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A comparative study of methanol as a supplementary carbon source for enhancing denitrification in primary and secondary anoxic zones

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Abstract A comparative study on the use of methanol as a supplementary carbon source to enhance denitrification in primary and secondary anoxic zones is reported. Three lab-scale sequencing batch reactors (SBR) were operated to achieve nitrogen and carbon removal from domestic wastewater. Methanol was added to the primary anoxic period of the first SBR, and to the secondary anoxic period of the second SBR. No methanol was added to the third SBR, which served as a control. The extent of improvement on the denitrification performance was found to be dependent on the reactor configuration. Addition to the secondary anoxic period is more effective when very low effluent nitrate levels are to be achieved and hence requires a relatively large amount of methanol. Adding a small amount of methanol to the secondary anoxic period may cause nitrite accumulation, which does not improve overall nitrogen removal. In the latter case, methanol should be added to the primary anoxic period. The addition of methanol can also improve biological phosphorus removal by creating anaerobic conditions and increasing the availability of organic carbon in wastewater for polyphosphate accumulating organisms. This potentially provides a cost-effective approach to phosphorus removal from wastewater with a low carbon content. New fluorescence in situ hybridisation (FISH) probes targeting methanol-utilising denitrifiers were designed using stable isotope probing. Microbial structure analysis of the sludges using the new and existing FISH probes clearly showed that the addition of methanol stimulated the growth of specific methanol-utilizing denitrifiers, which improved the capability of sludge to use methanol and ethanol for denitrification, but reduced its capability to use wastewater COD for denitrification. Unlike acetate, long-term application of methanol has no negative impact on the settling properties of the sludge.

Keywords Denitrification · External carbon · Methanol · Methanol utilizing denitrifiers

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Introduction

Owing to eutrophication in Logan and Brisbane rivers and in Moreton Bay, local councils in Queensland, Australia have agreed to enforce stringent effluent limits on all wastewater treatment plants, to maintain an average effluent total nitrogen concentration of

5 mg l^{-1} or less. According to a study by six local councils in QLD, Australia (Dennison and Abal 1999), two large wastewater treatment plants (WWTPs) were identified to contribute more than half of the total point source nutrient loading into Brisbane River. A further 45% increase in nutrient loading is anticipated to occur from these plants during the next 70 years. Hence, these WWTPs need to be upgraded to achieve extended nitrogen removal. Achieving very low nitrogen discharge levels (inorganic nitrogen concentrations in effluent of 1–3 mg N l^{-1}) is often problematic when the influent wastewater is limited in a suitable supply of biodegradable COD (or biodegradable chemical oxygen demand). One of the cost effective alternatives to enhance denitrification in activated sludge is to add external carbon sources such as methanol (Nyberg et al. 1996; Purtschert et al. 1996). This approach has been employed by many plants in Australia.

In determining the type of carbon source to enhance denitrification, criteria such as cost, denitrification rate, degree of carbon utilization, sludge production, handling & storage and content of unfavourable toxic compounds are taken into consideration (Aesoy et al. 1998). Among commercially available carbon sources, such as methanol, ethanol, acetate, as well as other alternative carbon sources, such as hydrolysed sludge and solid organic wastes from households, methanol is commonly used mainly because it satisfies many of the above-mentioned criteria. In particular, methanol is known to support lower biomass yield, and thus reduces sludge production (Hallin et al. 2006; Purtschert et al. 1996). However, the need for a period of acclimatization of biomass to methanol addition has often been reported (Ginige et al. 2004; Louzeiro et al. 2002; Hallin et al. 2006). Louzeiro et al. (2002) observed deterioration of sludge settleability after methanol addition, but speculated that it was a result of excess nitrogen gas production caused by increased denitrification rates due to methanol addition.

The practical application of methanol as an external carbon source to enhance denitrification varies. Often, external carbon sources are added directly to the anoxic zone of a single-sludge system (i.e. either to the primary (Purtschert et al. 1996) or the secondary anoxic tank (Nyberg et al. 1996)). A systematic experimental comparison, in terms of carbon consumption and overall system performance, is still lacking on single-sludge systems employing either a primary or a secondary anoxic tank. The primary anoxic zone receives a variety of readily biodegradable influent carbon compounds, along with the external carbon source. In comparison, methanol would be the primary carbon source to support denitrification in the secondary anoxic zone, due to the oxidation of most influent COD in the primary anoxic zone and the aerobic zone. Such a difference could potentially impact on the microbial communities in the two systems, as previously highlighted by Hallin et al. (2006). Some benefits of a single sludge system with methanol addition to the secondary anoxic zone to enhance denitrification have already been reported (Louzeiro et al. 2003). However, a systematic comparison of the two options has not been reported to date.

This study investigates and compares the effects of long-term methanol addition to a single-sludge system in terms of the denitrification performance as well as the structure and properties of the microbial community. Three lab-scale sequencing batch reactors (SBRs) receiving domestic wastewater were operated in parallel. Methanol was added to two of the three SBRs and to the primary and secondary anoxic zones, respectively, while the third SBR received no methanol (a control). The comparison was made based on monitoring the long-term performance of the reactors, the development of the methanol utilizing denitrifiers and the capability of the sludges to use different carbon sources for denitrification.

Materials and methods

Lab reactor operation

Reactor setup

Three lab-scale SBRs were operated in two different configurations. SBR-1 and SBR-3 were operated as single sludge systems with primary anoxic zones, while SBR-2 was operated with both a primary and a secondary anoxic zone. All reactors were cylindrical (manufactured using Perspex) with holding volumes of 12 l, and were operated at working volumes of 9 l. Polarographic DO probes (YSI model 5739, Yellow Springs, Ohio) and pH electrodes (Ionode IJ44, TPS,

Australia) were fitted on all reactors. Mixing was achieved at 200 rpm in all reactors using overhead mixers (RZR2020, Heidolph, Germany). Air curtains were used to sparge compressed air during the aerobic phases, to maintain in-reactor DO concentrations from 1 to 3 mg l^{-1}. A PLC system (Opto 22, Temecula, USA) was used to fully automate the operation of all three reactors. Although online pH measurements were carried out and recorded, no pH control was performed in any reactors.

The reactors were seeded with recycled activated sludge (RAS) from Luggage Point sewage treatment plant (Brisbane, Australia), with starting mixed liquor volatile suspended solid (MLVSS) concentrations of approximately 3.0 g l^{-1}. All reactors were operated on a 4 h cycle for a period of 140 days at 22 ± 2°C. The 4 h cycle of SBR-1 and 3 was composed of a 75 min primary anoxic period with continuous feeding of wastewater (2 l) and mixing, 120 min of aerobic period and mixing, 30 min of settling and 15 min of decanting (2 l). The 4 h cycle of SBR-2 was composed of a 45 min primary anoxic period with continuous feeding of wastewater (2 l) and mixing, 120 min of aerobic period and mixing, 30 min of secondary anoxic period and mixing, 2 min of aeration (to strip N_2 produced in the secondary anoxic period) and mixing, 28 min of settling and 15 min of decanting (2 l). The hydraulic retention time for all reactors was 18 h. Mixed liquor of 100 ml was wasted during the last 5 min of each cycle, giving rise to a theoretical sludge age of 15 days in all reactors. However, the actual sludge age was estimated to be on average 9.5 days, taking into consideration the solids present in the effluent.

Wastewater collection and storage

Approximately 252 l of wastewater was collected from the effluent of a primary sedimentation tank at the Luggage Point sewage treatment plant (Brisbane, Australia). The collected wastewater was stored in a cold room at 4°C, and was used as the feed to all SBRs. Wastewater was collected weekly.

Methanol to supplement COD deficiencies

Methanol was introduced to the primary anoxic zone of SBR-1 and to the secondary anoxic zone of SBR-2, to enhance denitrification. Table 1 illustrates the different periods of methanol addition and the amount of COD that was externally introduced in each of these periods. SBR-3 was the control reactor and received no methanol. Methanol was fed at a flow rate of 1 ml min^{-1} to both SBR-1 and 2. SBR-1 received methanol throughout the 75 min primary anoxic period with the operation of a dosage pump intermittently (turned on for 60 s every 90 s). SBR-2 received methanol continuously throughout the 30 min secondary anoxic period.

Lab reactor performance monitoring and experimental studies

Long term monitoring

The overall reactor performance was monitored by routine measurements of influent and effluent organic and inorganic substances of all reactors during the entire period of reactor operation. On collecting, the

Table 1 Periods of methanol addition in the experimental study

Days	Period	Comments
1–30	Stage I	No methanol addition–Adaptation of sludge to operational conditions applied.
31–43	Stage II	Methanol was introduced to SBR-1 and SBR-2. The addition was progressively increased from 33.3 mg COD per litre of wastewater (week 1) to 66 mg COD per litre of wastewater (week 2). Stage II was included to gradually adapt the sludge to methanol.
44–99	Stage III	Methanol addition was increased to 133.5 mg COD per litre of wastewater. This level of dosage was calculated to be required to achieve a high degree of nitrogen removal from the wastewater used.
100–140	Stage IV	Methanol addition was reduced to 16.0 mg COD per litre of wastewater. Stage IV was included to compare the effect of methanol addition in SBR-1 and 2 when only a small amount of methanol is added.

(Methanol was added directly to the reactors. The methanol concentration given in the table refers to the equivalent concentration of methanol if it was directly added to the wastewater)

influent and effluent samples were immediately filtered using 0.22 μm pore size MILLEX-GP sterile filter units (Cat. No. SLGPO33RS, Millipore, Australia Pty. Ltd.). The soluble inorganic nitrogen (NH_4^+–N, NO_2^-–N, NO_3^-–N) and phosphorus (PO_4^{-3}–P) concentrations in all samples were determined photometrically, using flow injection analysis (FIA, Lachat QuickChem 8000 Analytical, Milwaukee, Wisconsin, USA). Volatile fatty acids (VFA) ranging from C_2 to C_5 were measured using high performance liquid chromatography (HPLC, Shimadzu HPLC system (SIL-10ADVP), Shimadzu, Scientific Instruments, Inc., Columbia USA). The HPLC system was fitted with a HPX-87H 300 mm × 7.8 mm, BioRad Amin-ex ion exclusion HPLC column and was operated at 65°C. 0.008 N H_2SO_4 sparged with helium was used as carrier solution at a flow rate of 0.6 ml min^{-1}. The detector used was a Waters 410 differential refractometer and was operated at 35°C. The VFAs that were measured individually were converted to their respective COD values and were summed up and given as a cumulative VFA-COD measurement. The mixed liquor suspended solids (MLSS) and mixed liquor volatile suspended solids (MLVSS) in all reactors were measured using methods detailed in the Standard Methods for examination of Water and Wastewater (American Public Health Association American Water Works Association and Water Environment Federation 1995), whilst the sludge volume index (SVI) measurements were carried out as detailed in Seviour and Blackall (1999).

Cycle studies

Cycle studies were performed on reactors on a weekly basis. Each cycle study involved withdrawing samples every 15 min during the anoxic phases (both primary and secondary) and every 30 min during the aerobic phases. All samples were analysed for NH_4^+–N, NO_2^-–N, NO_3^-–N, PO_4^{-3}–P and VFA. MLSS and MLVSS of all reactors were measured at the end of each cycle study (American Public Health Association American Water Works Association and Water Environment Federation 1995).

Denitrification properties

(a) In the presence of acetate, ethanol or methanol as COD: These studies were conducted on methanol adapted SBR-1 and 2 and non-adapted SBR-3 sludges during days 66–99. These comparative studies were designed to elucidate the capabilities of the sludges to use different carbon sources for denitrification. The titration and off-gas analysis (TOGA) sensor (Pratt et al. 2003) was used to create a controlled environment to study the denitrification properties of SBR-1, 2 and 3 when exposed to the three different carbon sources. The TOGA sensor maintained an in-reactor pH of 7.0 ± 0.01 (using 1 M HCl and 0.02 M NaOH) and a dissolved oxygen (DO) concentration of 0.0 mg l^{-1} (through sparging non-O_2 containing gas through the reactor). The gas emissions from the bioreactor were measured using a mass spectrometer (MS, OmniStar, Balzers AG, Liechtenstein). The pre-mixed gas used for the MS calibration and for sparging through the bioreactor was composed of 4.96% N_2, 1.99% Ar, 0.5% CO_2 and 92.55% He (Linde Gas Pty. Ltd., Australia). 1% Nitrous oxide (Linde Gas Pty. Ltd., Australia) was also used for MS calibration, to enable monitoring any possible production of nitrous oxide during denitrification. An additional carrier gas (99.996% He, Linde Gas Pty. Ltd., Australia) was used to dilute the reactor gas to a concentration range suitable for MS measurement.

In each test, a 3 l mixed liquor sample was collected from an SBR at the beginning of a cycle and was placed in the TOGA bioreactor. Just prior to initiating the experiment, 10 ml of a 3 g l^{-1} NO_3^-–N stock solution was injected into the TOGA reactor to provide a minimum in-reactor NO_3^-–N concentration of 10 mg l^{-1}. The bioreactor was then operated until a constant off-gas N_2 signal was recorded on the MS, which is demonstrative of an endogenous respiration status in the reactor. Subsequently, 10 ml of carbon source (acetate, ethanol or methanol) was "dumped fed" into the reactor to apply an initial COD:N ratio of 10. The reactor was monitored for denitrification until the previous endogenous respiration rates were observed on the MS, indicating that the externally added COD was completely consumed. The sludge was returned to the parent reactor at the end of the batch test. At least one week was allowed for the reactor to recover and attain steady state before another test was carried out using the same sludge.

During the TOGA experiment, offline samples were obtained, both throughout the initial

endogenous respiration period, and the subsequent period where COD was made available for denitrification. During the endogenous respiration period, 10 ml mixed liquor samples were withdrawn from the reactor every 10 min. On adding COD, 10 ml mixed liquor samples were withdrawn from the reactor every 5 min during the first 30 min, and subsequently, samples were withdrawn every 10 min until the end of the experiment. The mixed liquor samples were immediately filtered (Whatman nitrocellulose membrane, 0.2 μm pore size), to be analysed for NH_4^+–N, NO_2^-–N, NO_3^-–N, PO_4^{-3}–P and the carbon source added using methods detailed previously. Finally, prior to concluding the experiment, the MLVSS in the TOGA bioreactor was measured using standard methods.

(b) In the presence of naturally occurring COD in wastewater: During the 140 days of reactor operation, 4 batch studies (on days 37, 59, 72 and 93) were performed on each of the SBR-1, 2 and 3 sludges, to elucidate the capabilities of all three reactor sludges to utilize wastewater COD for denitrification.

These batch studies were performed at the end of decant of a reactor cycle. The subsequent reactor cycle was interrupted manually and 3 l of freshly collected wastewater was fed together with 135 mg of NO_3^-–N (13.5 mg NO_3^-–N per litter of reactor volume) into all three reactors maintaining mixed anoxic conditions. Subsequently, 10 ml mixed liquor samples were withdrawn from all three reactors every 5 min for a duration of 1 h, and filtered and analysed for NH_4^+–N, NO_2^-–N, NO_3^-–N, PO_4^{-3}–P and VFAs.

Expression of denitrification rates

The denitrification rates were calculated as detailed in Sozen and Orhon (1999). The rate of inorganic nitrogen reduction was evaluated in terms of electron equivalence (or rate of electron donor consumption) and could be expressed as follows.

$$\Delta N = \left(\Delta NO_3^- + 0.6\Delta NO_2^-\right)$$

ΔN is the electron acceptor consumption rate by anoxic respiration represented as nitrate equivalent (mg l^{-1}). ΔNO_3^- (positive) is the nitrate consumption rate, ΔNO_2^- (negative or 0) is the nitrite accumulation rate. ΔN was used in order to compare the denitrification rate in the presence of nitrite accumulation.

Quantification of denitrifying communities

(a) Bio-marking denitrifying microbial communities capable of utilizing methanol as a carbon source using stable isotope probing (SIP): A full-scale sludge (St Mary's, Sydney, Australia) fully adapted to methanol as a supplementary carbon source for enhancing denitrification was used for stable isotope probing. Prior to applying SIP, the activated sludge was assessed for its denitrification capacity with methanol using the TOGA sensor. The TOGA sensor was operated to assess the denitrification properties as detailed in section "Denitrification properties (a)". On confirming denitrification in the presence of methanol, the sludge was diluted to a volume of 300 ml (MLVSS 3.5 g l^{-1}) in preparation for SIP. The mixed liquor was placed in the 500 ml TOGA bioreactor and exposed to ^{13}C-methanol (99% $^{13}CH_3OH$) for 48 h under strict anoxic conditions following the experimental procedure detailed in Ginige et al. (2005) to isotopically label the target microbial community. A COD:NO_2^-–N ratio of 10:1 was maintained by feeding 12.5 ml $NaNO_2$ solution (17.3 g l^{-1}) and 12.5 ml ^{13}C-methanol solution (24.2 g l^{-1}) at a flow rate of 0.1 ml min^{-1}. Nitrite was used instead of nitrate for denitrification to specifically target microbial communities that possessed a more complete denitrification pathway.

(b) Full cycle rRNA analysis including probe design: Methodology for DNA extraction, separation of bio-marked DNA, 16S rRNA clone library analysis, sample fixation, probe design and optimisation was carried out as previously described (Ginige et al. 2004). To optimise the newly designed MEDEN 635 oligonucleotide probe, the pure cultures *Methylovorus glucosotrophus* (DSM 6874) and *Azoarcus oryzae* (DSM 13638) were used as negative controls. Due to a lack of pure cultures whose 16S rRNA would bind the designed MEDEN 635 probe, the optimum formamide concentration for the probe was determined using fixed enriched sludges (obtained from SBR-1 and SBR-2 during stage III of reactor operation) as positive controls. A similar approach of using mixed microbial biomass as positive controls for probe optimisation has been reported previously (Crocetti et al. 2000; Erhart et al. 1997; Schmid et al. 2000; Ginige et al. 2004, 2005).

(c) Quantification of methanol utilizing microbial communities: Biomass samples from SBR-1, SBR-2 and SBR-3 were fixed with 4% paraformaldehyde for fluorescence in situ hybridization (FISH) (Amann et al. 1990; Bond et al. 1999; Manz et al. 1992). FISH was performed routinely on biomass of all three reactors to determine the changes in methanol-utilizing denitrifying populations. Cy3 labelled DEN67 (Ginige et al. 2004), HYP1241 (Layton et al. 2000) and MEDEN 635 (sequence 5′ AGCC TCGCAGTTTCAAAC 3′, designed in this study—see the Results section) oligonucleotide probes were used to target methanol utilizing denitrifiers. Cy3 labelled PAOMIX (a combination of PAO462, PAO651 and PAO846) (Crocetti et al. 2000) probes were used to target polyphosphate accumulating organisms (PAOs). Cy5 labelled EUBMix (a mixture of EUB338 (Amann et al. 1990), EUB338-II and EUB338-III (Daims et al. 1999) probes) oligonucleotide probe was used to target all bacteria in the sample. FISH samples were observed using a Bio-Rad Radiance 2000 confocal laser-scanning microscope. Cy3 and Cy5 were excited with, respectively, a 1 W 543 nm and a 638 nm HeNe laser and captured using a 580 ± 16 nm BP and a 660 nm LP filter. FISH quantification of the denitrifiers, in proportion to the total Bacteria, was carried out using previously published methods (Ginige 2003; Crocetti et al. 2000; Bouchez et al. 2000).

Results and discussion

Long-term reactor performance and impact of reactor configuration on the effectiveness of methanol addition

SBR-1, 2 and 3 reactor effluent

The effluent NOx-N concentrations of SBR-1, 2 and 3 during the entire experimental period and the VFA-COD (sum of all measured VFAs converted to COD) and NH_4^+–N present in influent wastewater is shown in Fig. 1 The average effluent NO_x–N, NH_4^+–N and PO_4^{-3}–P concentrations for all three SBRs, during Stages I, III and IV, are summarised in Table 2, along with the average influent NH_4^+–N, VFA-COD and PO_4^{-3}–P concentrations during the same periods.

The NH_4^+–N concentration contained in the wastewater was relatively stable (34.8 mg l^{-1} with a standard deviation (SD) of 8.5), while the VFA-COD concentrations in the influent demonstrated considerable variability maintaining an average value of 48.5 mg l^{-1} (SD = 27.5) during the entire period of study. This likely explains the relatively large standard deviations reported in Table 2. A clear correlation can be observed in Fig. 1 between the effluent NO_x–N concentration of SBR-3, which received no external carbon sources, and the VFA-COD concentration in the influent wastewater.

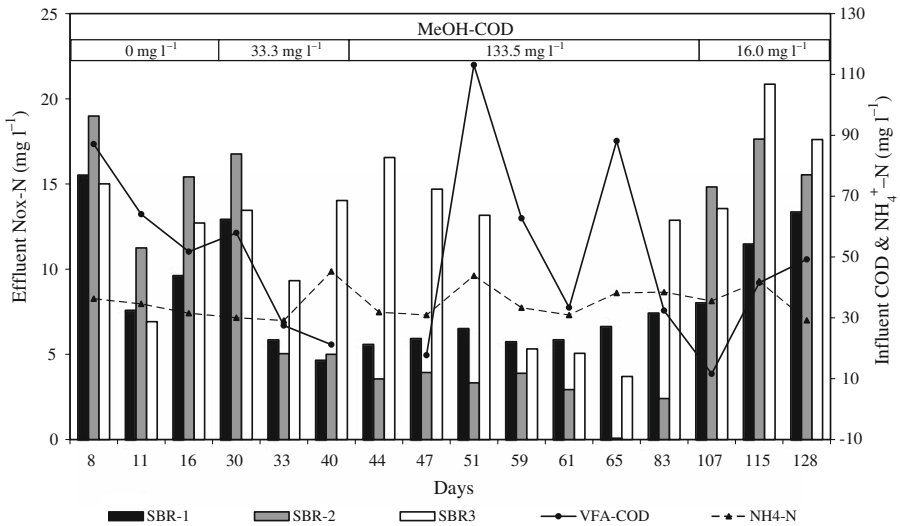

Fig. 1 Effluent NO_x–N concentrations of SBR-1, 2 and 3 in the presence of varying VFA-COD and NH_4^+–N in influent wastewater and varied methanol addition

Table 2 Average influent VFA-COD, NH_4^+–N and PO_4^{-3}–P, effluent NH_4^+–N, NO_x–N and PO_4^{-3}–P concentrations during Stages I, III and IV of reactor operation

Stages	Influent (mg l^{-1})			SBR-1 Effluent (mg l^{-1})			SBR-2 Effluent (mg l^{-1})			SBR-3 Effluent (mg l^{-1})		
	VFA-COD	NH_4^+–N	PO_4^{-3}–P	NH_4^+–N	NO_x–N	PO_4^{-3}–P	NH_4^+–N	NO_x–N	PO_4^{-3}–P	NH_4^+–N	NO_x–N	PO_4^{-3}–P
I	65.2 ± 15.4	33.1 ± 2.8	6.9 ± 0.6	0.2 ± 0.2	11.4 ± 3.5	6.1 ± 1.0	0.2 ± 0.1	15.6 ± 3.3	6.4 ± 0.4	0.3 ± 0.3	12.0 ± 3.5	6.3 ± 1.1
III	51.5 ± 35.3	36.1 ± 9.8	7.6 ± 1.6	0.9 ± 1.1	6.5 ± 1.8	6.8 ± 1.8	0.5 ± 0.4	2.8 ± 1.8	4.1 ± 2.3	1.4 ± 1.6	10.8 ± 5.3	7.6 ± 1.5
IV	40.1 ± 19.0	33.0 ± 7.5	6.7 ± 1.3	2.3 ± 3.0	11.9 ± 2.9	6.3 ± 1.6	1.4 ± 1.4	15.8 ± 2.7	6.3 ± 1.8	0.8 ± 0.6	17.5 ± 3.2	8.2 ± 3.8

According to Table 2, SBR-1 and SBR-3 performed very similarly in Stage I. While having similar nitrification performance, SBR-2 displayed poorer denitrification performance in comparison to the other two reactors, even though the total anoxic period in all three reactors was identical. Cycle studies on SBR-2 showed that the second anoxic period (30 min) was not effective, with little denitrification occurring in the absence of easily biodegradable COD (data not shown). On initiating methanol addition, the denitrification performance of both SBR-1 and SBR-2 improved quickly and dramatically, reaching an effluent NO_x–N concentration in Stage III of 6.5 mg l^{-1} (SD = 1.8) and 2.8 mg l^{-1} (SD = 1.8), respectively. Both were considerably lower than that of SBR-3 (10.8 mg l^{-1} (SD = 5.3)). Contrary to Stage I, however, SBR-2 had a much better denitrification performance than SBR-1, although both reactors received the same amount of methanol. The cycle study data in Fig. 2 show that the denitrification performance of SBR-1 was limited by its configuration. As a predenitrification system with a volumetric exchange ratio of 0.22 (corresponding to a recycle ratio of 3.5 in a continuous system), the nitrogen removal efficiency of SBR-1 was limited to below 80%. In the anoxic period, nitrate, rather than COD, was the limiting factor. Indeed, Fig. 2 shows that there was leakage of MeOH to the aerobic period in SBR-1. In contrast, SBR-2 could remove nitrogen to a very low level, as long as an adequate amount of methanol was added.

During stage IV, when methanol addition was reduced to a very low level (16.0 mg COD per litre of wastewater), SBR-1 and SBR-2 achieved an average effluent NO_x–N concentration of 11.9 mg l^{-1} (SD = 2.9) and 15.8 mg l^{-1} (SD = 2.7), respectively. While both performed better than SBR-3, which demonstrated an average effluent NO_x–N concentration of 17.5 mg l^{-1} (SD = 3.2), SBR-1 significantly out-performed SBR-2, as occurred in Stage I. The cycle study in this period (data not shown) revealed that in SBR-2 methanol was primarily used to reduce nitrate to nitrite. This led to nitrite accumulation and hence no N removal. The underlying reason for nitrite accumulation in this case is not clear. It is worthwhile to also observe that the nitrification performance of SBR-1 deteriorated slightly in this stage compared to the previous stages. The good nitrification performance by SBR-3 in the

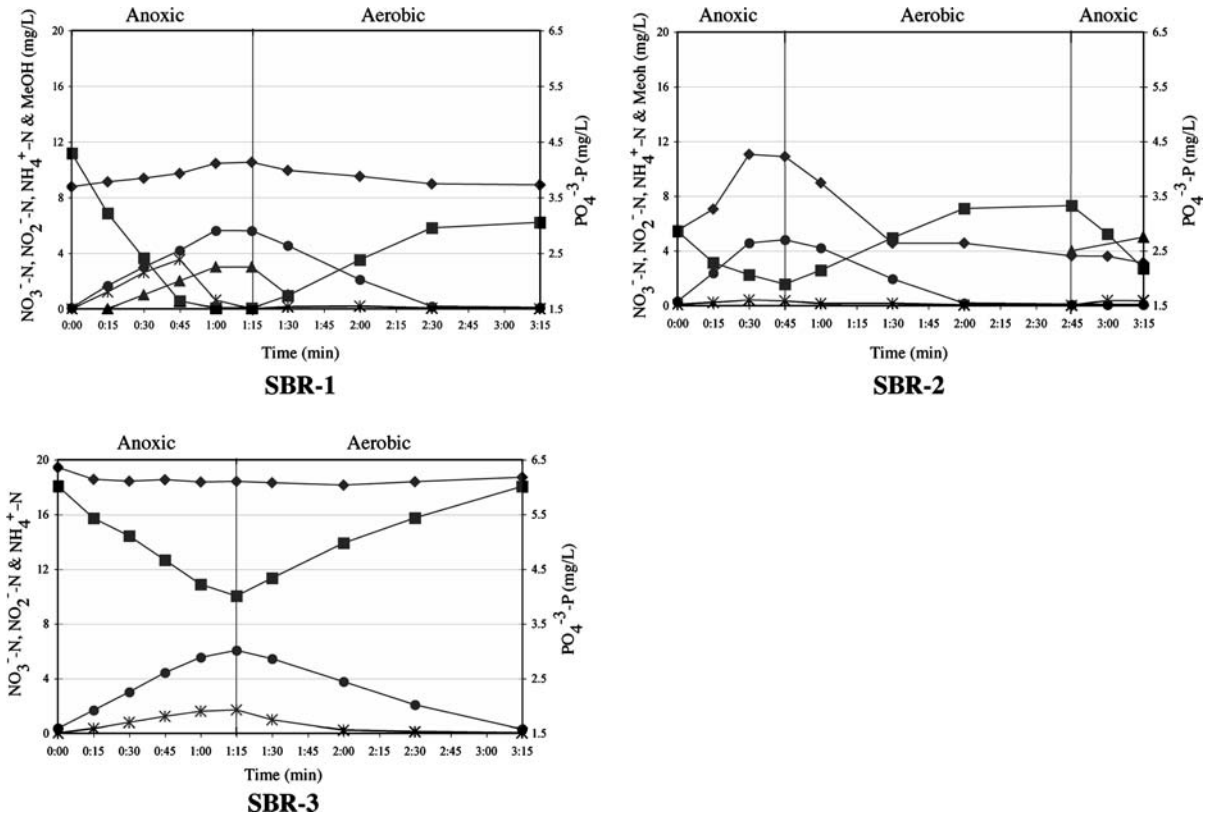

Fig. 2 Profiles of NH_4^+–N (•), NO_2^-–N (*), NO_3^-–N (■), PO_4^{-3}–P (◆) and MeOH (▲) of a reactor cycle on day 82 of SBR-1, 2 and 3 operation

same period suggests that the deterioration was unlikely caused by the presence of any inhibitors in wastewater. It is not clear if there was a link with the change in methanol addition.

The results showed that the reactor configuration has a major impact on the effectiveness of methanol as an external carbon source in enhancing denitrification. When a very low effluent nitrate concentration is desired, a second anoxic zone should be introduced, where methanol should be added. In other cases, it is more effective to add methanol to the primary anoxic zone. One limitation of the former case, as Fig. 2 shows, is that a limited amount of nitrate would be recycled to the primary anoxic period, thus resulting in poorer use of the wastewater COD for denitrification. This may, however, not be a problem in a continuous system. Among other differences between a continuous system and a SBR, recycling of nitrate from the aerobic zone to the primary anoxic zone can easily be implemented in a continuous process and indeed typically exists in full-scale wastewater treatment plants (Tchobanoglous et al. 1991).

Another important observation is that SBR-2 achieved a comparatively higher phosphorus removal in Stage III (Table 2). While methanol is not a carbon source of choice for polyphosphate accumulating organisms (PAOs) (Martin et al. 2006), its addition to the second anoxic period created anaerobic conditions in the primary anoxic period, enabling the use of VFAs (contained in influent wastewater) by PAOs for achieving phosphorus removal. The development of *Accumulibacter*, a widely reported PAO, in SBR-2 was confirmed by microbial analysis of the sludge using FISH (data not shown). This indirect impact of methanol addition on phosphorus removal could be exploited in full-scale BNR systems, in cases where the influent wastewater does not contain an adequate amount of easily biodegradable COD to enable satisfactory N and P removal. This approach is expected to be more cost-effective than adding acetate (Thomas et al. (2003)) considering the

relatively lower price for methanol. As shown in Fig. 2, phosphorus release and uptake also occurred in SBR-1, indicating that phosphorus removal may also be achieved by adding methanol to the primary anoxic zone. However, denitrifiers compete with PAOs for the wastewater VFAs, which could be detrimental to P removal performance.

Sludge settling properties and mixed liquor volatile suspended solids

Over the study period, SBR-1, 2 and 3 showed relatively good settling characteristics, maintaining sludge volume index (SVI) values between 84 and 143 ml g^{-1}. The reactors maintained clear effluent with an average total suspended solid (TSS) concentration of 43 mg l^{-1}. During all stages, SBR-3 maintained an average MLVSS value of 1.21 g l^{-1} (SD = 0.14), while the MLVSS concentrations in SBR-1 and SBR-2 varied considerably with a strong correlation to the amount of methanol received, reaching 1.80 g l^{-1} (SD = 0.47) and 1.94 g l^{-1} (SD = 0.43), respectively in stage III.

The addition of methanol had no apparent effect on the overall settling properties of the biomass. After 80 days of acclimatization to methanol, SBR-1 and 2 continued to demonstrate good settling properties. This observation is in contrast to the very poor settling properties observed by Ginige et al. (2005), when acetate was used as an external source of carbon to enhance denitrification. FISH images revealed that methanol facilitated an enrichment of methanol utilizers with good flocculation properties. In comparison, Ginige et al. (2005) reported that non-flocculating planktonic cells increased when acetate was a sole source of carbon. The impact of methanol on other biological processes, such as nitrification, also appeared minimal, as both SBR-1 and 2 achieved very low ammonia concentrations in their effluent (see Table 2).

The methanol utilising microbial communities in SBR-1, 2 and non-adapted SBR-3 sludges

Full cycle rRNA analysis of St Mary's sludge

During SIP, the TOGA sensor reported denitrification rates as high as 8 mg N_2–N mg $MLVSS^{-1}$ h^{-1}. Ninety (90) 16S rRNA bacterial clones obtained from the [^{13}C] DNA fragment were grouped in to 9 operational taxonomic units (OTU) according to restriction fragment length polymorphism (RFLP). 73% of bacterial clones (65 out of 90) formed two distinct banding patterns in the RFLP analysis. Partial DNA sequencing of 16S rRNA gene sequences revealed that 6 out of the 9 OTUs (76%) had sequences closely related to members of the *Betaproteobacteria*, whilst one OTU (8%) was closely related to *Hyphomicrobium* spp. of the *Alphaproteobacteria*. The remaining clones were affiliated with *Bacteroides* 16S rRNA gene sequences. Chimeric 16S rRNA gene sequences were detected on 9% of the bacterial clones analysed, and were omitted from further analyses. Evolutionary distance analysis of 16S rRNA gene sequences affiliated with the *Betaproteobacteria* identified a monophyletic clustering of sequences within the obligate methylotrophs in the *Methylophilaceae* of the *Betaproteobacteria* closely associated to 16S rRNA gene sequences targeted by the FISH probe DEN 67 (Ginige et al. 2004). FISH probe design focused on these sequences due to their high abundance (76%) in the clone library and their phylogenetic association with other methanol utilising bacteria. The probe MEDEN 635 was designed to be specific for 16S rRNA gene sequences obtained from this clone library study affiliated with *Methylophilaceae*. The optimal formamide concentration for MEDEN 635 ranged from 25 to 40% and at 25% formamide both negative controls, *M. glucosotrophus* (one mismatch) and *A. oryzae* (two mismatches) were not capable of binding the probe, and MEDEN 635 targeted cells began to loose probe intensity at 45% formamide. The OTU closely related to the genera *Hyphomicrobium*, was already targeted by the available FISH probe, HYP1241 (Layton et al. 2000) and other 16S rRNA gene sequences were not investigated for probe design due to their low abundance in the clone library and poor affiliation with other cultured bacteria.

The abundance of the probe targeted microbial communities

The MEDEN 635 probe designed in this study correlated well with increase in numbers of target population and denitrification rates. Cells targeted by this probe were only occasionally detected during stage I of SBR-1, 2 and 3 operation. Upon

introducing methanol in stage II, SBR-1 and 2 demonstrated a steady increase in abundance of MEDEN 635 target organisms. With increased concentrations of methanol in stage III, the target population reached an abundance of 18 and 10% in SBR-1 and 2, respectively at the end of stage III. During stage IV MEDEN 635 targeted cells dropped dramatically to as low as 8 and 6% in SBR-1 and 2, respectively. During stages II, III and IV a significant positive correlation (Pearson's correlation $r(4) = 0.961$, $P < 0.01$) was observed between the abundance of MEDEN 635 target cells and the increase in denitrification rates. The MEDEN 635 target population in SBR-3 was approximately 1%. The low occurrence of MEDEN 635 targeted cells in SBR-3 suggests that these organisms are highly selective users of methanol.

The DEN 67 target organisms were found in all three SBR reactors, with poorer correlation to denitrification rates. DEN 67 targeted cells were not highly abundant and ranged between 1 and 6%. This is extremely low compared to the study conducted by Ginige et al. (2004) where DEN 67 targeted cells were found to be as high as 50% in a methanol fed denitrifying reactor operated using a synthetic feed. As highlighted by Ginige et al. (2005), this observation further demonstrates the importance of culture and enrichment independent methods to identify microbial communities directly responsible for biological processes in a specific eco-system.

Hyphomicrobium spp. were found to be approximately 1% in all SBR reactors, with SBR-2 demonstrating an abundance of 3% on day 59. Although the abundance was very low, SBR-2 did show some positive correlation between the abundance of *Hyphomicrobium* spp. and increase in denitrification rates. Even though previous studies have demonstrated the abundance of *Hyphomicrobium* spp. in anoxic environments where methanol was the primary carbon source, it is inconclusive whether these organisms in fact could use methanol as a sole source of carbon for denitrification. Since only 8% of the clones were found to be closely related to *Hyphomicrobium*, it is unclear whether *Hyphomicrobium* spp. is a subset of the denitrifying community capable of utilising methanol as a sole source of carbon in SBR-1 and 2 reactors.

Denitrification capacities of methanol adapted SBR1 and 2 and non-adapted SBR-3 sludges with various carbon sources

Denitrification capacities in the presence of methanol, ethanol or acetate as sole sources of carbon

The maximum specific denitrification rates of SBR-1, 2 and 3 sludges in Stage III with methanol, ethanol or acetate as the sole carbon source are presented in Fig. 3 Also present in the figure are the measured

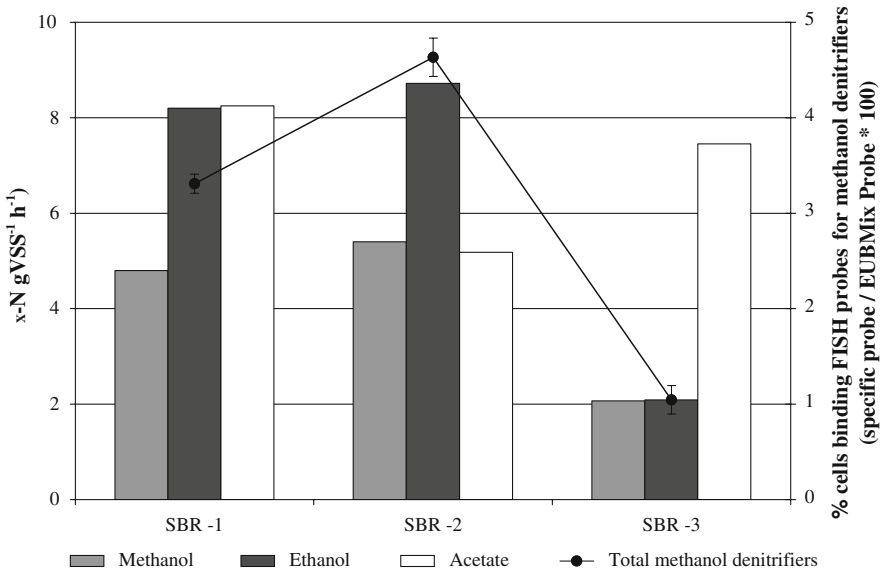

Fig. 3 Denitrification rates of SBR-1, 2 and 3 sludges in the presence of methanol, ethanol or acetate as sole source of carbon and the community analysis of methanol utilizing denitrifiers using FISH biovolume measurement. The total methanol denitrifier population is a cumulative area of cells targeted by probes DEN67 and MEDEN 635 is expressed as a percentage of cell area detected with EUBMix

total methanol-utilizing denitrifier populations in the three reactors during stage III. The nitrogen mass balances on all batch experiments revealed a high degree of accuracy, where the margin of error was <10% between the NO_x–N consumption measured in the liquid phase and the corresponding N_2 production in the gas phase (N_2O emission was not detected in all experiments).

The FISH quantification data revealed that the abundance of known methanol utilizers was considerably higher in SBR-1 and SBR-2 than in SBR-3. The results confirm the previous hypothesis that the addition of methanol to a single-sludge system would cause changes to the microbial community in the sludge, by supporting the development of specific methanol-utilizing denitrifying populations. The methanol-supported denitrification rates observed with the three sludges correlate well with the microbial data. The methanol-adapted sludges (SBR-1 and SBR-2) demonstrated denitrification rates with methanol that were two fold higher than that of the non-adapted SBR-3 sludge. The SBR-1 and SBR-2 rates were considerably higher than those reported by Louzeiro et al. (2002, 2003). These differences could be due to the presence of a large number of methanol utilizing denitrifiers in our test reactors.

While not shown in Fig. 3, the reduction of methanol dosage to SBR-1 and 2 by over 80% during stage IV resulted in a considerable decrease (44 and 35%, respectively) in denitrification rates by the two sludges with methanol. This correlated well with the decreased methanol-utilizing populations in both reactors (data not shown). In comparison, the methanol-driven denitrification rate observed with SBR-3 sludge remained almost unchanged and continued to be considerably lower than the rates observed with SBR-1 and 2.

SBR-1 and 2 demonstrated denitrification rates approximately four times that of SBR-3, when ethanol was used as a sole source of carbon (Fig. 3). Hallin et al. (2006) and Hallin and Pell (1998) also demonstrated that, when compared with a non-adapted sludge, methanol adapted sludges have a higher capacity to denitrify with ethanol as a carbon source. Considering that SBR-1 and 2 sludges have had no previous exposure to ethanol and that SBR-3 failed to demonstrate a comparable denitrification rate with ethanol, it could be assumed that majority of the methanol utilizers in SBR-1 and 2 are likely also able to utilize ethanol for denitrification. From an operational point of view, the observations made in this and previous studies reveal an opportunity for the operators to switch between methanol and ethanol, from time to time, depending on market pricing and availability. However, the long-term application of ethanol on the capability of the sludge to denitrify with methanol as the carbon source requires further investigation.

The fact that the non-adapted SBR-3 sludge demonstrated very low denitrification rates with ethanol and methanol indicates that neither ethanol nor methanol is ideally suited for intermittent addition to a non-adapted sludge to deal with peak nitrogen loading. Our observation is consistent with past research (Louzeiro et al. 2002, 2003; Hallin et al. 1996; Hallin and Pell 1998).

All reactors achieved good denitrification with acetate (Fig. 3). SBR-1 and SBR-3 demonstrated similar denitrification rates, which were significantly higher when compared with the rate of SBR-2. There could be two possible reasons for this observation. First, much of the denitrification in SBR-2 took place in the secondary anoxic zone in the presence of methanol. Table 2 shows an average nitrate concentration of 2.8 mg l^{-1} (SD = 1.8) in SBR-2 effluent compared to 6.5 mg l^{-1} (SD = 1.8) and 10.8 mg l^{-1} (SD = 5.3) in SBR-1 and SBR-3, respectively. Nitrate was therefore likely a limiting factor for denitrification in SBR-2 during the primary anoxic period, limiting the use of VFAs in wastewater for denitrification in this period. This may have negatively impacted on the development of acetate utilizing denitrifiers, resulting in reduced denitrification rates with acetate. The second reason could be attributed to a possible competition for acetate between denitrifiers and PAOs, thus leading to a reduced denitrification rate. During the batch experiments, SBR-1 and 2 both demonstrated phosphorus release (24.6 and 37.7 mg l^{-1}, respectively) despite the presence of nitrate, suggesting that some of the acetate was in fact taken up by PAOs, limiting the availability of acetate for the denitrifiers in SBR-2. This would result in the observed lower denitrification rates.

Denitrification capacities with wastewater COD

Figure 4 shows that SBR-1 and 2 sludges experienced a significant reduction in their capability to oxidise wastewater COD with acclimatization to

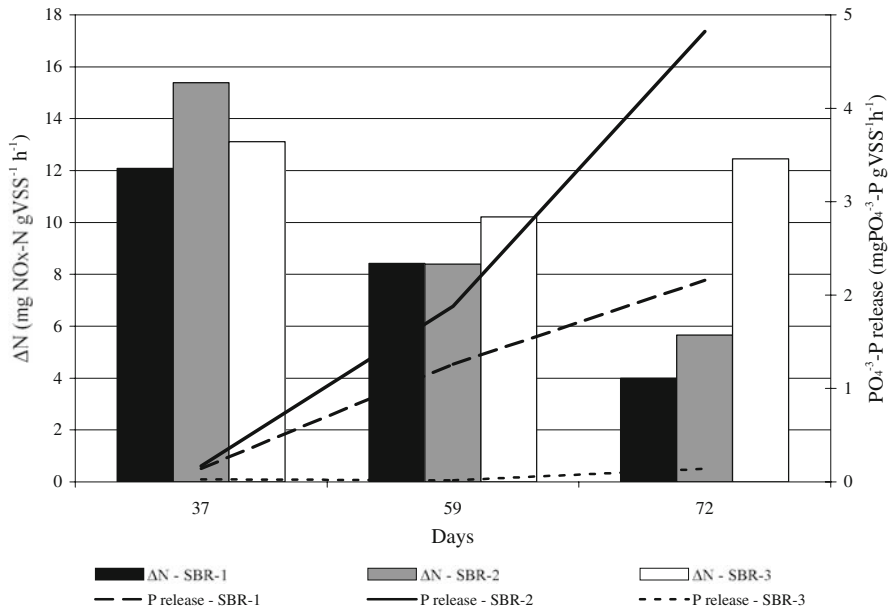

Fig. 4 Denitrification rates (ΔN) and PO_4^{-3}–P release of SBR-1, 2 and 3 sludges in the presence of wastewater COD as the sole source of carbon

methanol. During a short period of 35 days of continuous methanol dosing (from days 37 to 72), the denitrification capacity of SBR-1 and SBR-2 sludges with wastewater COD decreased by over 60%. In contrast, the denitrification capacity of SBR-3, the control reactor, with real wastewater remained steady and the rates observed on average were similar to values reported by Carucci et al. (1996). This comparison indicates that methanol supplementation negatively impacts on the capability of the sludge to denitrify with wastewater COD. As discussed in the previous section, this could be related to the competition between denitrifiers and PAOs for the wastewater COD. Figure 4 also illustrates the capacity of all three reactors to remove phosphorus in the presence of wastewater COD. SBR-1 and 2 demonstrated an increase in phosphorus removal with decreasing denitrification rates. By day 72, the methanol supplemented SBR-1 reactor demonstrated a PO_4^{-3}–P release that was 15-fold higher compared to SBR-3, whilst SBR-2 demonstrated a PO_4^{-3}–P release that was 35-fold higher. FISH results with the PAOMIX probes showed an enrichment of PAOs in SBR-1 and 2, compared to very low numbers (<1%) found in SBR-3. The reduced denitrification rates and the enhanced phosphorus removal observed with wastewater COD indicates that, in addition to the anaerobic environment created with methanol supplementation, the reduced capability of SBR-1 and 2 adapted sludges to utilize wastewater COD may have facilitated the increase of PAO population in SBR-1 and 2 reactors.

Conclusions

A comprehensive study on the use of methanol as an external carbon source to enhance denitrification in a primary and secondary anoxic tank of single sludge systems is reported. The following conclusions can be drawn from this study.

- The addition of methanol stimulates the growth of specific methanol-utilizing denitrifiers, which improves the capability of sludge to use methanol and ethanol for denitrification, but reduces its capability to use wastewater COD for denitrification.
- Reactor configuration has a major impact on the effectiveness of methanol as an external carbon source for enhancing denitrification. Methanol should be added to the secondary anoxic zone when a large amount is required to achieve very low effluent nitrate levels. On the other hand, methanol should be added to the primary anoxic zone when a small amount is needed. Adding a small amount of methanol to the secondary anoxic zone may cause nitrite accumulation,

which does not result in complete nitrogen removal.

- The addition of methanol may indirectly induce/improve biological phosphorus removal, by creating anaerobic conditions and increasing the availability of wastewater COD for polyphosphate accumulating organisms. This potentially provides an alternative approach to phosphorus removal from wastewater with low COD content.
- Unlike acetate, long-term application of methanol has no negative impact on the settling properties of the sludge.

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