DENITRIFICATION OF ACTIVATED SLUDGE IN THE PRESENCE OF DIFFERENT ORGANIC SUBSTRATES

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ABSTRACT

The effect of organic carbon on denitrifying activity was studied in batch reactor. Four reactors were operated in parallel under anoxic condition in four different donor electrons, which were acetic acid (Reactor A), methanol (Reactor M), phenol (Reactor P), and glucose (Reactor G). The reactors were fed with the artificial waste, which contain 721.8 mg/l $NaNO_3$. The concentration of organic carbon added to the reactors were varied from TOD:N ratio of 0.5:1; 1:1, 1.5:1, to 2:1. The denitrification activity was estimated by measuring the reduction rate of nitrogenous oxide and N_2O gas production. The denitrification capacity of adapted-sludge was also investigated, and the rates were estimated from the cumulative N_2O (without acetylene inhibition) and N_2 gas production. Reduction rate of nitrogenous oxide in all reactors increased during the investigation; the increase reduction rate were correlated to the increase of organic carbon concentration. The maximum reduction rate of nitrogenous oxide in reactor A was higher than those of the others. However, reduction rate in reactor M was more constant, so that nitrogenous oxides existed in this reactor was removed faster. The highest potential denitrification rate (N_2O production) was observed in sludge of reactor A. However, N_2 gas recovery from nitrate and nitrite transformed by sludge of reactor M was the highest. Linear correlation between nitrogenous oxide reduction with gas production was observed in reactor A, M and P, but not in reactor G.

Keywords: Denitrifying microorganisms, denitrification, nitrogenous oxide, activated-sludge.

ABSTRAK

Studi mengenai pengaruh karbon organik terhadap aktivitas denitrifikasi telah dilakukan dengan menggunakan reaktor unggun tetap (batch-reactor). Empat reaktor dioperasikan secara paralel dalam kondisi anaerobik dengan pemberian karbon organik yang berbeda, yaitu asam asetat (reaktor A), metanol (reaktor M), fenol (reaktor P), dan glukosa (reaktor G). Keempat reaktor tersebut diberi umpan limbah tiruan yang mengandung 721,8 mg/l $NaNO_3$. Pemberian konsentrasi karbon organik bervariasi dari rasio TOD/N 0,5; 1,0; 1,5; dan 2,0. Aktivitas denitrifikasi di dalam reaktor diukur berdasarkan kecepatan reduksi nitrogen oksida (NO_3^- + NO_2^-) dan pembentukan gas N_2O. Aktivitas denitrifikasi dari lumpur (sludge) yang telah beradaptasi dengan masing-masing karbon organik diukur berdasarkan pembentukan gas N_2 dan N_2O (tanpa penghambatan asetilen). Reduksi nitrogen oksida di semua reaktor meningkat sejalan dengan meningkatnya konsentrasi karbon organik. Kecepatan maksimum reduksi nitrogen oksida di reaktor A lebih tinggi dibandingkan dengan di reaktor lainnya. Namun kecepatan reduksi nitrogen oksida di reaktor M lebih konstan sehingga waktu untuk mereduksi seluruh nitrogen oksida di reaktor M lebih cepat. Aktivitas denitrifikasi (produksi N_2O) tertinggi dicapai oleh lumpur di reaktor A. Namun produksi gas N_2 sebagai hasil transformasi nitrat dan nitrit dari lumpur di reaktor M lebih tinggi dibandingkan dengan lumpur di reaktor lainnya. Hubungan linier antara aktivitas reduksi nitrogen oksida dengan produksi gas terjadi di reaktor A, M dan P, namun tidak di reaktor G.

Kata kunci: Mikroorganisme denitrifiksi, denitrifikasi, nitrogen oksida, lumpur aktif.

INTRODUCTION

Denitrifying bacteria are mostly heterotrophs; they require organic carbon sources for cell growth and nitrate reduction. The effects of external and internal carbon sources on denitrification rates have been investigated (McCarty *et al.*, 1969; Barnard & Meiring, 1977; Gerber *et al.*, 1986; Tam *et al.*, 1992; Fass *et al.*, 1994). It had been reported that acetate was found to give the highest denitrification rate in almost all cases. However, methanol had often been used in practice since it was less expensive. Ethanol was also commonly used as an organic substrate to provide the reducing power for nitrate elimination (Bockle *et al.*, 1986; Liessens *et al.*, 1993). The potential of some denitrifiers to degrade hazardous pollutants such as aromatic hydrocarbons, phenols and benzoate derivatives had also been reported (Braun & Gibson, 1984; Nozawa & Maruyama, 1988; Dolfing et al., 1990; Fries et al., 1994; Frazer et al., 1995; Rabus & Widdel, 1995; Heider & Fuchs, 1997; van Schie & Young, 1998; Rockne et al., 2000; Yamagishi et al., 2001; Bae et al., 2002). Thus, denitrifying processes could be an alternative strategy for remediating polluted area. Furthermore, many organic compounds, which were non-biodegradable under aerobic conditions or slowly degradable under anaerobic conditions, could be effectively utilized by denitrifiers as a carbon source (Zoh et al., 1999) and therefore removed from its environment.

Adaptation of the denitrifying microbes to carbon sources may involve both the induction and synthesis of enzymes in the existing microflora. McCarty *et al.* (1969) and Nyberg *et al.* (1992) demonstrated a lag period before denitrification to occur with methanol addition. On the contrary, there was an immediate response to acetate in an alternating type activated sludge process (Isaacs *et al.*, 1994). Akunna *et al.* (1994) and Quevedo *et al.* (1996) suggested that both the nature of the carbon source and the C/N ratio affect the competition between denitrification and ammonification. Cole (1991) reported that abundant source of carbon and strictly anaerobic conditions mainly resulted in the production of ammonia, instead of other gases. Consequently, efficient biological denitrifications require availability of adequate carbon to nitrogen ratios and easily degradable carbon sources.

To improve the performance of removal in nitrogen reactors, the effect of organic carbon sources on denitrification activity are important to be studied. The present study was undertaken to characterize the denitrification activity in activated sludge with different kind of organic carbon substances as donor electron. The effect of C:N ratio of the organic carbon used on dissimilative nitrate reduction of denitrifying microbes was also studied.

MATERIALS AND METHODS

Operation of denitrifying batch reactors

Sludge samples from the Kohoku domestic sewage plant were used as inocula. Four glasses of 1.27 l batch reactors filled with 1 l of synthetic medium were operated in parallel at 22-24° C for 78 d. Each reactor had a cyclic reaction time of 24 h. One cycle consisted of reaction (0-23 h), sedimentation (23-23.5 h), decanting and feeding (23.5-24 h). After the sedimentation, 0.5 l of the supernatant was decanted and replaced with fresh feed. The medium was agitated with a magnetic stirrer at 200 rpm. The reactors were operated with four different carbon sources: 1) acetic acid (Reactor A), 2) methanol (Reactor M), 3) phenol (Reactor P), and 4) glucose (Reactor G). The reactors were fed with an artificial waste, which contained $NaNO_3$ (721.8 mg/l), $MgSO_4.7H_2O$ (42 mg/l), $CaCl_2$ (12 mg/l) and NH_4Cl (10 mg-N/l). The addition of acetate, methanol, phenol and glucose into the reactor was varied with

TOD:N-ratio of 0.5:1; 1:1; 1.5:1, to 2:1. The mixture in the reactor was buffered with 0.2 M phosphate buffer at pH 7.0. Table 1 shows the concentration of organic carbon and nitrogen added to the reactor.

Denitrification activity of bioreactors (based on N_2O production rate)

Denitrification activity was determined according to acetylene inhibition technique (Yoshinari & Knowles, 1976) with slight modification. Portions (5 ml) of activated sludge was transferred to a serum bottle and added with 0.25 ml of 500 mg nitrate-N/l. Stirrer bar was added to the serum bottle and sealed with gas-tight aluminum caps equipped with a butyl rubber septum for gas sampling. The headspace was made anoxic by evacuating and flushing with N_2. Acetylene of 10% (v/v) was injected to the reactors and the headspace gas was measured 10 mins thereafter. Organic substrate was added by injecting 0.25 ml organic chemical at 20 mins thereafter and measurement of the headspace gas was continued. Gas samples of 40 ml were withdrawn from the headspace by a gas-tight syringe with stopper at 5 mins intervals and injected directly to Gas Chromatography for nitrous oxide analysis.

Denitrifying activity of adapted-sludge (based on gas production)

After the anoxic system of the four batch reactors had been steadily operated for over 2 months (73 d), the denitrifying capability of adapted-sludge were investigated. The adapted-sludge was sampled from reactor A, M, P and G and re-suspended in buffer solution on small bottles. Feeding medium and operation were identical to all reactors (described above). The headspace was rendered anaerobic by evacuating and flushing with argon twice and then venting to atmospheric pressure. Substrate addition was made by injecting 0.5 ml of stock solution to reach an initial concentration of 100mg/l $NaNO_3$-N, 401.79 mg/l of acetic acid, 286.09 mg/l of methanol, 179.85 mg/l of phenol and 401.79 mg/l of glucose. Samples were incubated on stirrer plate at 200 rpm for 24 h. At a given time interval over 24-h period, the mixed liquid was sampled and the concentration of nitrate, nitrite, were analyzed. The gas was sampled

Table 1. Composition of carbon and nitrogen used as feed in the reactors

Conditions	Days	Carbon source as the mode of reactor			
		Acetic acid (mg/L)	Methanol (mg/L)	Phenol (mg/L)	Glucose (mg/L)
TOD/N=0.5 Phase I	1-14	133.93	95.36	59.95	133.93
TOD/N=1.0 Phase II	15-24	267.86	190.73	119.90	267.86
TOD/N=1.5 Phase III	25-73	401.79	286.09	179.85	401.79
TOD/N=2.0 Phase IV	74-78	535.71	381.45	239.80	535.71

and the concentration of N_2O and N_2 were analyzed.

Analyses

Bacteria growth yields were monitored by measuring the volatile suspended solids (VSS) and mild liquor suspended solid (MLSS), which was determined according to Gesui Shiken Hou-Hou (Japanese Sewage Works Association, 1974). The TOC was measured as dissolved organic carbon using TOC analyzers (TOC-500 Shimadzu Seisakusho, Kyoto, Japan). The concentrations of NO_2-N, and NO_3-N were determined by ion chromatography (IC7000, Yokogawa Analytical Systems, Tokyo, Japan). Ammonia-nitrogen (NH_4-N) concentration was determined by ion-chromatography (HIC6A, Shimadzu Corporation, Japan). Nitrous oxide gas (N_2O-N) and N_2 gas concentrations were determined by Gas Chromatography (5890A with ECD detector, Hewlett-Packard Co.Ltd.USA and GC-8A, Shimadzu Corporation, Japan, respectively).

Measurement of denitrification rate

Denitrification rates measured as eliminated NOx-N (mg/l/minute), were calculated from the time course data of nitrogenous oxide changing pattern during 24 h reaction.
Average rate was calculated as:

$$\frac{(\text{NOx-N concentration at } t_0 - \text{NOx-N concentration at } t_n)}{\text{the time(tn)}}$$

where:
t_0 = time 0
tn = time in which the concentration of NOx-N become 0
The maximum rate is the highest rate value within 24 h reaction.

RESULTS AND DISCUSSION

Performance of denitrification activity in the reactors (based on nitrogenous oxide reduction)

During the first 14 d, the reactors were operated at a low TOD:N ratio of 0.5:1 (phase I) in order to provide time for denitrifying microbes in activated sludge to adapt to the organic compound added. As illustrated in Fig. 1, the nitrogenous oxide reduction of all reactors have not yet stabilized at the first day

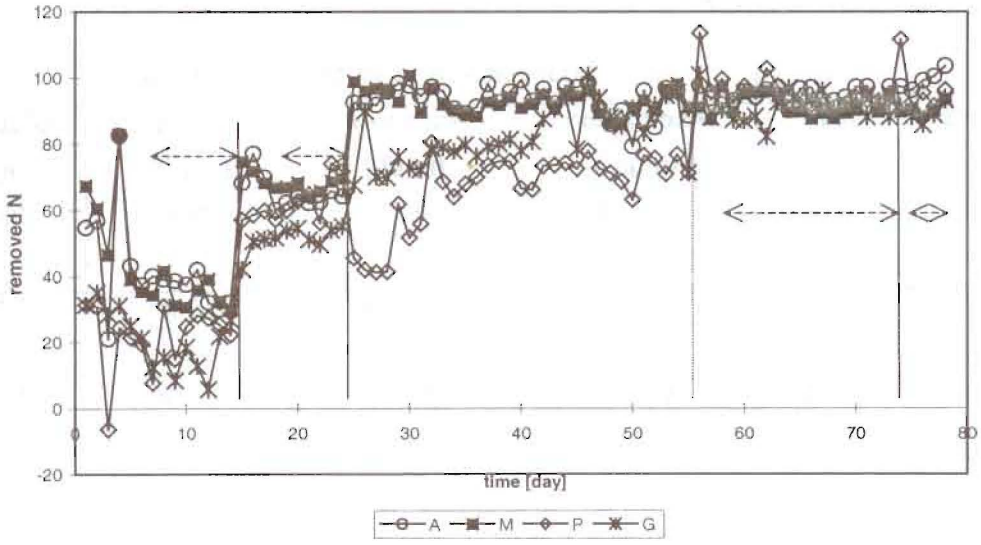

Figure 1. The daily dynamic of removed nitrogenous oxides (NO_2+NO_3)-N for each reactor as affected by TOD:N ratios for total periode of 78 d.

operation. It started to become stable on the 5th day. The amount of nitrogenous oxide removed in reactor A and M were higher than those in reactor P and G. The efficiency of nitrate reduction in reactor A was about 38%, whereas in reactor M, P and G were 35%, 22% and 17%, respectively (Fig. 2). However, the concentration of TOC removed in all reactors were higher, more than 80% (Fig. 3). The low capacity of nitrogenous oxide reduction during this period might be due to low organic concentration in the influent.

In the second 10 d period, the TOD:N ratio was increased to 1:1 (Phase II). The removed concentration of nitrogenous oxide increased to about 60-70 mg/l. The efficiency of nitrate reduction in the reactor A, M, P and G, were 64%, 67%, 66 % and 65%, respectively (Fig. 2). Since the efficiencies of reduced TOC were high in all reactors (90%), the lowest capacity to reduce nitrate was due to low concentration of organic carbon added in this stage. The concentration of organic carbon has not enough capacities for reducing power. During this period, denitrifying activity in reactor P increased and reached the similar value as in reactors A and M. It suggests that the denitrifying microbes

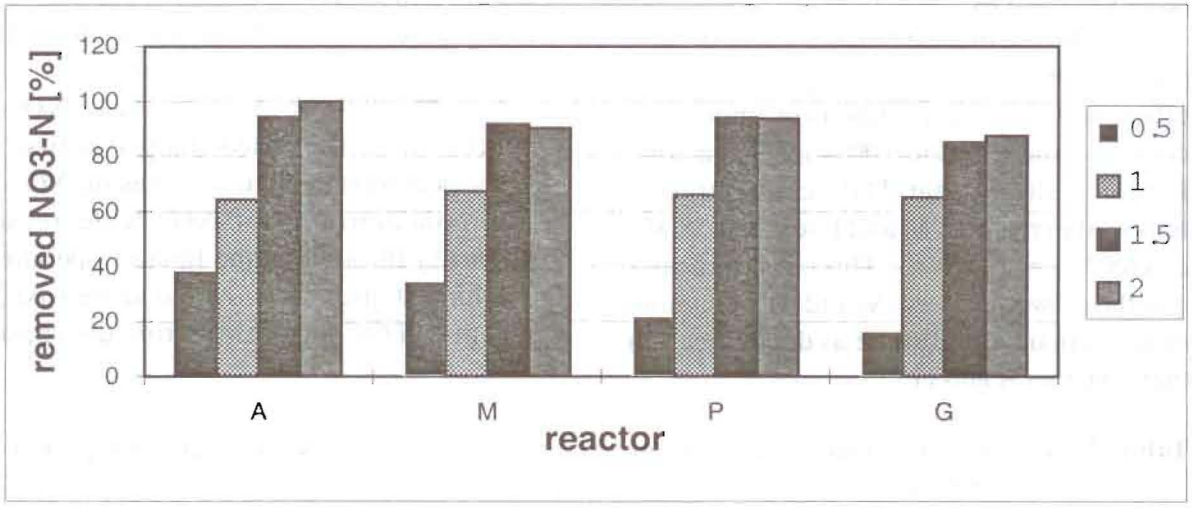

Figure 2. Average value of nitrate reduction.

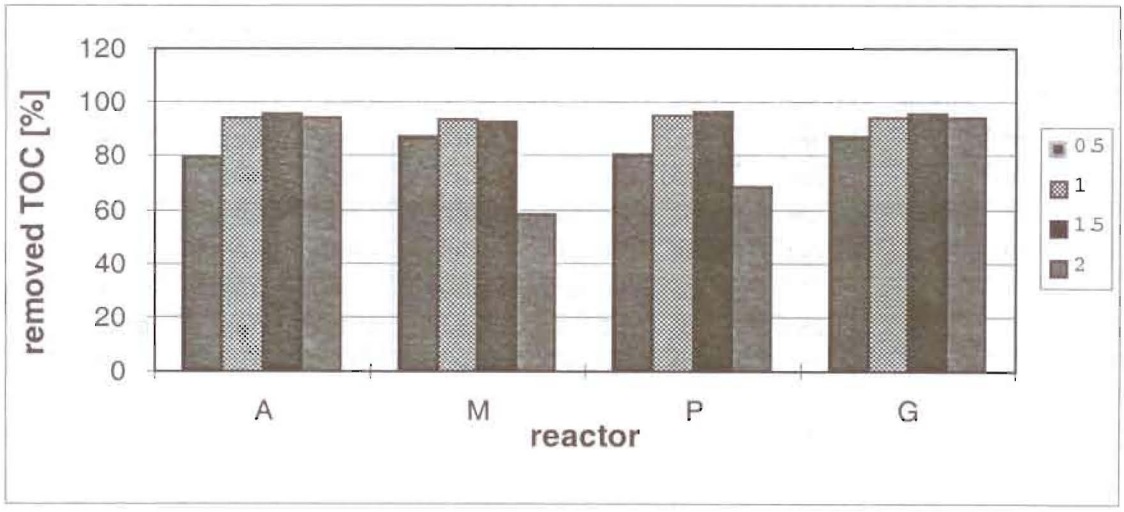

Figure 3. Average value of TOC reduction

in this reactor has already adapted to the phenol.

During the first 6 d of operation under TOD:N ratio of 1.5 (phase III), the concentration of nitrogenous oxide removed in the reactor P dropped, lower than observed in the phase II. This indicates that microbes in the reactor P were still not able to use high concentration of phenol. For the next few days, the concentration of phenol added to the reactor was reduced to 119.90 mg/l (TOD:N ratio of 1:1). At the 56th day, phenol concentration was increased again to TOD:N ratio of 1.5:1. The capability to remove nitrogenous oxide increased from the 57th day and the value became similar to those in reactors A and M.

During the last 5 d, the TOD: N ratio was further increased to 2:1 (phase IV). The efficiency of nitrate reduction under this condition increased to 100% in all reactors. Figure 3 shows that TOC concentration removed in reactors M and P reached 58.8% and 68.4%, respectively. This result indicates that those two reactors (M and P) were more efficient in using substrate as donor electron than reactors A and G.

Denitrification rate (Nitrogenous oxide reduction rate)

In this study, we determined denitrification rate as average and maximum values. In all reactors, the denitrification rates increased during the investigation period (Table 2). The average denitrification rate in the reactor M was higher than in reactor A, P or G. However, the maximum rate of denitrification was higher in reactor A than in other reactors. This fact shows that there are different patterns of nitrogenous oxide reduction in each reactor. Those differences were affected by different organic carbon used. The changing pattern of nitrogenous oxides and organic carbon concentration as illustrated in Fig. 4, might explain the differences between each reactor.

In reactor A (Fig. 4a), NO_3-N concentration decreased sharply at first 30 mins, accompanied with increasing NO_2-N. The concentration of NO_2-N decreased gradually throughout the time of operation, reaching 0 after 10 h. At the same first 30 minute, TOC concentration decreased

Table 2. Nitrogenous oxide reduction rate in reactors A, M, P and G during the period of investigation (78d)

Condition	day	reactor A (mg-N/l/min)		reactor M (mg-N/l/min)		reactor P (mg-N/l/min)		reactor G (mg-N/l/min)	
		average	max	average	max	average	max	average	max
Phase I	2	0.040	0.212	0.042	0.193	0.021	0.008	0.024	0.028
	7	0.028	0.340	0.024	0.182	0.005	0.040	0.009	0.023
	10	0.026	0.389	0.021	0.138	0.017	0.054	0.013	0.126
Phase II	16	0.054	0.284	0.050	0.199	0.041	0.090	0.035	0.106
	23	0.046	0.538	0.048	0.263	0.052	0.052	0.038	0.269
Phase III	29	0.145	0.843	0.216	0.371	0.043	0.062	0.053	0.411
	31	0.142	0.773	0.295	0.397	0.039	0.039	0.050	0.406
	37	0.161	0.859	0.506	0.455	0.051	0.132	0.088	0.306
	44	0.161	1.127	0.506	0.530	0.049	0.216	0.036	0.343
	58	0.209	1.323	0.581	0.581	0.158	0.244	0.093	0.361
Phase IV	78	0.837	1.219	0.870	1.291	0.224	0.409	0.156	0.748

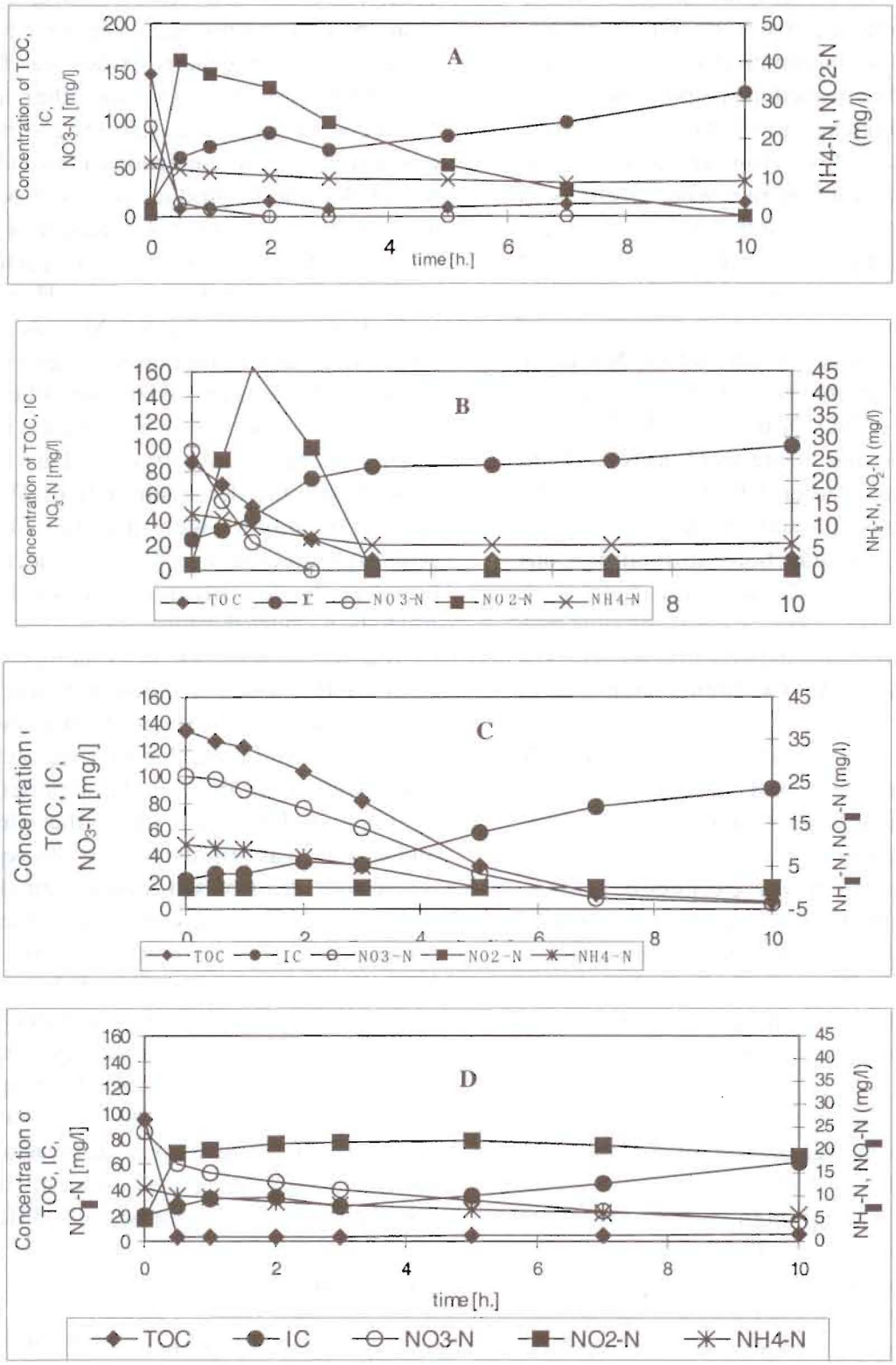

Figure 4. Time cours of nitrogen and organic carbon conversion in: (A) reactor A; (B) reactor M; (C) reactor P and (D) reactor G

sharply. This indicates that denitrifying microbes in reactor A immediately used acetic acid as reducing power to reduce nitrate, result in high maximum denitrification rate (Table 2). The immediate response to acetate has been noted previously (Tam et al., 1992; Isaacs et al., 1994). However, a continuous decrease in reaction rate when acetic acid used as a sole carbon substrate was observed. This finding has not been reported before, and should be taken into consideration in application.

The reduction rate of NO_3-N sharply decreased at the first 1 h, followed by increasing of NO_2-N in reactor M (Fig. 4b). Both nitrate and nitrite were depleted from the bulk liquid after 3 h. At the same time TOC decreased gradually during 3 h. It is showed that the reduction rate of nitrogenous oxide in reactor M was not as high as in reactor A. However, a constant rate was observed. It might explain that the average rate in reactor M was higher than that in reactor A.

In reactor P (Fig. 4c), NO_3-N concentration decreased gradually for the first 10 h. A similar pattern was observed for TOC concentration. In this reactor, nitrite concentration never exceeded 1 mg/l, suggesting that nitrite was not accumulated and nitrate was effectively converted to nitrogen gas. This result also indicates that the conversion from nitrate to nitrite was the rate-limiting step in this denitrification process.

In reactor G (Fig. 4d), NO_3-N concentration decreased gradually, followed by increasing of NO_2-N. The concentration of NOx-N was still high after 10 h reaction. On the contrary, TOC concentration decreased sharply within 30 mins, which was more rapid than that of nitrogenous oxides.

Since the experimental conditions were identical in the four bioreactors, it was suggested that the different rates of denitrification was mainly due to differences in organic carbon used as electron donor. Acetate is easily to metabolize by bacterial cells, resulting faster energy build up for reducing power. This might explain why the reduction of nitrogenous oxides was faster at the first 30 mins. On the other hand, bacteria, which are growing on C1 compounds such as methanol, cannot employ the TCA cycle to produce reducing power. This might explain why the reduction of TOC in reactor M was slower than in reactor A, resulting low denitrification rate. However, reduction rate in reactor M was more constant, so that nitrogenous oxides existed in this reactor was removed faster. Phenol is aromatic compound, which make it not readily accessed by enzymes. This might explain why the decreasing rate of TOC in reactor P was slower than the others. Although glucose was faster to decrease, however, the denitrification rate was low. It might be because the population of microbes in reactor G was not only denitrifying bacteria. Reduction of nitrate in this reactor was assumed to be conducted by both, nitrate respiring bacteria and denitrifying bacteria. The result shown in Fig (4d) supported this assumption. The figure shows that glucose and nitrate was simultaneously decreased, whereas nitrite increased gradually during the first 5 h, which suggest that the nitrite was produced by nitrate respiring bacteria or other biological process. After 5 h, nitrite concentration began to decrease gradually down to 0 after 24 h, which suggests that the denitrification process occurred during this period. The fact that pathway of nitrate, nitrite, and organic carbon in each reactor was different, suggesting that the denitrifying population in each reactor was also different.

Biomass production

A linear relationship between biomass (Fig.5) and concentration of reduced nitrogenous oxide was observed for reactors A, M and P, except for the G reactor where biomass appeared to be very high. Enhanced

growth yield due to NO_3^- or NO_2^- reduction would be expected for any microbial reduction processes that was coupled to electron transport phosphorylation, such as denitrification, nitrate respiration, and nitrite respiration (Thauer et al., 1977). Both denitrifiers and nitrate respirers showed enhanced growth from nitrate or nitrite reduction. The difference between respiratory denitrifiers and the latter group came from the fact that growth yield was not proportional to NO_3^- or NO_2^- concentration for the nitrate respirers, but it was for denitrifiers, especially when they were grown on NO_3^-. Figure 5 shows the indication that biomass yield in reactor G was not proportional to NO_3^- or NO_2^- reduction, suggesting that the main population in this reactor was not denitrifying bacteria.

Potential denitrification rate (N_2O gas production rate)

Potential denitrification rate was evaluated by measuring the N_2O gas production with acetylene inhibition method. The denitrification activity in reactors A, M and P were increased throughout the period of investigation (Fig. 6). Relatively similar value of denitrification rate was observed on

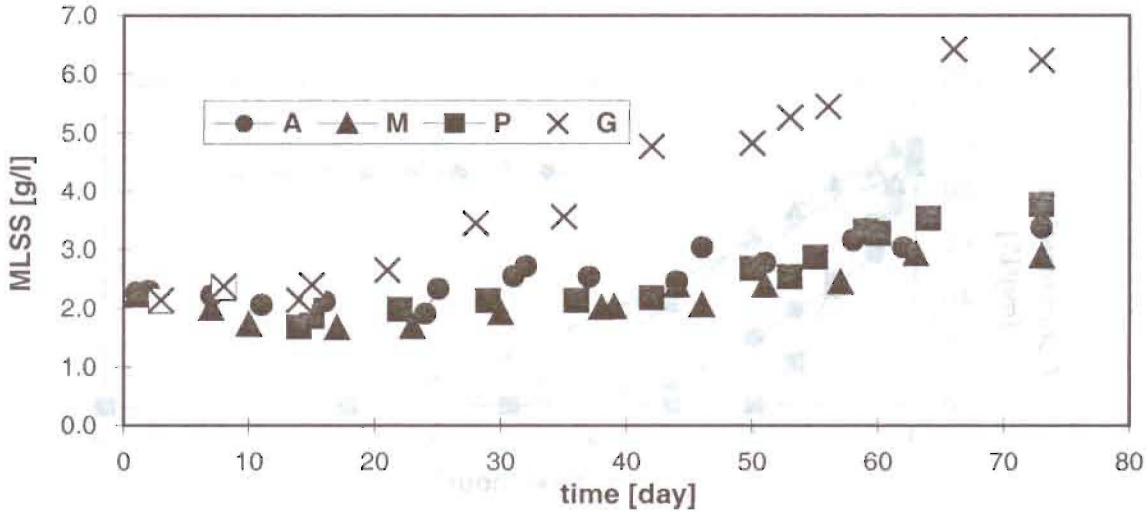

Figure 5. Biomass concentration (MLSS) in reactors A, M, P and G during operation under TOD:N ratio 0.5, 1.0, 1.5 and 2.0

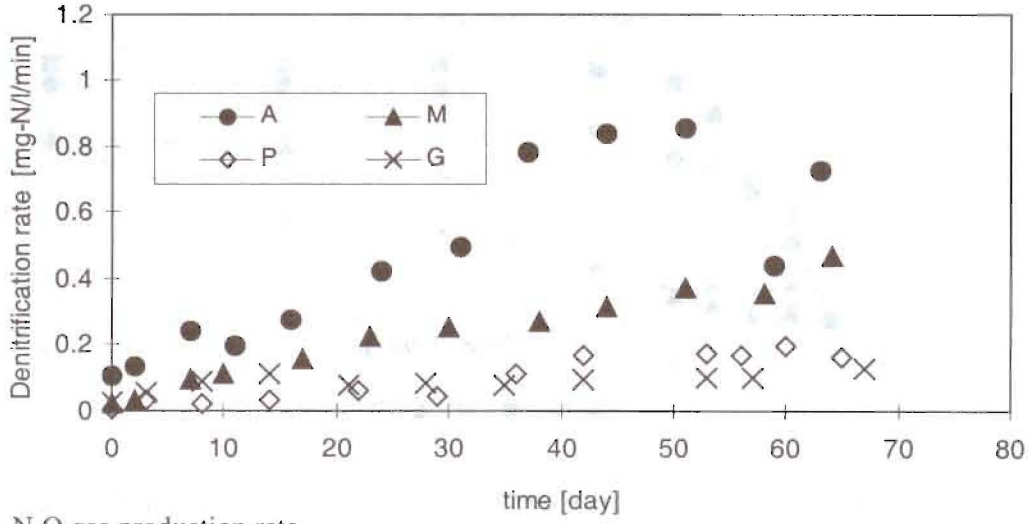

Figure 6. N_2O gas production rate

the reactor G. Higher specific denitrifying activity with acetic acid as substrate (reactor A) was observed than the others. These results were in agreement with the data on reactor performance that the maximum denitrification rate in reactor A was higher than that in reactors M, P or G. The potential denitrification rate in reactor G was the lowest. This fact indicates that the high capacity to reduce nitrogenous oxide in reactor G was mostly due to activity of nitrate respiring or other biologically processes, instead of denitrifying microbes.

Denitrification activity (gas production) of adapted-sludge

The characteristic feature of respiratory denitrification is that the reduction of nitrogenous oxides is coupled to electron transport phosphorylation where N_2O and/or N_2 is a major product (Thauer et al., 1977). The pattern of N_2 gas production (Fig. 7) from nitrous oxide reduction (Fig.8) was linear. The maximum recovery of N gases were 80.1% 87%, 64.1% and 59.5% on acetic acid, methanol, phenol, and on glucose-adapted sludge, respectively. The maximum N_2O gas production on acetic acid-adapted sludge was 6%, almost 0% observed on methanol-

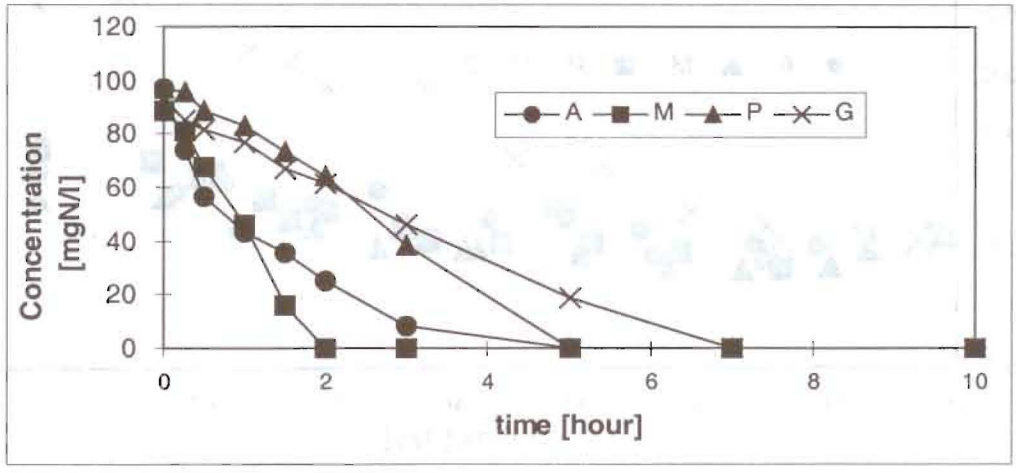

Figure 7. Time course of N_2 gas production in the headspace of small batch culture (adapted-sludge)

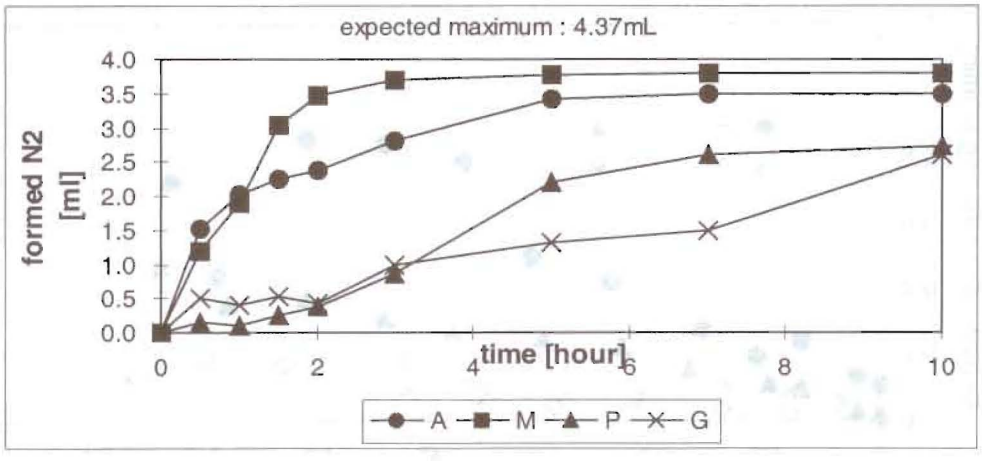

Figure 8. Time course of nitrogenous oxide reduction in small batch culture (adapted-sludge)

adapted sludge, 14.7% on phenol-adapted sludge and 24.4% in the glucose-adapted sludge (Fig. 9). The highest concentration of N_2O gas in A, P and G sludge observed at the first 7 h reaction, indicates that N_2O gas was an intermediate product of denitrifiers activity which then continuously converted to N_2 gas. In case of glucose-adapted sludge, the highest concentration of N_2O gas was suggested produced by denitrifiers and also by microorganisms of nonrespiratory N_2O production. Since assimilation of some nitrate or nitrite-N to NH_4 is not expected under these conditions, thus approximately 100% recovery of N as gases would be expected. As reported in previous study (Kaspar, 1982), at least 80% of the nitrate or nitrite N transformed should be found as N_2O plus N_2 to establish respiratory denitrification. Since in A and M adapted-sludge produced more than 80% N_2 gas, this stoichiometry is sufficient to claim that nitrate reduction in A and M adapted-sludge (reactors A and M) were due to respiratory denitrification. These results were in agreement with the data on reactor performance.

CONCLUSIONS

Denitrification occured in acetic acid (A), methanol (M), phenol (P) and glucose (G) reactors with the rates increased as the concentration of carbon source increased. The highest rates were at the highest TOD:N ratio tested, i.e., 2:0. The linear correlation between nitrogenous oxide reductions with gas production was observed for acetic acid, methanol and phenol adapted-sludge, but not for glucose adapted-sludge. Methanol was more efficient as electron donor for denitrification, since the denitrification rate with methanol was highly enough and constant rate was observed. Production of N_2 gas was high and CO_2 gas was low with methanol. However, if the high rate and limited time were the purpose of the operation, acetic acid might be better to be used than methanol. Phenol was also used efficiently as alternative donor electron for denitrification, but the denitrification rate was slow, and longer time was needed for denitrifying population to adapt to the phenol addition. Glucose was not recommended to be used as electron donor for denitrification, since glucose may support growth of various microbes in the population. Therefore, complexes biologically processes occurred in

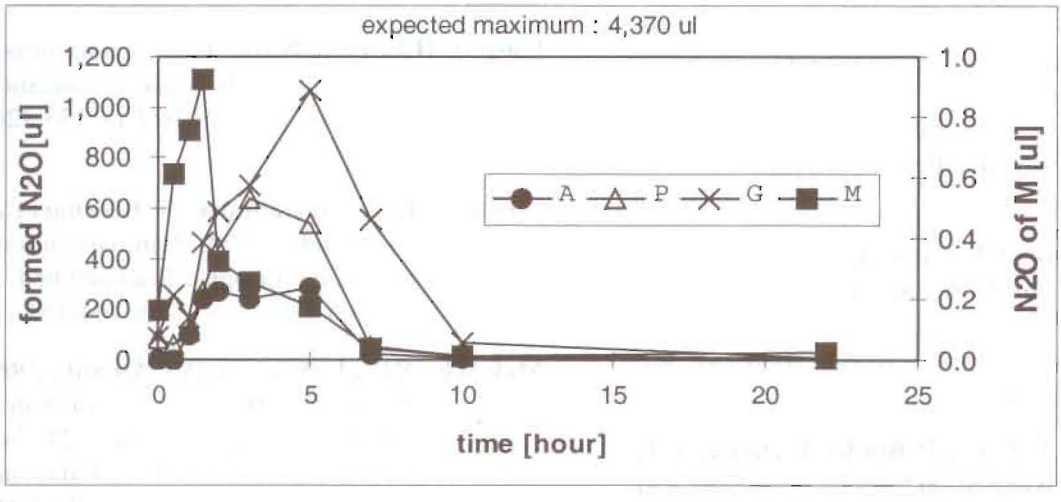

Figure 9. Time course of N_2O gas production in the headspace of small batch culture (without acetylene inhibition)

the reactors may result in inefficient denitrification processes.

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