylene inhibition technique (Lindau et al. 2011), and flow-through reactors (FTR) (Evrard et al. 2013). The last method was developed by Northby (1976) and adapted for microbiological processes by Esteves et al. (1986). The FTR system consists of the continuous flow of a solution enriched with a known nitrate concentration through a mass of sediment, measuring the concentration of ammonium, nitrite, and nitrate in the reactor outlet at fixed time intervals (more details in Torres et al. 2016). This method allows reaching steady-state conditions, and determining the kinetic parameter of the nitrate reduction rate: maximum potential reduction rate (R_{max}) and half-saturation constant (K_m).

In the present study, we propose the use of a novel matrix in order to study the nitrogen dynamics with the FTR method, as is the sludge of a wastewater stabilization pond (WSP). The treatment carried out in WSP results from the complex symbiosis between bacteria and microalgae species. Most of the scientific research on stabilization ponds has focused on the processes that occur in surface water, while an important part of the treatment takes place at the bottom of the pond. The sludge is the result of organic matter sedimentation, both of the settleable solids present in the raw wastewater and of phytoplankton developing inside the pond. In the bottom water, various processes take place either in absence or with low content of dissolved oxygen. The effective height of the sludge column will depend on the interaction of several factors as concentration of settleable solids in the raw wastewater, microalgae development, anaerobic degradation rate, compression, sludge composition, pond geometry, season, and the position relative to the inlet. Several studies have been addressed to determine the impact of sludge accumulation on the stabilization ponds performance (Ouedraogo et al. 2016; Coggins et al. 2017; Li et al. 2018). In general, the sludge from a stabilization pond is characterized by an impressive and diverse content of organic matter, nutrient concentrations, and microorganisms as well as a widely negative redox potential (Faleschini and Esteves 2014).

In our study, we characterized both surface and bottom water, and the sludge from the municipal stabilization pond from Puerto Pirámides town, in winter and summer. Also, sludge for use in FTR experiments was collected, in order to determine the NRR kinetics as well as the nitrite and ammonium production. In laboratory experiments, we replicated the same temperatures recorded in the field, during winter and summer surveys.

Materials and methods

Study site and sampling

Puerto Pirámides is the only urban settlement (~600 inhabitants) within Península Valdés (latitude: 42° 35′ S–longitude: 64° 17′ W), in the Province of Chubut (Patagonia, Argentina). With the goal of conserving biodiversity and natural resources, Península Valdés has been declared Natural Heritage of Humanity by UNESCO in 1999 (Fig. 1).

Puerto Pirámides has a strong freshwater deficit, with a rainfall of 230 mm·year^{-1} and an evaporation rate of 2000 mm·year^{-1} (data from the CCT CONICET-CENPAT weather station). In addition, the town has not a freshwater body nearby, and the main source of drinking water is obtained through seawater desalination. During the tourism season, there is a great water demand due to the arrival of 150,000 tourists/year (reaching a peak of 6000 people per day in summer), mainly associated with its two attractions: beach activities in the warm months and the famous whale watching in the cold season. Consequently, drinking water must be transported by trucks from Puerto Madryn city, located 100 km away.

The town has a sewage network that covers 85% of all households; the collected liquid receives a primary treatment through sieves. Then, the wastewater has three stages of pumping to reach the treatment system, which consists of four facultative stabilization ponds working in parallel (Fig. 1). The generated flow was estimated in 270 m^3·day^{-1} during summer and 170 m^3·day^{-1} during the rest of the year, and since the flow is divided into 4 ponds, each one has a theoretical hydraulic retention time (HRT) of 25 and 39 days in summer and in the rest of the year, respectively. The design depth of each pond is 1.5 m.

To achieve the proposed objectives, each pond was subdivided in two areas: the “A” zone corresponds to the sector near the raw wastewater entrance and the “B” zone is near the outlet of the treated wastewater. We select these points by the extreme impact between the two zones regarding contact with raw wastewater; therefore, different physical-chemical composition and of microbiological structure is expected. In each sector, we define the following samples, both in winter and summer 2015: (1) the surface water was collected between 0 and 0.1 m depth; (2) the bottom water was taken at 1.3 m depth; (3) sludge was taken 1.5 m depth. To ensure the depth of the sampling site, we used a buoy and a hose of

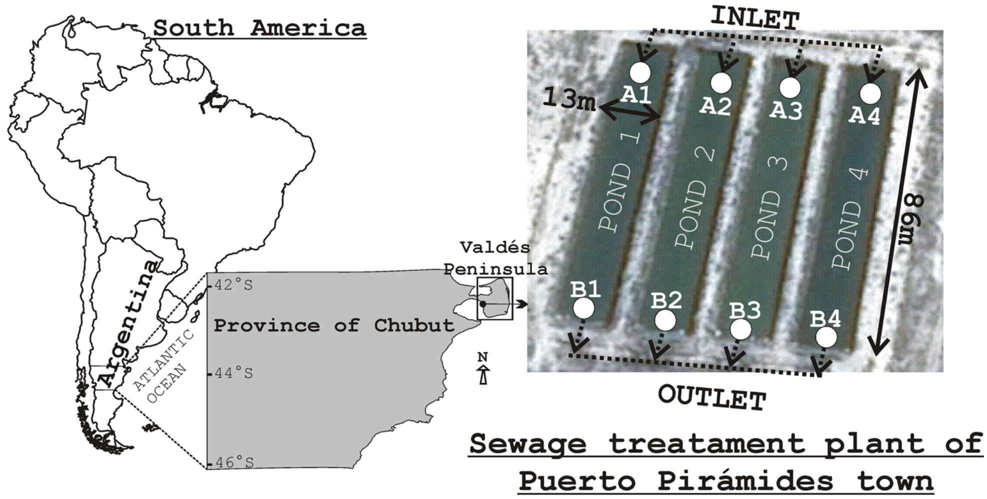

Fig. 1 Location of Puerto Pirámides town within Península Valdés (Patagonia Argentina). Detail of the treatment system, the white circles indicate the sampling points

the length required for each sample. The sample was suctioned using a manual bilge pump. The samples were arranged in plastic containers, kept cold until the arrival at the laboratory. In particular for sludge samples, its oxidation by sealing it over with bottom water was avoided, which in all cases had a redox potential less than −250 mV. In surface and bottom waters at the sampling points, in situ parameters with a multiparameter probe (YSI-556 MPS) were recorded, and in laboratory were determined: NO_3^- and NO_2^- concentrations using an Autoanalyzer Technicon II, NH_4^+ concentration applying the manual phenol technic, suspended solids and pigments (chlorophyll "a" and phaeophytin) according to APHA (1980).

Nitrate reduction and ammonium and nitrite production experiments

Once in the laboratory, the sludge samples of each sampling point were unified to form a composite sample, thus obtaining an Inlet sample (resulting of collecting sludge samples A1, A2, A3, and A4) and an Outlet sample (same procedure but with the B sampling sites) for winter and summer. In the sludge samples, the moisture (calculated after sludge drying at 105 °C until constant weight) and total organic matter content (determined by calcination of dry sludge at 550 °C in a muffle furnace for 4 h) were analyzed in triplicate according to APHA (1980).

Experiments in flow-through reactor (FTR) were carried out in triplicate. The wet sludge previously weighed (~30 g), was disposed inside the reactor (diameter: 45 mm; height: 55 mm), and retained on a glass-frit (10 μm porosity). Above the sludge, a thin layer of bottom water from the sampling site was added. The anaerobic atmosphere in each reactor was maintained supplying nitrogen gas. The experiments were run in dark conditions to avoid the activity of photosynthetic microorganisms. Each reactor was supplied with NO_3^- enriched water at a constant flow (36 ml·h^{-1}) through a Technicon peristaltic pump, and the feed flux direction was from top to bottom. The experiments lasted 9 h, far surpassing other FTR research that reached a steady state after 4 h with sediment from natural and impacted aquatic environments (Esteves et al. 1986; Torres et al. 2012, 2016). In our study, the equilibrium state was established as the time in which no modifications in the output nutrients concentrations were detected.

Standard solutions of sterilized deionized water enriched with nitrate (KNO_3) were prepared: 300, 600, 1200, 2000, and 4000 μM. The selection of the experimental concentrations was not related to the nitrate concentrations measured in the surface water of the Puerto Pirámides treatment system, but was based on nitrate range possible to find in wastewater treatment systems. In addition, the stock solutions were analyzed for initial concentration of ammonium and nitrite. The experiments were carried out at the same temperature registered in the water at the time of sampling (winter: 6 °C and summer: 24 °C), and it was maintained by water circulation using a thermostatic bath (Mgw Lauda Kzr). After passing the solution through the reactor and every 15 min, a fraction of 6 ml for analysis of NH_4^+, NO_2^-, and NO_3^- concentrations was collected.

Reaction rates and calculations

The net rate of NO_3^- consumption and NH_4^+ and NO_2^- productions inside the FTR, defined as function of the dry weight of sludge into the reactor, were obtained considering the difference between inflow and outflow concentrations. The rates (R_c) were determined under steady-state conditions as follows:

$$\mathrm{R}_c\ \left(\mathrm{NO_3^-, NO_2^-, NH_4^+}\right)\left(\mu\mathrm{moles\ N}\cdot \mathrm{g}^{-1}\cdot \mathrm{h}^{-1}\right) = \frac{F_0{*}(C_0{-}C_\mathrm{i})}{W} \quad (1)$$

where F_0 is the flow of initial solution enriched with NO_3^- ($ml{\cdot}h^{-1}$); C_0 and C_i are the N_{inorg} input and output concentrations (μM N-N_{inor}); and W is the dry weight of sludge in the reactor (g).

The NO_3^- reduction rates were plotted as a function of initial NO_3^- concentrations. According to the shape of the relationship between NO_3^- reduction rates and NO_3^- concentration, the Michaelis–Menten function was fitted to the data by least-square regression:

$$R_c NO_3^- = \frac{R_{max} * C_{eq}}{K_m + C_{eq}} \quad (2)$$

where R_{max} is the maximum uptake rate (μmoles N-$NO_3^-{\cdot}h^{-1}{\cdot}g^{-1}$); C_{eq} is the output NO_3^- concentration at the steady state (μM N-NO_3^-); and K_m is the half-saturation constant (expressed in mg N-$NO_3^-{\cdot}L^{-1}$). The optimized values of R_{max} and K_m were obtained using the Lineweaver-Burk linearization, which involves a double reciprocal, plotting the inverse of the NO_3^- reduction rates ($1/R_c$) with the inverse of NO_3^- input concentrations ($1/C$) (Pallud et al. 2007).

Results and discussion

Characterization of surface water and sludge from the stabilization ponds

Surface and bottom water characteristics are presented in Fig. 2. Regarding the surface water analysis, during summer was characterized by a higher concentration of suspended solids and pigments in the Inlet zone. The ammonium concentration was similar between the Inlet and Outlet zones, and low nitrification was recorded (< 0.1 mg N-NO_2^-+NO_3^-). In winter, we did not find differences between the Inlet and Outlet for all the measured parameters. However, there were differences between seasons: a greater nitrification and lower ammonium concentration was detected in winter, as well as higher pigments content in the Outlet sector. Inorganic nitrogen presented a pattern which was different from that normally observed in stabilization ponds operating in temperate climates (e.g., Picot et al. 2009; Faleschini et al. 2012). We associated this unusual behavior with the fact that the town of Puerto Pirámides is characterized by the arrival of a large number of tourist in the summer season already described, which reduces HRT by 36% compared to winter, which would affect the adequate conditions to promote nitrification, and ammonium consumption by phytoplankton.

Regarding the bottom water analysis, we observed an important increase in those parameters that reflect the sedimentation processes, phytoplankton concentration, and organic nitrogen degradation. The suspended solids ranged between 115 and 309 times, chlorophyll "a" varied between 15 and 63 times, phaeophytin between 177 and 353 times, and ammonium concentration between 1.3 and 2.5 times higher than in surface water. These processes increased during the summer, due to a greater development of phytoplankton biomass and higher organic matter degradation rates associated with the increased temperature.

The level of dissolved oxygen saturation in the surface water was in all cases greater than 60%, reaching values above 120% in summer. In bottom water, the dissolved oxygen level remained below 20% (summer) and below 34% (winter).

Regarding the sludge used in FTR experiments, all the samples presented high water content (moisture greater than 94%) and organic matter concentration (greater than 37%) (Table 1). These general characteristics suggest that the sludge is an extremely complex matrix and different from that reported in natural sediments where their reduction activity has been determined (Laverman et al. 2006; Laverman et al. 2007; Pallud et al. 2007). Due to the high organic matter level, it was expected that the electron donor concentration during the FTR experiments was not limiting. Although not all this organic matter may be bioavailable, BOD_5 measurements have been carried out in bottom water (data unpublished), which showed high values: between 406 and 980 $mg{\cdot}L^{-1}$ (Winter) and between 520 and 1200 $mg{\cdot}L^{-1}$ (Summer), and this will be even higher in the sludge samples.

Nitrate reduction activity

The results obtained after reaching the equilibrium state in the FTR experiments are presented below (Fig. 3). In the experiment using the sludge collected in summer and for the three initial NO_3^- concentrations (300, 600, and 1200 μM), we did not find significant differences in the nitrate consumed between Inlet and Outlet, being 68.3±4.0, 109.5 ±8.1, and 242.6±9.0 μmol N-NO_3^-, respectively. However, for the solutions with an initial concentration of 2000 and 4000 μM, the Outlet clearly presented higher nitrate consumption with respect to the sludge from the Inlet (p< 0.05; Friedman nonparametric test), reaching a maximum consumption of 410.2±58.5 μmol. Regarding the percentage of removal in each treatment, a tendency to a reduction with the increase in the stock solution concentration was observed. A maximum percentage of removal for the Outlet sludge (23.8% with 300 μM) and the minimum for the Inlet (5.2% with 4000 μM) were determined.

In winter experiments, the NR activity was clearly lower than that observed in summer: the maximum nitrate consumption was 161.2±24.3 μmol (with 2000 μM) and the percentage of removal was in all cases less than 11%, reflecting that the nitrate reduction activity is affected by the temperature, according to Laverman et al. (2006, 2007).

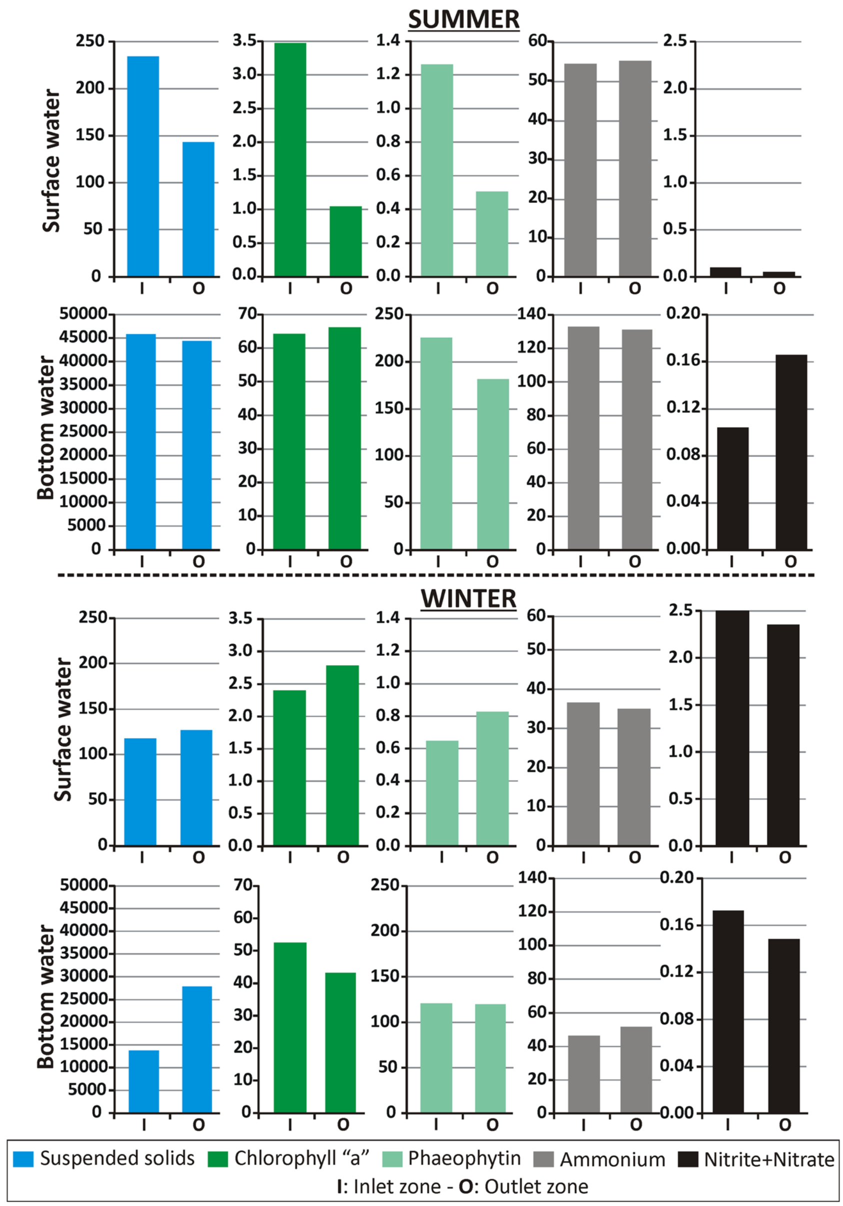

Fig. 2 Concentration (in mg·L^{-1}) of measured parameters in surface and bottom water inside the Puerto Pirámides stabilization ponds, in summer and winter seasons

Ammonium and nitrite at the reactor outlet

In the stock solution, the average of the initial ammonium concentration was 0.02±0.01 mg·L^{-1} and for nitrite was 0.2 ±0.05 μM. The release/production of ammonium and nitrite during the stationary state is shown in Fig. 4. Initially, the presence of ammonium at the output of the reactors can be mainly associated with their washing from the porewater and desorption from the attached sites. Therefore, its concentration was very high during the first 2 h (ranged between 58 and 113 mg·L^{-1}), and then a gradual decrease was observed. At the equilibrium state, the ammonium measured at the output of the reactors has been associated with the anaerobic degradation rate of the organic nitrogen present in the sludge, because in the course of 9 h a wash equivalent to 5.6 times the reactor volume was carried out. Roychoudhury et al. (1998) reported a similar pattern in the ammonium generation due to the degradation of organic matter in FTR experiment. At the steady state in summer experiment, we always observed higher

Table 1 Moisture and organic matter content in the sludge collected in two sites of the stabilization pond system from Puerto Pirámides

Season	Site	Moisture (%)	Organic matter (%)
Winter	Inlet	96.13±1.18	37.57±3.52
	Outlet	96.87±0.83	47.03±2.31
Summer	Inlet	95.59±0.40	45.51±1.92
	Outlet	94.32±1.56	38.25±4.44

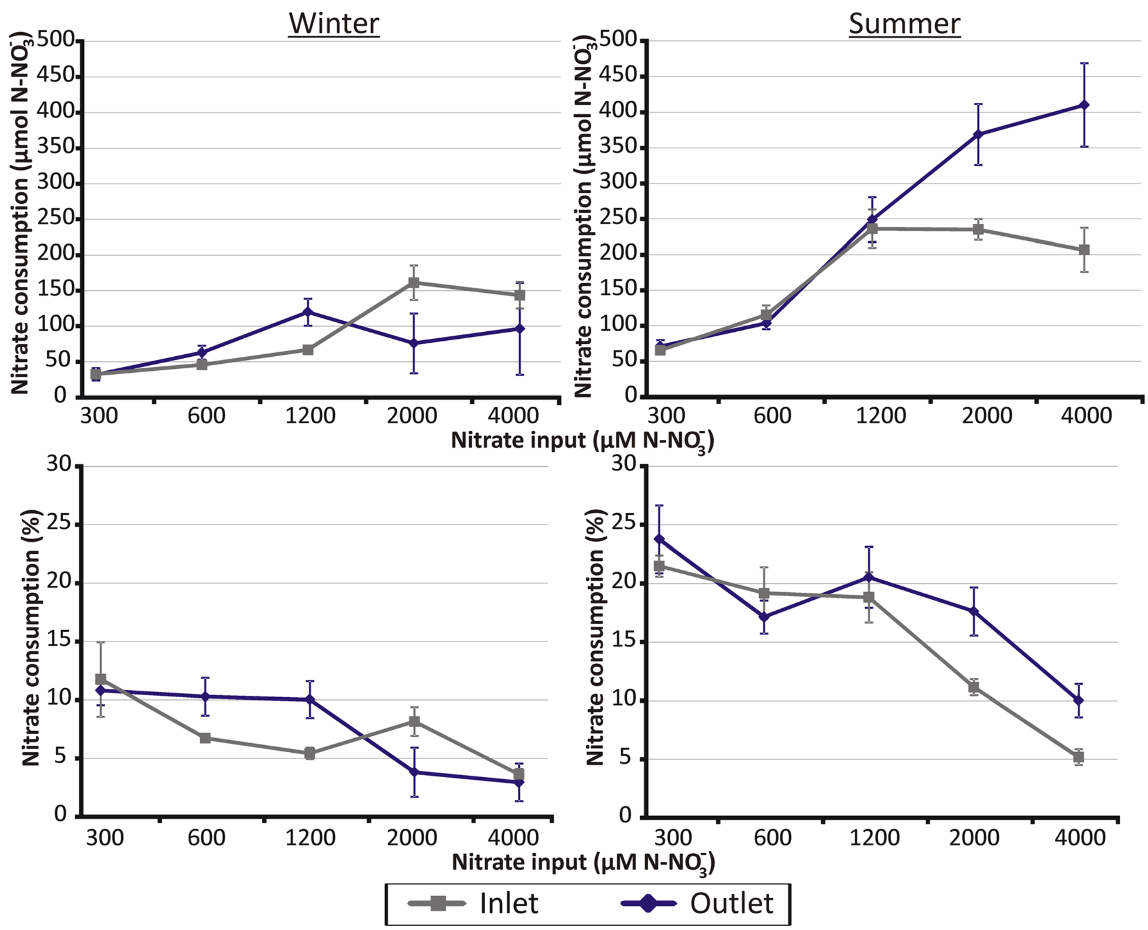

Fig. 3 Nitrate reduction activity as function of the absolute nitrate consumption (top graphics) and as function of the percentage consumed from the stock solution (down graphics) in winter (left) and summer (right)

concentrations in the Outlet sludge, with a maximum value of 12.5 mg·L^{-1}, while in the Inlet, there were no marked differences for the ammonium release between the different concentrations of nitrate in the stock solutions, with an average value of 3.7±0.9 mg N-NH_4^+·L^{-1}. During winter experiments, the average of the ammonium concentration produced in the Outlet was 6.2±1.3 mg N-NH_4^+·L^{-1}, while at the Inlet was 3.2 ±1.2 mg N-NH_4^+·L^{-1}. Expressing the ammonium production rate (APR) as function of dry weight of sludge per hour, the following results were obtained: 0.075±0.029 mg N-NH_4^+·gss^{-1}·h^{-1} (Inlet-Winter); 0.14±0.04 mg N-NH_4^+·gss^{-1}·h^{-1} (Outlet-Winter); 0.12±0.05 mg N-NH_4^+·gss^{-1}·h^{-1} (Inlet-Summer); 0.13±0.06 mg N-NH_4^+·gss^{-1}·h^{-1} (Outlet-Summer). These APRs were 36 and 170 times higher than the maximum value reported by Laverman et al. (2006, 2007). The APR can thus be used as a proxy for the rate of organic matter degradation (Laverman et al. 2012); therefore, our high APR values can be related to the elevated biodegradable organic matter present in the sludge from Puerto Pirámides ponds.

Another possible way of ammonium generating involves the dissimilatory nitrate reduction to ammonium (DNRA). However, the prevailing conditions in our research decreased the chances of development of this metabolic pathway, since DNRA is favored when nitrate is limiting (Kelso et al. 1997), and in our experiments we ensure their constant presence. In the same way, Chutivisut et al. (2018) using activated sludge argued that DNRA was development under a high ratio of organic carbon to nitrate, indicating an environment with high electron donor. Regardless of our experimental conditions, in-depth studies of DNRA have been reported as a widespread process but with a lower contribution in NO_3^- reduction than denitrification (Wang et al. 2019).

The nitrite production with the Outlet sludge showed a tendency to enhance with the increase of nitrate feed concentration, reaching a maximum of 11.9 µM N-NO_2^- (Summer with 4000 µM N-NO_3^-) and 11.0 µM N-NO_2^- (Winter with 4000 µM N-NO_3^-) (Fig. 4). The same pattern was registered in the Inlet during winter, with a maximum production of 12.5 µM N-NO_2^- (with 4000 µM N-NO_3^-). However, during summer with the Inlet sludge, the nitrite production remained relatively constant and representing half concentration of the other trials, with an average production of 5.3±1.8 µM N-NO_2^-.

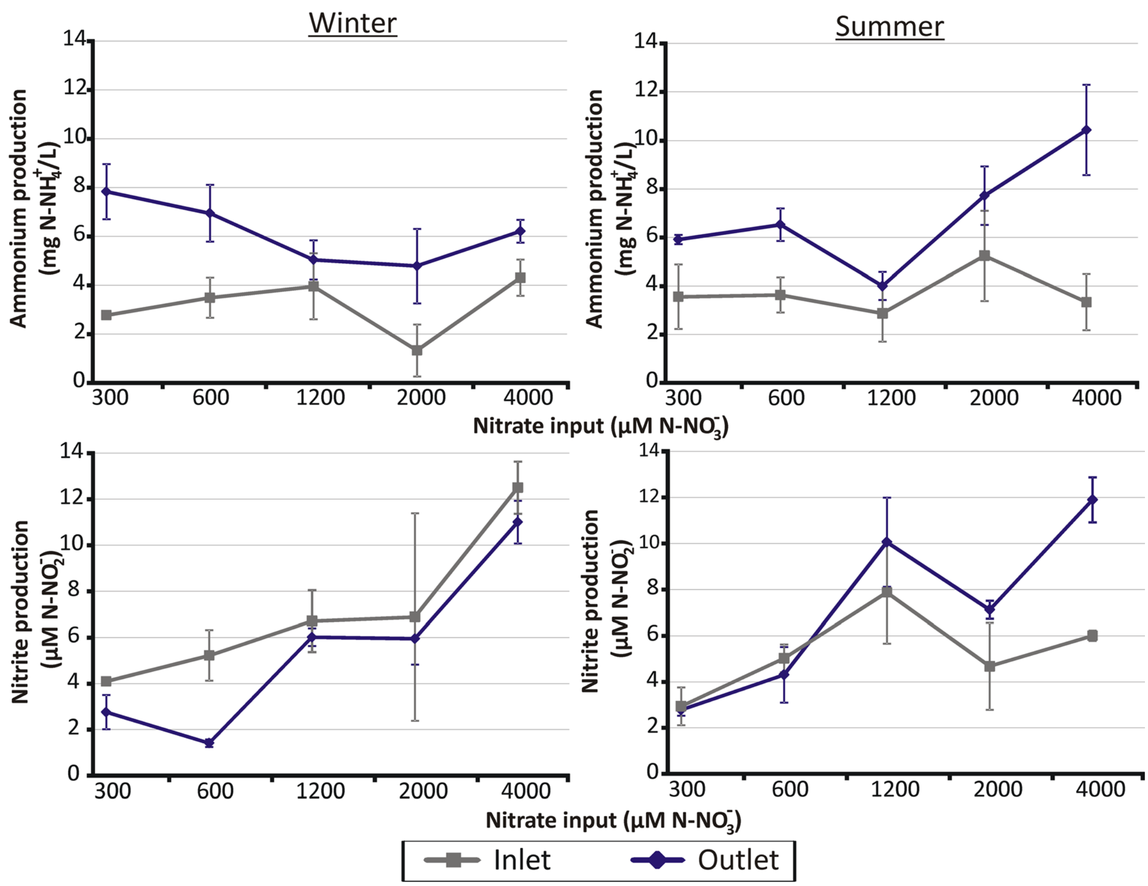

Fig. 4 Production of ammonium (top graphics) and nitrite (down graphics) in winter (left) and in summer (right) registered at the steady state during the experiments using Inlet and Outlet sludges as function of the nitrate feed concentration

Taking into account that the nitrification process was discarded given the reduced condition in the sludge and the N_2 incorporation from the feed solution, and also the reduced possibility of DNRA discussed above, the presence of NO_2 must be a consequence of the denitrification pathway. Is remarkable that in others' FTR experiment, the nitrite was detected sporadically and never approached steady state (Laverman et al. 2006). Researching the nitrogen removal using sludge from a SBR for a treatment based on partial nitrification coupled with Anammox, the non-accumulation of nitrite was attributed to the activity of anammox bacteria, even at temperatures below 15 °C (Hu et al. 2013). However, in studies of nitrate reduction with batch reactors inoculated with bacteria acclimated from activated sludge, significant concentrations of nitrite were recorded over prolonged periods of experimentation time (Cao et al. 2013; Durban et al. 2020).

The percentages of nitrate consumed that could be explained by the nitrite detected in the outlet of the reactor are plotted in Fig. 5. Nitrite is an intermediate in denitrification and DNRA processes, and an input for Anammox, thus the environmental conditions and the presence/dominance of the different bacterial groups, determines the amount of nitrite that will accumulate and the amount of elements that result from the nitrite reduction (NO, N_2O, N_2 or NH_4^+) will be produced.

A marked difference between winter and summer seasons was detected; under lower temperature the presence of nitrite was clearly higher in relation to the missing nitrate. This would indicate an incomplete nitrate reduction resulting in the accumulation of nitrite. While in summer season, taking into account that the nitrate reduction was the greatest, the presence of nitrite did not explain more than 4.5% of the missing nitrate. This would indicate that the higher temperature increases not only the nitrate reduction but also the efficiency of nitrite reduction, either by the generation of gaseous forms of nitrogen, that escape from the water (denitrification or Anammox) or NH_4^+ (DNRA). In our experiments, the maximum values of missing nitrate explained by nitrite production (12.3%) were lower than in other studies, e.g., 17% in Laverman et al. (2007); between 25 and 45% in Laverman et al. (2012), which would indicate that using sewage sludge a greater proportion of the nitrite generated followed, at least, one more step in its reduction.

Nitrate reduction rates and adjustment to the Michaelis-Menten model

Analyzing the summer data of the Inlet sludge, and considering the five nitrate feed points, a non-significant

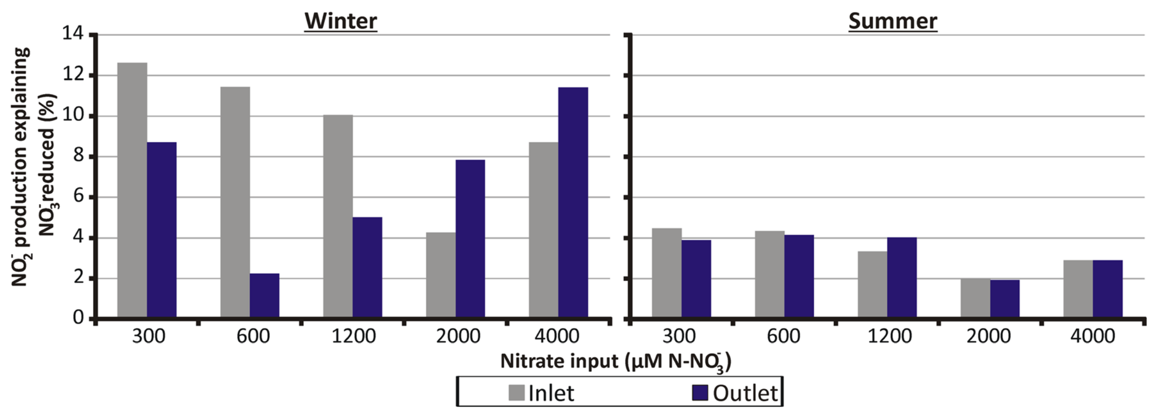

Fig. 5 Production of nitrite in winter (left) and summer (right) as function of the missing nitrate at the reactor outlet

adjustment (R^2 = 0.63) to the Michaelis-Menten function was obtained (Fig. 6). The adjustment increased if the maximum nitrate feed concentration (4000 μM) is discarded (R^2 = 0.95), which could indicate a saturation in the NR activity at the maximum feed nitrate concentration. Another explanation would imply that the enzymatic activity levels present in the Inlet sludge require a longer adaptation time when exposed to the maximum concentration of nitrate, as described by Nair et al. (2007). The data for the Outlet sludge presented an optimal adjustment, including the five nitrate feed concentrations (R^2= 0.95). The maximum NRR value in the Inlet sludge was 7.7 μmol N-NO_3^-·gdw^{-1}·h^{-1}, and in the Outlet sludge was 10.8 μmol N-NO_3^-·gdw^{-1}·h^{-1} (Fig. 6).

Regarding to winter season, we observed a marked decrease in the maximum values of NRR: 3.0 μmol N-NO_3^-·gdw^{-1}·h^{-1} for the Inlet and 4.5 μmol N-NO_3^-·gdw^{-1}·h^{-1} for the Outlet (Fig. 7). These values represent a decrease of 61.0% (Inlet) and 58.2% (Outlet) with respect to the maximum activities found in summer.

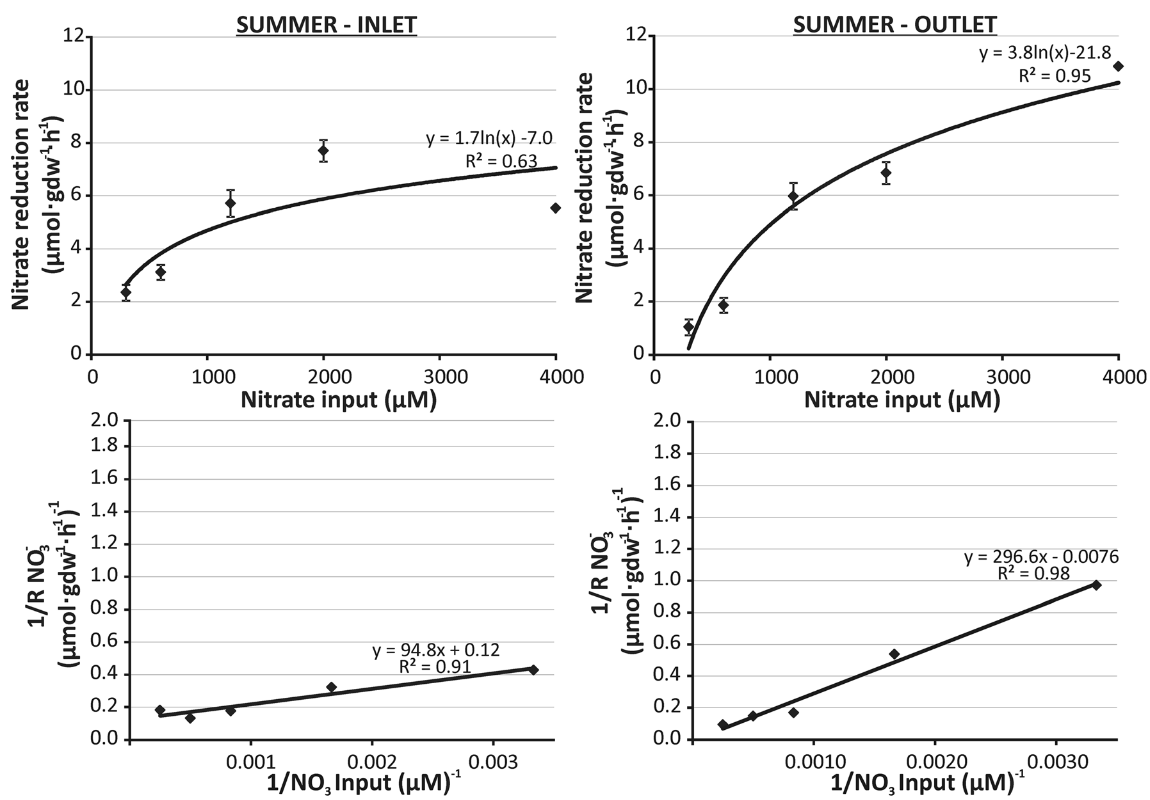

Fig. 6 Nitrate reduction rate vs initial nitrate concentration (black point), and the line was fitted by use of the Michaelis-Menten equation (top graphics). Double reciprocal plot of the nitrate concentration vs nitrate reduction activity (down graphics), for Inlet sludge (left) and Outlet sludge (right) in summer

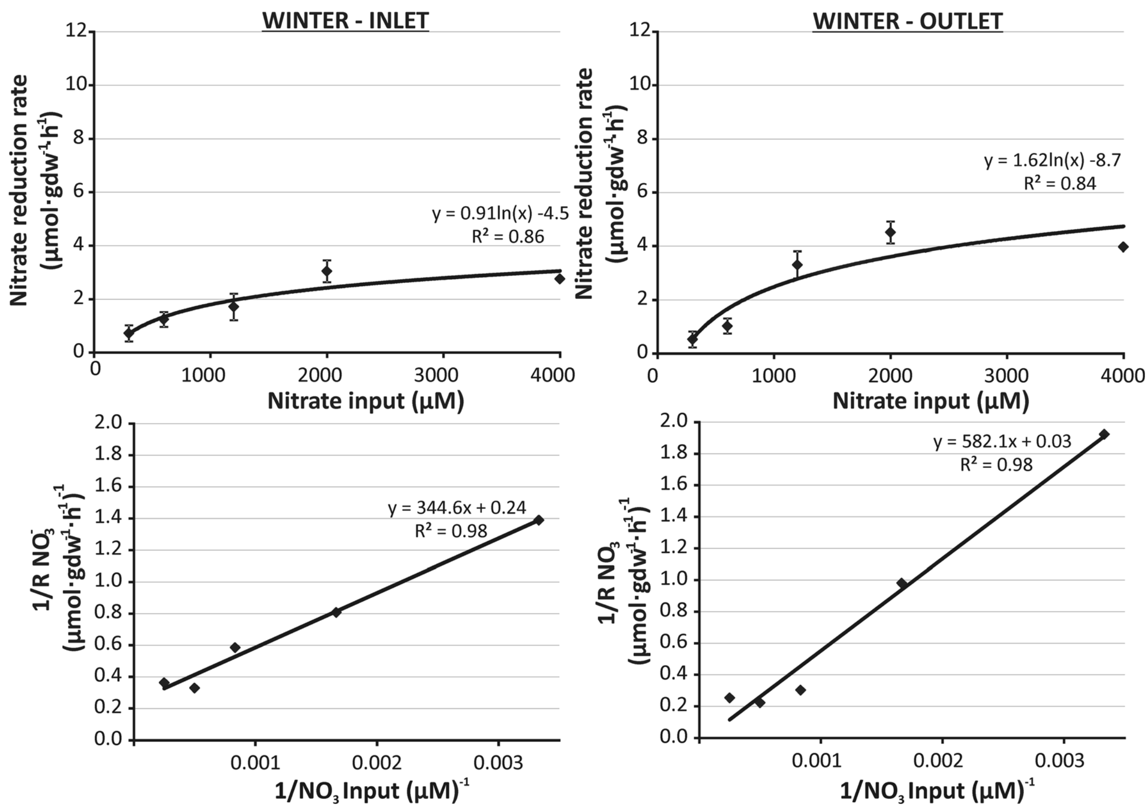

Fig. 7 Nitrate reduction rate vs initial nitrate concentration (black point), the line was fitted by use of the Michaelis-Menten equation (top graphics). Double reciprocal plot of the nitrate concentration vs nitrate reduction activity (down graphics), for Inlet sludge (left) and Outlet sludge (right) in winter

Although the adjustments for both Inlet and Outlet data were adequate, more flattened curves than in summer were obtained. This reflects the way in which lower temperature influences the delay of microbiological processes.

The kinetic parameter values using the double-reciprocal plot were calculated and are indicated in Table 2. The maximum nitrate reduction activities were obtained with the Outlet sludge and with the summer temperature. The lowest activity was recorded in winter season with the Inlet sludge, which could be conjugating a lower activity due to the low temperature along with a microbial structure that would have a lower presence of nitrate-reducing bacteria.

Table 2 Kinetic parameters calculated through in vitro FTR experiment using sewage sludge

Season	Sludge site	R_{max} (µmol-N·gdw^{-1}·h^{-1})	K_m (mg-N·L^{-1})
Winter	Inlet	4.1	6.1
	Outlet	32.6	47.7
Summer	Inlet	8.2	12.0
	Outlet	131.6	192.7

The Inlet sludge presented a greater affinity for nitrate (<K_m values), while in the Outlet sludge were recorded the lowest affinities (>K_m values). In addition, Reay et al. (1999) found a reduction in the ability of the organisms to sequester inorganic nitrogen from the surrounding environment with the decrease of temperature.

Dalsgaard and Bak (1994) studied the effect of the sulfide as an inhibitor of the NO_3^- reductive pathway. This could be differentially affecting the Inlet sludge, due to its proximity to the raw sewage entrance; it could contain a higher sulfide concentration than the Outlet, explaining in part the lower NO_3^- reduction activity found in the Inlet sludge.

In the literature, the NRR has been studied through laboratory experiments using microbial consortium from activated sludge. There are marked methodological differences with respect to our study, since these studies have been carried out in fed-batch reactors, inoculating with previously acclimatized microbial consortium (mainly by stepwise nitrate increase) or with concentrated microorganisms (by centrifugation or using an specific bacteria from a strain collection), and with the supplement of an external source of carbon as electron donor. Furthermore, these experiments have been of much longer duration (from 1 to 8 weeks) than the residence time (1.6 h) inside our reactors. However, taking into account these experimental conditions, it is possible to take them as reference values, which have NRR values that are widely higher than those reported in our study with sludge of a sewage stabilization pond (Table 3).

Comparing the kinetic parameters obtained in our study with those registered in FTR experiment using sediment from natural environments, it is clearly observed that the R_{max} obtained with the sludge from WSP far exceed those registered in natural sediments (2.7 to 3300 times higher; compared with Laverman et al. 2006, 2009, 2012; Torres et al. 2016).

Regarding the half-saturation constant, in the most cases, our K_m values were higher than those reported in the literature (Laverman et al. 2006, 2009; Torres et al. 2016). This situation implies a lower affinity for nitrate; in FTR studies, it has been associated with matrices with a great diversity of benthic communities of denitrifying organisms (Laverman et al. 2007, 2009). It is due to its origin that the bacterial complexity is expected to be greater in the sludge collected from wastewater treatment systems. The bacterial community structure has been studied in sewage sludge, presenting a high bacterial diversity (Nascimento et al. 2018) and in particular of denitrifying bacteria (Lee et al. 2002).

Our research recorded a significant nitrate reduction activity on a laboratory scale using sludge from a sewage stabilization pond. As a future challenge, it still remains to study in depth how much of the reduction of nitrite and nitrate could be replicated in a facultative pond at full scale, as a consequence of vertical mixing caused by wind effect. To our knowledge, there are no studies of the wind effect on nutrient concentrations in the water column, but there are researches on thermal, dissolved oxygen, chlorophyll "a," and BOD_5 stratifications: Meneses et al. (2005) detected vertical mixing processes with wind speed between 1 and 4 $m \cdot s^{-1}$ and Ukpong (2013) with 1.64, 1.88, and 2.27 $m \cdot s^{-1}$. In the specific case of the Patagonian coastal region, it is characterized by strong winds, with monthly average speed of 4.3 $m \cdot s^{-1}$ and events every month that are around 23 $m \cdot s^{-1}$ (Frumento and Contrera 2017). This is a reason why oxidized nitrogen forms can reach the bottom pond.

In recent years, several studies have addressed the use of new technologies which take advantage of different metabolic pathways of the nitrogen cycle, for sustainable removal of nitrogen from municipal wastewater, at the same time that search for saving in aeration energy, in cost of incorporating external organic matter, and reduce the production of sludge. These studies have evaluated the following topics: (1) Potential dissimilatory nitrate reduction by anammox bacteria, using nitrite as intermediate coupled to the oxidation of volatile fatty acids (Castro-Barros et al. 2017). (2) Partial nitritation-anammox wastewater treatment plants, ammonium-oxidizing bacteria (AOB) convert approximately half of the supplied ammonium to nitrite under O_2 limitation, and in turn, nitrite together with the remaining ammonium is converted to N_2 by anammox bacteria (Hu et al. 2013; Cao et al. 2016). (3) Nitrite production incentive via partial denitrification coupled with anammox process (Wang et al. 2019). These treatments perform a "domestication of sludge" from conventional treatment systems, looking for sludge of absolute dominant bacteria (anammox or denitrification bacteria) (Miao et al. 2018; You et al. 2020). Based on our results, stabilization pond sludge could be a novel and interesting matrix for use in these innovative treatments.

Conclusions

Our study presents the first in vitro records of nitrogen dynamics using the flow-through reactor technique, with sludge from a full-scale WSP, which represents a simple and low-cost method compared to other complex techniques.

- The sludge had an impressive water content with extremely fine particles and high organic matter content, much greater than in natural sediment used to determine the kinetics of nitrate reduction.

Table 3 Reducing nitrate activity under different experimental conditions

Microbial consortium	C source	Range of NR rate—nitrate removal efficiency	Reference
Thickened activated sludge	Acetate	1.78–3.86 mmol $N\text{-}NO_3^- \cdot g\text{-}MLSS^{-1} \cdot h^{-1}$	Glass and Silverstein 1998
Sludge from a fertilizer industry plant	Acetate	1.49–2.3 mmol $N\text{-}NO_3^- \cdot g\text{-}MLSS^{-1} \cdot h^{-1}$	Nair et al. 2007
Pure culture of *Paracoccus denitrificans* and *P. fluorescens*	Ethanol	1.87–21.2 mmol $N\text{-}NO_3^- \cdot Kg^{-1} \cdot h^{-1}$	Vacková et al. 2011
Mixed microbial culture with activated sludge			
Denitrification bacteria isolated from activated sludge	Glucose Acetate Citrate	56.6±3.8% 74.8±0.8% 80.7±1.6%	Yang et al. 2012
Pure culture of *Halomonas desiderata*	Acetate	0.063 mM $NO_3^- \cdot L^{-1} \cdot h^{-1}$	Alquier et al. 2014
Pellet from activated sludge	Acetate	0.06–0.97 mM $NO_3^- \cdot h^{-1}$	Durban et al. 2020

- Nitrate reduction rates followed the Michaelis-Menten function. The maximum nitrate reduction activity was obtained with the summer temperature and using the sludge collected from the outlet of the treatment system.
- The differences recorded in terms of the nitrate reduction activity and the affinity constant could be explained by the temperature range tested, and also, by the differential composition of sludge from the two sampling sites. The high nitrate reduction rates along with a low recovery of nitrite in the percolated water would indicate a great tendency to the nitrogen reduction, due to the generation of gaseous forms of nitrogen that escape from the water (denitrification or Anammox) or ammonium production (DNRA).
- In addition, the nitrate reduction activity with sewage sludge was considerably higher than those recorded in sediments from natural aquatic ecosystems. This fact provides a perspective of a relevant participation of nitrate/nitrite reduction processes in full-scale wastewater ponds, when by mixing processes the oxidized nitrogen forms reach the bottom zone. Finally, more research is needed to determine the potential use of sludge from stabilization ponds as an inoculum in innovative systems for nitrogen removal.

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Author contribution MF participated in samplings, laboratory analysis, data interpretation, and the writing of the original draft. AT participated in the experiments design, nutrient analysis, and the manuscript review and edition.

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Declarations

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