Characteristics of granular sludge in a single upflow sludge blanket reactor treating high levels of nitrate and simple organic compounds

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Abstract Simultaneous denitrification and methanogenesis were accomplished in a single upflow sludge blanket (USB) reactor. More than 99% and 95% of nitrate and chemical oxygen demand (COD) removal rates were obtained at a loading of 600 mg NO_3-N/L·d and 3,300 mg COD/L·d, respectively. The specific denitrification rate (SDR) increased as COD/NO_3-N ratios decreased. Maximum SDR with acetate could reach 1.05 g NO_3-N/gVSS·d. Significant sludge flotation was observed at the top of the reactor due to the change of microbial composition and the formation of hollow granules. Granules became fluffy and buoyant due to the growth of denitrifiers. Microscopic examination showed that granules exhibited layered structure and they were mainly composed of *Methanosarcina* sp., *Pseudomonas* sp., and rod-shaped bacteria.
Keywords Denitrification; hollow granules; methanogenesis; *Methanosarcina* sp.; specific denitrification rate

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Introduction

Anaerobic processes have been widely used for treating various high-strength wastewaters. Although they have various advantages over aerobic processes, the anaerobic processes are generally required for effluent treatment to reach the discharge standards for residual organic and nitrogen compounds. In particular, nitrogen removal is necessary otherwise its discharge into the aquatic environment might result in several problems such as surface water eutrophication, groundwater contamination, and public health. Therefore, the effluents of anaerobic processes should be treated by any means including physicochemical and/or biological processes before being released into the receiving environment. Biological nutrient removal processes including nitrification and denitrification have been generally used for removal of ammonia nitrogen.

Some researchers have attempted denitrification using anaerobic processes since the early 1980s. Kasper *et al*. (1981) suggested that anaerobic microorganisms have been found to exhibit higher ammonification (60–70%) than denitrification (30–40%) potentials. Tiedje (1988) revealed that dissimilatory nitrate reduction to ammonium is the major pathway in anaerobic process. Akunna *et al*. (1993) revealed that nitrate reduction to ammonium depended on the availability of carbon. The application of a single integrated denitrifying/methanogenic process has been proposed by several researchers (Hanaki and Polprasert, 1989; Akunna *et al*., 1992) because of the better understanding of denitrification and development of high-rate anaerobic digesters using self-immobilization. Most of the researches have been focused on the effect of carbon sources for denitrification using anaerobes, determination of optimum C/N ratio and the effect of nitrous oxide on methanogenesis. Recently, other researchers have suggested that an integrated denitrifying /methanogenic process has several advantages including low external carbon requirement, and low space requirement over traditional nitrification-denitrification processes (Hendriksen and Ahring, 1996). Furthermore, the integrated process is known to increase the life of the anaerobic process for treating leachate which contains high concentrations of organic matter and ammonia nitrogen.

Although some research has been conducted on the simultaneous treatment of nitrate and organic compounds, there are few studies on the characteristics of microorganisms. The objectives of this investigation were to assess the performance of a USB reactor with various C/N ratios and to characterize microorganisms in a USB reactor treating high levels of nitrate and acetate simultaneously as well as understanding more on the interactions between denitrifiers and methanogens.

Materials and methods

Experimental conditions and reactor operations

The single USB reactor used in this study was 5.25 L in effective volume. The reactor was placed in a temperature controlled room at 35°C. For the feed to the USB reactor, a basal nutrient medium was prepared with 270 mg/L of KH_2PO_4, 350 mg/L of K_2HPO_4, 75 mg/L of $CaCl_2{\cdot}6H_2O$, 100 mg/L of $MgCl_2{\cdot}6H_2O$, 20 mg/L of $FeCl_2{\cdot}4H_2O$, 5 mg/L of $MnCl_2{\cdot}4H_2O$, 0.05 mg/L of $ZnCl_2$, 0.03 mg/L of $CuCl_2$, 0.01 mg/L of $NaMoO_4{\cdot}2H_2O$, 0.5 mg/L of $CoCl_2{\cdot}6H_2O$, 0.05 mg/L of $NiCl_2{\cdot}6H_2O$, and 0.05 mg/L of Na_2SeO_3. Granular sludge was obtained from an upflow anaerobic sludge blanket (UASB) reactor treating brewery wastewater and seeded to the reactor. The USB reactor started at the organic loading rates (OLR) of 1.3 kgCOD/m^3·d with hydraulic retention time (HRT) of 1.7d. Nitrate was added in the form of a mixed solution of KNO_3 and $NaNO_3$ up to 1,600 mg NO_3-N/L.

Analytical methods

Gas composition was analyzed in a gas chromatograph (Gow Mac series 580) with the thermal conductivity detector (TCD). Acetate was measured by a gas chromatograph (HP 5890 series II). Anions were determined using a Dionex 120 ion chromatograph with a Dionex conductivity detector and Ionpac AS4A-SC column. Microstructures of the granules were examined using a scanning electron microscope (SEM) and transmission electron microscope (TEM). The SDR of the granules was measured in serum bottles. The substrate used for the SDR measurements was acetate. Other parameters were analyzed according to Standard Methods (APHA, 1992).

Results and discussions

Performance of USB reactor

The single USB reactor was continuously operated for 460 days. Figure 1 shows the variations of the influent and effluent COD concentrations with various C/N ratios.

As shown in Figures 1 and 2, COD removal rate was more than 98% during the phase without nitrate addition except for the initial acclimation period. In the initial period, acetate was hardly detected in the effluent.

When the nitrate was added to the reactor (C/N = 60), the COD removal rate decreased slightly. When the nitrate concentration increased to 133 mg NO_3-N/L (C/N = 30), the COD removal rate decreased to about 75% due probably to the change of environmental conditions including pH, pE, and so on. Chen and Lin (1993) suggested that the inhibitory effect of the nitrogen oxides on the methanogenesis was attributed to an elevation of the redox potential of the culture as well as the toxic effect of the nitrogen oxides themselves. They also reported that the optimum pH for denitrification was 7.0–8.0 and 6.5–7.5 for

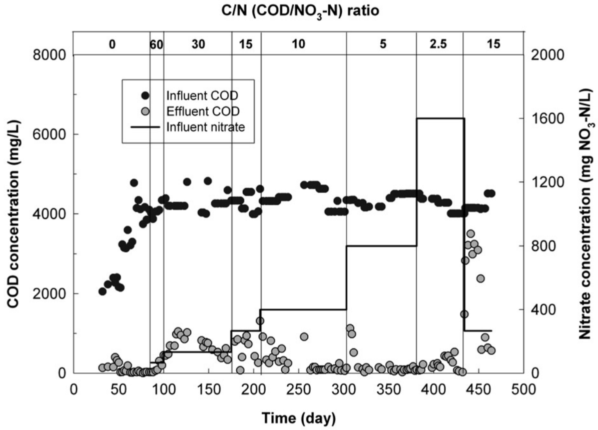

Figure 1 Influent and effluent COD concentration at various C/N ratios

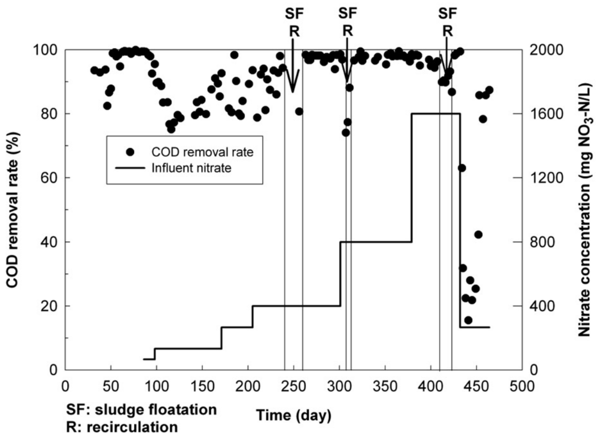

Figure 2 Performance of USB reactor

methanogenesis. The denitrification and methanogenesis also could sink hydrogen generated during the reaction, increasing pH by producing bicarbonate.

At a C/N ratio of 10, the COD removal rate increased gradually as microorganisms became acclimated. In this period, the COD removal rate recovered up to more than 90%. But the COD removal rate decreased rapidly due to the operational problems such as sludge flotation. A large number of granules floated to the top of the reactor due to the increase of denitrifiers. Hendriksen and Ahring (1996) suggested that flotation and occasional washout of sludge occurred probably by development of filamentous sludge and by formation of gas bubbles trapped in the sludge. They also suggested that the recirculation could be a solution for reduction of the sludge flotation. In this research, recirculation was attempted to enhance mixing and to increase shear force so as to prevent agglomeration of granules.

When the nitrate concentration increased to 800 mg NO_3-N/L (C/N = 5), the COD removal rate decreased temporarily due to the sludge flotation, but recovered and was maintained at more than 95% by increasing recirculation ratio up to 300%.

At a C/N ratio of 2.5 (1,600 mg NO_3-N/L), the USB reactor achieved over 90% of COD removal rate. Although low levels of nitrate (< 1.5 mg NO_3-N/L) were occasionally detected, nitrate was hardly detected in the effluent during the experimental periods except for phase VI (C/N = 2.5) as shown in Figure 3. During this phase, nitrite build-up was also detected temporarily. McCarty (1966) also observed this phenomenon whereas Moore and

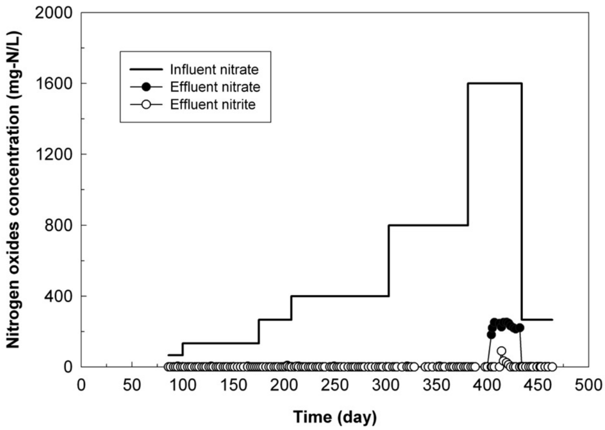

Figure 3 Nitrogen oxides concentration with operational time

Schroeder (1970) did not observe it. They also suggested that many species of bacteria reduced nitrate to nitrite but apparently fewer species reduce nitrite. Thus, in batch or plug flow systems, nitrite would be expected. Although the nitrite build-up was observed, it is due mainly to the high level of nitrate but not to the flow pattern. This result also indicated that the influent did not have sufficient quantities of substrate for complete denitrification. In this period, all organics were utilized by denitrification, as evidenced by the end of methane production.

When the nitrate concentration was increased from 67 (C/N = 60) to 1,600 mg NO_3-N/L (C/N = 2.5), electron flow to denitrification was more prevalent. This is because denitrification is thermodynamically more favorable than methanogenesis as the following Eq. (1) and Eq. (2) show:

$$5CH_3COO^- + 5H_2O \rightarrow 5CH_4 + 5HCO_3^- \qquad \Delta G^0 = -33.84 \text{ kcal/mole} \quad (1)$$

$$5CH_3COO^- + 8NO_3^- + 8H^+ \rightarrow 5CO_2 + 4N_2 + 5HCO_3^- + 14H_2O \quad \Delta G^0 = -951.48 \text{ kcal/mole} \quad (2)$$

where ΔG^0 = change of standard Gibbs free energy at pH 7.

The value of denitrification was considerably lower than that of methanogenesis, indicating the former was the more favorable reaction. When the C/N ratios were low, meaning a shortage of electron supply, denitrifiers would utilize all the electrons available, and only the leftovers would be utilized by methanogens (Fang and Zhou, 1999).

Using Eq. (2), the acetate/N and C/N mass ratios for complete dentrification are 2.63 and 2.86, respectively. The practical ratios should be higher than the above ratios because of the assimilation of acetate for biomass formation. Reported values for the amount of minimum COD required for denitrification vary depending on substrate and growth conditions. For denitrification with methanol as carbon sources, values ranging from 3.45 to 5.34 mg COD/mg NO_3-N have been found (Narkis *et al*., 1979; Hanaki and Polprasert, 1989; Chen and Lin, 1993). Christensson *et al*. (1994) reported that the COD required for denitrification ranged from 3.85 to 6.1 mg COD/mg NO_3-N for ethanol, but 3.6 mg COD/mg NO_3-N for acetate (Narkis *et al*., 1979).

Sudden decreases in COD removal rate corresponded to the periods of recycle pump failure and low pH caused by decrease of alkalinity at the end of the experiment (COD/NO_3-N = 15). At the same time, NH_4-N_{eff}/NH_4-N_{inf} ratio increased to 1.4. This result indicated that the activity of ammonia forming bacteria exceeded that of the denitrifier under the acidic pH range. Many researchers suggested that reduction of nitrate to nitrogen gas was the major pathway in the acetate substrate. But this result showed that ammonification as well as denitrification could occur when using acetate as a substrate.

As shown in Figure 4, effluent solids concentration increased as C/N ratios decreased. Granules became fluffy and buoyant due to the enhanced growth of denitrifiers on the surface of the methanogenic granules. This was confirmed by the change in color of the denitrifying/methanogenic granules from black to yellowish gray. It was probably also caused by the growth of denitrifiers. Other researchers also observed a similar color change (Hendriksen and Ahring, 1996).

As shown in Figure 5, the granules had layered structures. The denitrifiers could grow primarily on the granular surface with sufficient nitrate while the methanogens grew in the inner part where nitrate was deficient. The densely growing denitrifiers on the surface of the granules could remove nitrogen oxides. The removal of nitrogen oxides on the surface could induce a drop of redox potential and eliminate the inhibitory effect of nitrogen oxides on the methanogens. This sequential degradation of acetate by the denitrifiers and the methanogens would result in the substrate diffusion limitation. As a consequence of substrate diffusion limitation, the inner parts would receive a much lower substrate concentration. This phenomenon would result in the formation of hollow granules as shown in Figure 5 (c).

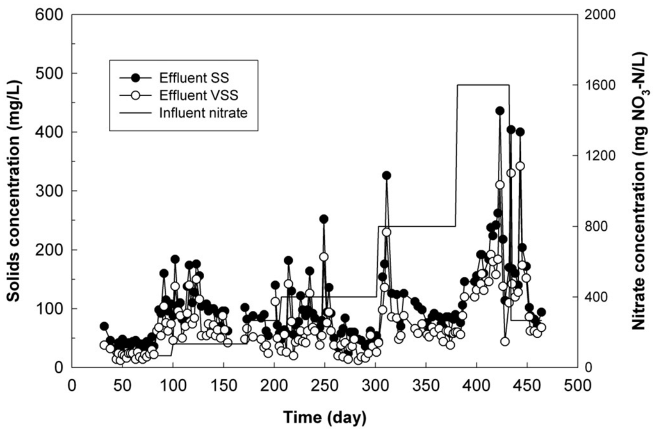

Figure 4 Solids concentration with operational time

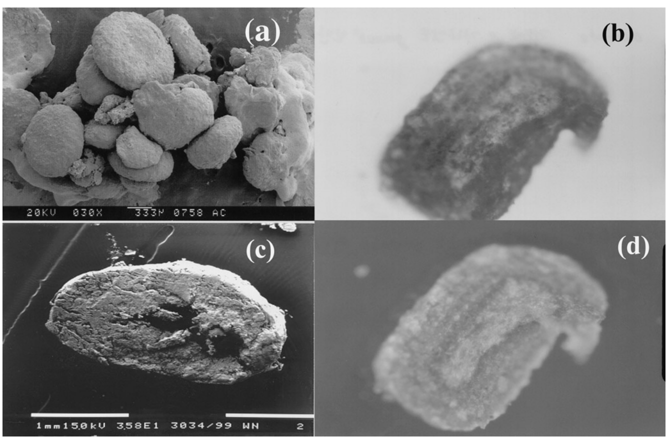

Figure 5 Microscopy of granules (a) scanning electron microscope of agglomerated granules; (b) section of granules under light microscope; (c) scanning electron microscope of hollow granule at the top of the reactor; (d) section of granules under the epi-fluorescent excitation

Competition for electrons between denitrification and methanogenesis

In the single USB reactor treating nitrate and acetate, denitrifiers and methanogens competed for electrons to produce nitrogen and methane, respectively, according to the reduction half-equations suggested by Fang and Zhou (1999). Therefore, fractions of electron flow to methanogenesis and denitrification could be estimated from the amount of denitrifier and methane produced. Figure 6 shows that electron flows to denitrification and methanogenesis were dependent on the C/N ratios. The percentage of the electron flow to denitrification increased as the C/N ratios decreased. At a C/N ratio of 2.5, all of the electron flow was assigned to the denitrification. During this period, methane gas was not produced although most of COD was removed indicating that the methanogenic bacteria were not active because most of the fed acetate was consumed for the denitrification. This result indicated that the denitrifiers became dominant over the methanogenesis in the presence of excess nitrate as mentioned previously. That is, when the C/N ratios were low, which implied a shortage of electron supply, denitrifiers would utilize all the electrons available and only the leftovers would be utilized by methanogens.

Characteristics of granules

Morphological structures of granules formed during acetate and nitrate treatment in the USB reactor were examined with SEM, and TEM microscopes. As shown in Figure 7 (d)

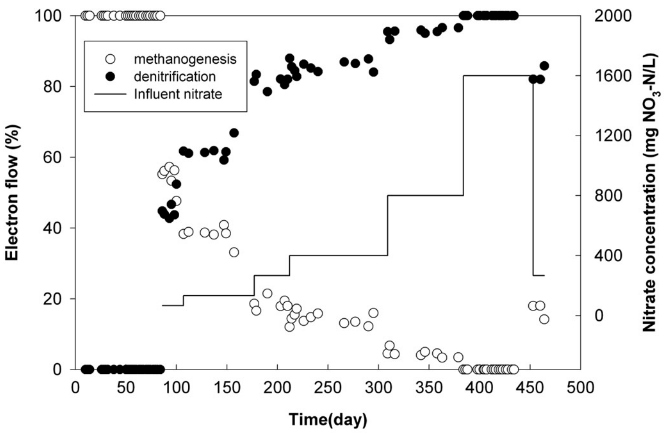

Figure 6 Percentage of electron flow by methanogenesis and denitrification

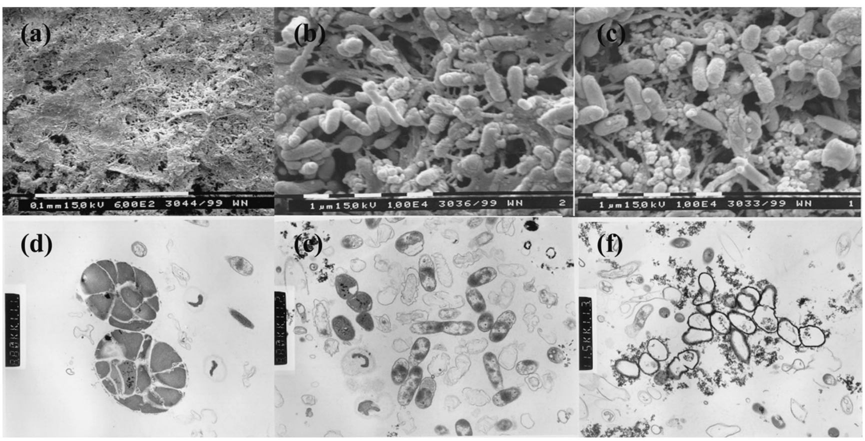

Figure 7 Microscope photographs of granules: (a, b, c) SEM of granules from the reactor; (d, e, f) TEM of granules from reactor

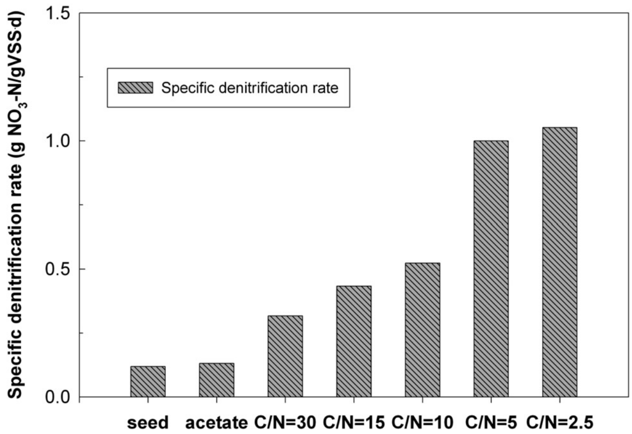

Figure 8 Specific denitrification rate with various operating conditions

and (f), highly magnified fine structured as well as roughly structured intracellular materials were observed after ruthenium-red staining. Between colonies, glycocalyx was found encapsulating the cells, morphologically resembling *Methanosarcina* sp. (Figure 7d). Bacteria attached to each other by fine structured polysaccharides covered more electron dense cell walls. The granules were colonized by a mixed population that included long rods, chain forming cocci, thin filaments, and small rods and cocci (Figure 7 a, b, c). A portion of granules showed a dense integument of several layers of rod-shaped bacteria. These bacteria had similar shapes and dimensions to *Pseudomonas* sp. Several researchers reported that *Pseudomonas* sp. were some of the bacteria with high denitrifying capabilities (Akunna *et al*., 1992; Payne, 1981).

The SDR increased as microorganisms acclimated to the nitrate as shown in Figure 8. Maximum SDR with acetate could reach 1.05 g NO_3-N/gVSS·d, which was compared to the reported values of 0.96 g NO_3-N/gVS·d with methanol (Henze, 1991).

Conclusions

- Simultaneous denitrification and methanogenesis could be achieved in a single USB reactor. Unremoved carbon in denitrification was utilized by methanogenesis. More than 99% and 95% of nitrate and COD removal rate were obtained at a loading of 600 mg NO_3-N/L·d and 3,300 mg COD/L·d, respectively.
- The characteristics of granules were changed with decreasing C/N ratios. The specific denitrification rate increased as C/N ratios decreased. Maximum SDR with acetate could reach 1.05 g NO_3-N/gVSS·d. In particular, significant sludge flotation was observed at the top of the reactor due to the change of microbial composition. Granules became fluffy and buoyant due to the growth of denitrifiers. The recirculation could be the alternative for solving the excess sludge flotation problem.
- The surface of granules was colonized by a mixed population including long rods, chain forming cocci, thin filaments, and small rods and cocci. Granules were mainly composed of microcolonies of *Methanosarcina* sp., *Pseudomonas* sp. with high denitrifying capabilities and rod-shaped bacteria.
- An integrated denitrifying/methanogenic process had several advantages over traditional nitrification-denitrification processes such as low external carbon requirement and low space requirement. Furthermore, it could increase the life of the anaerobic process treating leachate and manure which contain high concentrations of organic matter and ammonia nitrogen.

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