

# Improvement of denitrification by denitrifying phosphorus removing bacteria using sequentially combined carbon

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**Abstract** The effects of sequentially combined carbon (SCC) using a symbiotic relationship of methanol and acetic acid on biological nutrient removal were investigated in both the continuous bench scale process consisting of an anoxic, an aerobic and a final settling tank and intensive batch tests. Compared to the use of respective sole carbon sources, methanol and acetic acid, the use of SCC showed superior removal efficiency of nitrogen (98.3%) and phosphorus (approximately 100%). Furthermore, the use of SCC enhanced simultaneous denitrification and phosphorus uptake by denitrifying phosphorus removal bacteria (DPB), resulting in the highest specific denitrification rate (SDNR) of 0.252 g NO<sub>3</sub>-N/g VSS/d achieved from the first anoxic zone with methanol of 30 mg COD/l. From batch tests performed under carbon limited anoxic conditions, 1 g of nitrate was used by DPB for P-uptake of 1.19 g. According to this result, 0.205 g NO<sub>3</sub>-N/g VSS/d was accomplished by normal denitrifiers using methanol, and 0.047 g NO<sub>3</sub>-N/g VSS/d was achieved by DPB. This research also demonstrated that the increase of poly-β-hydroxybutyrate (PHB) stored by phosphorus accumulating organisms (PAOs) could be of importance in improving aerobic denitrification. The use of SCC produced the highest P-release in the anoxic zone, indicating the amount of PHB would be higher compared to the use of other sole carbons. Therefore, the SCC could be a very effective carbon source for the enhancement of aerobic denitrification as well.

**Keywords** Aerobic denitrification; denitrifying phosphorus removal bacteria; poly-β-hydroxybutyrate; sequentially combined carbon; specific denitrification rate

## Introduction

Denitrification is generally produced by facultative heterotrophic bacteria oxidizing carbon substrate as an energy source under the absence of oxygen. In recent years, many studies (Kern-Jespersen and Henze, 1993; Mino *et al.*, 1995; Barker and Dold, 1996; Meinhold *et al.*, 1998) have demonstrated that denitrification can take place by DPB, a fraction of PAOs, capable of utilizing nitrate as oxidant for phosphate uptake under anoxic conditions. Mino *et al.* (1995) proposed that a fraction of PAOs have a denitrification capability using nitrate and PHB as an electron acceptor and donor, respectively. Recently, Chuang *et al.* (1996) found that when the available external carbon source had been depleted, low rate denitrification and phosphorus uptake started to occur at the same time, while the presence of available organic substrate under the anoxic condition causes a higher simultaneous denitrification and phosphorus release. Their preliminary metabolism model of DPB showed that once the available acetate had been completely consumed, the only way for the generation of ATP is to degrade PHB using nitrate, leading to concurrent phosphorus uptake and denitrification. Kern-Jespersen and Henze (1993) reported that the rate of phosphorus uptake occurring under anoxic conditions was dependent on the size of PHB stored by DPB; when PHB is depleted, phosphorus uptake did not take place even though nitrate is available.

It is also widely accepted that some microbes can simultaneously utilize nitrates and oxygen as terminal electron donors, leading to denitrification under fully aerobic conditions (Anderson and Levine, 1986; Robertson *et al.*, 1989; Kugelman and Spector,

1992; Kim, 1993; Ho, 1994). This phenomenon is termed aerobic denitrification. Therefore, taking advantage of DPB or aerobic denitrifiers could result in the overcoming of organic carbon shortage and aeration demand.

In the present study, the effects of SCC on denitrification by DPB and aerobic denitrifiers were investigated and compared with the use of respective sole carbon sources, methanol and acetic acid. The aim of the use of SCC was to minimize the adverse effect of nitrate on P-release due to substrate competition. Thus methanol that does not affect phosphorus removal was used for nitrogen removal first and then acetic acid was used only for phosphorus removal.

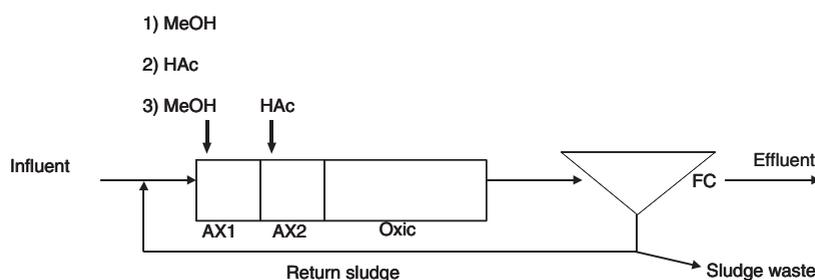
## Materials and methods

### Operation of continuous bench scale process

The flow scheme of the bench scale pilot plant for this research is depicted in Figure 1 to treat the effluent of the MLE (Modified Luzack-Ettinger) activated sludge pilot plant at the Rockland County Sewer District No. 1 wastewater treatment plant, NY. The anoxic zone was compartmentalized into two zones for the addition of SCC. Methanol of 30 mg COD/l was added to the first anoxic zone and acetic acid of 50 mg COD/l was added to the second anoxic zone. When a sole carbon source was used, it was supplemented to the first anoxic zone at a quantity equivalent to 80 mg COD/l, and the results related to the anoxic zone were obtained from the second anoxic zone. The characteristics of the influent are shown in Table 1. Since organic substrate present in the influent was mostly non-biodegradable, nitrogen and phosphorus removal in this research was affected only by the supplemented external carbon source. The process HRT for the bench scale reactors was 30 minutes for each anoxic zone and 80 minutes for the aerobic zone. The influent flow was 450 L/day and the sludge return ratio ( $Q_r/Q$ ) was 1. The MLSS was in the range of 4,000–5,000 mg/l, corresponding to SRT of 7 to 15 days.

### Batch experiments

Intensive batch experiments were also carried out to study the activity of biomass derived from the continuous bench scale process operated with the addition of SCC. These



1) For the use of sole methanol added to the 1st anoxic zone, 2) For the use of sole acetic acid added to the 1st anoxic zone, 3) For the use of SCC, methanol and acetic acid added to the 1st and 2nd anoxic zones, respectively.

AX 1: 1st anoxic zone, AX 2: 2nd anoxic zone, FC: Final clarifier

**Figure 1** Flow scheme of the continuous bench scale pilot plant

**Table 1** Characteristics of the influent fed to the bench scale process

TIN, mg N/l	Ortho-P, mg P/l	*SCOD, mg/l	*TCOD, mg/l	pH
24–37	4–6	105–110	160–170	7–7.4

\* The concentration of COD represented the sum of external carbon sources at a level of 80 mg/l as COD and influent carbon substrate that was mostly non biodegradable

experiments included determination of denitrification rate at various concentrations of methanol and the capacity of simultaneous denitrification and phosphorus uptake under anoxic and carbon limited conditions.

Batch tests for denitrification with methanol were carried out with 1.5 litres of sludge taken from the first anoxic tank of the continuous bench scale process in which the phosphorus concentration was below 1 mg P/l. A solution of sodium nitrate was added in the batch reactor to adjust the initial nitrate concentration to around 18 mg N/l. After mixing for 30 seconds, the initial concentration of nitrate, phosphorus, COD and VSS was measured, and then three different methanol dosages of 20, 40 and 60 mg COD/l were added respectively. Samples were taken from the reactor as a function of HRT, and the concentrations of COD and nitrate were measured.

In order to investigate the interaction between P-uptake and  $\text{NO}_3$  reduction under carbon limited anoxic conditions, the tests were conducted in 1.5 litres of sludge taken from the second anoxic tank where the concentration of phosphorus release was about 24 mg P/l. The 4 experimental runs were tested with various initial nitrate concentrations of 0.7, 5.3, 9.2 and 13.8 mg N/l, respectively, by adding a solution of sodium nitrate to the reactor. None of the external carbon substrates was added to the batch reactor. The concentrations of nitrate and phosphorus were measured as a function of HRT.

#### **Analytical methods**

Samples were collected and analyzed three times per week under steady state conditions. The samples taken at various sampling sites were analyzed for nitrogen, phosphorus, COD, SS, DO and pH. Ammonia was determined by the Nessler method using a Hach Nessler Reagent and a Hach spectrophotometer (DR-2000). Nitrite and nitrate were measured by a colorimetric method using the Hach ready-made reagent (NitriVer 3 nitrite Reagent powder and NitraVer 6 nitrate Reagent powder) and a Hach spectrophotometer (DR-2000). Ortho-P was measured by the Ascorbic acid method using the Hach ready-made reagent (Posver 3 reagent) and a Hach spectrophotometer (DR-2000). COD was determined by the Closed reflux colorimetric method using Hach Digestion Vials-Low Range and a Hach spectrophotometer (DR-2000). DO meter (YSI, Model 58, USA) and a glass electrode pH meter (Orion, Model 525A, UAS) were used for the determination of dissolved oxygen (DO) concentration and pH, respectively. Suspended solids (SS) and volatile suspended solids (VSS) were analyzed according to Standard Methods (APHA, 1995).

## **Results and discussion**

### **Performance of the continuous bench pilot plant**

The continuous experiments were carried out to investigate the effects of carbon substrate on 1) biological nitrogen and phosphorus removal efficiency and 2) aerobic denitrification.

*Biological nitrogen and phosphorus removal efficiency.* The use of SCC can be compared with the use of respectively sole carbon sources, methanol and acetic acid at a quantity equivalent to 80 mg COD/l in terms of nitrogen and phosphorus removal. As indicated in Table 2, the highest overall TIN removal of 98.3% was observed in the SCC addition run with the final effluent concentration of 0.41 mg N/l. The efficiency of TIN removal slightly decreased to 95.5% with the use of sole acetic acid as a carbon source. The methanol addition run achieved 84.3% of TIN removal.

The o-P removal efficiency increased from 95.1% in the sole acetic acid addition run to approximately 100% in the SCC addition run with a non detectable o-P concentration in the final effluent. A negligible o-P removal efficiency of 0.8% was found in the methanol addition run, which probably results from microbial synthesis not associated with the

biological P-removal mechanism. This result confirms that methanol does not affect P- release, P-uptake and not even secondary release in the system (Gerber *et al.*, 1987; Tam *et al.*, 1992). The demonstration of superior nitrogen and phosphorus removal with the use of SCC might be due to a symbiotic relationship of methanol and acetic acid. In general, denitrifiers have priority over PAOs with regard to the uptake of acetic acid that is the favorable substrate for both bacteria. Thus, the use of sole acetic acid might induce a significant substrate competition between PAOs and denitrifiers to utilize acetic acid, and denitrifiers would consume much more of the acetic acid compared to PAOs. On the other hand, nitrate in the SCC addition run was mostly removed in the first anoxic zone with methanol so that almost all the acetic acid added in the second anoxic zone could be used only for P-release and stored in the form of PHB for luxury uptake of phosphorus in the oxic zone. This could explain the observation of the higher amount of P-release in the anoxic zone of the SCC addition run even though acetic acid was 30 mg COD/l less and anoxic HRT was 15 minutes shorter compared to the addition of sole acetic acid 80 mg COD/l.

*Comparison of aerobic denitrification.* During the experiments in order to determine the capacity of aerobic nitrogen removal, a low influent nitrate concentration entered the system without recycling of nitrified nitrogen from the aerobic zone to the anoxic zone. According to the results of a mass balance presented in Table 3, substantial amounts of nitrogen were removed in the aerobic zone. The respective percentages of TIN removal in the aerobic zone on the basis of influent TIN were 18.9% in the sole methanol addition run, 34.7% in the sole acetic addition run and 36% in the SCC addition run. These results imply that acetic acid might be the more favorable carbon substrate to aerobic denitrifiers compared to methanol.

**Table 2** Comparison of the SCC with the sole carbon source under aspects of nitrogen and phosphorus removal

Methanol/acetic acid, mg COD/l		80/0	0/80	30/50
TIN, g N/d	Influent	11.295	10.994	10.895
	Final effluent	1.778	0.5	0.185
NOx-N removal, g N/d	In the anoxic zone	8.325	8.838	8.447 <sup>a</sup>
o-P, g P/d	Influent	2.138	1.913	2.421
	Final effluent	2.120	0.095	0
o-P release, g P/d	In the anoxic zone	-0.063	18.360	-1.818 <sup>a</sup>
o-P uptake, g P/d	In the aeration zone	0.108	20.124	22.086 <sup>b</sup>
				22.725

a: result obtained from the first anoxic zone with methanol during an anoxic HRT of 15 minutes

b: result obtained from the second anoxic zone with acetic acid during an anoxic HRT of 15 minutes

**Table 3** Comparison of the SCC with the sole carbon source under aspects of aerobic denitrification

Methanol/acetic acid, mg COD/l		80/0	0/80	30/50
TIN, g N/d	Influent	16.398	16.434	16.497
	Effluent	4.676	2.871	2.475
TIN Removal, g N/d	In the anoxic zone	8.766	8.253	7.578 <sup>a</sup>
	In the aeration zone	3.096	5.706	0.486 <sup>b</sup>
o-P, g P/d	Influent	2.174	2.048	5.967
	Effluent	2.214	1.04	2.097
o-P release, g P/d	In the anoxic zone	0.234	6.876	0.072
				-1.134 <sup>a</sup>
o-P uptake, g P/d	In the aeration zone	0.531	7.830	14.805 <sup>b</sup>
				15.669

a: from the first anoxic zone with methanol, b: from the second anoxic zone with acetic acid

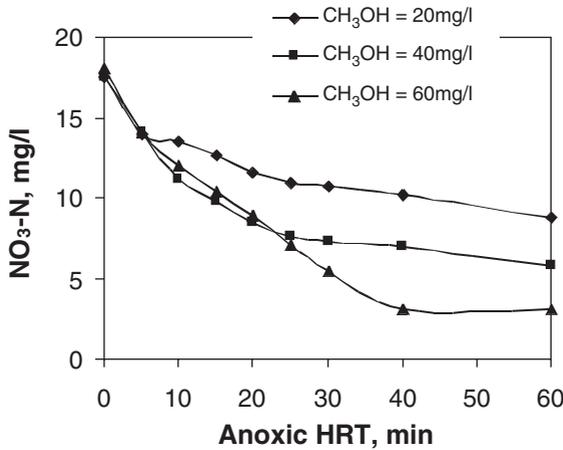
**Batch experiments**

Seven experimental runs were divided into two groups of batch tests in terms of 1) denitrification with methanol and 2) simultaneous denitrification and phosphorus uptake under carbon limited anoxic conditions. The sludge used for the batch tests was taken from the continuous bench scale pilot plant being operated with the SCC addition.

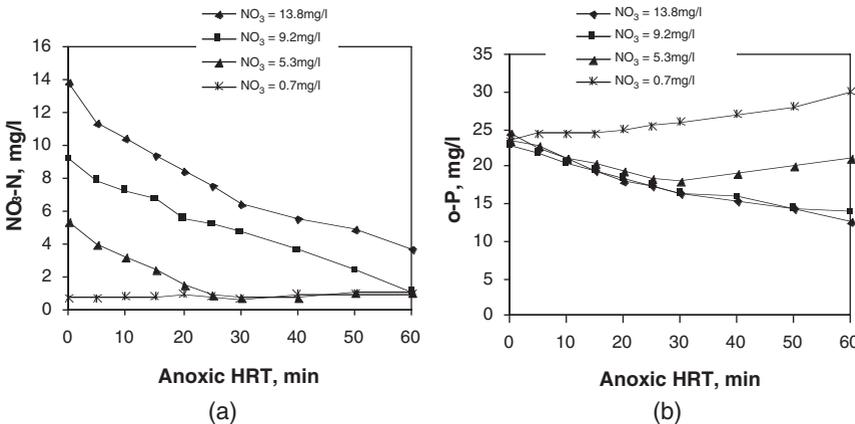
*Denitrification with methanol.* Figure 2 shows the batch test results of denitrification under anoxic conditions with three different levels of methanol. The SDNR was calculated by linear regression using data points obtained before methanol was limited, and the range was between 0.190–0.193 g NO<sub>3</sub>-N/g VSS/d. This indicates that an increase of methanol dosage did not significantly enhance the denitrification rate.

*Simultaneous denitrification and phosphorus uptake under carbon limited anoxic conditions.* The 4 experimental runs were carried out to quantify the extent of denitrification by DPB capable of utilizing nitrate as an electron acceptor. The initial COD originated from testing sludge taken from the continuous bench scale process was about 25 mg/l. There was an insignificant change in COD during a HRT of 60 minutes in all runs. This indicates that initial COD was mainly non-biodegradable. Thus, both nitrate and phosphorus were not affected by the initial COD.

Figure 3 shows the concentration of nitrate slowly decreased even though no readily



**Figure 2** Variation of nitrate as a function of HRT at various methanol concentrations



**Figure 3** Variation of (a) nitrate and (b) phosphorus as a function of HRT under anoxic and carbon limited conditions

biodegradable carbon substrate is available, and the denitrification rate was much lower compared to when methanol was available. Phosphorus uptake was also observed when nitrate was present in a certain amount, which indicates DPB might degrade PHB as an internal carbon using nitrate as an electron donor instead of DO. The simultaneous SDNR and specific P-uptake rate were 0.056–0.058 g NO<sub>3</sub>-N/g VSS/d and 0.066–0.069 g P/g VSS/d, respectively, which occurred by DPB with the degradation of PHB stored resulting from an acetate uptake of 50 mg COD/l.

#### **Effects of carbon source on denitrification**

The value of SDNR obtained from the anoxic zone was 0.147 g NO<sub>3</sub>-N/g VSS/d in the sole methanol addition run and 0.122 g NO<sub>3</sub>-N/g VSS/d in the sole acetic acid addition run.

Compared to these values, the highest SDNR of 0.252 g NO<sub>3</sub>-N/g VSS/d was achieved from the 1st anoxic zone with methanol in the SCC addition run. This value is higher than the average SDNR of 0.192 g NO<sub>3</sub>-N/g VSS/d obtained in the batch test which was carried out with the same sludge taken from the continuous bench pilot plant. It could be presumed that the enhanced denitrification rate with methanol in the continuous bench pilot plant might result from simultaneous denitrification and phosphorus uptake by DPB. The PHB stored in PAOs taking up the acetic acid in the 2nd anoxic zone would be degraded for P-uptake in the oxic zone and then, if still present, be possibly available to the 1st anoxic zone by sludge return. As noted, methanol added to the 1st anoxic zone would not be available as a carbon source for PAOs. Therefore, DPB would degrade PHB remaining after utilization for P-uptake in the oxic zone, using nitrate as an electron acceptor in the 1st anoxic zone. Although the amount of PHB was not measured in this study, the P-uptake of 1.818 g N/d (Table 2) in the 1st anoxic zone could possibly prove the above assumption. Based on this explanation, a SDNR of 0.252 g NO<sub>3</sub>-N/g VSS/d can be divided into a denitrification rate by normal denitrifiers using methanol as a carbon source and a denitrification rate by DPB. From the batch test (Figure 3), 1 g of nitrate was used by DPB to take up phosphorus of 1.19 g under anoxic and the absence of acetic acid conditions. According to this result, the denitrification rate by normal denitrifiers using methanol was 0.205 g NO<sub>3</sub>-N/g VSS/d in the continuous bench pilot test since phosphorus concentration of 2.02 mg P/l (1.818 g N/d) was taken up, indicating that the nitrate at 1.7 mg N/l was removed by DPB. Thus, 0.047 g NO<sub>3</sub>-N/g VSS/d of SDNR was achieved by DPB.

#### **Effects of carbon source on aerobic nitrogen removal**

The nitrogen removal in the aerobic zone was observed at a significant amount when high influent ammonia concentration was applied to the system with no internal recycle. The ammonia stripping was not the case for nitrogen removal in the aeration zone since the pH was in the range of 6.56–6.72 during the experimental period. In addition, the DO level was maintained at above 3.5 mg/l, which can fully penetrate a bacterial floc. Thus the nitrogen removal in this research was not due to anoxic microzone denitrification. Therefore it could be stated that nitrogen removal in the aerobic zone appeared to be accomplished by aerobic denitrifiers under well-aerated conditions.

At the sole acetic acid addition runs, the aerobic nitrogen removal increased from 3.3 g N/d (data not shown) to 5.7 g N/l (Table 3) with increasing acetic acid dosage from 50 to 80 mg COD/l. Kugelman and Spector (1992) suggested a modified activated sludge process employing a fed anaerobic selector followed by an aerobic selector with the off-line storage of the return sludge under anaerobic conditions to enhance the nitrogen removal in the aerobic conditions. A storage period of 4 hours and 8 hours with SBR laboratory unit resulted in 10 mg/l and 15 mg/l nitrogen removal, respectively, under aerobic conditions. According to an example of volatile fatty acids (VFAs) production

(e.g., acetate) from sludge fermentation in a laboratory scale process (Molof and Kim, 1991), the amount of VFAs production obtained was 930 mg/l and 1,160 mg/l as acetic acid at 4 hours and 8 hours, respectively. Therefore, the improvement on aerobic nitrogen removal reported by Kugelman and Spector (1992) might be attributed to an increase of VFAs production with a longer storage period. Kim (1993) also demonstrated that the nitrate removal in the oxic zone increased with increasing acetate levels.

However, compared to 80 mg COD/l of the sole acetic acid addition, the SCC addition achieved a similar amount of nitrogen removal in the oxic zone even though acetic acid added in the 2nd anoxic zone was 30 mg COD/l less. Based on the amount of P-release presented in Table 3, the amount of PHB stored by PAOs is believed to be higher in the SCC addition run with 50 mg COD/l of acetic acid than in the 80 mg COD/l of the sole acetic acid addition run. These results might be ascribed to the minimization of substrate competition between PAOs and denitrifiers by using SCC. The PHB metabolism investigated by many researchers (Dawes and Senior, 1973; Kern-Jespersen and Henze, 1993; Chuang *et al.*, 1996) is that PHB is synthesized in the presence of carbon substrate whereas PHB is oxidized by DPB in the presence of oxygen or nitrate when the carbon substrate is limited. Accordingly, it could be stated that DPB are able to denitrify not only under anoxic conditions but also under oxic conditions if carbon substrate was not available. Therefore, the increase in the amount of carbon reserves, PHB, rather than the increase of acetic acid dosage itself, could be more important for the enhancement of aerobic denitrification.

Aerobic denitrification achieved in the sole methanol addition run was twice as low as in the other two runs. Some researchers (Bang *et al.*, 1995; Neef *et al.*, 1996; Zhao, 1998) noted that methanol was found to be a suitable substrate for aerobic denitrification under discontinuously aerated conditions. Zhao (1998) reported that 20% of denitrification was obtained in the 3-Bardenpho aerobic zone, whereas 80% of denitrification in the IACM (intermittently-aerated, completely-mixed) tank was achieved. Therefore, a possible explanation for low removal efficiency of aerobic nitrogen is that methanol is more effective in aerobic denitrification under an alternating anoxic/aerobic environment than under a continuously aerated one.

## Conclusion

The sequentially combined carbon using a symbiotic relationship of methanol and acetic acid was investigated to enhance the BNR efficiency and its performance was compared with respectively sole carbon sources of methanol and acetic acid. Sludge derived from the SCC addition run demonstrated the highest BNR efficiency, and improved the rate of denitrification in the anoxic zone not only by normal denitrifiers with methanol but also by DPB capable of using nitrate as an electron donor instead of oxygen. One g of nitrate was reduced by DPB for phosphorus uptake of 1.19 g under anoxic and carbon limited conditions. The enhancement of nitrogen removal in the aerobic zone was affected by the type of carbon substrate. Aerobic denitrifiers appeared to prefer acetic acid to methanol. In addition, it seems that PHB stored by PAOs taking up the acetic acid could be a contributory factor for aerobic denitrification. Compared to the use of each sole carbon, the use of SCC, which induced the highest amount of P-release in accordance with the highest amount of PHB, could be a very effective carbon source for aerobic denitrification as well.

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