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The role of nitrate and nitrite in a granular sludge process treating low-strength wastewater

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ABSTRACT

Granular sludge has recently come under study as an efficient technology in wastewater treatment. Different microorganisms coexisting within the granules allow simultaneous nitrification, denitrification and phosphorus removal (SNDPR). However, the behaviour of the process varies when nitrogen and phosphorus removal are coupled. The purpose of this paper is to study the SNDPR process in granular sludge with low-strength wastewater, focusing on the effect of nitrification products such as nitrite and nitrate on phosphorus removal. Results showed that aerobic granules allowed anoxic phosphorus uptake using either nitrite or nitrate as an electron acceptor. Phosphorus uptake rates had similar values using either product of nitrification (13.64 mg P g^{-1} VSS h^{-1} in the presence of nitrate and 12.20 mg P g^{-1} VSS h^{-1} in the presence of nitrite) without any observed nitrite inhibition of phosphorus uptake. However, different P uptake-N removal ratios were found in simultaneous denitrification and phosphorus removal. Nitrate was the best option for phosphorus-rich wastewater treatment (3.04 mg P mg^{-1}N), while nitrite was more beneficial with the SNDPR of nitrogen-rich influents (1.68 mg P mg^{-1}N).

1. Introduction

Nitrogen and phosphorus removal is one of the latest objectives of wastewater treatment under the European Water Framework Directive (2000/60/CE). Alternating anaerobic–aerobic–anoxic conditions for classical nitrification–denitrification and phosphorus removal processes allow complete biological nutrient removal (BNR). When different microorganisms coexist in the same tank (i.e. a sequencing batch reactor or SBR) simultaneous nitrification–denitrification (SND) can take place as a result of an existing oxygen gradient, with denitrification occurring in the areas of low dissolved oxygen (DO) concentrations [1]. Of particular interest in the SND process is the fate of nitrite, which is an intermediate of both nitrification and denitrification. For the most effective nitrogen removal, any nitrite that is produced by nitrification should be reduced to N_2 rather than oxidized to nitrate, which represents an unnecessary use of oxygen [2] and a reduction of nearly 40% of the organic matter demand for denitrification [3]. Phosphorus removal is accomplished by polyphosphate accumulating organisms (PAOs) and at least a fraction of which have the capacity to denitrify (these are known as denitrifying PAOs, or DPAOs) using nitrate and/or nitrite instead of oxygen as an electron acceptor for P removal [4]. Denitrification by DPAOs, therefore, would enhance simultaneous nitrification, denitrification and phosphorus removal (SNDPR) in a single reactor [5].

In light of these simultaneous processes, granular sludge has recently been proposed as a compact and dense accumulation of biomass for wastewater treatment [6], and its qualities, such as high settling velocities, high biomass retention, high activity and an ability to withstand high loading rates, have been widely described in the literature [7,8]. Nutrient removal has also come up as a topic in aerobic granulation [9,10] but no reports linking it with low-strength wastewater have been presented. In terms of biochemical reaction rates, the biggest and densest granules present a more significant mass transfer limitation [11]. Carvalho et al. [12] found that oxygen concentration profiles within the granule had a linear profile at depths greater than 300 μm. The apparent physical separation of microorganisms could potentially affect mass transfer, but would not impact negatively on the overall removal efficiency. Hence, oxygen and substrate mass transfer within an aerobic granule could enhance SNDPR.

In granular sludge, factors such as diffusion coefficients, conversion rates, granule size and biomass spatial distribution affect the concentration profiles of each compound in a BNR system. The effect of separate factors cannot be studied experimentally as they all strongly influence each other [13]. Bearing this in mind, the

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Table 1
Influent concentrations, loads applied and removal efficiency of the granular SBR.

	Influent ($mg\,L^{-1}$)	Load ($Kg\,m^{-3}\,d^{-1}$)	Removal (%)
Organic matter (COD)	616	0.76	88
Nitrogen	74.2	0.09	92
Phosphorus	5.65	0.007	99

main objective of this paper is to experimentally analyse SNDPR in a granular sludge system. The basis of the study is the effect of products of nitrification such as nitrite and nitrate on phosphorus removal to enhance SNDPR when treating low-strength wastewater. In addition to an attempt to study the separate factors affecting nutrient removal, analyses of cycle studies and batch tests were carried out to identify the interactions contained within all the biological reactions in the same reactor.

2. Materials and methods

2.1. Experimental set-up

A 30 L cylindrical glass SBR with an internal diameter of 28 cm was used in this study. The maximum working volume applied (V_{max}) was 27 L, resulting in a height to diameter ratio (H/D) of 1.6. The reactor was operated using a two step-feed strategy (total volume per cycle, V_{in}, of 11 L) in 8-h cycles. The volumetric exchange ratio (VER, V_{in}/V_{max}) and hydraulic retention time (HRT) were set at 41% and 0.81 days respectively. The dissolved oxygen set point for aerobic conditions was fixed at $1.5\,mg\,O_2\,L^{-1}$ by an on/off control. The applied cycle consisted of, first, a fill-anaerobic–aerobic sequence (0.25 h–2.3 h–3.1 h); second, a fill-anoxic–aerobic sequence (0.1 h–1.25 h–0.72 h); third, settling (0.03 h); and finally, the effluent discharge (0.25 h). The SBR was fed with enriched ethanol synthetic wastewater (described in Puig et al. [14]) with average COD, ammonium and phosphate concentrations similar to those of typical domestic wastewater and summarised in Table 1.

The reactor was seeded with floccular sludge from an urban wastewater treatment plant (Sils-Vidreres, Girona, Spain). To achieve microorganism diversity, the process was run for 30 days under the conditions previously described for the enhancement of nitrogen and phosphorus removal, with 40 min of settling time. After this acclimation period, the system was stimulated for granulation by reducing the settling time from 40 to 2 min in 20 days, using the methodology described in Coma et al. [15]. The settling time decrease forced a reduction of the minimum settling velocity allowed for the sludge to be kept in the reactor from 0.28 to $5.5\,m\,h^{-1}$. Using this procedure, small and slow-settling flocs were removed from the system through the effluent. That allowed diminishing the competition from substrate uptake from the sludge mixture and hence the substrate was made more available for large and compact flocs and granules [16]. Cycle studies were carried out periodically after the granulation process in order to evaluate the nutrient removal performance.

2.2. DPAO batch experiments

Batch experiments were carried out in sealed glass reactors using 2 L of granular sludge taken from the parent SBR at the end of the aerobic phase. The sludge was exposed to anaerobic conditions for 190 min and then aerobic or anoxic conditions for 150 min to determine phosphorus release and uptake rates, using oxygen, nitrite or nitrate as electron acceptors. The reactors were fed with synthetic wastewater composed of acetate (as organic matter), ammonium, phosphate and microelements. Batch tests were performed at the same initial concentrations of organic matter, ammonium and phosphorus (40 mg TOC L^{-1}, 4.9 mg N-NH_4^+ L^{-1} and 3.5 mg P-PO_4^{3-} L^{-1} on average). After exposure under anaerobic conditions, dissolved oxygen was fixed at $1.5\,mg\,O_2\,L^{-1}$ for one experiment and at $7\,mg\,O_2\,L^{-1}$ for the second test under aerobic conditions. Nitrite or nitrate solutions were added as a pulse for the anoxic phases. The nitrate and nitrite concentration inside the reactors after the pulse was $20\,mg\,N\,L^{-1}$. This concentration was chosen because it was the theoretical maximum achieved in the parent SBR with low-strength wastewater. On the nitrite test, the maximum free nitrous acid (FNA) calculated at the working pH [17] was $0.0026\,mg\,N\text{-}HNO_2\,L^{-1}$ when the pulse was introduced. The system was thermostated at $20.0 \pm 0.5\,^{\circ}C$. The pH range varied from 7.3 to 8.2. The ratio between the anoxic phosphate uptake rate (PUR) and the aerobic PUR was used as an index of DPAO activity [18].

2.3. Analyses

Analyses of the ammonium (N-NH_4^+), total Kjeldahl nitrogen (N-TKN), chemical oxygen demand (COD), mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) were performed according to *Standard Methods for the Examination of Water and Wastewater* [19]. Total organic carbon (TOC) was measured with a Shimadzu TOC-VCSH analyser. Nitrite (N-NO_2), nitrate (N-NO_3) and phosphate (P-PO_4^{3-}) were analysed using ionic chromatography (Metrohm 761-Compact; 4110B). The granule morphology was investigated using a stereomicroscope SteREO Discovery V12. Fluorescent in situ hybridisation (FISH) was performed to quantify the composition of phosphorus and nitrogen removal bacteria. Granules were previously smashed before fixation to obtain homogeneous samples. The probes and methodology used for the detection of population involved in enhanced biological phosphorus removal (EBPR), known as PAOs (PAO462, PAO651 and PAO846), GAOs (GAO431 and GAO989) and alphaproteobacterial GAOs (ALF969, SBR9-1a, TFO-DF218, TFO-DF618, DF988 and DF1020), have been described in Puig et al. [20]. To identify nitrifying bacteria, a Fluos-labelled NSO1225 probe was used for ammonium oxidising bacteria [21], and for nitrite oxidising bacteria a Cy3-labelled probe (consisting of NIT3, compNIT3 [22] and Ntspa-0662 and compNtspa-0662 [23]) was used. The probed sludge was examined using a Leica® confocal laser microscope. The area containing specific labelled probe cells was quantified as a percentage of the area of the entire bacterial population (EUBMIX).

3. Results

3.1. Granular nutrient removal performance

An SBR was started up for biological nutrient removal and granulation purposes. After the settling time was fixed at 2 min, granules appeared in the reactor and it took 3 months to obtain a fully granulated system. Only when stable granules were the whole biomass present in the system, organic matter, ammonium and phosphate were removed from it (Table 1). The mean sludge retention time (SRT) presented during this period was 12 days (6 days under aerobic conditions, SRT_{AER}). Effluent concentrations were below the discharge limits allowed ($125\,mg\,O_2\,L^{-1}$; $15\,mg\,N\,L^{-1}$ and $2\,mg\,P\,L^{-1}$; 91/271/CEE). Fig. 1 presents the effluent concentrations of nitrite, nitrate and phosphate during the period of study when granules were completely formed and BNR achieved.

As previously noted, ammonium was not present in the effluent during the granular nutrient removal performance. However, nitrites and nitrates as products of nitrification were detected at low concentrations in the effluent ($3 \pm 1\,mg\,N\,L^{-1}$). Fig. 1 shows that two periods can be clearly differentiated. In Period I nitrite was the sole product of nitrification, while in Period II nitrate was

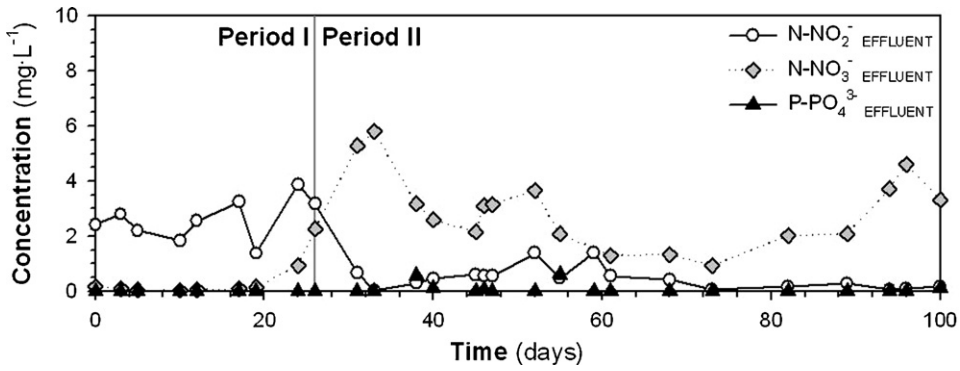

Fig. 1. Nitrite, nitrate and phosphate effluent concentrations from granular nutrient removal SBR.

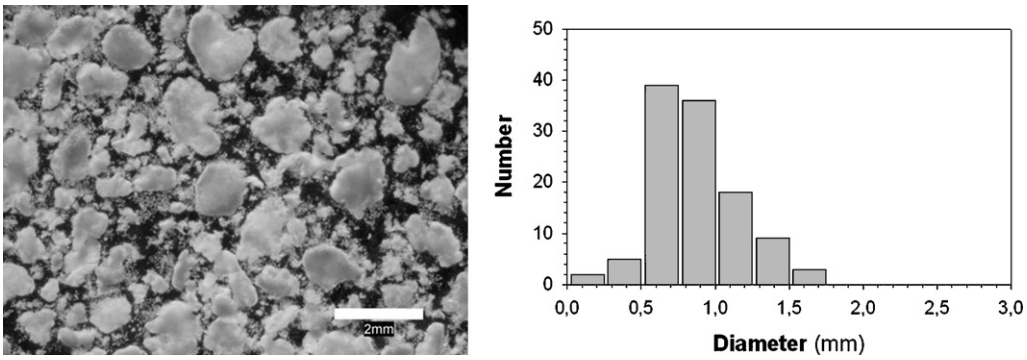

Fig. 2. Stereomicroscope image (left) and size distribution (right) of the granule.

the main nitrogen compound. Phosphorus effluent was less than 0.5 mg N L^{-1}. Once a stable granular system had been achieved at the end of the performance, the mean carbon, nitrogen and phosphorus removal efficiencies were 88%, 92% and 99% respectively.

In terms of physical analysis, Fig. 2 shows a stereomicroscope image and the size distribution of the granules obtained. The surface of the granules was anamorphous, with a mean diameter of 0.8 ± 0.3 mm, and the granules' settling abilities were corroborated by a sludge volumetric index (SVI) of 59 ± 7 mL g^{-1}. In terms of the microbial population, Fig. 3 depicts micrographs obtained for nitrifying and phosphorus removal bacteria. FISH results showed an enrichment of EBPR organisms during and after the granulation period, with 58% *Accumulibacter* (PAO) and 18% *Competibacter* (GAO) being achieved. For nitrogen removal bacteria, the percentages were 9% for ammonia oxidising bacteria (AOB) and 6% for nitrite oxidising bacteria (NOB).

Cycle studies were carried out to evaluate nutrient removal performance with granules. Fig. 4 shows a typical cycle profiles for Period I (nitrite production) and Period II (nitrate production).

During the anaerobic phase, PAOs took up the organic matter, thereby reducing the organic carbon concentration (Fig. 4A), while phosphate was being released into the bulk liquid (Fig. 4C) in both periods. In the subsequent aerobic phase, the phosphate uptake rate (PUR) was 8.24 mg P g^{-1} VSS h^{-1} in Period I and

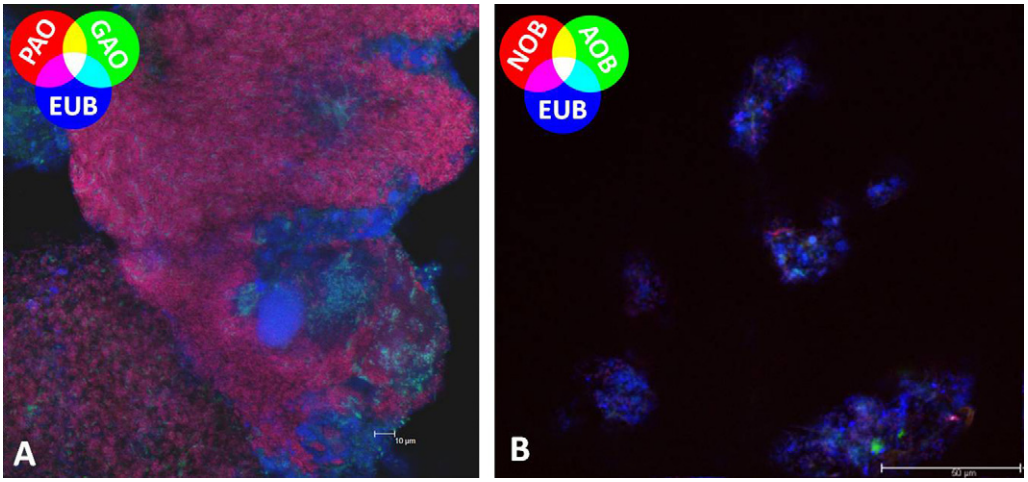

Fig. 3. Confocal laser microscope images for *Accumulibacter* (in red) and *Competibacter* (in green) on micrograph A (scale bar 10 μm) and ammonia oxidising bacteria (in green) and nitrite oxidising bacteria (in red) on micrograph B (scale bar 50 μm). All bacteria were stained in blue. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

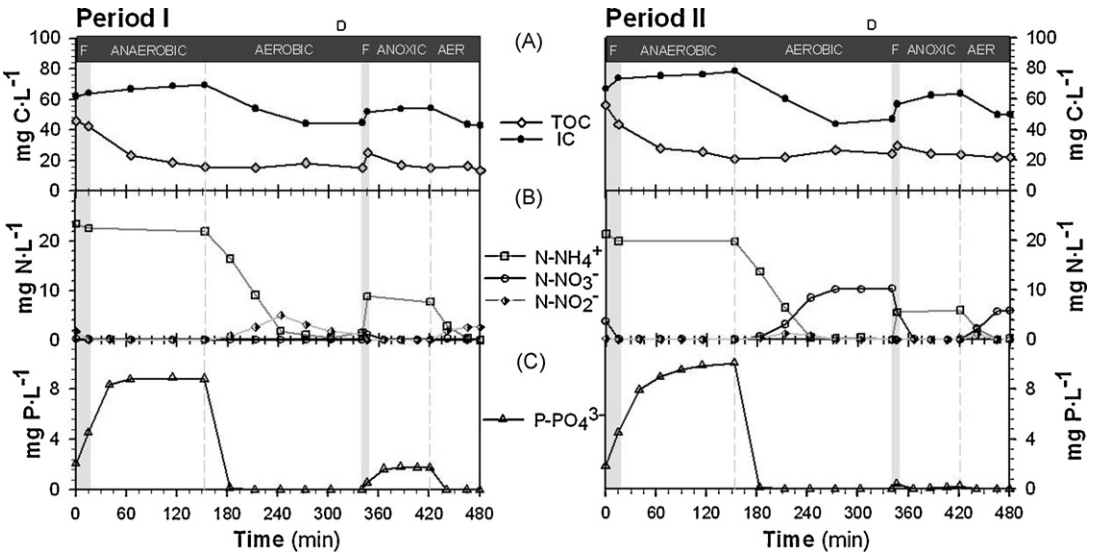

Fig. 4. Typical cycle profiles concentrations of total organic carbon and inorganic carbon (A), ammonium, nitrite and nitrate (B) and phosphate (C) from Period I (nitrite accumulation, left) and Period II (nitrate accumulation, right) of the granular nutrient removal SBR.

6.2 mg P g^{-1} VSS h^{-1} in Period II. At the same time, nitrification took place leading to a clear reduction in inorganic carbon (IC, Fig. 4A). In the first period, not the nitrate but the nitrite detected during ammonium removal indicated decoupling in the nitrification population.

The ammonium depletion (20.1 mg N L^{-1}) at minute 240 of the cycle and the nitrite release (5.1 mg N L^{-1}) were unbalanced. This indicated that not only nitrification but also a denitrification process had taken place under aerobic conditions and without any soluble organic matter, as TOC remained constant in the aerobic phase (Fig. 4A, Period I). Nitrite did not appear in the bulk liquid until phosphate was practically removed (i.e. minute 180). Furthermore, the nitrite concentration decreased after the available ammonia had been completely oxidised, so that at the end of the first aerobic phase (minute 340) only 1.2 mg N-NO_2^- L^{-1} were present in the media. In Period II, nitrate rather than nitrite appeared during the nitrification process (Fig. 4B, Period II), but only after phosphate had been totally removed from the system (minute 180, Fig. 4C, Period II). From this point on, no nitrate reduction was achieved and at the end of the first aerobic phase only 10.3 mg N-NO_3^- L^{-1} were present, representing just 52% of the ammonium oxidised (Period II, Fig. 4B).

After the second feeding (minute 340), the residual nitrogen from the previous aerobic phase was rapidly denitrified. In Period I, anaerobic conditions were achieved faster and phosphate was released using the surplus organic matter supplied in the second feed. Nevertheless, in Period II a higher denitrification was necessary during the anoxic phase because of the 10.3 mg N-NO_3 L^{-1} present at the beginning of the phase compared to the 1.2 mg N-NO_2 L^{-1} observed in Period I. Due to these high nitrate levels, not enough biodegradable organic matter was available for EBPR in the subsequent phases.

Both cycle performances (Fig. 4) evidenced nitrogen and phosphorus removal. The fact that neither nitrate nor nitrite was released in the media until phosphorus was depleted to zero seems to suggest the occurrence of aerobic nitrification coupled with denitrification and phosphorus removal (DPAO activity). The major differences in cycle performance in the two periods were found while nitrification and phosphate uptake were taking place in the first aerobic phase. The highest PUR (8.24 mg P g^{-1} VSS h^{-1}) was achieved using nitrite as the electron acceptor, with SND reaching 61% in the first aerobic phase of Period I (Fig. 4). Conversely, when nitrate was the main nitrification product (Period II, Fig. 4) SND only increased to 22% in the first aerobic phase and neither SND nor phosphorus removal was detected in the second phase.

Results obtained from the granular SBR's evolution indicated different behaviours depending on the product of nitrification released into the media (either nitrite or nitrate). For this reason, batch tests were carried out to evaluate the SNDPR activity of the granular sludge using different electron acceptors (i.e. nitrite, nitrate and oxygen).

3.2. Batch tests for simultaneous nitrogen and phosphorus removal

Cycle analysis showed unbalanced ammonium oxidation (to nitrite or nitrate) under aerobic conditions. Moreover, nitrite and nitrate production was not observed until phosphorus was totally taken up. As a result of this, the hypothesis that denitrifying PAOs (DPAOs) were the major phosphorus removal population was proposed.

In order to confirm the hypothesis and quantify the activity of DPAOs in the system, batch tests under anaerobic–anoxic conditions were performed and compared with anaerobic–aerobic tests in terms of the activity of the entire phosphorus removal population. In addition, nitrite and nitrate were evaluated as different electron acceptors for DPAOs under anoxic conditions, and different oxygen concentrations (1.5 and 7 mg O_2 L^{-1}) were tested to study the performance of SNDPR in granular sludge. Fig. 5 shows the data obtained under nitrate (A), nitrite (B) and low and high oxygen (C and D) concentrations during the batch tests.

The anaerobic–anoxic tests with a nitrate (Fig. 5A) and nitrite (Fig. 5B) pulse were fed with 20 mg N L^{-1} at the beginning of anoxic conditions. This concentration was chosen because it was the maximum amount of nitrogen achieved inside the granular SBR during the whole performance. Phosphorus and nitrate were completely taken up in the nitrate test (Fig. 5A). However, some phosphorus remained in the media at the end of the second test when nitrites were completely consumed (Fig. 5B, around 2 mg P g^{-1} VSS), probably due to the lower electron transference using nitrite instead of nitrate as electron acceptor. Both experiments showed a smooth reduction of 0.6 mg N-NH_4^+ g^{-1} VSS in the ammonium concentration, which can be attributed to biomass assimilation processes.

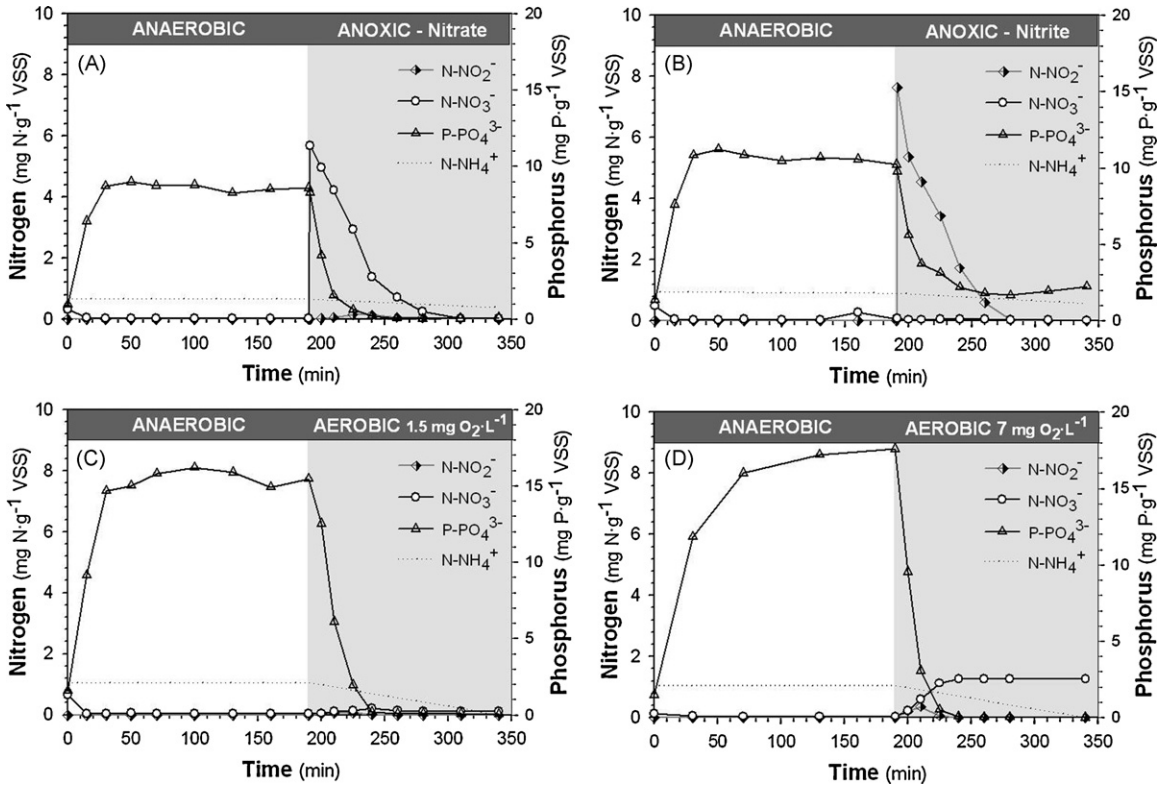

Fig. 5. Specific profiles of batch test for simultaneous nitrogen and phosphorus removal under nitrate (A), nitrite (B), low oxygen concentration (C) and high oxygen concentration (D).

Tests carried out under anaerobic–aerobic conditions showed some differences in nitrogen compounds at different DO set points (1.5 and 7 mg O_2 L^{-1}). In both cases phosphorus was quickly taken up from the bulk liquid. Ammonium was completely oxidised during the aerobic phase in both tests. However, the low oxygen concentration test (Fig. 5C) did not show any nitrogen compounds at the end of the experiment, as low DO allowed nitrogen removal by SND [1]. The test for high oxygen concentration (Fig. 5D) registered a nitrate concentration at the end (1.27 mg $N\text{-}NO_3^-$ L^{-1}) as a result of nitrification during the aerobic period.

Table 2 summarises the parameters obtained during the batch test experiments. On the one hand, the PUR obtained from anaerobic–aerobic tests showed higher values than those obtained from anaerobic–anoxic tests. This could be explained by DPAOs having enzymes that allow them to accomplish aerobic EBPR metabolism, whereas PAOs require a long time to synthesise the necessary enzymes for anoxic EBPR metabolism [24]. On the other hand, anaerobic–anoxic tests resulted in similar PUR values using nitrate and nitrite as electron acceptors (13.64 mg P g^{-1} VSS h^{-1} and 12.20 mg P g^{-1} VSS h^{-1} respectively), as well as similar ratios for DPAO activity (47% in the nitrate test, 42% in the nitrite test). Nevertheless, in terms of the ratio of phosphorus uptake during nitrogen removal (P_{UP}/N_{REM}), a higher amount of phosphate can be removed using nitrate (3.04 mg P mg^{-1}N) rather than nitrite (1.68 mg P mg^{-1}N) as the electron acceptor.

4. Discussion

The SBR cycle studies involved simultaneous nitrogen and phosphorus removal. According to the literature, DPAOs in BNR systems are highly beneficial in terms of a lower COD requirement (because the same carbon source is used for both N and P removal) and a reduced aeration cost [25]. However, reliable termination of nitrification at nitrite (nitritation) has proved difficult to achieve in the treatment of domestic wastewater [26]. The growth of nitrite oxidising bacteria (NOB) can be limited by numerous factors as temperature, SRT and the combination of both (AOB grow faster at 20 °C), oxygen limitation (enhanced by diffusion through the granule) and free ammonia (FA) and free nitrous acid (FNA) [27], both of them insignificant at domestic wastewater concentrations. The use of granular sludge in domestic treatment could benefit nitritation and subsequent denitrification and phosphorus removal via nitrite thanks to the diffusion of nutrients and oxygen through the granule. In this hypothetical case, according to Carvalho et al. [12], AOB would be positioned on the outer part of the granule where oxygen is less limited and denitrifiers and/or PAOs would remain in the inner part (at depths greater than 300 μm) under anoxic or anaerobic conditions. This hypothesis was accepted in this study as the mean granular size was between 0.5 and 1 mm.

However, the results raised various questions about SND via DPAOs, such as the use of nitrite as an electron acceptor when

Table 2
Phosphorus and nitrogen ratios during batch test.

Batch operation	PUR (mg P g^{-1} VSS h^{-1})	P_{UP}/N_{REM} (mg P mg^{-1}N)	PUR/PURaer
Test 1(nitrate) – Fig. 5A	13.64	3.04	0.47
Test 2 (nitrite) – Fig. 5B	12.20	1.68	0.42
Test 3 (1.5 mg O_2 L^{-1}) – Fig. 5C	23.14	–	–
Test 4 (7 mg O_2 L^{-1}) – Fig. 5D	29.26	–	–

treating low-strength wastewater. Results obtained from batch tests showed that PAOs use both nitrite and nitrate for phosphorus removal in granular sludge. Nevertheless, a higher P_{UP}/N_{REM} ratio was found when using nitrate rather than nitrite as the electron acceptor (3.04 and 1.68 mg P mg^{-1}N respectively). This fact is because of the higher electron transfer from nitrate rather than nitrite during denitrification. At the same time, similar anoxic PUR were obtained from both nitrate and nitrite test. Although nitrite has been considered a potential inhibitory compound for P uptake (0.4 mg N g^{-1} VSS and 2 mg N g^{-1} VSS for aerobic and anoxic phosphate uptake respectively according to the literature [4,28,29]), Zhou et al. [17] have suggested nitrous acid rather than nitrite as the true inhibitor in the anoxic phosphorus uptake (0.02 mg N-HNO_2 L^{-1} completely blocks phosphate uptake). When working with low-strength wastewater, nitrogen does not usually reach high concentrations. Therefore, even if nitrite is formed in granular sludge, FNA will not be enough to completely inhibit phosphorus removal (maximum FNA of 0.0026 mg N-HNO_2 L^{-1} tested in this study).

In light of simultaneous removal, according to Metcalf and Eddy [3], nitrite would require a smaller organic matter consumption for denitrification (e.g. 1.99 g bsCOD g^{-1} N-NO_2^-) compared to denitrifying nitrate (e.g. 3.3 g bsCOD g^{-1} N-NO_3^-). This, together with the smaller P_{UP}/N_{REM} ratio obtained using nitrite, would lead to an enhancement of SNDPR when treating nitrogen-rich wastewater (low P/N ratios). Conversely, nitrate would be the best option when dealing with phosphorus-rich wastewater (high P/N ratios) for SNDPR purposes.

5. Conclusions

Biological nutrient removal by granular sludge can be achieved for low-strength wastewater (i.e. domestic sewage) treated in an SBR working at an exchange ratio of 41%. Granular sludge allows interactions between different microorganisms. PAOs can take advantage of that, using both nitrite and nitrate as products of nitrification under aerobic conditions because of oxygen diffusion into the particles. Both electron acceptors (nitrite and nitrate) have similar rates for phosphate uptake. However, nitrate will be more beneficial in phosphorus-rich wastewater treatment (high P/N ratio) as it shows a P_{UP}/N_{REM} ratio of 3.04 mg P mg^{-1}N, higher than the one obtained using nitrite (1.68 mg P mg^{-1}N). Furthermore, nitrite could be used in nitrogen-rich wastewater treatment (low P/N ratio) when working with granular sludge for SNDPR purposes. Nitrite would not cause inhibitions on phosphate uptake due to the characteristics of the low-strength wastewater and the granule morphology. Additional studies need to be carried out in order to take advantage of anoxic phosphorus removal via nitrite in a granular sludge process.

Acknowledgments

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