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Denitrification Characteristics and Microorganism Composition of Acclimated Denitrifier Consortium

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Abstract The effect of the COD/N ratio on denitrification characteristics was evaluated for the development of a denitrification process. Activated sludge, acclimated to an anoxic condition, was used as the denitrifier consortium (mixture of denitrifying organisms) for enhanced nitrogen removal in a recirculating aquarium system. Synthetic wastewater containing nitrate was used as the influent solution and glucose was used as the carbon source for denitrification. The COD/N ratio varied within a range of 1.5-7.2. The denitrification efficiency was higher than 97% even at a COD/N ratio of 1.5. Under a theoretical COD/N ratio of 3.0, nitrite was detected, however, the amount was less than 1% of the total influent nitrogen. The number of both nitrate-reducing bacteria and denitrifying bacteria reached 3.5×10^5/ml with a COD/N ratio of 1.5 after 45 days of operation.

Key words: Denitrification, acclimation, COD/N ratio, denitrifier consortium

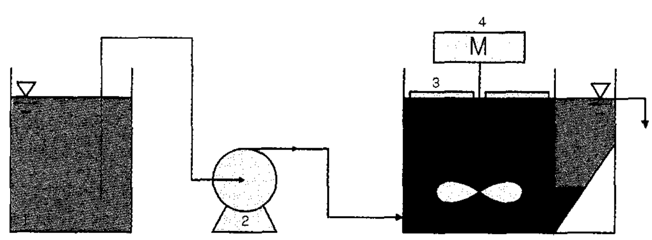
Nitrate is the end product of biological nitrification and accumulates unless a denitrification or hydroponic system is connected to the water treatment process [10]. Although nitrate is not generally regarded as an acutely toxic compound to aquatic organisms [3, 22], it should not be left to accumulate because phytoplankton blooms, the inhibition of nitrification, and toxicity problems will eventually occur at certain nitrate concentrations. Many processes for denitrification have been in operation worldwide or under development [4, 12, 18, 21], and the organic materials in wastewater and methanol as external carbon sources for the denitrification were used in these processes. Based on stoichiometric parameters of denitrifiers [16], the theoretical amount of COD needed for the removal of nitrate (COD/N ratio) was proposed to be 3.42 g COD per g nitrate nitrogen, and the COD/N ratio was reported to be maintained at 6.5 as an optimum value for the activated sludge process [9].

However, the COD/N ratio in the fish aquarium ranges between 1 and 10 depending on the water exchange rate, and denitrification is difficult to take place because organic carbon sources are present in smaller amounts than the values mentioned above [9, 16]. Addition of organic materials like methanol and glucose is required, and chemicals have to be consumed for complete denitrification. Since the current water treatment system of fish aquariums consists of a sand filter or sponge filter, desirable removal of nitrogen compounds cannot be expected.

Therefore, as a preliminary study for the development of a compact, cost-effective, and efficient recirculating fish aquarium system, the denitrification characteristics were investigated for a wide range of COD/N ratios, and the microorganism composition at an extreme condition was evaluated to determine the degree of acclimation of activated sludge.

Denitrification Reactor

The system consisted of a denitrification tank and sedimentation tank, as shown in Fig. 1, and the working volumes of each tank were 35 l and 10 l, respectively. Plates to prevent any surface aeration were installed on the top of the denitrification tank.



1. Influent tank
2. Peristaltic pump
3. Prevention plate of surface aeration
4. Agitator
5. Denitrification tank
6. Sedimentation tank

Fig. 1. Schematic diagram of a denitrification reactor.

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Denitrifier Consortium

Activated sludge obtained from the Jang Lim sewage treatment facility (Pusan, Korea) was acclimated to the denitrifier consortium by feeding nitrate and glucose. The activated sludge had a total solid (TS) content of 3.8 % (w/v) and 1 l of the acclimated sludge was added as an inoculum. The final concentration of the total suspended solids (TSS) was approximately 1,000 mg/l.

Operation of Reactor

The reactors were operated in a continuous mode and in an anoxic condition at room temperature. The hydraulic retention time (HRT) was 1 day. The COD/N ratio was reduced stepwise from 7.2 (corresponding to a loading rate of 95.5 g COD/m^3/day, 13.2 g NO_3^--N/m^3/day), to 4.8 (94.9 g COD/m^3/day, 20.5 g NO_3^--N/m^3/day), then to 2.8 (93.5 g COD/m^3/day, 33.5 g NO_3^--N/m^3/day), and finally to 1.5 (94.8 g COD/m^3/day, 63.5 g NO_3^--N/m^3/day).

The composition of the synthetic recirculating water used as the feed solution was glucose, KNO_3, Na_2HPO_4, and $MnSO_4$. Glucose was used as an electron donor (organic carbon source) [6, 15, 20]. The amount of each material was varied as the COD/N ratio changed. Glucose was maintained at 100 mg/l. In proportion to the COD/N ratios of 7.2, 4.8, 2.8, and 1.5, all the constituents except for glucose were increased as follows: KNO_3 to 10, 20, 30, and 60 mg NO_3^--N/L; Na_2HPO_4 to 40, 80, 120, and 240 mg/l; and $MnSO_4$ to 2, 4, 6, and 12 mg/l, respectively.

After filtering the samples through 1-μm glass microfibre filters, all chemical analyses were conducted according to Standard Methods [1]. The dissolved oxygen and pH were measured by a DO meter (YSI 550, Ohio, U.S.A.) and pH meter (Mettler Toledo MP 220, Schwerzenbach, Switzerland), respectively.

Acclimation of Activated Sludge to Denitrifier Consortium

During acclimation of the sludge to the denitrifier consortium, the nitrate loading and removal rates, the nitrite concentration, and the COD/N ratio were determined as shown in Fig. 2. In the early stage of the start-up period, when the initial COD/N ratio was 2.54 and the DO concentration was 1.4 g/m^3, the system was unstable and no nitrate was removed at all. After the COD/N ratio was increased to 3, the system became stable and the nitrate removal rate reached to 7.5 g/m^3/day and the denitrification efficiency increased by 55%. The intermediate product resulting from denitrification, nitrite, built up to about 1.0 g/m^3, however, it gradually decreased to below 0.1 g/m^3 after 15 days of operation. The nitrite build-up could have occurred due to a limitation of organic carbon and deficiency of nitrite reductase activity [20]. The nitrite concentration decreased to 0 after 15 days of operation. The initial low nitrite reductase activity may have been the reason for the nitrite detection during the start-up period. As the COD/N ratio was increased to

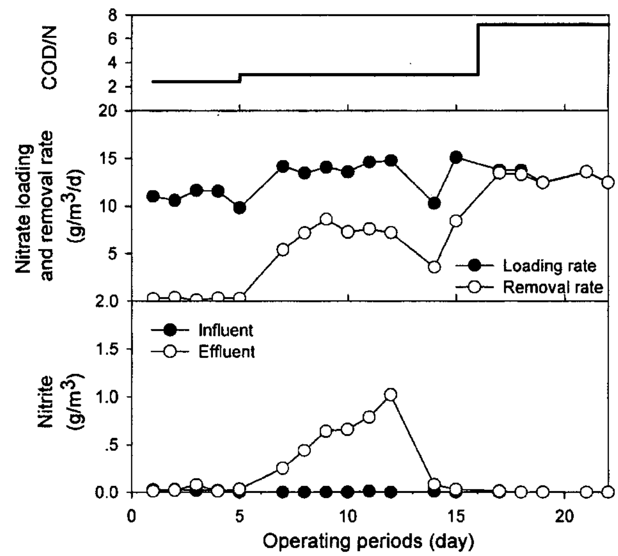

Fig. 2. Characteristics of nitrate reduction and denitrification activity during initial acclimation of activated sludge to the denitrifier consortium.

COD/N ratio was increased to 7.2 to achieve a 95% nitrate removal efficiency.

7.2, the nitrate removal rate increased to 13.2 g/m^3/day and the denitrification efficiency became almost 100%. These results are in good agreement with Jung *et al.*'s report [9] which showed that, with activated sludge, the denitrification efficiency was higher than 95% and the process was stable with ratios of COD/N higher than 6.5.

Effect of COD/N Ratio

When the denitrification system reached a steady state, the COD/N ratio was reduced by the addition of nitrate. The operational COD/N ratio was reduced stepwise from 7.2 to 1.5 as the loading rates of nitrate nitrogen correspondingly increased. The range of the loading rate for nitrate nitrogen was 13.2 to 63.5 g NO_3^--N/m^3/day. Figure 3 shows the nitrate loading and removal rates, the nitrite concentration, and the COD/N ratio during 90 days of operation. As the COD/N ratio decreased, the denitrification efficiency also decreased slightly. However, even at a COD/N ratio of 1.5, which was a low value compared to previous studies [9, 13], the denitrification efficiency was higher than 97%.

When glucose is used as the organic carbon source, the stoichiometric expression is:

$$C_6H_{12}O_6+2.8NO_3^-+0.5NH_4^++2.3H^+ \rightarrow 0.5C_5H_7O_2N+1.4N_2+3.5CO_2+6.4H_2O \quad \text{(Eq. 1)}$$

Here, the requirement of glucose for denitrification is 3.9 g glucose per g nitrogen and the stoichiometric COD/N ratio is 4.2 [14]. In this study, even at a lower COD than the stoichiometric value for the removal of nitrate, a high efficiency of nitrate removal was achieved. Carbon sources for denitrification could be obtained from the external energy

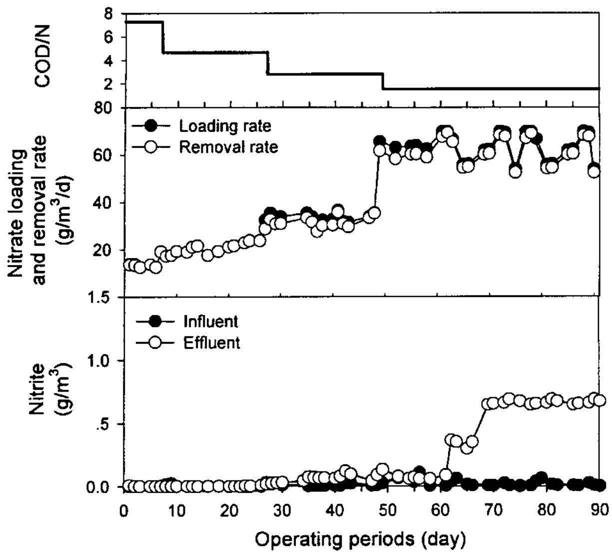

Fig. 3. Characteristics of nitrate removal and nitrite accumulation relative to COD/N ratio.

COD/N ratio was reduced stepwise to 1.5 and then the nitrite concentration in the influent and effluent was determined.

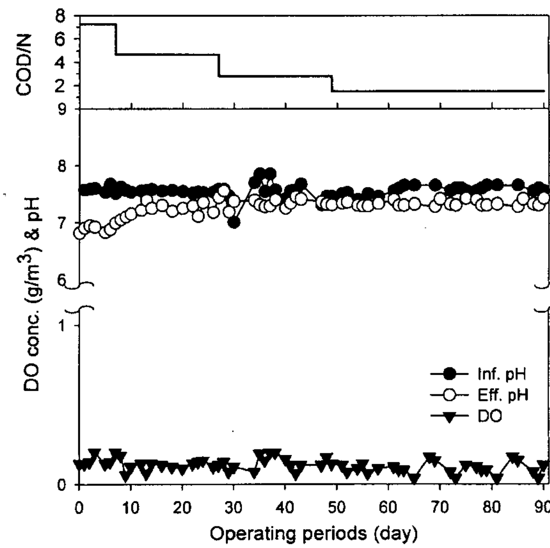

Fig. 4. pH and DO profile of the denitrification process relative to COD/N ratio variation.

sources like glucose, methanol, and organic materials in wastewater and in the cell mass of the sludge, the so-called internal energy source [7]. Burdick *et al.* [2] proposed the following experimental equation for the denitrification reaction:

$$q_D=0.03\ (F/M)+0.029 \qquad \text{(Eq. 2)}$$

Here, q_D is the specific denitrification rate (mg NO_3^--N/g MLSS/day), F/M is the food to mass ratio (g BOD/g MLSS/day), 0.03 is the coefficient for the denitrification by methanol, and 0.029 is the constant for denitrification by endogenous respiration. The following experimental equation was obtained in this study:

$$q_D=0.0357\ (F/M)+0.0479 \qquad \text{(Eq. 3)}$$

In Eq. 2 and Eq. 3, the coefficient for denitrification by external energy sources (glucose), 0.0357, was similar to the 0.03, the result of Burdick *et al.* [2] but the constant for the denitrification by endogenous respiration was higher than the result of Burdick *et al.* [2]. This indicates that the organic materials in the cell mass could be used as the internal energy sources under glucose-limitation conditions.

According to Wickins [23], nitrite toxicity is more severe than ammonia toxicity in an intensive fish culture facility, especially in a recirculation culturing system. Nitrite can accumulate in a recirculating system as a result of incomplete bacterial reduction. The intermediate product resulting from denitrification, nitrite, is toxic and undesirable. It also inhibits the growth of microoganisms [7]. Under the COD/N ratio of 2.8, nitrite was detected. With COD/N ratios of 2.8 and 1.5, the nitrite concentrations were 0.1 g/m^3 and 0.63 g/m^3, respectively. With a COD/N ratio of 2.8, the nitrite concentration was under the standard aquacultural criteria of 0.1 g/m^3 [8]. With a COD/N ratio of 1.5, the nitrite concentration was higher than the maximum criteria of 0.55 g/m^3 [11], however, the amount was less than 1% of the total influent nitrogen which could be reduced if the denitrification and nitrification processes were connected. This indicates that the limitation of organic carbon would appear to be the reason for the nitrite accumulation due to the lower energy input for the full extent of activation of the denitrification process.

Figure 4 shows the dissolved oxygen (DO) concentration in the denitrification tank and the pH of the influent and effluent. The DO concentration was less than 0.2 mg/l, and the influent and effluent pHs were within a range of 6.7-7.6. According to Surampalli [18], DO concentrations greater than 1 mg/l inhibit denitrification, and the rate of denitrification is reduced at pH below 6.0 or above 9.0. The optimum pH is within a range of 6.5-8.0. This indicates that the denitrification process in this study was within the optimum range. Furthermore, the effluent pH was lower than the influent pH in contrast to the results with methyl alcohol and acetates. Fermentation reactions may have been responsible for the observed low pH level, suggesting the presence of microorganisms in the biocommunity capable of performing both fermentation and nitrate reduction. This fact was supported by observations made by Tomozawa *et al.* [20], Ermel [5], and Simpkin and Boyle [17].

Microorganism Composition

With a COD/N ratio of 1.5, the composition of the microorganisms in the denitrification tank was determined by the most probable number (MPN) and plate counter methods. The initial medium color (pH 7.0- 7.2) before

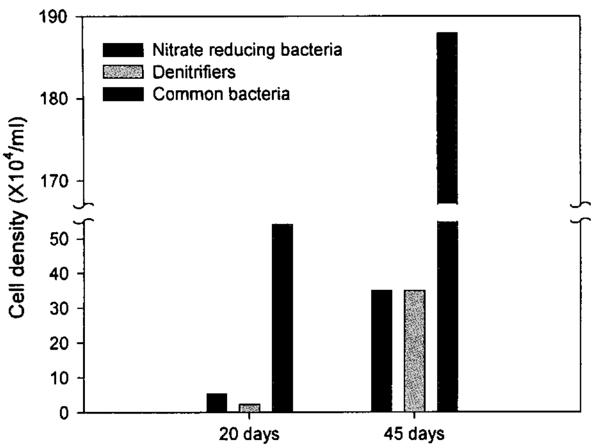

Fig. 5. Microorganism composition at a COD/N ratio of 1.5.

the nitrate was reduced was green due to the BTB (Bromotimolblue) indicator. The color changed to dark blue in the alkali solution as the denitrification progressed. The change of color was monitored, and the nitrogen gas produced was collected in a tube. The change to a dark blue color indicated that the nitrate-reducing bacteria had grown, and the color change and gas production indicated that the denitrifiers had grown. The number of nitrate-reducing bacteria, denitrifiers, and common bacteria were determined after incubation at 30°C for 2 weeks and 10 days, respectively.

As shown in Fig. 5, the cell densities for the nitrate-reducing bacteria, denitrifiers, and common bacteria, were determined to be 5.4×10^4/ml, 2.4×10^4/ml, and 5.4×10^5/ml after 20 days of reactor operation, and 3.5×10^5/ml, 3.5×10^5/ml, and 1.88×10^6/ml after 45 days of reactor operation, respectively.

According to Thauer [19], the pathway of denitrification is:

$$NO_3^- + H_2 \xrightarrow{\text{Nitrate reducing bacteria}} NO_2^- + H_2O + 39\ \text{kcal} \qquad \text{(Eq. 4)}$$

$$2NO_2^- + 3H_2 + 2H^+ \xrightarrow{\text{Denitrifiers}} N_2 + 4H_2O + 190\ \text{kcal} \qquad \text{(Eq. 5)}$$

When glucose is used as the organic carbon source in the denitrification process by activated sludge, fermentative conditions increase the cell density of facultative anaerobic bacteria, which can reduce nitrate to nitrite [24]. This might increase the nitrite concentration in the early period after decreasing the COD/N ratio to 1.5. However, in this study, the cell densities of nitrate-reducing bacteria and denitrifiers became almost the same and the nitrite concentration was maintained at 0.63 g/m^3 after 15 days of adjusting the COD/N ratio to 1.5.

Therefore, in this study, even at a COD/N ratio of 1.5, which was low compared to previous studies on the denitrification [9, 13] carried out at the COD/N ratio higher than 3.0, the denitrification efficiency could be maintained at 97%. This could beneficially contribute to developing the fish aquarium for the raw fish restaurants, which have a wide range of COD/N ratios. Even though glucose was used as the carbon source, the densities of nitrate reducing bacteria and denitrifier became the same at the COD/N ratio of 1.5 and the effective denitrification was carried out without accumulation of nitrite.

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REFERENCES

1. APHA. 1995. Standard methods for the examination of water and wastewater. 19th ed. American Public Health Administration. Washington DC, U.S.A.
2. Burdick, C. R., D. R. Refling, and H. D. Stensel. 1982. Advanced biological treatment to achieve nutrient removal. *J. Water Pollut. Control Fed.* **54:** 1078-1086.
3. Colt, J. and G. Tchobanoglous. 1976. Evaluation of the short-term toxicity of nitrogenous compounds to channel catfish, *Ictalurus punctatus. Aquaculture* **8:** 209-24.
4. Constantin, H. and M. Fick. 1997. Influence of C-sources on the denitification rate of a high-nitrate concentrated industrial wastewater. *Water Res.* **31:** 583-589.
5. Ermel, G. 1982. Stickstoffentfernung in einstufigen Belebungsanlagen: Steuerung der Denitrifikation. Diss. Universität Braunschweig, Germany.
6. Hendriksen, H. V. and B. K. Ahring. 1996. Integated removal of nitrate and carbon in an upflow anaerobic sludge blanket (UASB) reactor; Operating performance. *Water Res.* **30:** 1451-1458.
7. Henze, M., P. Harremoes, Jes la Cour Jansen, E. Arvin. 1995. *Wastewater Treatment Biological and Chemical Process.* pp. 55-111. Springer-Verlag. Berlin. Germany.
8. Jo, J. Y. and B. H. Lee. 1996. *Aquacultural Engineering.* pp. 71-94. 1st ed. Research Center for Ocean Industrial Development. Pukyong National University. Jung Myoung Dang Press. Pusan. Korea.
9. Jung, K. H., J. H. Shon, J. B. Park, and Y. H. Lee. 1998. Nitrogen removal of municipal wastewater with 2-stage activated sludge system at low temperature. *Kor. J. Water Qual.* **14:** 29-35.
10. Kim, N. S. and M. Y. Park. 1993. Removal of inorganic nitrogen and phosphorus from Cow's liquid manure by batch algal culture. *J. Microbiol. Biotechnol.* **3:** 214-217.
11. Klontz, G. W., P. C. Downey, and R. L. Focht. 1979. *A Manual for Trout and Salmon Production.* Stering H. Nelson and Sons. Murray. Utah, U.S.A.

12. Kuroda, M., T. Watanabe, and Y. Umedo. 1996. Simultanuous oxidation and reduction treatments of polluted water by bio-electro reactor. *Water Sci. Technol.* **34:** 101- 108.
13. Lim, S. I. 1996. Recycling water treatment in aquaculture system by using fluidized bed process. Master Thesis. Pukyong National University. Pusan, Korea.
14. Montheith, H. D. 1980. Industrial waste carbon source for biological denitrification. *Prog. Water Technol.* **12:** 127- 141.
15. Piyapawn, S. 1995. Interaction between nitrate reducing bacteria and methane forming bacteria in anaerobic reactors. Master thesis. Asian Institute of Technology. Bangkok, Thailand.
16. Sedlak, R. I. 1991. *Phosphorous and Nitrogen from Municipal Wastewater.* p. 19. 2nd ed. Lewis publisher. London, U.K.
17. Simpkin, T. J. and W. C. Boyle. 1985. The regulation of the synthesis of the denitrifying enzymes in activated sludge. *Proc. 40th Indust. Waste Conf. Purdue University,* U.S.A.
18. Surampalli, R. Y., R. D. Tyagi, O. Scheible, and J. A. Heidman. 1997. Nitrification, denitrification and phosphorus removal in sequential batch reactors. *Bioresour. Technol.* **61:** 151- 157.
19. Thauer, R. K., K. Jungermann, and K. Decker. 1977. Energy conservation in chemotrophic anaerobic bacteria. *Bacteriol. Rev.* **41:** 100- 180.
20. Tomozawa, T., Y. Saito, and T. Hoaki. 1989. Characteristics of denitrification process using USB methods. *J. Water Waste* **31:** 35- 41.
21. van Benthum, W. A. J., J. M. Garrido, J. P. M. Mathijssen, J. Sunde, M. C. M. van Loosdrecht, and J. J. Heijnen. 1998. Nitrogen removal in intermittently aerated biofilm airlift reactor. *J. Environ. Eng.* **124:** 239- 248.
22. Westin, D. T. 1974. Nitrate and nitrite toxicity to salmonid fishes. *Prog. Fish. Cult.* **36:** 86- 89.
23. Wickins, J. F. 1981. Water quality requirements for intensive aquaulture: A review. pp. 17- 37. *In* Tiews, K. (ed.), *Proc. World. Symp. on Aquaculture in Heated Effluents and Recirculation Systems.* Vol. **1**. Berlin.
24. Wilderer, P. A., W. L. Jones, and U. Dau. 1987. Competition in denitrification systems affecting reduction rate and accumulation of nitrite. *Water Res.* **21:** 239- 245.