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Response of Poly-phosphate Accumulating Organisms to Free Nitrous Acid Inhibition under Anoxic and Aerobic Conditions

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ABSTRACT

The response of free nitrous acid (FNA)-adapted poly-phosphate accumulating organisms (PAOs) to FNA inhibition under aerobic and anoxic conditions was studied. Anoxic P-uptake was 1-6 times more sensitive to the inhibition compared to aerobic P-uptake. The aerobic nitrite reduction rate increased with FNA concentration, accompanied by an equivalent decrease in the oxygen uptake rate, suggesting under high FNA concentration conditions, electrons were channeled to nitrite reduction from oxygen reduction. In contrast, the nitrite reduction rate decreased with increased FNA concentration under anoxic conditions. Anaerobic metabolism of PAO under both anoxic and aerobic conditions was observed at high FNA concentrations. Growth of PAOs decreased sharply with FNA concentration and stopped completely at FNA concentration of 10 μg HNO_2-N/L. This study, for the first time, investigated the function of nitrite/FNA in an aerobic denitrifying phosphate removal process by evaluating electron as well as energy balances, and provides explanation for FNA inhibition mechanisms.

Keywords: Poly-phosphate Accumulating Organism (PAO), Free Nitrous Acid (FNA), Phosphate Uptake, Aerobic Denitrification, Detoxification

1. INTRODUCTION

Biological phosphorus removal can be combined with nitrogen removal in wastewater treatment plants (WWTPs). In a simultaneous nitrogen and phosphorus removal process, poly-phosphate accumulating organisms (PAOs) play an important role in denitrification and phosphate removal by using stored carbon as energy source (Meinhold et al. 1999; Zeng et al. 2003). Nitrogen removal via nitrite has a number of advantages over traditional nitrification process, including lower carbon source requirements for denitrification, lower oxygen consumption, a higher denitrification rate and less sludge production (Turk and Mavinic, 1986). These advantages are even more notable when wastewater contains high ammonium or low organic carbon contents.

However, nitrite accumulation is an unfavourable occurrence in a WWTP, as it has been reported that nitrite can slow down, or even completely stop microbial activities and reconfigure the microbial community structure (Saito et al., 2004; Pijuan et al., 2010). Indeed, nitrite has been reported to inhibit both aerobic and anoxic phosphate uptake by PAOs in enhanced biological phosphorus removal (EBPR) processes (Meinhold et al., 1999; Yoshida et al., 2006). Free nitrous acid (FNA i.e. HNO_2), the protonated species of nitrite, is likely the true inhibitor of phosphate uptake (Zhou et al., 2007). Pijuan et al. (2010) and Zhou et al., (2010) reported that aerobic and anoxic metabolism of PAOs are seriously affected by FNA.

Meinhold et al. (1999) reported that aerobic P-uptake by a PAO culture that was never exposed to nitrate or nitrite is less sensitive to FNA than anoxic P-uptake whereas Saito et al. (2004) concluded that aerobic P-uptake was more affected than anoxic P-uptake by nitrate-adapted phosphate removal culture. While Yoshida et al. (2006) also demonstrated aerobic PAO as being more sensitive to nitrite, they also pointed out that a nitrite-adapted culture would have stronger tolerance to nitrite inhibition.

It is well known that oxygen inhibits the activity of denitrifying enzymes and suppresses their synthesis, and nitrite and nitrate are less preferred electron acceptors with lower energy yields when compared with oxygen respiration (Thauer et al. 1977); however, aerobic denitrification occurs in many cases (Kucera and Dadak, 1983; Robertson and Kuenen, 1984; Wan et al. 2011) including during aerobic phosphate removal. Pijuan et al. (2010) observed nitrite reduction under aerobic conditions. They suggested that PAOs reduce nitrite for detoxification instead of energy generation. In contrast, Yoshida et al. (2006) suggested that PAOs can reduce nitrite for energy production rather than for detoxification under aerobic conditions.

Through batch tests, the present study investigates the response of an FNA-adapted PAO culture to different FNA concentrations under aerobic as well as anoxic conditions. The metabolisms of PAOs under these two conditions, namely phosphate uptake, nitrite reduction, poly-hydroxyalkanoate (PHA) oxidation, glycogen production, growth, and oxygen uptake rate (OUR), were measured and compared. This paper presents, for the first time, a comparative study conducted using an enriched PAO culture adapted to FNA, to examine PAOs' response to FNA inhibition under aerobic and anoxic conditions. This study further investigates and discusses the function of nitrite/FNA in the aerobic denitrifying phosphate removal process by evaluating electron as well as energy balances.

2. MATERIALS AND METHODS

2.1. Sludge source

The PAO culture was withdrawn from a sequencing batch reactor (SBR) fed with synthetic wastewater containing organic sources, ammonium, orthophosphate, and a trace nutrient supplement. The reactor had been operated about 18 months before the tests. The carbon source was a mixture of acetate and propionate providing 200 mg COD/L

($COD_{Acetate}/COD_{propionate}$ is 3). Ammonium and phosphate were applied at 20 mg $N\text{-}NH_4^+/L$ and 10 mg $P\text{-}PO_4^{3-}/L$, respectively. The reactor had a working volume of 4 L and was operated with a cycle time of 4 h comprising 54 min anaerobic, 60 min aerobic, and 25 min anoxic periods, followed by a second phase of anaerobic (35 min), aerobic (25 min), and anoxic (20 min) periods, and thereafter a 10 min settling and decanting period. In each cycle, 1 L synthetic wastewater was fed to the reactor in the first 8 min of the first anaerobic period and again in the first 3 min of the second anaerobic period. This resulted in a hydraulic retention time of 16 hours. Sludge retention time (SRT) was maintained at 13 days. pH in the reactor was maintained in the range of 7.0-8.0 by PLC controlled acid and alkaline dosing pumps. In order to achieve simultaneous nitrification, denitrification and phosphate removal (SNDPR), the dissolved oxygen (DO) concentration during the aerobic periods was controlled at 1.0-1.5 mg/L. Nitrite accumulated to 11.6 mg NO_2^--N/L during the aerobic phase. The SBR was displaying excellent phosphate and nitrogen removal performance when its biomass was withdrawn for the batch experiments described below. The results of a cyclic study of the parent reactor can be found in supplementary information (SI, Figure SI0). Sludge was adapted to a FNA concentration of 0.9 HNO_2-N/L.

2.2. Batch tests

2.2.1. *Sludge pretreatment*

Sludge for all batch experiments, unless otherwise described, was obtained according to the following pretreatment procedures. Mixed liquor biomass was withdrawn from the SBR at the end of the cycle (anoxic phase). An anaerobic phase was allowed for one hour with addition of sodium acetate at 150 mg COD/L to generate the PHA pool for PAOs. Any residual COD at the end of the anaerobic phase was removed by a series of washing steps. During these washing steps, mix liquor was allowed to settle and supernatant was removed

and replaced with phosphate buffer solution (PBS). Above steps were repeated three times. Washed biomass was distributed into 27 batch reactors operated in parallel, each with a working volume of 100 mL. Five mg/L of ATU (Aldrich, USA) was added to the mixed liquor to inhibit nitrification. Ammonium and phosphate were injected resulting in initial N and P concentrations of 10 mg NH_4^+-N/L and 20 mg PO_4^{3-}-P/L, respectively.

2.2.2. Sampling and reactor operation

Sampling and reactor operation protocols, unless otherwise described, were as follows. Each batch test lasted for one hour. During each inhibition experiment, mixed liquor samples were taken every 10 min and immediately filtered through disposable Millipore filters (0.45 μm pore size) for analysis of ammonium, nitrite and phosphate. Solids samples for the analysis of PHA and glycogen were taken every 20 min and fixed with formaldehyde (Oehmen et al., 2005). Mixed liquor samples for total suspended solids (TSS) and volatile suspended solids (VSS) measurements were withdrawn at the beginning and end of each experiment. Results obtained were normalized for biomass concentrations which were calculated using VSS subtracting PHA and glycogen content. During aerobic inhibition tests, air was supplied intermittently by an on-off controller at dissolved oxygen (DO) levels between 2 – 6 mg O_2/L. Anoxic condition was maintained by applying nitrogen continuously during the inhibition tests from the headspace of the batch reactor.

2.2.3. FNA inhibition tests

FNA inhibition tests were carried out with 27 FNA concentrations achieved by varying the initial nitrite concentration (0-65 mg NO_2^--N/L) and pH (6.5-8), as summarized in Table 1. The FNA concentration was calculated using $\frac{S_{N-NO_2}}{K_a * 10^{pH}}$, with the Ka value determined using

$e^{-2300/(273+T)}$ for any given temperature T (°C) (Anthonisen et al. 1976). FNA concentration of zero in the aerobic test means that the test was performed in the absence of nitrite, in which the maximum rates of each metabolic pathway was obtained. The aerobic and anoxic inhibition tests were initiated by the injection of a 60 mM nitrite stock solution into pretreated sludge. Each test lasted for one hour. pH was controlled at the preset set-point (Table 1) using 0.1 M HCl. Reactor operation and sampling followed the protocol described in section 2.2.2. Maximum oxygen uptake rate (OURmax) was determined using the maximum net oxygen consumption rate from each aerobic inhibition test. Detailed calculation can be found in SI.

2.2.4. Maximum P-uptake rate under anoxic condition

The maximum P-uptake rate under anoxic conditions was obtained through continuous feeding of nitrite at various loading rates in parallel reactors. This approach was chosen because a pulse addition of nitrite resulting in a non-limiting nitrite concentration could induce an inhibitory effect preventing maximum rates from being measured. The feeding rates were designed to enable different FNA loading rates in the reactors so that bacteria activity at extremely low FNA concentration can be determined. Detailed operating conditions and the experimental design can be found in SI.

2.3. Chemical and Microbial Analyses

Ammonia (NH_4^+), nitrite (NO_2^-) and orthophosphate (PO_4^{3-}-P) concentrations were determined in accordance with Standard Methods (APHA 1995). VFAs were measured with a gas chromatograph and a DB-FFAP 15m x 0.53mm x 1.0μm (length x ID x film) column, while the injector block and FID detector were operated at 250 and 300 °C, respectively. High purity helium was used as carrier gas at a constant pressure of 103 kPa. Filtered sample (0.9 mL) was transferred into a GC vial and 0.1 mL of formic acid was added. For analysis, 1

μL of sample was injected in splitless mode. Mixed liquor suspended solids (MLSS) and volatile MLSS (MLVSS) were analyzed in accordance with Standard Methods (APHA, 1995).

Glycogen was determined using the method of Zeng et al (2003). Briefly, 5 mL of 0.6 M HCl was added to each weighed freeze-dried biomass in screw-topped glass tube. The tubes were heated at 105 °C for 6 hours. After cooling and centrifugation at 2057 g for 10 min, 1 mL of the supernatant was transferred to a high performance liquid chromatography (HPLC) vial for glucose analysis. PHA analysis was performed using the method of Oehmen et al. (2005) to determine poly-β-hydroxybutyrate (PHB), poly-β-hydroxyvalerate (PHV), and poly-β-hydroxy-2-methylvalerate (PH2MV). Weighed freeze-dried biomass and PHB/V and PH2MV standards were placed into screw-topped glass tubes. The tubes were heated at 100 °C for 20 hours after the biomass was suspended in 2 mL methanol acidified with 3% H_2SO_4 and 2 mL chloroform. After cooling, 1 mL Milli-Q water was added and the sample was mixed. When the phases separated, around 1 mL of the bottom organic layer was transferred to the GC vials for analysis.

Fluorescence in situ hybridization (FISH) of PAO was performed as described by Amann (1995) with Cy5-labelled EUBMIX probes (for most Bacteria; Daims et al., 1999) and Cy3-labelled PAOMIX probes (for Candidatus *Accumulibacter phosphatis* or *Accumulibacter*, comprising equal amounts of probes PAO462, PAO651 and PAO846, Crocetti et al., 2000). The presence of the major groups of GAOs currently known was tested using Cy5-labelled GAOMIX probes (for Candidatus *Competibacter phosphatis* or *Competibacter*, comprising equal amounts of probes GAOQ431 and GAOQ989, Crocetti et al., 2002), Cy5-labelled DF1MIX (for *Defluviicoccus vanus* cluster 1-related bacteria or -GAO, comprising equal amounts of probes DEF218 and DEF618, Wong et al. 2004), and Cy5-labelled DF2MIX (for *Defluviicoccus vanus* cluster 2-related GAOs, comprising equal amount of probes DF988 and

DF1020 plus helper probes H966 and H1038, Meyer et al., 2006) against FITC-labeled EUBMIX probes. Briefly, FISH preparations were visualized with a Zeiss LSM 510 Meta confocal laser-scanning microscope (CLSM) using a Plan-Apochromat 63x oil (NA1.4) objective. Thirty images were taken from each sample for quantification. The images were 8-bit, 512x512 pixel with 0.1 μm pixel size. The area containing cells targeted by the Cy3 or Cy5-labelled specific probes (PAOMIX, GAOMIX or -GAO) was quantified as a percentage of the area of Cy5 or FITC-labeled Bacteria probe (EUBMIX) within each image using a pixel counting program (Image J). The final quantification result was expressed as a mean percentage obtained from 30 images along with the standard error.

3. RESULTS AND DISCUSSION

3.1. Microbial communities

Accumulibacter, a known PAO, was enriched in described SNDPR SBR, accounting for approximately 45% of the whole community as measured with fluorescence in-situ hybridization. Glycogen accumulating organisms (GAOs) belonging to *Competibacter* were less than 5% of the total population in the same culture. The *Defluviicoccus vanus*-cluster 1 and cluster 2 groups were not detected.

3.2. Batch test results

Profiles of intracellular (PHA and glycogen) and extracellular (ammonium, nitrite and phosphate and dissolved oxygen) compounds measured during Test 3 (see Table 1) are displayed in Figure 1 as an example. Phosphate uptake was observed in the presence of oxygen and nitrite with simultaneous consumption of PHA and production of glycogen. The consumption of nitrite and oxygen are displayed as well. Biomass growth expressed as

ammonium consumption can also be found in Figure 1. The conversions rates of all compounds were calculated through linear regression of each profile, and standardised against the biomass concentration. For non-linear regression compounds (e.g phosphate in Figure 1), the rates were determined using the linear regression phase. OUR was calculated and displayed as maximum OUR (see Figure SI1). The results obtained in all twenty seven tests are summarised in Figure 2.

3.3. Response of P-uptake to FNA

PAOs expressed a lower P-uptake activity using nitrite as sole electron acceptor under anoxic conditions as compared to using both oxygen and nitrite under aerobic conditions (Fig. 2a). The maximum anoxic P-uptake rate (0.18 mg PO_4^{3-}-P/(min*gBiomass)), which was obtained through a continuous feeding test (Figure SI2), is less than 50% of the maximum aerobic P-removal rate. Fig. 2a also shows that anoxic P-uptake was 100% inhibited at an FNA concentration of 5 µg HNO_2-N/L, which is much lower than the FNA concentration (approximately 10 µg HNO_2-N/L) that resulted in complete inhibition of aerobic P-uptake. P-release was found at FNA concentration of approximately 5 µg HNO_2-N/L and 10 µg HNO_2-N/L under anoxic and aerobic conditions, respectively. These findings are in agreement with the observations by Meinhold et al. (1999) that anoxic P-uptake is more sensitive to nitrite/FNA inhibition; however, these results contradict the results reported by Saito et al. (2004), which indicated that aerobic P-uptake was more sensitive than anoxic P-uptake. The contradictory results could be due to the difference in microbial populations and their different tolerance to FNA. Unfortunately, the microbial populations were not reported in Meinhold et al. (1999) while substantial amounts of GAO were detected by Saito et al. (2004).

The higher sensitivity of anoxic PAO metabolism to FNA inhibition compared to its aerobic

metabolism could be due to the following reasons:

(1) Nitrite was the sole electron acceptor during the anoxic P-uptake tests while two types of electron acceptors (nitrite and oxygen) coexisted in the aerobic P-uptake tests. It has been reported that some, if not all, denitrifying strains can use these two electron acceptors simultaneously leading to a higher metabolic rate than when only one electron acceptor is supplied (Robertson and Kuenen, 1990).

(2) It is known that nitrate, nitrite and other oxides of nitrogen serve as electron acceptors in their own right, and that the respiratory pathways to oxygen or to the nitrogen oxides are to some extent separate (Stouthamer, 1980). If the oxygen uptake pathway is inhibited to some extent by high FNA concentrations, the cytochrome chain would become more reduced. More electrons would then be forced to flow to nitrite to create an alternative energy generating pathway, although this would require the redox state of the cytochrome chain to be reduced to a certain level (Robertson and Kuenen, 1992). Cells may have less flexibility when only one electron acceptor is available.

(3) Before FNA completely inhibits the electrons flow to oxygen, oxygen would still be the preferred electron accepter due to its higher energy potential. This may explain the generally higher aerobic P-uptake rates compared to anoxic P-uptake rates at the low FNA concentration range (Fig. 2a). Meanwhile, oxygen respiration under low FNA concentrations may inhibit nitrite respiration due to the influx competition of redox equivalents into the cytochrome chain, which may result in low denitrification rates.

It has been reported that FNA-adapted and non FNA-adapted PAOs have different response to FNA inhibition (Meinhold et al., 1999; Saito et al., 2004; Yoshida et al., 2006). The results of current study were obtained from an FNA-adapted culture. A comparison of the inhibitory effects of FNA on aerobic and anoxic P-uptake by FNA-adapted and non-FNA-adapted PAOs is given in Table 2, which reveals that FNA-adapted PAOs were generally more

tolerant to FNA inhibition. This finding is in agreement with results of Yoshida et al. (2006) that a non-FNA-adapted PAO culture was much more severely inhibited by FNA.

Further, P-release, which is part of the anaerobic metabolism of PAOs, was observed under both anoxic and aerobic conditions at higher FNA concentrations (Fig. 2a).

3.4. Response of glycogen and PHA transformation to FNA

The highest glycogen production activity was observed in the aerobic control test (no nitrite addition); however, similar glycogen production rates were observed under both anoxic and aerobic conditions at all other FNA levels (Fig. 2c). Glycogen degradation was detected at FNA concentrations higher than 10 μg HNO_2-N/L under both aerobic and anoxic conditions. The degradation rates were observed to be relatively higher under aerobic conditions.

Aerobic PHA degradation rates were also affected adversely by FNA starting from very low FNA concentrations (0.26 μg HNO_2-N/L) (Fig. 2d), while remaining stable from FNA concentration of 5 μg HNO_2-N/L onwards; however, complete inhibition of PHA degradation was not reached even at the highest FNA concentration tested (50 μg HNO_2-N/L). Anoxic PHA degradation rates followed a similar trend as those under aerobic conditions. PHA was continually degraded during the aerobic and anoxic reaction phases when FNA concentrations were lower than 20 μg HNO_2-N/L, whereas PHA production rather than consumption was observed at higher FNA concentrations (Table 3). Furthermore, glycogen was degraded simultaneously. It is obvious that anaerobic metabolism of PAO was activated at high FNA concentrations without the presence of any external carbon sources. Table 3 also shows that the level of aerobic PHA production and glycogen degradation was generally higher than that of anoxic conditions.

At this stage it is not clear if the degraded glycogen was directly transformed to PHA under high FNA concentration conditions. O'Leary et al. (1976) reported that nitrite in the form of

FNA reacts with SH groups at a high rate. Zhou et al. (2010) proposed that the SH-containing enzyme, CoA-SH, an essential intermediate catalyst for PAOs' metabolic pathways, e.g. the tricarboxylic acid (TCA) cycle, also contains SH groups which could be denatured by FNA. In the TCA cycle, CoA-SH is involved in the three steps of reactions of acetyl-CoA, α-ketoglutarate, and succinyl-CoA recirculation. If any of these three CoA-SH enzymes is inactivated due to the interaction of FNA and CoA-SH, acetyl-CoA, succinyl-CoA or propionyl-CoA will be accumulated. It is reasonable to assume that the CoA compounds can be condensed into PHA. This process is possible with the reducing power supplied from glycogen degradation (Table 3). Further, glycolysis may also provide pyruvate as a source of propionyl-CoA formation.

3.5. Response of NO_2^- reduction, growth and OUR to FNA

Fig. 2b displays aerobic and anoxic denitrification rates at different FNA concentrations. Anoxic and aerobic nitrite reduction rates increased as FNA concentration increased in the range of 0-10 μg HNO_2-N/L. Anoxic nitrite reduction rates decreased gradually in the FNA concentration range of 10-40 μg HNO_2-N/L and remained constant when the FNA concentration exceeded 40 μg HNO_2-N/L. This finding indicates that anoxic nitrite reduction may also be affected by high concentrations of FNA. In contrast, aerobic nitrite reduction rates were not negatively affected by the increase in FNA concentration. It is noteworthy that the PAOs' aerobic denitrification rates were higher than the anoxic denitrification rates when the FNA concentration was higher than 30 μg HNO_2-N/L. A similar trend was observed by Pijuan et al. (2010), although a relatively lower FNA concentration range (0- 16 μg HNO_2-N/L) was used in that study.

Given that nitrite can freely cross the cell membrane in the form of FNA and the intracellular accumulated FNA is known to inhibit a broad range of enzymes, it had been proposed that

reduction of nitrite under aerobic conditions is for detoxification rather than supporting growth (Robertson and Kuenen, 1990; Moir and Wood, 2001); however, it had also been reported that the capacity of aerobic denitrification is determined by the redox state of the cytochrome chain (Robertson and Kuenen, 1992). Under certain conditions, nitrite reductase can perform both detoxification and respiratory functions, supplementary to, or competitive with, aerobic respiration (Stein, 2011).

Both aerobic and anoxic growth stopped when the FNA concentration was higher than 10 μg HNO_2-N/L, as shown by the ammonium assimilation rates (Fig. 2e). Once growth ceased, other anabolic activities of the PAOs, i.e. P-uptake and glycogen production, stopped as well (Fig. 2a, 2c), while catabolic activities of denitrification (Fig. 2b) and PHA degradation still continued. These results are consistent with those of Zhou et al., (2010) and Pijuan et al., (2010) in which anabolic activities (P-uptake and glycogen production) of PAOs were shown to be more sensitive to FNA inhibition than catabolic activities (PHA degradation and nitrite reduction).

Maximum OUR obtained in aerobic tests at different FNA concentrations are presented in Fig. 2f. Maximum OUR decreased sharply in the FNA concentration range of 0-20 μg HNO_2-N/L and remained relatively constant in the higher FNA concentration range. Yang (1985) reported that nitrite interacts directly with the terminal oxidase and inhibits the oxygen-binding reaction, thus retarding the oxygen uptake rate. Given the fact that the consumption of the second electron acceptor, NO_2^-, was increased as FNA concentration increased under aerobic conditions (Fig. 2b), the PAOs had probably switched the metabolic pathway from utilizing O_2 as the main electron acceptor to NO_2^-. Fig. 2 also illustrates that the loss of oxygen respiration ability was lower than that of phosphorus uptake, glycogen production and growth. This finding suggests that the PAO's oxygen respiration ability is more tolerant to FNA inhibition as compared to P-uptake, glycogen production and growth.

3.6. Simultaneous nitrite and oxygen reduction

Aerobic denitrification has been commonly found in simultaneous nitrification and denitrification (SND) systems (Bernat and Baryla, 2007; Wan et al., 2011). Through this co-respiration system, electrons from a slowly degraded carbon source, such as PHA, flow to both oxygen and nitrite reduction enzymes. Aerobic denitrification has also been found in phosphorus removing systems (Yoshida et al., 2006; Pijuan et al. 2010); however, it is not clear that reduction of nitrite in these studies is a function of detoxification or co-respiration.

3.7. Electron shuttling

The following assumption is made in the electron balance calculation performed in Table 4: (1) electron donor (PHA) was sufficient in the first 20 min of the inhibition studies. Thus, the calculations are based on data from first 20 min; (2) the portion of PHA oxidized by oxygen for energy and as carbon source for P-uptake, glycogen production, growth and maintenance follows the model of Smolders et al. (1995); (3) reduction of nitrite may be supplementary to oxygen respiration.

PHA consumption in the first 20 min of each FNA inhibition test was measured. The results from five FNA concentrations in terms of low, medium and high ranges were evaluated for electron balance calculation. Assuming degraded PHA was completely oxidized by oxygen, the required oxygen amount is shown in Table 4. In the control test (FNA was absent) and in tests at low FNA concentrations, the required oxygen amount was generally corresponding to the measured oxygen consumption values; however, in medium and high FNA concentration ranges, measured oxygen consumption was far lower than the required amount. An alternative electron acceptor, nitrite, was assumed to support the oxidation further. The required nitrite amounts are described in three forms with final products of denitrification

assumed to be nitrogen gas (N_2-required), nitrous oxide (N_2O-required) and nitric oxide (NO-required), respectively. At medium-high FNA concentrations, the measured nitrite amount was close to the N_2-required values, while it seems shifted to N_2O-required values at higher FNA concentration. Zhou et al. (2008) had reported that high FNA concentrations inhibit N_2O reduction severely. It is noteworthy that NO could be the final product of denitrification at the highest FNA concentration (46 μg HNO_2-N/L). Unfortunately, N_2O and NO were not measured in this study.

3.8. Energy requirement

Most of the PAOs' anabolic activities were stopped at high FNA concentrations including growth, P-uptake, and glycogen production. A portion of energy generated from PHA oxidization would be required for biomass maintenance in the absence of these anabolic processes. According to Smolders et al. (1994), ATP consumption for PAOs' maintenance is 0.019 mmol ATP/(Cmmol*h), which is equivalent to 0.244 mmol ATP/gBiomass in a 20-min period (assuming a biomass formula of $CH_{2.09}O_{0.54}N_{0.20}P_{0.015}$). Energy generated through PHA oxidization by oxygen, possible P-release and glycogen degradation, at high FNA concentrations, was calculated using the model of Murnleitner et al. (1996) (Table 5). The portion of PHA oxidized by nitrite is also displayed in Table 5, while energy generation from this pathway was not calculated.

At a FNA concentration of 30 μg HNO_2-N/L, energy produced through PHA oxidization by oxygen was sufficient for biomass maintenance. Additional energy produced from nitrite reduction would certainly be used for other processes. At a higher FNA concentration of 46 μg HNO_2-N/L, energy generated from PHA oxidation by oxygen was not sufficient to support biomass maintenance. The extra energy could be provided from PHA oxidation by nitrite (Table 5). Phosphorus release and glycogen consumption were observed at high FNA

concentrations, as reported by Meinhold et al. (1999) and Zhou et al. (2010). Under anaerobic conditions these two pathways are energy production processes. When these processes take place under aerobic conditions to generate energy, the possible energy generated from P-release and glycogen degradation was calculated as shown in Table 5.

It is clear that energy produced through aerobic P-release and glycogen degradation processes would not only be for maintenance process. Zhou et al. (2010) observed that intracellular ATP of the PAOs decreased dramatically after incubation in high FNA concentrations. FNA has been reported to be able to passively diffuse across the membrane of the cell, and as a consequence of the neutral intracellular pH, become trapped inside the cell in its anionic form (Hinze and Holzer 1985). To counteract the acidification effect of the weak organic acid (HNO_2), the cell will pump out protons via the energy-requiring plasma H^+-ATPase, leading to the uncoupling of energy generation from growth (Brul and Coote 1999). It is possible that biomass would need extra energy to carry out such a detoxifying process.

In summary, the results from this study indicate that PAOs may use nitrite as energy source through the reduction process to support their maintenance and other activities including detoxification via expelling of protons under inhibiting conditions. Anaerobic metabolism of PAOs, for example, P-release, glycogen degradation and PHA production was observed at high FNA concentrations (Table 3 and 5). These phenomena could be attributed to the requirement of extra energy and accumulation of acetyl-CoA and propionyl-CoA resulting from inhibition on certain enzymes.

4. CONCLUSIONS

The finding that some part of the metabolism of poly-phosphate accumulating organism continued when the growth stopped at FNA concentration of 10 μg HNO_2-N/L, may indicate that free nitrous acid can be used as an operating parameter to carry out waste and/or

wastewater treatment, at desired rates with the least excess sludge production. The identification of the thresholds of FNA inhibition on certain metabolic pathways and particular functional microbes require more studies. The investigation of intracellular activities of microbes, e.g. ATP generation and consumption, activities of functional enzymes, would help researchers to further understand the response of microbes to FNA inhibition.

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FIGURE CAPTIONS

Figure 1. Level of intracellular (PHA (▼) and glycogen (Δ)), extracellular (ammonium (●), nitrite (○) and phosphate (×)) compounds, and oxygen (•) at an FNA concentration of 0.79 µg HNO_2-N/L

Figure 2. Comparison of aerobic (○) and anoxic (Δ) P-uptake (a), nitrite reduction (b), glycogen production (c), PHA consumption (d) and ammonium consumption (e) rates, and aerobic OURmax (f) at different FNA concentrations

Figure 1.

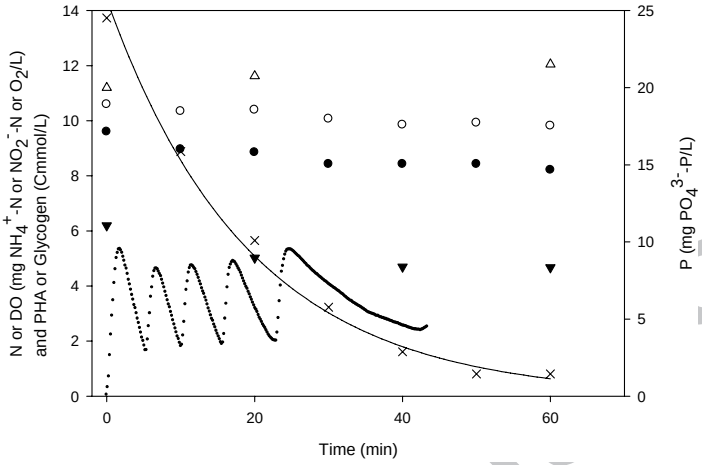

Figure 2.

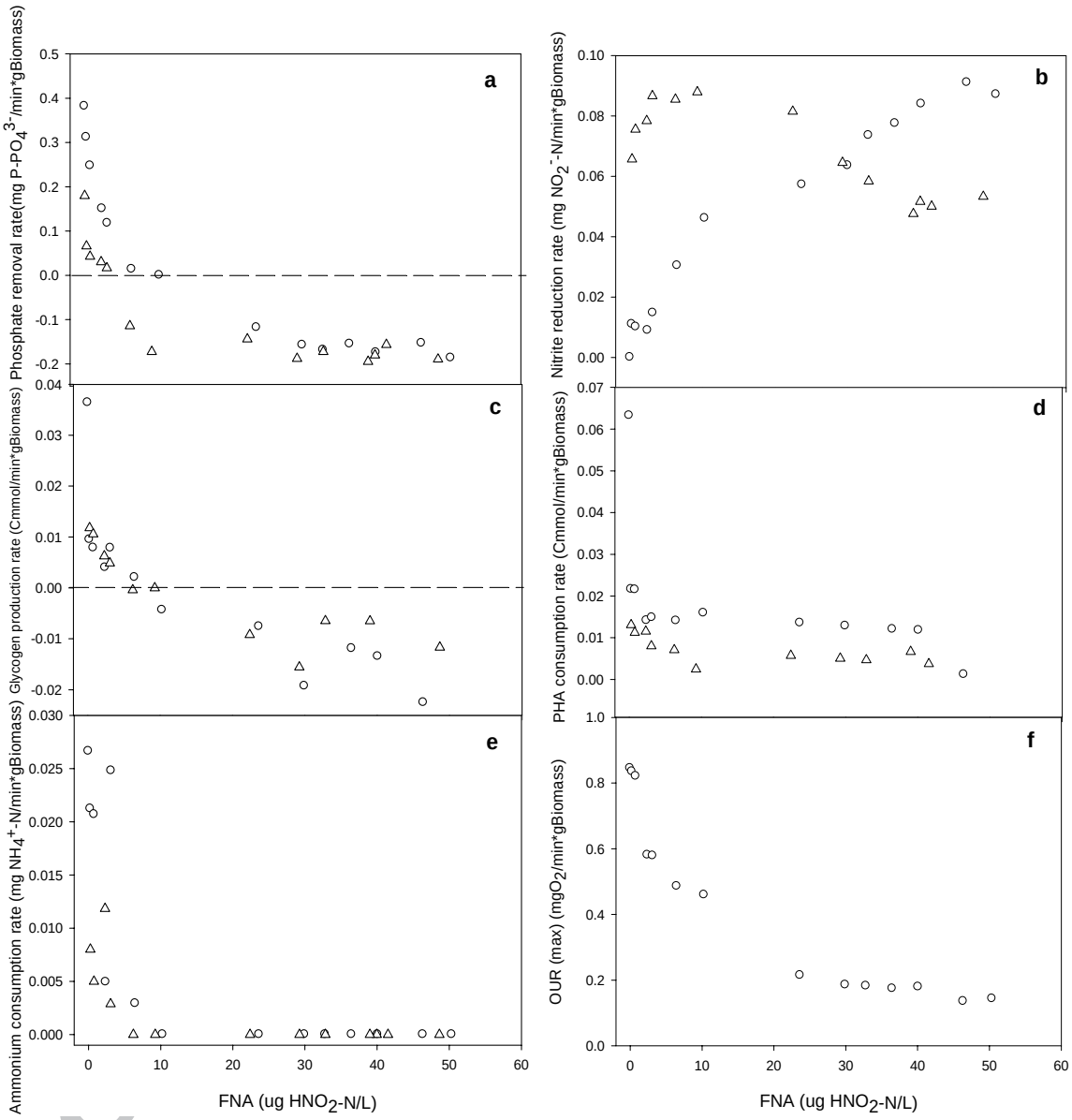

Table 1. Experimental conditions applied in batch tests

Tests No. (Aerobic)	**pH**	**NO_2^- ($mgNO_2$-N/L)**	**FNA ($\mu gHNO_2$-N/L)**
1	8	0	0
2	8	11.0	0.26
3	7.5	10.6	0.79
4	7	43.5	10.3
5	6.5	61.9	46.4
6	7.5	32.2	2.4
7	7.5	42.0	3.1
8	7	27.4	6.5
9	6.5	31.6	23.7
10	6.5	40.0	30.0
11	6.5	49.0	32.8
12	6.5	51.3	36.5
13	6.5	55.1	40.1
14	6.5	67.3	50.4
Tests No. (Anoxic)	**pH**	**NO_2^- ($mgNO_2$-N/L)**	**FNA ($\mu gHNO_2$-N/L)**
15	8	10.7	0.25
16	7.5	10.0	0.75
17	7	39.0	9.2
18	6.5	53.3	40.0
19	7.5	30.4	2.3
20	7.5	40.7	3.1
21	7	26.3	6.2
22	6.5	29.9	22.4
23	6.5	39.0	29.2
24	6.5	46.0	32.8
25	6.5	52.1	39.0
26	6.5	55.4	41.5
27	6.5	64.9	48.6

Table 2. Comparison of FNA inhibition on P-uptake by FNA-adapted and non FNA-adapted PAOs

Culture type	FNA Inhibition (%)	FNA (μg HNO_2-N/L)	Reference
non FNA-adapted PAO	72 (Oxic)	0.7	Yoshida et al. (2006)
	100 (Oxic)	1.5	Saito et al. (2004)
	100 (Oxic)	4	Pijuan et al. (2010)
FNA-adapted PAO	20 (Oxic)	0.7	Yoshida et al. (2006)
	100 (Oxic)	10	This study
	100 (Anoxic)	5	

Note: Oxic- experiments were carried out under aerobic conditions; Anoxic- experiments were carried out under anoxic conditions

Table 3. Glycogen conversion to PHA aerobically and anoxically

FNA (μg HNO_2-N/L)	PHA production (Cmmol/g Biomass)	PHB production (Cmmol/g Biomass)	PHV production (Cmmol/g Biomass)	PH2MV production (Cmmol/g Biomass)	Glycogen degradation (Cmmol/g Biomass)
Aerobic					
20	0.090	0.019	0.071	-	0.278
30	0.323	0.174	0.149	-	0.386
44	0.367	-	0.374	0.034973	0.451
Anoxic					
20	0.014	0.042	-	-	0
33	0.059	0.044	0.015	-	0.129
40	0.094	0.063	0.031	-	0.190

"-": Consumption instead of production, data are not shown here.

Table 4. Calculated required and measured amount of electron acceptors to oxidize measured amount of PHA

FNA (μg HNO_2-N/L)	Required O_2 (mmol/g Biomass)	Measured O_2 (mmol/g Biomass)	Required NO_2^- (mmol/g Biomass)	Required NO_2^- (mmol/g Biomass)	Required NO_2^- (mmol/g Biomass)	Measured NO_2^- (mmol/g Biomass)
Denitrification final product			N_2	N_2O	NO	
0	0.400	0.385	0	0	0	-
0.26	0.332	0.329	0	0	0	0.016
10.3	0.256	0.192	0.085	0.128	0.255	0.083
30.0	0.126	0.073	0.073	0.109	0.218	0.091
46.4	0.080	0.054	0.034	0.051	0.102	0.130

Table 5. ATP generation calculation

ATP for maintenance	0.244 mmolATP/g Biomass			
	PHA>O_2	PHA>NO_2^-	P-release >ATP	Glycogen>ATP
FNA (μgHNO_2-N/L)	30.0			
C or P mmol/g Biomass	0.064	0.048	0.102	0.386
ATP (mmolATP/g Biomass)	0.289	N.A.	0.102	0.193
FNA (μg HNO_2-N/L)	46.4			
C or P mmol/g Biomass	0.048	0.023	0.099	0.451
ATP (mmol/g Biomass)	0.219	N.A.	0.099	0.226

Note- PHA>O_2: amount of PHA oxidized by oxygen and energy generated through this pathway; PHA>NO_2^-: amount of PHA oxidized by nitrite; P-release>ATP: P-release amount and possible ATP generation from P-release; Glycogen>ATP: glycogen degraded amount and possible ATP generation from glycogen degradation; N.A. not available

> FNA-adapted culture is more tolerant to FNA inhibition > Anoxic P-uptake is more sensitive to the inhibition compared to aerobic P-uptake > Electrons were channeled to nitrite reduction from oxygen reduction under inhibition > Anaerobic metabolism of PAO was observed at higher FNA concentrations > The anoxic and aerobic growth of PAOs decreased sharply with FNA concentration