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Nitrous oxide emission by denitrifying phosphorus removal culture using polyhydroxyalkanoates as carbon source

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Abstract

Nitrous oxide (N_2O) emission has been reported to be enhanced during denitrification when internally-stored compounds are used as carbon sources. However, negligible N_2O emissions have been detected in the few studies where polyhydroxyalkanoates (PHA) were specifically used. This study investigated and compared the potential enhancement of N_2O production, based on utilization of an internally-stored polymer and external carbon (acetate) by a denitrifying phosphorus removal culture. Results indicated that at relatively low chemical oxygen demand-to-nitrogen (COD/N) ratios, more nitrite was reduced to N_2O in the presence of an external carbon source as compared to an internal carbon source (PHA). At relatively higher COD/N ratios, similar N_2O reduction rates were obtained in all cases regardless of the type of carbon source available. N_2O reduction rates were, however, generally higher in the presence of an internal carbon source. Results from the study imply that when the presence of an external carbon source is not sufficient to support denitrification, it is likely competitively utilized by different metabolic pathways of denitrifying polyphosphate accumulating organisms (DPAOs) and other ordinary denitrifiers. This study also reveals that the consumption of PHA is potentially the rate-limiting step for N_2O reduction during denitrification.

Key words: N_2O; denitrification; carbon source; enhanced biological phosphorus removal; DPAOs; PHA

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Introduction

Nitrous oxide is a greenhouse gas. Although atmospheric concentrations of N_2O are low, its influence on global-warming is 320 times stronger than that of carbon dioxide (CO_2). Anthropogenic activity has been determined to be a significant contributor of atmospheric N_2O. In particular, wastewater treatment processes incorporating biological nutrient removal are major sources of N_2O emissions. N_2O is a by product of microbial nitrification and denitrification, the main mechanisms for nitrogen removal in wastewater treatment processes (Chung and Chung, 2000; Fux and Siegrist, 2004; Kishida et al., 2004). Complete denitrification from nitrate (NO_3^-) to molecular nitrogen (N_2) consists of four reduction steps, with nitrite (NO_2^-), nitric oxide (NO) and N_2O as reaction intermediates. However, under certain conditions, N_2O has been found to be the final product instead of an intermediate (Otte et al., 1996; Schulthess et al., 1995; Hanaki et al., 1992; Zhou et al., 2008; Lemaire et al., 2006).

Environmental and operating conditions, e.g., dissolved oxygen (Otte et al., 1996), nitrite (Schulthess et al., 1995), pH (Hanaki et al., 1992), free nitrous acid (FNA) (Zhou et al., 2008), and carbon sources (Hanaki et al., 1992; Schulthess and Gujer, 1996; Chung and Chung, 2000; Zeng et al., 2003b; Lemaire et al., 2006), may cause N_2O-accumulation during denitrification processes. Among these parameters, carbon sources have a relatively large impact on N_2O accumulation (Kargi and Uygur, 2003).

It has been shown that denitrifying bacteria prefer volatile fatty acids (VFAs) as a carbon source over complex organic molecules (Elefsiniotis and Wareham, 2007). Polyhydroxyalkanoates (PHA) have been reported to be a possible inducer of N_2O emissions when utilized as growth substrate and the availability of external growth substrate is limited (Schalk-Otte et al., 2000). Itokawa et al. (2001) suggested that at relatively low COD/N ratios, a higher N_2O concentration could be observed due to incomplete denitrification caused by the lack of external carbon sources. However, Adouani et al. (2010) found a lack of correlation between the types of carbon source with the amount of N_2O produced. It has also been observed that COD-limited conditions did not necessarily increase N_2O accumulation (Zhang and Wang, 2009). Further, Itokawa et

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al. (2001) pointed out that endogenous denitrification did not result in N_2O accumulation.

This study investigated a special group of denitrifiers commonly found in the enhanced biological phosphorus removal (EBPR) process. Typically EBPR is achieved by subjecting polyphosphate accumulating organisms (PAOs) to alternating anaerobic and aerobic conditions. Previous works (Meinhold et al., 1999; Zeng et al., 2003a) have shown that some PAOs are able to oxidize their intracellular PHA with nitrate and/or nitrite as the terminal electron acceptor, providing energy for phosphorus uptake. This would mean the carbon source taken up by PAO in the anaerobic phase is used for both denitrification and the removal of phosphorous, and is advantageous when a wastewater contains relatively low levels of organic carbon.

N_2O accumulation can be a potential problem in such a system as PHA had been suggested to promote N_2O production from denitrifying activated sludge (Zeng et al., 2003a). However, no appreciable N_2O accumulation could be deduced based on mass-balance calculations of a denitrifying PAOs (DPAOs) reactor reported by Kuba et al. (1996) where poly-β-hydroxybutyrate (PHB) was used as carbon source for denitrification by DPAOs. Zhou et al. (2008) proved that N_2O accumulation may occur in a DPAOs system under certain conditions. They pointed out that FNA may be the inducing factor for N_2O accumulation.

Overall, the abovementioned studies on N_2O accumulation occurring under COD-limited conditions have not addressed the role of PHA as a carbon source. This study investigated the impact of relatively low COD/N ratios and PHA utilization on N_2O accumulation by a denitrifying phosphorus removal culture. To investigate the effect of the type and quantity of carbon source on N_2O accumulation, unfavorable environmental conditions for denitrification to occur were established in this study, i.e., relatively low COD/N ratios and high concentrations of nitrite.

1 Materials and methods

1.1 Seed biomass source

The DPAOs culture was drawn from a sequencing batch reactor (SBR) fed with synthetic wastewater containing organic sources, ammonia, orthophosphate, and a trace nutrients supplement. The carbon source was a mixture of acetate and propionate providing 200 mg COD/L with a $COD_{acetate}$ to $COD_{propionate}$ ratio of 3:1. The influent concentrations of ammonia and phosphate were 20 mg NH_4^+-N/L and 10 mg PO_4^{3-}-P/L, respectively. The trace nutrients supplement solution was prepared according to the method of Smolders et al. (1994). Details of reactor design, operation and performance are available in Zhou et al. (2010). The SBR was displaying excellent EBPR (> 99%) and nitrogen (> 90%) removal performance when its biomass was drawn for the batch experiments described below. Fluorescence *in-situ* hybridization (FISH) results showed that *Accumulibacter* was at an abundance of 39.8% (± 5.1%) of all bacteria bound to the EUBMIX probes. GAOs belonging to *Competibacter* were less than 1%. The *D. vanus*-cluster 1 and cluster 2 groups were not detected.

1.2 PHA as internal carbon sources ("PHA")

Pretreatment of sludge started with withdrawing 2000 mL of biomass from the parent reactor at the end of the aerobic phase and evenly distributing it into four batch reactors. The biomass was washed with phosphate buffer solution (PBS) to remove any remaining nitrite from the aerobic phase, and subsequently sparged with nitrogen gas to remove residual oxygen.

The "PHA" batch experiment comprised anaerobic (where external carbon was accumulated as PHA in DPAOs), and anoxic phases (where nitrite was added and used as an electron acceptor in denitrification). Four COD/N ratios of 0.625, 1.25, 1.875, and 2.5 were investigated with COD pre-stored as PHA during the anaerobic phase. It has been reported that the optimal COD/N ratio for complete nitrite reduction is 2.7–4.3 (Zhang and Wang, 2009). Therefore, the maximum COD/N ratio chosen of this study was 2.5 to create unfavorable conditions.

Different volumes of sodium acetate stock solution were introduced into the batch reactors to achieve the desired COD concentrations. The anaerobic phase lasted for an hour in the completely sealed batch reactors. At the end of this anaerobic phase, the biomass was washed with phosphate buffer solution (PBS) twice to remove any remaining sodium acetate and topped up with 20 mg PO_4^{3-}-P/L phosphorus solution to 500 mL in each batch reactor. N_2 was sparged through the biomass for 5 min before each reactor was sealed. A 60 mL gas-tight syringe filled with N_2 was then connected to each reactor to balance the gas pressure within the reactor during the following experiment. The two-hour anoxic phase was initiated by injecting sodium nitrite solution into the reactors. The concentration of nitrite at the start of the anoxic phase in each batch reactor was about 40 mg NO_2^--N/L. The pH in the reactor was adjusted and controlled at 7.5 ± 0.05 with addition of 0.1 mol/L NaOH or 0.1 mol/L HCl. Liquid and solid samples drawn at the beginning and end of the anaerobic phase were analyzed for VFAs and subjected to PHA and glycogen analysis. Liquid, solid, and gas samples were taken at 30-min intervals during the anoxic phase, and analyzed for phosphate, nitrite, ammonia, PHA, glycogen, NO and N_2O. Mixed liquor suspended solids (MLSS) and volatile MLSS (MLVSS) were measured at the beginning and end of the anoxic phase.

1.3 Sodium acetate as external carbon sources ("SA")

The "SA" batch experiments applied sodium acetate as the external carbon source for denitrification. The electron donor (carbon source) and electron acceptor (nitrite) were injected into the sealed batch reactors simultaneously. Pretreatment of the biomass followed the same procedures as described earlier. Four batch reactors with working volumes of 500 mL each were sealed immediately after such biomass pretreatment. Different volumes of sodium acetate solution with sodium nitrite solution were then injected into the batch reactors, resulting in COD/N ratios of 0.625, 1.25, 1.875, and 2.5. The anoxic phase lasted two hours. Control of pH and sampling strategies were the similar to those used in the "PHA" experiment.

1.4 Analysis

NH_4^+, NO_2^-, PO_4^{3-}-P, and MLSS and MLVSS concentrations were determined in accordance with Standard Methods (APHA, 1995). Acetate was measured with an Agilent gas chromatograph (Model No. 6890) equipped with a capillary column (DB-FFAP 15 m × 0.53 mm × 1.0 μm), with the injector and flame ionization detector (FID) operating at 250 and 300°C, respectively. High purity helium was used as the carrier gas at a constant pressure of 103 kPa. A 0.9-mL of the filtered sample was transferred into a GC vial to which 0.1 mL of formic acid was added. In each analysis, a sample volume of 1 μL was injected in splitless mode.

The concentration of glycogen was determined using the method of Zeng et al. (2003b). Briefly, a volume of 5 mL of 0.6 mol/L HCl was added into a known weight of freeze-dried biomass in screw-topped glass tubes, and then heated at 105°C for 6 hr. After cooling and centrifugation, 1 mL of the supernatant was analyzed for glucose using high performance liquid chromatography (HPLC). PHA analysis was performed using the method of Oehmen et al. (2005) to determine PHB, poly-β-hydroxyvalerate (PHV), and poly-β-hydroxy-2-methylvalerate (PH2MV). Freeze-dried biomass of known weights, and PHB/V and PH2MV standards were introduced into screw-topped glass tubes. The tubes were heated at 100°C for 20 hr after the biomass was suspended in 2 mL methanol (2 mL chloroform and acidified with 3% H_2SO_4). After cooling, 1 mL Milli-Q water was added and the sample was mixed. When the phases separated, approximately 1 mL of the bottom organic layer was transferred for analysis via gas chromatography.

Both dissolved N_2O (within the liquid phase) and emitted N_2O (in the reactor headspace) samples were collected in vacuum tubes (BD Diagnostics, USA). In order to extract the dissolved N_2O from the liquid phase, the liquid samples and the tubes were shaken overnight to allow for the equilibration of gas and liquid phases. The liquid samples were transferred to new vacuum tubes to be re-extracted and analysed for any residual N_2O, of which none was detected in all cases. Accumulated N_2O concentrations at the sampling point were quantified by the sum of N_2O concentrations arising from the analysis of both the liquid and gas samples. N_2O emission is the phenomenon of the balance of N_2O in both the gas- and liquid-phases under equilibrium conditions. In order to evaluate total N_2O emissions, total accumulated N_2O from the biosystem was considered; this can indirectly indicate the N_2O emission potential. N_2O and NO were determined using an Agilent GC 6890 coupled with a capillary column (GS Gaspro 60 m × 0.32 mm × 1.0 μm). Gas samples of 100 μL were manually injected into the GC operated with an oven temperature of 35°C. Inlet pressure was kept constant at 141.3 kPa. Helium was used as the carrier gas at a flow rate of 3 mL/min. The Micro-ECD detector was operated at 340°C. The limits of detection of the GC were 0.3 ppm and 0.1 ppm for N_2O and NO, respectively. Further, no NO was detected in all cases in the current study.

Fluorescence *in situ* hybridization (FISH) of PAO was performed as described in 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 α-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. FISH preparations were observed with a Zeiss LSM 510 Meta confocal laser-scanning microscope (CLSM) using a Plan-Apochromat 63× oil (normal aperture 1.4) objective. Thirty images were taken from each sample for quantification. All the images were 8-bit, 512 × 512 pixels and of 0.1 μm pixel size. The area containing cells targeted by the Cy3- or Cy5-labelled specific probes (PAOMIX, GAOMIX or α-GAO) was quantified as a percentage of the area of Cy5- or FITC-labelled Bacteria probe (EUBMIX) within each image using a pixel-counting program. The final quantification result was expressed as a mean percentage (with standard errors) obtained from the 30 images. The standard error of the mean (SE_{mean}) was calculated as the standard deviation of the percentage area divided by the square root of the number of images analyzed.

2 Results and discussion

Transitions of PHA, glycogen, nitrite, phosphate and N_2O compounds from both "PHA" and "SA" tests at the same COD/N ratio of 1.25 are displayed in Fig. 1. The rates of transition of the compounds were determined against the biomass concentration and described as mg N, P/(min·g biomass), or mg C/(min·g biomass). Details involving the calculations can be found in Zhou et al. (2007). N_2O accumulation rates were obtained from linear regression of N_2O concentrations (within both liquid and gas phases) during the 2 hr anoxic phase. As an example, Fig. 1b shows the N_2O and NO_2^- concentration profiles with regression trendlines. At pH 7.5 and an initial nitrite concentration of 38 mg NO_2^--N/L, nitrite reduction (N_2O production) and N_2O accumulation occurred simultaneously. The rates of the two processes were determined as 0.056 and 0.034 mg NO_2^--N or N_2O-N/(min·g biomass), respectively, through linear regression. The N_2O reduction rate was calculated as the difference between the measured NO_2^- reduction

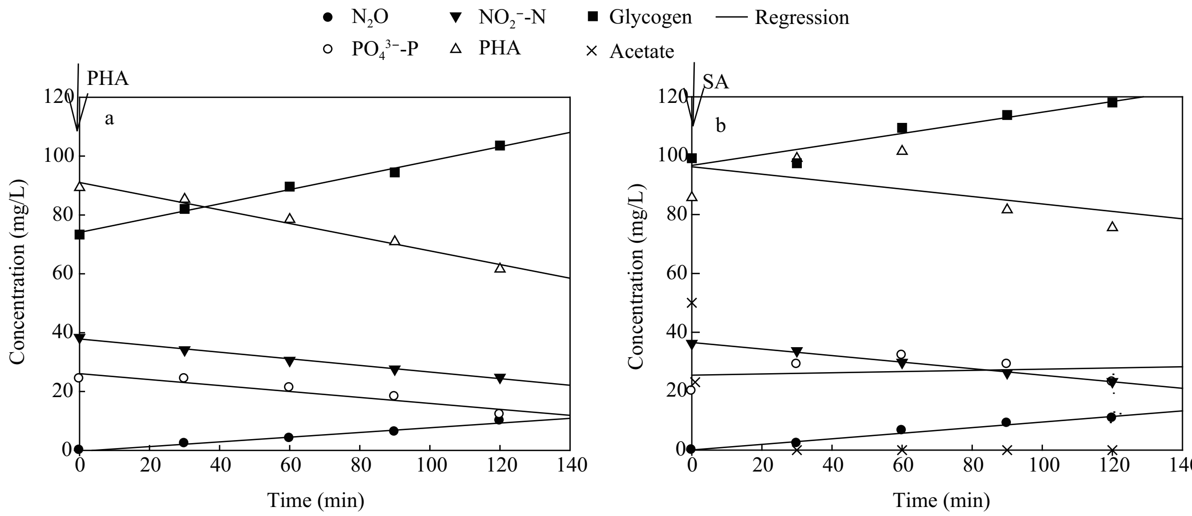

Fig. 1 Nitrite, phosphorous, N_2O, acetate, PHA, and glycogen concentration profiles during denitrification by internal carbon PHA (a) and external carbon sodium acetate (b) (COD/N = 1.25).

rate and N_2O accumulation rate. The N_2O reduction rates in other batch experiments were determined in a similar fashion. The extent of N_2O accumulation was determined as the percentage of denitrified nitrite accumulated as N_2O.

As shown in Fig. 1a, the biomass expressed a typical anoxic P-removal phenotype of DPAOs. Phosphate was taken up by utilizing the energy generated from PHA degradation and denitrification, with the replenishment of glycogen. The rate of accumulation of N_2O was constant (Fig. 1b). When COD was added as an external carbon source (acetate), anaerobic DPAOs metabolism with P-release, PHA generation and glycogen consumption was observed. Once the external carbon source was depleted (Fig. 1b), anoxic metabolism of DPAOs was employed. Nitrite reduction and N_2O accumulation were at relatively constant rates (R^2 > 0.98) during the entire test.

Figure 2 shows a comparison, under different COD/N ratios, of N_2O accumulation (Fig. 2a), production

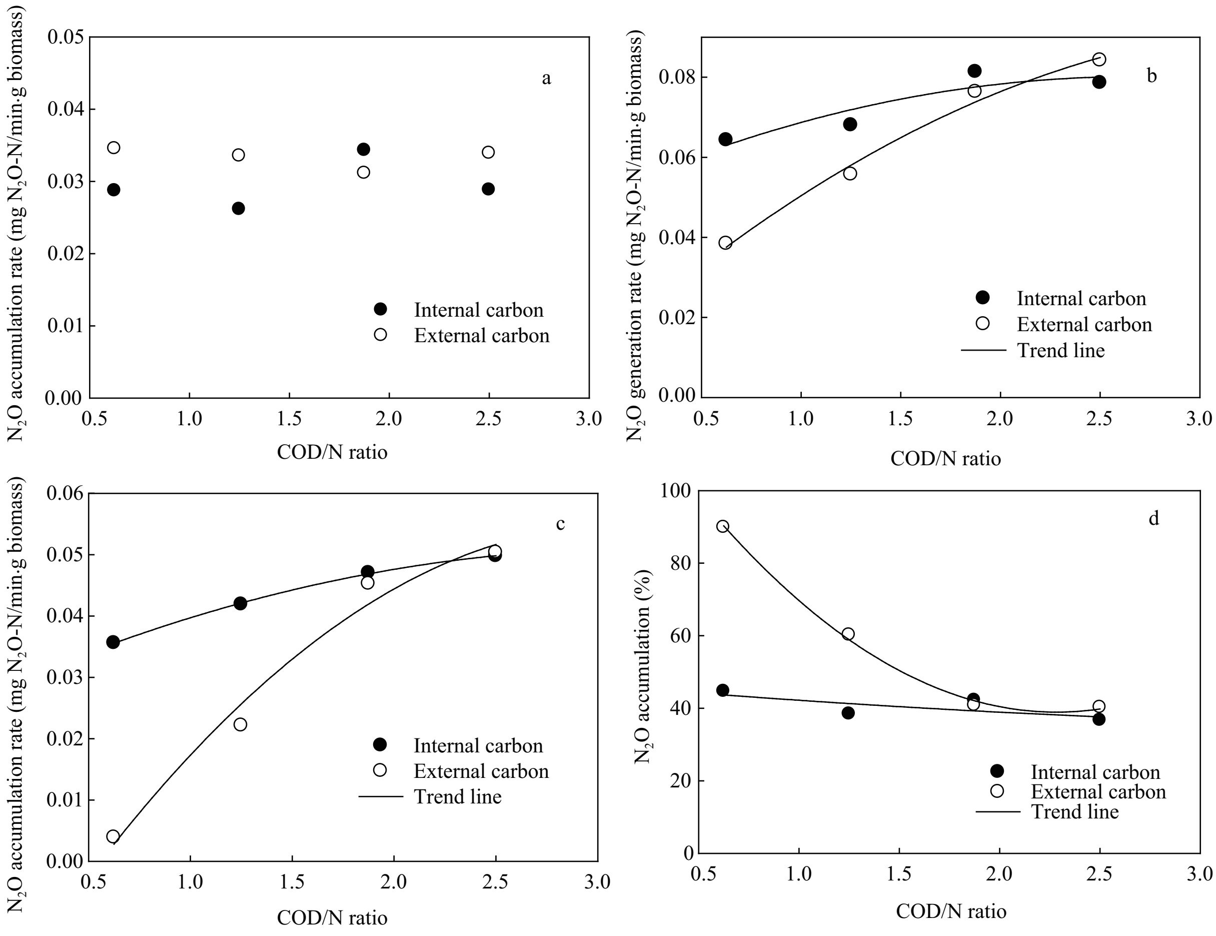

Fig. 2 N_2O accumulation (a), production (b), reduction (c) rates and percentages of N_2O accumulation (d) at different COD/N ratios.

(Fig. 2b), reduction (Fig. 2c) rates, and accumulation percentages (Fig. 2d) between the use of PHA and sodium acetate as the internal and external carbon source, respectively. In considering a particular COD/N ratio, the rates of accumulation of N_2O in the presence of an external carbon source were generally higher (c.f. an internal carbon source) (Fig. 2a). The study also suggested that the COD/N ratio did not significantly affect the rate of N_2O accumulation in the presence of both external and internal carbon source conditions. Furthermore, this indicated that in the considered range of COD/N ratios between 0.625 to 2.5, relatively higher COD/N ratios may not necessarily reduce N_2O accumulation rates from a denitrifying P-removal system.

The N_2O production rate was determined by the nitrite reduction rate as shown in Fig. 2b. With increasing COD/N ratios, the rates of production of N_2O increased slowly with PHA as carbon source. This suggested that when PHA was used as the sole electron donor, the COD/N ratio might not have been the major factor influencing nitrite reduction or N_2O production. In addition, the rate of PHA utilization was potentially the rate-limiting (Beun et al., 2002) step of denitrification. On the other hand, the rates of production of N_2O in the presence of external carbon increased sharply with increasing COD/N ratios. Therefore, availability of external carbon source and the COD/N ratio are likely important conditions for complete denitrification to occur externally (Chung and Chung, 2000; Kishida et al., 2004). Figure 2b also shows that nitrite reduction or N_2O production rates were generally higher with an internal carbon source compared to the external carbon source situation. This suggested that PHA may be a more efficient and accessible carbon source for DPAOs under carbon-limited conditions.

Figure 2c shows that higher N_2O reduction rates were obtained at higher COD/N ratios and this was more pronounced when an external carbon source was available. The N_2O reduction rates with an internal carbon source at relatively lower COD/N ratios (0.625–1.25) were higher than those in the presence of an external carbon source. The results added evidence that at very low COD/N ratios, stored PHA would be the preferred carbon source in DPAOs' denitrification process. At a COD/N ratio of 0.625, N_2O was hardly reduced in the presence of an external carbon source (Fig. 2c). It is clear that a very limited amount of external carbon source was used for N_2O reduction under this condition. It is noted that as long as the external carbon source was sufficient to support nitrite reduction processes (based on a COD/N ratio of 1.875 and above), N_2O reduction rates were comparable for both carbon types (Fig. 2c).

Low N_2O reduction activity results in increased N_2O accumulation. Figure 2d shows that with the external carbon sources, nearly 90% of denitrified nitrite accumulated as N_2O under low COD/N ratio conditions. The extent of this accumulation decreased as COD/N ratios increased. This trend is consistent with the study of Chung and Chung (2000) and Itokawa et al. (2001). Both groups reported there was less N_2O emission at higher COD/N ratios but this sharp decrease was only observed in studies involving external carbon sources. Using PHA as a carbon source, the extent of N_2O accumulation was not significantly reduced with greater carbon source supply. Approximately 40% of reduced nitrite accumulated as N_2O regardless of the COD/N ratios.

Comparing the extents of N_2O accumulation for the different types of carbon source and at the same COD/N ratio, a significant difference was observed only at the lower COD/N ratios. This limitation of an external carbon source supplied for denitrification may potentially introduce competitive utilization of carbon sources in a DPAO culture. The competition for electron donors would occur in the different metabolic pathways of DPAOs in terms of denitrification and PHA storage, and between ordinary denitrifiers and DPAOs.

Kuba et al. (1994) also observed that an external carbon source, sodium acetate, was partially utilized for denitrification and also for P-release in the presence of nitrate. They proposed that the reducing power for the formation of PHA is supplied through the tricarboxylic acid (TCA) cycle, and that the energy is produced by denitrification and P-release. Kuba et al. (1994) reported that 80% of the acetate is utilized for PHA production and phosphorus release, and that remaining is used in denitrification. Therefore, with the simultaneous presence of a carbon substrate and nitrite, some of the carbon substrate was likely to be stored as PHA in DPAOs, resulting in a further limitation of electron donors (necessary for nitrite and N_2O reduction).

Because of the large phylogenetic diversity of denitrifying bacteria, the commonly targeted rRNA genes are impractical for examining denitrifying communities (Wallenstein et al., 2006). In this study, the denitrifying population was not quantified. However, the existence of denitrifiers in this system cannot be discounted. It has been proposed that DPAOs may have *nirS* gene codes for nitrite reductase (Shoji et al., 2006). Hence, DPAOs may be considered a unique group of denitrifiers capable of utilizing both external (Kuba et al., 1994) and internal (Zhou et al., 2007) carbon for denitrification. Currently, the metabolism of DPAOs in using external carbon sources for the purpose of denitrification is not fully understood.

In the "PHA" study, PHA was used as the sole carbon source during the anoxic period (this period is hereafter referred to as PHAI). Most of the acetate in all "SA" tests was consumed in the first 30 min of the anoxic phase (Fig. 3). There was a period when acetate was depleted and PHA was used as the sole carbon source for denitrification (this period is hereafter referred to as PHAII). Figure 4 shows a comparison of N_2O reduction rates and percentage of N_2O accumulation between PHAI and PHAII. N_2O reduction rates and extent of N_2O accumulation during the PHAII period were determined from 30 min onwards (i.e., when acetate was absent). Figure 4a shows that at very low COD/N ratios (0.625 and 1.25), N_2O reduction rates were much lower in the PHAII tests as compared to those noted in the PHAI tests, resulting in N_2O as the main product of denitrification.

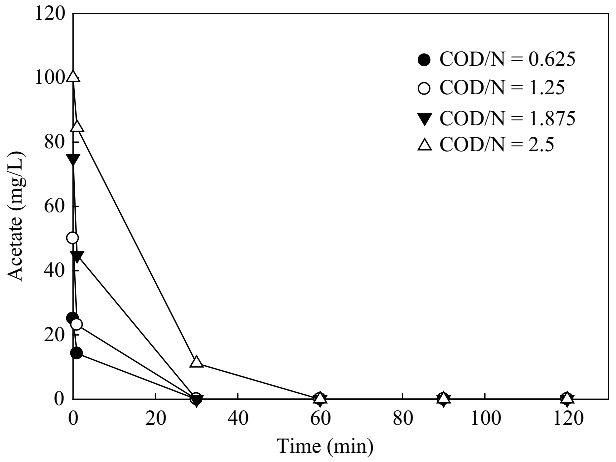

Fig. 3 Sodium acetate concentrations under different COD/N ratios in SA tests.

Table 1 Amount of accumulated PHA during the anaerobic phase* and comparison of ratios of PHA reduction rate to N_2O reduction rate of PHAI and PHAII tests with different COD/N ratios

COD/N ratio	PHA accumulation amount (mg C/g biomass)		PHA reduction rate/N_2O reduction rate (mg C/mg N)	
	PHAIII	PHAIV*	PHAI	PHAII
0.625	35.77	4.28	4.38	87.26
1.25	29.23	14.69	4.15	9.93
1.88	43.05	22.18	4.25	3.67
2.50	63.46	31.17	4.24	3.60

* in "PHA" study (hereafter referred to as PHAIII) as well as in the first 30 min of the "SA" study (hereafter referred to as PHAIV).
In PHAIV, acetate was present as external carbon source.

Table 1 shows the amount of PHA accumulated during the anaerobic phase under different starting concentrations of COD. The ratios of PHA reduction rate to the N_2O reduction rate of PHAI and PHAII tests under different COD/N ratios are also compared and shown in Table 1. It is noted that more PHA was stored in the PHAIII study. Hardly any PHA accumulation occurred at a COD/N ratio of 0.625 using external carbon in PHAIV. This may account for N_2O becoming the main denitrifying product during the PHAII period with COD/N of 0.625, due to a lack of carbon source (Fig. 4b). However, the results from the current study are in contrast with Kuba et al. (1994) where 80% of an external carbon source was accumulated as PHA by DPAOs. Given the fact that with such a low COD/N ratio and limited accumulation of PHA, the nitrite reduction rate could achieve 50% of the maximum rate obtained (COD/N of 2.5) in this study (Fig. 2b), nitrite reduction was certainly carried out through other pathways and/or by other microbial communities.

Table 1 also shows that in spite of the varying N_2O reduction rates from the PHAI tests (Fig. 4a), the ratios of PHA to N_2O had remained rather constant. This confirmed that the N_2O reduction rate is regulated and limited by the PHA degradation process (Beun et al., 2002). In addition, comparing the ratios of PHA to N_2O from the PHAI and PHAII studies at lower COD/N ratios (0.625–1.25), the ratios from the PHAII studies were considerably higher than those from the PHAI studies. It was clear further reduction of N_2O was significantly affected. It is however, unclear as to whether PHA utilization would be prioritized for phosphorus uptake at very low PHA levels.

In summary, this study applied different concentrations of carbon sources to a denitrifying phosphorus removal culture in two different ways. First, varied concentrations of COD in the form of sodium acetate were added to the culture during an anaerobic phase to generate different levels of PHA. Sodium acetate was also simultaneously applied with the electron acceptor nitrite, resulting in a much lower level of PHA accumulation as compared to the earlier case. The results indicated the available amount of intracellular PHA affected denitrification rates (Fig. 2b) (particularly N_2O reduction rates; Fig. 4a). However, if the available PHA was sufficient to support the multiple metabolic pathways of DPAOs, the PHA degradation rates controlled the N_2O reduction rates (Table 1). Increasing carbon source loading in both studies did not reduce N_2O accumulation. This could be due to the slow degradation kinetics of PHA and direct inhibition from FNA on N_2O reductase (Beun et al., 2002; Zhou et al., 2008).

It is noteworthy that although the simultaneous denitrifying phosphorus removal system has been proposed to be a carbon-source-efficient system and thus suitable

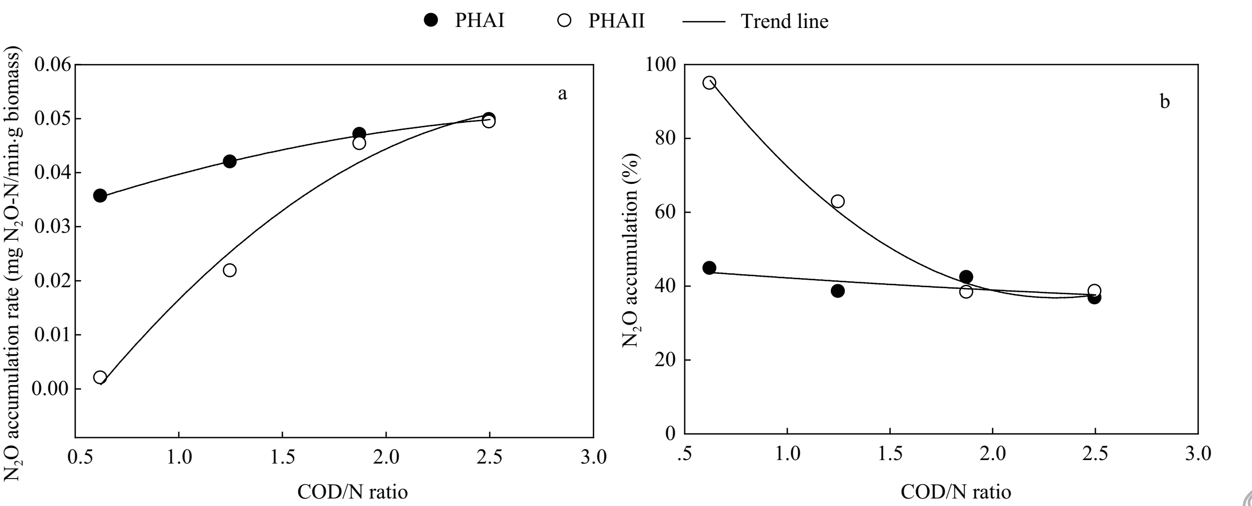

Fig. 4 N_2O reduction rates (a) and percentage of N_2O accumulation (b) in the internal carbon sources study (PHAI) and in the internal carbon sources period of the external carbon sources study (PHAII).

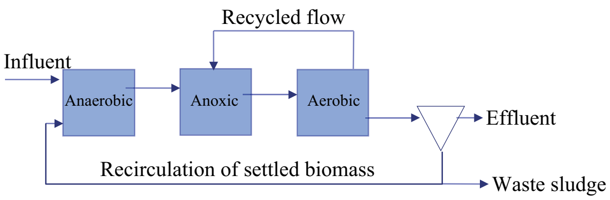

Fig. 5 Schematic of low N_2O emission wastewater treatment system for treating low carbon content wastewater.

for treatment of low-carbon-content wastewater, it may potentially induce the N_2O emission problem. In order to reduce N_2O emission from such a system, the current study suggests avoidance of competitive carbon sources (i.e., allowing a purely anaerobic phase to precede an anoxic phase). The recycled stream which may contain nitrite or nitrate can be fed into the anoxic phase (Fig. 5). Ordinary denitrifiers that may compete for carbon sources with DPAOs can therefore be gradually removed through this approach.

3 Conclusions

This study investigated the differences in N_2O accumulation caused by DPAOs in environments with internal (PHA) and external carbon (sodium acetate) sources. The following conclusions can be drawn:

At very low COD/N ratios (0.625–1.25), an external carbon supply may enhance N_2O accumulation when compared with the situation where the same level of carbon supply was applied as internal carbon (due to the special metabolism of DPAOs and potential carbon competition from ordinary denitrifiers). This phenomenon is likely to have an impact on simultaneous denitrifying phosphorus removal plant operation strategies in terms of avoiding N_2O accumulation.

At relatively higher COD/N ratios (1.875–2.5), the nature of the carbon source seemed not to affect N_2O accumulation. Similar percentages of N_2O accumulation were noted at higher COD/N ratios regardless of carbon source type.

The constant percentages of N_2O accumulation and ratios of PHA to N_2O drawn from the internal carbon source tests indicate that PHA consumption for denitrification is potentially the rate-limiting step for N_2O reduction.

At this stage, it is unclear if DPAOs can utilize an external carbon source for denitrification. A metabolic study on DPAOs utilizing external carbon and nitrite or nitrate simultaneously is required to provide a more detailed explanation of the impact of DPAOs on N_2O accumulation.

References

Adouani N, Lendormi T, Limousy L, Sire O, 2010. Effect of the carbon source on N_2O emissions during biological denitrification. *Resources, Conservation and Recycling*, 54(5): 299–302.

Amann R I, 1995. Fluorescently labelled, rRNA-targeted oligonucleotide probes in the study of microbial ecology. *Molecular Ecology*, 4(5): 543–554.

APHA (American Public Health Association), 1995. Standard methods for the examination of water and wastewater (19th ed.). Washiongton DC, USA.

Beun J J, Dircks K, Van Loosdrecht M C M, Heijnen J J, 2002. Poly-β-hydroxybutyrate metabolism in dynamically fed mixed microbial cultures. *Water Research*, 36(5): 1167–1180.

Chung Y C, Chung M S, 2000. BNP test to evaluate the influence of C/N ratio on N_2O production in biological denitrification. *Water Science and Technology*, 42(3-4): 23–27.

Crocetti G R, Banfield J F, Keller J, Bond P L, Blackall L L, 2002. Glycogen accumulating organisms in laboratory-scale and full-scale activated sludge process. *Microbiology*, 148: 3353–3364.

Crocetti G R, Hugenholtz P, Bond P L, Schuler A, Keller J, Jenkins D et al., 2000. Identification of polyphosphate-accumulating organisms and design of 16S rRNA-directed probes for their detection and quantitation. *Applied and Environmental Microbiology*, 66(3): 1175–1182.

Daims H, Brühl A, Amann R, Schleifer K H, Wagner M, 1999. The domain-specific probe EUB338 is insufficient for the detection of all bacteria: Development and evaluation of a more comprehensive probe set. *Systematic and Applied Microbiology*, 22(3): 434–444.

Elefsiniotis P, Wareham D G, 2007. Utilization patterns of volatile fatty acids in the denitrification reaction. *Enzyme and Microbial Technology*, 41(1-2): 92–97.

Fux C, Siegrist H, 2004. Nitrogen removal from sludge digester liquids by nitrification/denitrification or partial nitritation/anammox: Environmental and economical considerations. *Water Science and Technology*, 50(10): 19–26.

Hanaki K, Hong Z, Matsuo T, 1992. Production of nitrous oxide gas during denitrification of wastewater. *Water Science and Technology*, 26(5-6): 1027–1036.

Itokawa H, Hanaki K, Matsuo T, 2001. Nitrous oxide production in high-loading biological nitrogen removal process under low COD/N ratio condition. *Water Research*, 35(3): 657–664.

Kargi F, Uygur A, 2003. Effect of carbon source on biological nutrient removal in a sequencing batch reactor. *Bioresource Technology*, 89(1): 89–93.

Kishida N, Kim J H, Kimochi Y, Nishimura O, Sasaki H, Sudo R, 2004. Effect of C/N ratio on nitrous oxide emission from swine wastewater treatment process. *Water Science and Technology*, 49(5-6): 359–365.

Kuba T, Murnleitner E, Van Loosdrecht M C M, Heijnen J J, 1996. A metabolic model for biological phosphorus removal by denitrifying organisms. *Biotechnology and Bioengineering*, 52(6): 685–695.

Kuba T, Wachtmeister A, Van Loosdrecht M C M, Heijnen J J, 1994. Effect of nitrate on phosphorus release in biological phosphorus removal systems. *Water Science and Technology*, 30(6): 263–269.

Lemaire R, Meyer R, Taske A, Crocetti G R, Keller J, Yuan Z G, 2006. Identifying causes for N_2O accumulation in a lab-scale sequencing batch reactor performing simultaneous nitrification, denitrification and phosphorus removal. *Journal of Biotechnology*, 122(1): 62–72.

Meinhold J, Filipe C D M, Daigger G T, Isaacs S, 1999. Characterization of the denitrifying fraction of phosphate accumulating organisms in biological phosphate removal. *Water Science and Technology*, 39(1): 31–42.

Meyer R L, Saunders A M, Blackall L L, 2006. Putative glycogen-accumulating organisms belonging to the *Al-*

phaproteobacteria identified through rRNA-based stable isotope probing. *Microbiology*, 152(2): 419–429.

Oehmen A, Keller-Lehmann B, Zeng R J, Yuan Z G, Keller J, 2005. Optimisation of poly-β-hydroxyalkanoate analysis using gas chromatography for enhanced biological phosphorus removal systems. *Journal of Chromatography A*, 1070(1-2): 131–136.

Otte S, Grobben N G, Robertson L A, Jetten M S, Kuenen J G, 1996. Nitrous oxide production by *Alcaligenes faecalis* under transient and dynamic aerobic and anaerobic conditions. *Applied and Environmental Microbiology*, 62(7): 2421–2426.

Schalk-Otte S, Seviour R J, Kuenen J G, Jetten M S M, 2000. Nitrous oxide (N_2O) production by *Alcaligenes faecalis* during feast and famine regimes. *Water Research*, 34(7): 2080–2088.

Schulthess R V, Gujer W, 1996. Release of nitrous oxide (N_2O) from denitrifying activated sludge: verification and application of a mathematical model. *Water Research*, 30(3): 521–530.

Schulthess R V, Kühni M, Gujer W, 1995. Release of nitric and nitrous oxides from denitrifying activated sludge. *Water Research*, 29(1): 215–226.

Shoji T, Nittami T, Onuki M, Satoh H, Mino T, 2006. Microbial community of biological phosphorus removal process fed with municipal wastewater under different electron acceptor conditions. *Water Science and Technology*, 54(1): 81–89.

Smolders G J F, Van der Meij J, Van Loosdrecht M C M, Heijnen J J, 1994. Model of the anaerobic metabolism of the biological phosphorus removal process: Stoichiometry and pH influence. *Biotechnology and Bioengineering*, 43(6): 461–470.

Wallenstein M D, Myrold D D, Firestone M, Voytek M, 2006. Environmental controls on denitrifying communities and denitrification rates: Insights from molecular methods. *Ecological Applications*, 16(6): 2143–2152.

Wong M T, Tan F M, Ng W J, Liu W T, 2004. Identification and occurrence of tetrad-forming *Alphaproteobacteria* in anaerobic-aerobic activated sludge processes. *Microbiology*, 150(11): 3741–3748.

Zeng R J, Lemaire R, Yuan Z, Keller J, 2003a. Simultaneous nitrification, denitrification, and phosphorus removal in a lab-scale sequencing batch reactor. *Biotechnology and Bioengineering*, 84(2): 170–178.

Zeng R J, Yuan Z, Keller J, 2003b. Enrichment of denitrifying glycogen-accumulating organisms in anaerobic/anoxic activated sludge system. *Biotechnology and Bioengineering*, 81(4): 397–404.

Zhang J R, Wang S Y, 2009. Effect of influent COD/N ratio on nitrous oxide production during denitrification using different electron acceptors. In: Proceedings of the 2009 International Conference on Energy and Environment Technology (ICEET 2009). Piscataway, NJ, USA. 16–18 October. Vol. 2: 511–514.

Zhou Y, Ganda L, Lim M, Yuan Z G, Kjelleberg S, Ng W J, 2010. Free nitrous acid (FNA) inhibition on denitrifying poly-phosphate accumulating organisms (DPAOs). *Applied Microbiology and Biotechnology*, 88(1): 359–369.

Zhou Y, Pijuan M, Yuan Z G, 2007. Free nitrous acid inhibition on anoxic phosphorus uptake and denitrification by poly-phosphate accumulating organisms. *Biotechnology and Bioengineering*, 98(4): 903–912.

Zhou Y, Pijuan M, Yuan Z G, 2008a. Development of a 2-sludge, 3-stage system for nitrogen and phosphorous removal from nutrient-rich wastewater using granular sludge and biofilms. *Water Research*, 42(12): 3207–3217.

Zhou Y, Pijuan M, Zeng R J, Yuan Z G, 2008b. Free nitrous acid inhibition on nitrous oxide reduction by a denitrifying-enhanced biological phosphorus removal sludge. *Environmental Science and Technology*, 42(22): 8260–8265.

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