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Inhibition of free nitrous acid and free ammonia on polyphosphate accumulating organisms: Evidence of insufficient phosphorus removal through nitritation-denitritation

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ABSTRACT

The purpose of this study is to investigate the effect of Free Nitrous Acid (FNA) and Free Ammonia (FA) on enhanced biological phosphorus removal (EBPR) and in particular on the aerobic phosphorus uptake rate (PUR). To this end, a PAO-enriched biomass was developed at a lab-scale reactor in order to fuel a series of ex-situ batch experiments to test the effect of various nitrite or ammonium concentrations on the phosphorus uptake rate at different pH values. FNA was found to be a strong inhibitor of EBPR, in agreement with other studies with PUR being inhibited by 50 % under 1.5 μg HNO_2–N L^{-1} and 100 % at 13 μg HNO_2–N L^{-1}. FA was also found to inhibit EBPR with PUR being inhibited by 50 % under 6.4 mg NH_3–N L^{-1}. The results of this study suggest that EBPR under high nitrogen loading alongside nitritation-denitritation may not be a viable option.

1. Introduction

The effective removal of nutrients during wastewater treatment is considered essential to ensure good water quality and the prevention of eutrophication phenomena in the recipient water bodies. Ammonium nitrogen is commonly removed via nitrification and denitrification. Nitrification is accomplished by a series of consecutive biological reactions where ammonium is oxidized to nitrite by Ammonium Oxidizing Bacteria (AOB) and finally to nitrate by Nitrite Oxidizing Bacteria (NOB). In the presence of a carbon source and the absence of oxygen, nitrate is used as an electron acceptor by heterotrophic organisms and converted to nitrogen gas (denitrification).

Phosphorus is removed from wastewater either biologically or chemically. The application of Enhanced Biological Phosphorus Removal (EBPR) is considered preferable to chemical removal, due to lower operational costs. EBPR is carried out by Polyphosphate Accumulating Organisms (PAOs) and is achieved by implementing the alternation of anaerobic and aerobic/anoxic conditions. Under anaerobic conditions, PAOs can hydrolyze their intracellular polyphosphate chains and use the energy produced to uptake Volatile Fatty Acids (VFAs) and store them as polyhydroxyalkanoates (PHAs). Under aerobic and/or anoxic conditions, PAOs oxidize the stored PHAs, which serve as both the carbon and energy source for the uptake of orthophosphates and the formation of polyphosphate chains. Thus, phosphorus removal is achieved via the removal of the enriched sludge.

During the anaerobic stabilisation of sewage sludge, the reject water produced (especially from the dewatering unit) contains high concentrations of ammonium and phosphorus. In their recent study, Noutsopoulos et al. (2018) report total nitrogen and phosphorus concentrations in reject water of a dewatering unit downstream of an anaerobic digestion unit as high as 1300 mg L^{-1} and 72 mg L^{-1} respectively. These liquid streams are typically recycled to the WWTP's inlet, thus significantly increasing nutrient loading by up to 15–20 % (Gustavsson, 2010). Recently, the side-treatment of these streams prior to recirculation has emerged as an attractive strategy. It has been widely reported that AOB present a significantly stronger tolerance to high ammonium concentrations than that presented by NOB. As a result, in the presence of the high ammonium concentrations of sludge reject water, the second stage of nitrification would most likely be inhibited, therefore promoting NOB shunt. Besides NOB inhibition, nitrite, like nitrate may also be readily reduced by heterotrophic organisms. Nitrogen removal via nitritation-denitritation possesses several advantages over conventional

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nitrification-denitrification, specifically a reduced oxygen demand, lower carbon source requirements and a reduced biomass production (Guo et al., 2010).

Regarding phosphorus removal however, the achievement of EBPR under conditions prevailed in the nitritation-denitritation process may be problematic, as nitrite has been reported to inhibit the process (Saito et al., 2004). In reality, it has been reported that the protonated form of nitrite (Free Nitrous Acid, FNA) rather than nitrite is the actual inhibitor of PAOs (Zhou et al., 2007). Consequently, nitrite would have a significantly greater effect on EBPR at lower pH as its protonated form is in greater abundance. Zhou et al. (2011) suggested that the toxicity mechanism of FNA was associated with its effect on bacterial membranes and intracellular energy generation. FNA increasing concentrations have been found to positively correlate with reduced intracellular adenosine triphosphate (ATP) levels (Zhou et al., 2010). Accumulation of nitrite/FNA is known to inhibit P-uptake which is likely the result of harm to polyphosphate kinases (PPK) or PHAs consumption (Wang et al., 2011). The degrees of FNA inhibition on a specific process also vary in the literature. In their study, Saito et al. (2004) observed 100 % inhibition of aerobic phosphorus uptake rate (PUR) at the FNA concentration of 1.5 μg L^{-1}. Pijuan et al. (2010) observed aerobic PUR inhibitions of the order of 50 % and 100 % at the concentrations of 0.52 μg FNA L^{-1} and 4 μg FNA L^{-1}, respectively. In their experiments on biomass acclimated to the presence of nitrite, Zhou et al. (2012) observed 100 % inhibition of aerobic PUR at 10 μg FNA L^{-1}, while they also found anoxic PUR to be inhibited at 5 μg FNA L^{-1}. The differences in PAO's resilience to FNA that have been reported may be attributed to different degrees of acclimation to nitrite of the biomass used. This could be reflected in the distribution of the different PAO subtypes in the biomass. The main organism responsible for EBPR is *Candidatus Accumulibacter phosphatis* (Oehmen et al., 2007) and according to analysis of the polyphosphate kinase gene (ppk), which is thought to be primarily responsible for poly-P synthesis in bacteria, the most dominant clades of the species are: *Accumulibacter* type I (PAO I) and *Accumulibacter* type II (PAO II). Each clade may be further separated into individual strains. There are 5 known strains for PAO I (IA-IE) and 9 known strains for PAO II (IIA-III) although there is evidence to suggest a greater abundance (Song et al., 2019). Each type and their strains differ in their ability to use nitrate and nitrite as electron donors, as well as their tolerance to inhibitors such as FNA. In general, PAO II appear to be more resistant to FNA than PAO I. The most resistant strain to nitrite accumulation is PAO IID, which is the most abundant in nitrification systems (Zeng et al., 2016).

The inhibition of FNA on the growth of PAOs has also been reported. Pijuan et al. (2010) reported 50 % and 100 % inhibition for a non-acclimated biomass at the concentrations of 0.36 μg FNA L^{-1} and 7 μg FNA L^{-1} respectively. Zhou et al. (2012) studied the effect of FNA on a biomass acclimated to an FNA concentration of 0.9 μg L^{-1}. They reported that both aerobic and anoxic cell growth were fully inhibited at the FNA concentration of 10 μg L^{-1}.

Recently there have been reports that EBPR can also be inhibited by free ammonia (FA) (Zheng et al., 2013; Yang et al., 2018). The studies conducted up to this point mainly focused on the prolonged effect of FA on the growth of PAOs. Zheng et al. (2013) reported that the FA threshold concentration for PAOs was 17.76 mg NH_3–N L^{-1}. Analyses before and after treatment with such FA content showed that PAO population deteriorated significantly, while Glycogen Accumulated Organisms (GAOs) increased. In agreement to this, Yang et al. (2018) reported that PAOs were significantly inhibited (by over 90 %) after a 1-day exposure to 16 mg NH_3–N L^{-1}. Therefore, FA inhibition could provide a competitive advantage to GAOs over PAOs that would result in the deterioration of the EBPR process.

In view of the above, the purpose of this study is to investigate the inhibitory effect of FNA and FA on the aerobic PUR of PAOs and to propose kinetic models for aerobic phosphorus uptake focusing on FNA and FA inhibition. To this end, a PAO-enriched sludge was developed in an SBR operating in alternating anaerobic/aerobic/anoxic conditions and a series of ex-situ batch tests were performed on sludge retrieved from the SBR. The results would help highlighting the type of inhibition caused by FA and FA and to provide a greater understanding on the severity of inhibition along with useful information regarding the role of pH and nitrogen loading in EBPR systems. The data from these experiments would also be used in an attempt to construct inhibition models for both FNA and FA on PAOs growth.

2. Materials and methods

2.1. Experimental set-up

A sequencing batch reactor (SBR) was setup at the Sanitary Engineering Laboratory of the National Technical University of Athens and was operated for a total of 270 days. The SBR's working volume was 10 L and the seed sludge was collected from the activated sludge unit of the Psyttalia Wastewater Treatment Plant (PWWTP) in Athens. The system operated on two daily cycles, each consisting of an anaerobic phase of 3 h, an aerobic phase of 7 h and an anoxic phase of 1 h. Due to operational limitations, settling and decanting was carried out manually once per day over a 2 h timeslot. Feed consisting of synthetic wastewater containing organic carbon, ammonia and phosphorus was pumped into the SBR over a period of 2 min at the beginning of the anaerobic and anoxic cycles. The carbon source consisted solely of acetate and the dose was regulated in order to accomplish a Food to Microorganism (F/M) ratio of approximately 0.25 mg COD g^{-1}VSS d^{-1} (200 mg COD L^{-1} in the reactor at the start of each anaerobic and anoxic phase). Ammonium and phosphorus were added to the feed in the forms of ammonium chloride and potassium phosphate. Based on the feeding strategy the ammonium loading rate was 0.06 kg NH_4–N m^{-3} d^{-1} primarily to enhance biomass growth, while the loading rate for phosphorus was 0.02 kg PO_4–P m^{-3} d^{-1}, a dosage greater than the stoichiometric demand for growth in order to selectively promote the growth of PAOs. The Hydraulic Retention Time (HRT) was 2 d, while the Solid Retention Time (SRT) was kept constant at 10 d. Temperature in the SBR was kept stable at 20 ± 2 °C and pH was monitored throughout operation. Nitrite, nitrate, ammonium and COD concentrations in the SBR along with TSS and VSS were routinely analysed to ensure good performance and phosphorus concentrations were analysed throughout the anaerobic and aerobic phase. Oxygen was provided via air pump during the aerobic phase with DO concentrations in the SBR exceeding 3 mg L^{-1} within the first 30 min of operation, ensuring proper aerobic conditions. A schematic of the SBR configuration may be found in the Supplementary Material (Figure S1).

2.2. Batch experiments

Once the system had reached steady state operation (as evidenced from its performance with respect to MLSS concentration, effluent quality and aerobic phosphorus uptake rates, PUR), two different series of batch experiments were carried out to determine the effect of i) nitrite and ii) ammonium on the aerobic PUR.

2.2.1. Investigation on the effect of nitrite

The effect of nitrite on PUR was examined in a total of 18 batch tests at the pH values of 7, 7.5 and 8. According to the experimental protocol, a minimum of four batch tests were performed at each pH. The nitrite concentrations for each series were chosen as to provide a satisfactory range of inhibition degrees at each pH (Supplementary Material - Table S1). At the beginning of each experiment, sludge was extracted from the SBR prior to feed and divided equally into 3 bioreactors, each with a working volume of 0.5 L. An appropriate amount of acetate, in the form of sodium acetate solution, was added to each of the reactors in order to obtain an initial COD concentration of 200 mg L^{-1}. The pH of each container was adjusted to the targeted value just after feed and was kept stable for the remainder of the experiment. The reactors were then left stirring under anaerobic conditions over a 1 h period. At the end of

the anaerobic phase, the reactors were then aerated over a period of 3–4 h. One of the bioreactors served as a control while the other two reactors were fed with an equal amount of nitrite, in the form of a sodium nitrite solution at the start of aeration. Thus, each batch test examined the effect of a specific nitrite concentration on the duplicate reactors with respect to the control one. Starting on the beginning of aeration, samples of approximately 20 mL were obtained from each bioreactor every 30 min and immediately underwent centrifugation and filtration. The parameters of interest were PO_4–P, NO_2–N, NH_4–N, DO, T and pH. FNA concentration in mg N L^{-1} was calculated using the equation proposed by Anthonisen et al. (1976):

$$[HNO_2 - N] = \frac{[NO_2^- - N]}{10^{pH} \times \exp\left(-\frac{2300}{273+T}\right)} \quad (1)$$

where: NO_2–N, total nitrite concentration in mg N L^{-1}.

Throughout the experiments, nitrite concentrations in the control reactor were lower than 1 mg L^{-1} due to the occurrence of full nitrification. Concentrations this low would likely have no inhibitory effect on PAO activity. NH_4–N concentrations in all reactors averaged at about 10 mg L^{-1} that correlate to very insignificant FA concentrations at all pH's investigated.

In order to examine the recovery potential of biomass and the acute toxicity of nitrite, an additional experiment was carried out. The experiment was conducted similarly to the experiments mentioned above with one difference being that the aerobic phase was 2 h. The nitrite concentration was 35 mg N L^{-1} and the pH was 7.8 ± 0.1. After aeration, the original amount of acetate was once again added in order to remove the present nitrite and the reactors were left under stirring for an anoxic/anaerobic period of 2 h. Following this, the reactors were once again aerated for a period of 2 h. The performance of the reactors during the second aerobic phase was then compared to their original performance, obtained in the presence of 35 mg N L^{-1} nitrite, to determine the recoverability potential of EBPR.

Following the completion of the aforementioned series of experiments, ammonium loading in the SBR was gradually increased from 0.06 kg NH_4–N m^{-3} d^{-1} to 0.15 kg NH_4–N m^{-3} d^{-1} over a period of 30 days, while the SRT was lowered to 8 d. This was intended to inhibit NOB activity and allow greater nitrite concentrations in the SBR during operation. After 15 days of operation under these conditions, four additional batch experiments similar to the original series were conducted. The objective of these experiments was to determine if the biomass would exhibit a stronger tolerance to nitrite due to acclimation. The pH for these experiments was kept at 8 ± 0.1 and the nitrite concentrations investigated were NO_2–N = 40 mg L^{-1}, 70 mg L^{-1}, 120 mg $L^{-1}$and 300 mg L^{-1}.

2.2.2. Investigation on the effect of ammonium

The effect of ammonium on PUR was investigated over 19 batch tests at the pH values of 7, 7.5, 8 and 8.5.. According to the experimental protocol, a minimum of four batch tests were performed at each pH. The ammonium concentrations for each series were chosen as to provide a satisfactory range of inhibition degrees at each pH (Supplementary Material - Table S1). Similarly to the batch tests investigating the effect of nitrite, at the start of each experiment, sludge was retrieved from the SBR and divided equally into 3 containers, each with a working volume of 0.5 L. Each container was then fed an amount of acetate and was left stirring under anaerobic conditions over a 1 h period. The pH of each container was adjusted to the targeted value just after feed and was kept stable for the remainder of the experiment. At the end of the anaerobic phase, the reactors were then aerated over a period of 3–4 h. One of the bioreactors served as a control while the other two reactors were fed with an equal amount of ammonium, in the form of an ammonium chloride solution at the start of aeration. Each batch test examined the effect of a specific ammonium concentration on the duplicate reactors with respect to the control. Starting on the beginning of aeration, samples of approximately 20 mL were obtained from each bioreactor every 30 min and immediately underwent centrifugation and filtration. The parameters of interest were PO_4–P, NH_4–N, NO_2–N, DO, T and pH. FA concentration in mg N L^{-1} was calculated using the equation proposed by Anthonisen et al. (1976):

$$[NH_3 - N] = \frac{[NH_4^+ - N + NH_3 - N] \times 10^{pH}}{10^{pH} + \exp\left(\frac{6344}{273+T}\right)} \quad (2)$$

where: NH_4–N + NH_3–N, total ammoniacal concentration in mg N L^{-1}.

Like in the case of the experiments regarding the effect of nitrite, throughout these experiments nitrite concentrations in all reactors remained lower than 1 mg L^{-1}. NH_4–N concentrations in the control reactor averaged at 10 mg L^{-1}, meaning that PUR would not be affected by FA at all pH's investigated.

2.3. Chemical and microbiological analyses

Mixed liquor samples were immediately filtered through disposable Millipore filter units (0.22 μm). Total Solids (TSS), Volatile suspended solids (VSS), Chemical Oxygen Demand (COD), orthophosphates (PO_4^{3-}-P), ammonium (NH_4^+-N), nitrite (NO_2^--N) and nitrate (NO_3^--N) were analysed according to standard methods (APHA, 2005).

Fluorescence In Situ Hybridization (FISH) technique (Amann, 1995) was used to identify PAOs. Cy-3 labled PAOMIX was used to target PAOs in accordance to Crocetti et al. (2000), while Cy-3 labled EUBMIX was used to target most bacteria (Daims et al., 1999).

2.4. Modelling approach

A modification of the direct linear plot method proposed by Eisenthal and Cornish-Bowden (1974) was adopted to define the type of inhibition from the experimental results. Based on the linear plot method, the Michaelis-Menten equation is fitted for the case of aerobic PUR as follows:

$$PUR = PUR_{max}^{app} \frac{S}{K_m^{app} + S} \quad (3)$$

where PUR is the rate measured in the batch experiments, S is the concentration of phosphate at the beginning of the experiments and PUR_{max}^{app} and K_m^{app} are the maximum apparent PUR and the half saturation apparent concentration respectively, which account for the inhibitive effect of FNA or FA. The linear transformation of the above equation results in the following rearrangement:

$$PUR_{max}^{app} = \frac{PUR}{S} K_m^{app} + PUR \quad (4)$$

For example, In the case of non-competitive inhibition the extent of inhibition depends only on the concentration of the inhibitor so PUR_{max}^{app}. will decrease with increasing inhibitor concentration where K_m^{app} will remain the same (Supplementary Material - Figure S2). In order to examine the type of inhibition of FNA and FA on aerobic PUR, three series of batch experiments were performed for each inhibitor. In the batch experiments studying the effect of FNA, the first batch run was carried out with zero FNA presence for three PO_4–P initial concentrations and its results were compared to the other two batch runs carried out with FNA concentrations of 2.8 μg L^{-1} and 4.6 μg L^{-1} respectively (each batch run was repeated for three different initial PO_4–P concentrations). The same methodology was applied for batch experiments examining the effect of FA on aerobic PUR. In the first batch run the aerobic PUR was measured for three different initial PO_4–P concentrations in the absence of FA, while the other two runs were implemented for an initial FA concentration of 2 mg L^{-1} and 3.5 mg L^{-1} respectively.

Upon setting up axes K_m^{app} and PUR_{max}^{app} as the familiar x-y axes, for

each batch experiment, the measured PUR and the initial S are known and therefore creating a straight line with a slope of PUR/S and an intercept equal to PUR. By applying the same methodology for each replicate of each batch runs (each run corresponds to a certain FNA or FA concentration and different PO_4–P initial concentrations) three straight lines are produced (each corresponding to different initial PO_4–P concentration) which theoretically should intersect at a common point, whose coordinates gives the best fit values for PUR_{max}^{app} (y-axes) and K_m^{app} (x-axes) for each inhibitor concentration. However, almost always, there isn't any unique intersection point due to errors. In such cases it was proposed to use the median value of the apparent values of PUR_{max} and K_m instead (Eisenthal and Cornish-Bowden, 1974). In order to minimize the errors in the reading of the apparent values, the above procedure was combined with a simple statistical best fit determination of K_m^{app} value which resulted in the least sum of square errors between the calculated values of PUR_{max}^{app} for each batch experiment (according to equation (4)) and the average PUR_{max}^{app} of the three batch tests in each run.

3. Results and discussion

3.1. Establishment of a strong PAO culture

After a period of approximately 3 weeks, the SBR was already displaying appreciable PAO activity. The strong presence of PAOs was documented by FISH analyses (Fig. 1) and was also validated by the observed anaerobic COD consumption coupled with anaerobic P-release and aerobic P-uptake that characterize the biomass (Supplementary Material – Fig. S3). During operation, pH ranged from 7.5 to 8.5. Since changes in pH influence water chemistry and phosphorus dissolution/precipitation reactions, the results of which could lead to erroneous observations on biological P-removal, anaerobic P-release and aerobic P-uptake were recorded ex-situ under controlled pH. Throughout operation, the aerobic PUR ranged from 5 to 11 mg P g^{-1}VSS h^{-1} with average rates around 7 ± 1 mg P g^{-1}VSS h^{-1} during steady state conditions period (days: 60–210). As mentioned, NH_4^+, NO_2^- and NO_3^- were routinely analysed in the SBR. The maximum nitrite concentration observed was just below 1 mg N L^{-1}. During steady state period VSS averaged at 2500 ± 200 mg L^{-1} and TSS averaged at 2800 ± 200 mg L^{-1}.

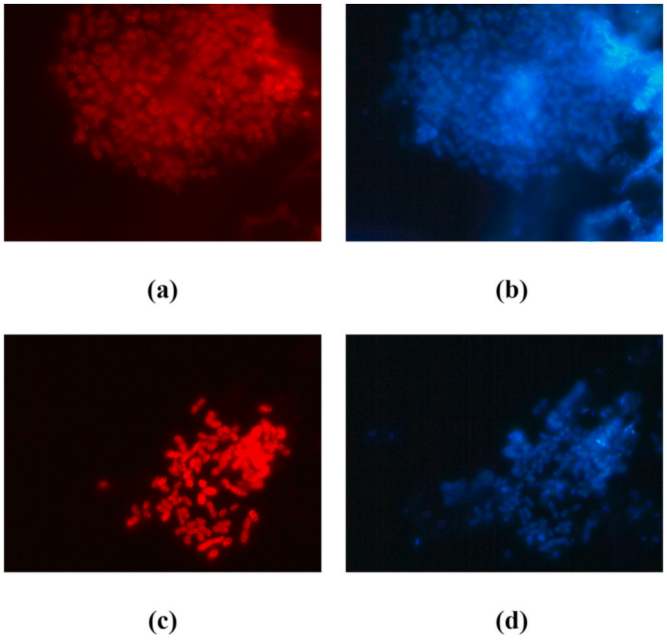

Fig. 1. In situ identification of PAOs using Cy-3 labled PAOMIX (PAOs depicted in red in 2a, 2c, all microorganisms stained with DAPI presented in blue in 2b, 2d). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

3.2. Effect of nitrite

The effect of nitrite on the aerobic phosphorus uptake rate of PAOs has been studied through several batch tests for several nitrite doses and pH. The results of the first series of batch experiments are presented in Fig. 2a. According to the results, the inhibitory effect of nitrite on aerobic PUR appears to be strongly dependant on pH. Aerobic PUR was found to be inhibited by 50 % under a NO_2–N concentration of 10 mg L^{-1} at the pH of 7, 20 mg L^{-1} at the pH of 7.5, whereas at the pH of 8, 50 % inhibition occurred under the nitrite concentration of 50 mg L^{-1}. Inhibition of PUR appears to be greater at lower pH values, as FNA concentration increases inversely to pH. The statistical difference of the inhibition experiments for the different pH values examined was determined through the paired *t*-test. A threshold p-value of 0.05 was selected for evaluating the statistical significance for each experimental series (each for a different pH). Based on the results the increase of pH from 7 to 8 resulted to a statistically significant decrease of the inhibition due to FNA (p-value equal to 0.00022). The FNA concentrations for each batch test were calculated with respect to temperature and pH (equation (1)) and the inhibitory effect of FNA on aerobic PUR is presented in Fig. 2b. These results suggest that FNA is most likely the actual inhibitor, in agreement with previous studies (Zhou et al., 2007). In these tests, PUR is shown to be inhibited by 50 % at an FNA concentration of 1.5 μg HNO_2–N L^{-1}, while the process seems to be fully inhibited at a concentration of 13 μg HNO_2–N L^{-1}. The degree of inhibition in relation to FNA appears to follow a logarithmic trend with PUR being less affected for higher FNA concentrations. For instance, a margin of 4–6 μg HNO_2–N L^{-1} practically equally inhibits PUR by 80 % as

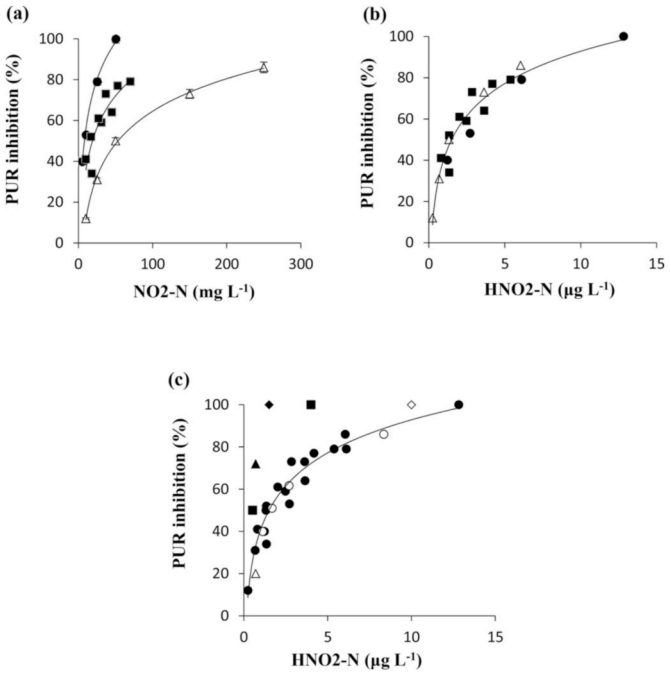

Fig. 2. (A): The effect of nitrite on aerobic PUR inhibition for pH = 7 (●), pH = 7.5 (■) and pH = 8 (Δ), (b): The effect of FNA on aerobic PUR inhibition for pH = 7 (●), pH = 7.5 (■) and pH = 8 (Δ), (c):.Comparison of studies on the effect of FNA on aerobic PUR. (●): This study, first series of experiments, (○): This study, second series of experiments, (▲): non-acclimatized biomass - Yoshida et al. (2006), (Δ): acclimatized biomass - Yoshida et al. (2006), (■): non-acclimatized biomass - Pijuan et al. (2010), (◆): non-acclimatized biomass - Saito et al. (2004), (◊): acclimatized biomass - Zhou et al. (2012).

shown in Fig. 2b. One point of interest is that the inhibitory effect of FNA observed in this study seems consistent with those observed in other studies where experiments were performed on biomass that was acclimatized to the presence of nitrite. As mentioned, during these experiments, nitrite concentrations in the SBR never exceeded 1 mg NO_2–N L^{-1} (less than 0.08 μg HNO_2–N L^{-1}; a concentration which according to our results would not inhibit the process). This observation was the reason to conduct further experiments on the biomass after the implementation of a higher nitrogen loading and consequently, a greater FNA presence in the SBR.

After the completion of this series of experiments, ammonium loading in the SBR was gradually increased from 0.06 kg NH_4–N m^{-3} d^{-1} to 0.15 kg NH_4–N m^{-3} d^{-1} over a period of 30 days, while the SRT was lowered to 8 d. The inhibition of NOB activity resulted in nitrite concentrations of up to 10 mg NO_2–N L^{-1} being observed during the aerobic phase. In regard to the SBR's temperature and pH, this would mean that PAOs were exposed to a maximum FNA concentration of 0.7 μg HNO_2–N L^{-1} (almost tenfold the maximum concentration observed in the SBR during the first series of experiments). Following the transitional period of 30 days and a further 15 days of operation under these new conditions, four batch experiments were conducted. In these experiments, the control's PUR averaged at a significantly lower value than the rate observed during the first series of experiments (3.5 ± 0.5 mg P g^{-1}VSS h^{-1} as opposed to 7 ± 1 mg P g^{-1}VSS h^{-1}). This would correspond to a lower PAO population in the biomass most likely due to inhibition from FNA. However the experiments showed that FNA inhibition on the biomass was almost identical to that observed during the first series of experiments meaning that either the biomass was incapable of getting acclimated to greater FNA concentrations or that in fact, PAOs may be acclimatized to FNA concentrations as low as 0.08 μg HNO_2–N L^{-1} that were observed during the first period of experiments. This would seem in agreement with other studies conducted on PAOs that were acclimatized to nitrite. The results of these experiments are compared to others in the literature in Fig. 2c. Even though the inhibitions observed in this study are lower than the ones previously reported, FNA appears to be a strong inhibitor of EBPR.

Regarding the possible acute toxicity of FNA on PAOs, an additional experiment was conducted as described in Materials and methods (Supplementary Material – Fig. S4). The reactor containing the nitrite concentration of 35 mg NO_2–N L^{-1} performed poorly in comparison to the control reactor. However following the anoxic removal of the nitrite present and the anaerobic release of phosphorus, the reactor appeared to aerobically remove phosphorus at an almost identical rate (5 % difference) to that displayed by the control reactor, meaning that the reactor had fully recovered from the presence of nitrite. This would lead to the conclusion that while FNA inhibits the growth rate of PAOs, EBPR is capable of recovering from nitrite shock loads.

3.2.1. Modelling FNA inhibition to aerobic PUR

By applying the modified direct linear plot method described in Materials and Methods, the values of PUR_{max}^{app} and K_m^{app} were calculated for all experimental runs. According to the results PUR_{max}^{app} decreased from 21.9 mg PO_4–P g^{-1}VSS h^{-1} at the zero FNA experiment to 7.3 mg PO_4–P g^{-1}VSS h^{-1} and 6.2 mg PO_4–P g^{-1}VSS h^{-1} for the experiments performed with an FNA content of 2.8 μg L^{-1} and 4.6 μg L^{-1} respectively. Furthermore, K_m^{app} value for all experiments was almost constant presenting an average value equal to 4.5 mg L^{-1} and a relative low coefficient of variation (6 %). The decrease of the PUR_{max}^{app} with the increase of FNA concentration and the steadiness of K_m^{app} value clearly indicates a non-competitive mode of inhibition.

A possible inhibition mechanism of FNA on PAOs activity may be described by the ability of FNA to bind equally well to both the ppk enzyme and the enzyme-substrate complex. In non-competitive inhibition it is assumed that both bindings occur at a site distinct from the active binding site that is being occupied by the substrate. Similarly, the effect of FNA on AOB and anammox activities has been regarded to follow the non-competitive inhibition model (Jiménez et al., 2012; Puyol et al., 2014).

In order to model FNA effect to aerobic PUR the non-competitive inhibition kinetics were adopted:

$$PUR = PUR_{max}\frac{K_{iFNA}}{S_{FNA} + K_{iFNA}} \quad (5)$$

where K_{iFNA} is the inhibition constant corresponding to the FNA concentration that inhibits the process by 50 %, S_{FNA} is the FNA concentration in the tank and PUR_{max} refers to the maximum aerated PUR at conditions with zero inhibition (practically refers to the PUR measured at the control system of each experimental batch series).

The ability of the aforementioned non-competitive inhibition model was also compared with the model proposed by Levenspiel (1980) (equation (6)), the Andrew's inhibition model (Andrews, 1968, equation (7)) and the model proposed by Zhou et al. (2007) to describe FNA effect on anoxic PUR (equation (8)). It is emphasized that both Andrew's and Levenspiel's models are substrate inhibition models which are widely used due to their simplicity. In the present study both models are used only for comparison with the simple non-competitive model.

$$PUR = PUR_{max}\left(1 - \frac{S_{FNA}}{S_{FNA}^*}\right)^n \quad (6)$$

where S_{FNA}* is the critical FNA concentration in the tank at which PUR is completely inhibited and n is a constant.

$$PUR = PUR_{max}\frac{S_{FNA}}{K_S + S_{FNA} + \frac{S_{FNA}^2}{K_{iFNA}}} \quad (7)$$

$$PUR = PUR_{max}\frac{S_{FNA}}{K_S + S_{FNA}}\, e^{\alpha S_{FNA}} \quad (8)$$

where S_{FNA}* is the critical FNA concentration in the tank at which PUR is completely inhibited, Ks is the affinity coefficient constant and n, α constants.

The Nash-Sutcliffe efficiency coefficient (NSE) and the percent bias (PBIAS) were used as statistic indices to evaluate models' performances as proposed by Moriasi et al. (2007). Performance criteria used to accept each model's predictivity were NSE >0.8 and PBIAS within ±20 %.

Models parametric values were calculated by performing best fit analysis to the experimental data in order to obtain the least sum of square errors (SSE). Based on this methodology an FNA inhibition constant of 1.5 μg L^{-1} was calculated for the simple non-competitive model (eq. (5)). Accordingly, by using a S_{FNA}* value of 13 μg L^{-1} (based on the experimental results), an n value equal to 4.4 gave the best fit of this model. Both Andrew's and Zhou's models were applied by adopting a Ks value of 0.031 μg L^{-1} as proposed by Zhou et al. (2007), while in Andrew's model, K_{iFNA} best fit value was equal to the value calculated for the simple non-competitive inhibition model (1.5 μg L^{-1}). Under best fit conditions the four models SSE were equal to 696, 1627, 807 and 1038 for the simple non-competitive, Levenspiel's, Andrew's and the model proposed by Zhou et al. (2007) respectively, thus highlighting that the former kinetic model is the more accurate one (eq. (5)) (Table S2 in Supplementary Material).

Fig. 3 illustrates the graphical comparison between the experimental results and the non-competitive model's output.

3.3. Effect of ammonium

The effect of ammonium on the aerobic phosphorus uptake rate of PAOs has been studied through a series of batch tests for several ammonium doses and pH. The results of the batch experiments regarding the inhibitory effect of ammonium on aerobic PUR are shown in Fig. 4a. Based on the results it appears that elevated ammonium concentrations result in significantly higher PUR inhibition at greater

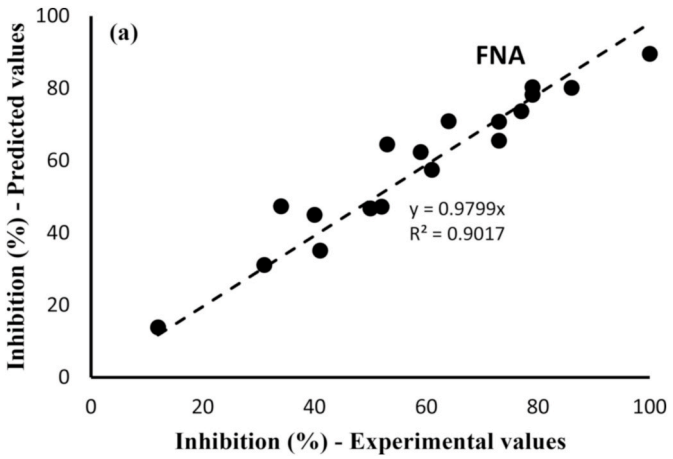

Fig. 3. Comparison of experimental and predicted results regarding the inhibitive effect of FNA on aerobic PUR.

pH values. At the pH of 7, PUR was inhibited by 10 % for a concentration of 300 mg L^{-1} NH_4–N, while at the pH of 8.5, PUR was inhibited by 50 % for a concentration as low as 45 mg L^{-1} NH_4–N. It is worth mentioning that complete inhibition of the process wasn't recorded even at ammonium-nitrogen concentrations as high as 1000 mg L^{-1}. The correlation of the degree of inhibition to pH provides a clear indication that FA rather than ammonium is the actual inhibitor of the process, as it is well known that FA concentrations increase with increasing pH. The statistical difference of the inhibition experiments for the different pH values examined was determined through the paired *t*-test. A threshold p-value of 0.05 was selected for evaluating the statistical significance for each experimental series (each for a different pH). Based on the results the increase of pH from 7 to 8.5 results in a statistically sound increase of inhibition due to FA (p-value equal to 0.001). Fig. 4b presents the inhibitions on aerobic PUR as observed for the corresponding FA concentrations (calculated for each ammonium dose based on equation (2)). According to the results it is concluded that PAOs aerobic activity related to phosphorus uptake (PUR) is inhibited by 50 % under a FA concentration of 6.4 mg NH_3–N L^{-1}, while FA concentrations above 30 mg L^{-1} appear to severely inhibit the process (>90 %). As mentioned, previous studies have demonstrated that EBPR would completely deteriorate after a prolonged exposure to FA concentrations greater than 18 mg NH_3–N L^{-1}. According to the results of this study, concentrations of this magnitude would inhibit aerobic PUR by 70–80 %. The diminished PUR observed under these FA concentrations would most likely support these observations when taking the SRT into account alongside a possible greater resilience to FA by other antagonistic microbial groups.

As mentioned, FA is a strong inhibitor of NOB as well and necessary in order to establish the nitritation-denitritation process. Vadivelu et al. (2006) reported that the growth of NOB is fully inhibited at the FA concentration of 6 mg L^{-1}. This concentration would also inhibit PUR by nearly 50 %.

3.3.1. Modelling FA inhibition to aerobic PUR

By applying the modified direct linear plot method described in Materials and Methods, the values of PUR_{max}^{app} and K_m^{app} were calculated for all experimental runs. According to the results PUR_{max}^{app} decreased from 21.9 mg PO_4–P g^{-1}VSS h^{-1} at the zero FA experiment to 18.8 mg PO_4–P g^{-1}VSS h^{-1} and 18 mg PO_4–P g^{-1} VSS h^{-1} for the experiments performed with an FA content of 2 mg L^{-1} and 3.5 mg L^{-1} respectively. Furthermore, the increase of FA resulted in a gradual decrease of the K_m^{app} value, from 4.5 mg L^{-1} in the control experiment to 4.2 mg L^{-1} and 3 mg L^{-1} for the experiments performed with an FA content of 2 mg L^{-1} and 3.5 mg L^{-1} respectively. The decrease of both apparent values of PUR_{max} and K_m is a clear indication of an uncompetitive mode of inhibition.

In order to model FNA effect to aerobic PUR the non-competitive inhibition kinetics were adopted:

$$PUR = PUR_{max}\frac{S}{S\cdot\left(1+\frac{S_{FA}}{K_{iFA}}\right)+K_S} \quad (9)$$

where K_{iFA} is the inhibition constant, S_{FA} is the concentration of FA in the tank, S is the concentration of PO_4–P in the tank, K_S is the half saturation coefficient for PO_4–P and PUR_{max} refers to the maximum aerated PUR at conditions with zero inhibition (practically refers to the PUR measured at the control system of each experimental batch series). The predictive capacity of the aforementioned inhibition model was tested against to the model proposed by Levenspiel (1980) and described by the following equation:

$$PUR = PUR_{max}\left(1-\frac{S_{FA}}{S_{FA}^*}\right)^n \quad (10)$$

where S_{FA}* is the critical FA concentration in the tank at which PUR is completely inhibited and n is a constant.

As already explained each model's parametric values were calculated by performing best fit analysis to the experimental data in order to obtain the least SSE. Based on this methodology an FA inhibition constant (K_{iFA}) of 10 mg L^{-1} was calculated for the simple model (eq. (9)). Accordingly, by using a S_{FA}* value of 40 mg L^{-1} (based on the experimental results), an n value equal to 2.3 gave the best fit of this model. By comparing the statistical indices of the two models it is concluded that the conventional un-competitive model described by equation (9)

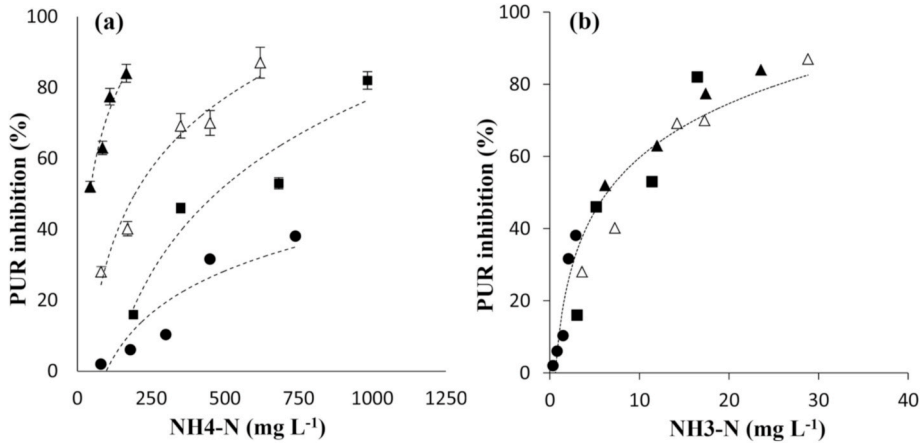

Fig. 4. The effect of ammonium nitrogen (a) and FA (b) on aerobic PUR inhibition for pH = 7 (●), pH = 7.5 (■), pH = 8 (Δ) and pH = 8.5 (▲).

exhibits better fit to experimental data than Levenspiel's model.

Both performance indices examined (NSE and PBIAS) showed very satisfactory values; NSE was equal to 0.90 while PBIAS was equal to −0.71 % (Table S2 in Supplementary Material). Fig. 5 illustrates the satisfactory comparison between the experimental results and the model's output. Therefore, it is anticipated that equation (9) can provide for an accurate simulation of the inhibition of FA on the aerated PUR of PAOs.

3.4. Implications for full-scale applications

Based on the results of this study, it appears that both nitrite and ammonium can inhibit EBPR, as demonstrated by their effect on PUR. FNA and FA appear to be the actual inhibitors, in agreement with other studies. The observed inhibitory effect of FA seems to validate the findings of Yang et al. (2018) while the effect of FNA seems consistent with that reported by Zhou et al. (2012) for an acclimated biomass. Fig. 6 displays the predicted inhibitions on PUR due to either nitrite or ammonium with respect to pH for the temperature of 20 °C. The corresponding inhibitions on PUR regarding nitrite or ammonium were calculated using equations (5) and (9). According to this nomograph, operating under a high pH could combat EBPR inhibition due to FNA, but the process would be inhibited by the high concentrations of FA. Nitrogen loading is a key factor as high FA concentrations are practically required to stimulate the NOB shunt and therefore the achievement of the nitritation-denitritation process, but at the same time may hinder EBPR due to the high FA and/or FNA content. Raising pH during the nitritation process may assist EBPR as ammonium is oxidized to nitrite. When the residual concentration of ammonium nitrogen becomes equal to the concentration of nitrite nitrogen, the optimal pH of operation is just over 8.2. The adjustment of pH would have to combat the biochemical drop of pH during nitritation. Therefore it seems that the use of an on-line monitoring strategy is urgently needed to avoid severe inhibition effect on PAOs activity in nitritation-denitritation systems. However even with the application of this optimal approach, nitrogen concentrations that are typically present in sludge liquors may prove to forbid EBPR under these conditions. For instance, according to the diagram, a nitrogen concentration of 200 mg L^{-1} (a concentration much less than the concentrations generally found in sludge liquors) would mean that when nitrite nitrogen and ammonium nitrogen are at equilibrium under optimal pH, EBPR would be inhibited by 50 % by the presence of FNA alone and that the presence of FA alone would inhibit the process also by 50 %. The combined effect of both inhibitors has not been studied as of this point. However, it is very likely that EBPR would be severely deteriorated under these conditions. It should be noted that the treatment of sludge liquors with EBPR is feasible if the reactor is operated with an appropriate volume exchange ratio in order to achieve low ammonium and nitrogen concentrations. These conditions however would also allow a greater NOB presence which would compromise the nitritation-denitritation pathway leading to greater oxygen demand and thus operation cost. Consequently, further research is needed in order to assess the optimal conditions with regard to nitrogen loading rate, volume exchange ratio and operational strategy particularly during start-up needed to achieve satisfactory EBPR through nitritation-denitritation.

4. Conclusions

EBPR under the nitritation-denitration process appears to be at the least challenging with pH and nitrogen loading playing a key role. FNA appears to be a strong inhibitor of EBPR with 50 % inhibition of PUR observed at 1.5 μg HNO_2–N L^{-1} and 100 % inhibition observed at 13 μg L^{-1}. FA was also found to inhibit PUR, with 50 % inhibition observed at a concentration of 6.4 mg NH_3–N L^{-1}. A non-competitive inhibition model gives a satisfactory description of FNA inhibition, while an uncompetitive model gives the best fit to experimental results regarding FA inhibition.

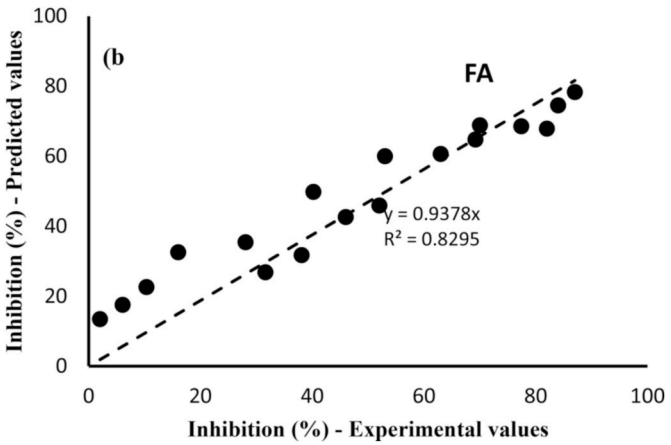

Fig. 5. Comparison of experimental and predicted results regarding the inhibitive effect of FA on aerobic PUR.

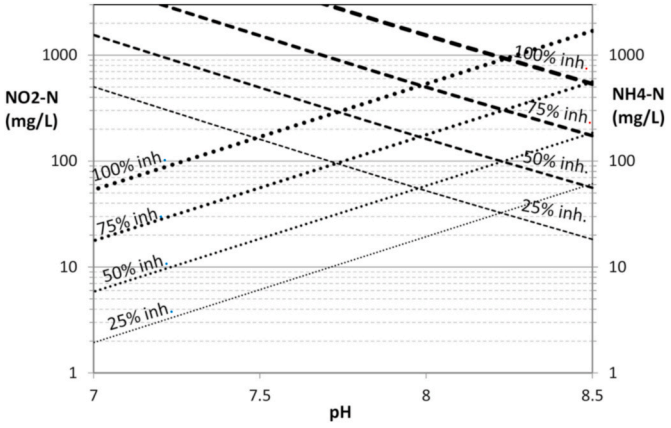

Fig. 6. Predicted effect of nitrogen loading on aerobic PUR with respect to pH and distribution between nitrite (···) and ammonium (—) nitrogen at T = 20 °C (Nitrogen concentrations for both nitrite and ammonium are presented in logarithmical scale).

Author contribution

The role of free nitrous acid and free ammonia in the inhibition of the aerobic phosphorus uptake rate: Evidence for insufficient phosphorus removal through nitritation-denitritation.Dimitris Andreadakis: design and Formal analysis of the results, writing, Constantinos Noutsopoulos: Conceptualization, inhibition models, writing, Gerasimos Fragkiskatos: FNA inhibition experiments, Daniel Mamais: experimental design, review, Theodora Misirli: FA inhibition experiments, Kyriaki Argyropoulou: FNA inhibition experiments, Eva Themeli: inhibition experiments in acclimatized biomass, Simos Malamis: experimental design.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2021.113390.

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