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Analysis of the Activated Sludge of a Municipal WWTP by Several Bio-Parameters

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The activated sludge from the aeration basin of the Su-yeong municipal wastewater treatment plant which has operated by a standard activated sludge process in Busan, Korea was investigated during April 2004 and January 2005 with several bio-indicators. The number of bacteria and fungi per gram of dry weight of MLSS were estimated to be 3.1×10^{6}~1.5×10^{8} and 1.1×10^{3}~1.1×10^{5} colony forming units, respectively, by the plate agar method. By cultivation-independent methods, such as 4',6-diamidino-2-phenylindole stain and fluorescence in situ hybridization, the ratio of eubacteria to the entire biomass was evaluated by more than 80% (v/v). The ratio of ammonia-oxidizing bacteria and nitrite-oxidizing bacteria to the total eubacteria was determined to be 7.0~9.8% and 3.3~6.2% without heavy variation in spite of a period of relatively low temperature in the basin. It would be expected that the nitrification would occur or at least co-exist throughout the year in the sludge of many municipal WWTP with influents that contain the sufficient nitrogen sources although the WWTP does not have any specialized processes for the removal of nitrogen.

Key Words : Activated sludge, Wastewater treatment plant (WWTP), Cultivation independent method

1. Introduction

Wastewater is a primary source of pollution of the natural water environment. Most modern municipal wastewater treatment plants (WWTP)s have one or more biological processes for the removal of pollutants from wastewater[1]. Because the biological processes are known to be the most economical and safe processes, they are used not only in classical activated sludge processes for removing organic materials from wastewater but also in the advanced treatment processes for removing nutrients, such as nitrogen or phosphorus[2~6]. Although numerous studies have been conducted on the ecology and microbiology of sludge, little is known about the sludge. The aims of this research were to investigate the monthly variation in the numbers of microorganisms in conventional activated sludge and the dynamics of the ratio of ammonia oxidizing- and nitrite-oxidizing bacteria in sludge from a WWTP. The investigation involved the following two categories of physico-chemical tests, such as the sludge volume index (SVI) and mixed liquid suspended solids (MLSS) as well as a biological tests such as the classical cultivation method[7] for detecting the dynamics of heterotrophic bacteria numbers and a cultivation independent method for identifying ammonia- and nitrite-oxidizing bacteria groups[8].

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2. Material and Methods

2.1. Sampling

Samples of mixed liquid from the activated basin of the S municipal WWTP were collected once per month between April 2004 and January 2005. The samples were immediately transported to the laboratory after sampling and physico-chemical factors and biological factors were examined.

2.2. Physico-chemical factors

The pH and temperature of the mixed liquid in the aeration basin were measured directly at the site with a pH probe. The sludge volume $(SV)_{30}$ devotes the percent value of the volume of which one liter of mixed liquid is settled during 30 minutes in an Imhoff cone cylinder. The MLSS measurement was measured

based on Standard Methods[9]. The SVI (sludge volume index) was calculated using the formula below.

$$SVI = SV_{30}\ (\%) / MLSS\ (mg/L)] \times 10{,}000$$

2.3. Biological test by classical cultivation method

For the biological test by the classical cultivation method, we counted the numbers of general bacteria and fungi per g of dry weight of MLSS by the standard plate method in triplicate experiments. Plate count agar (Merck) was used for counting the numbers of general aerobic and facultative anaerobic heterotrophic bacteria. Potato dextrose agar (Merck) was used for fungi. The pH of the PDA was adjusted 3.6±0.1 with 10% (w/v) tartaric acid[7] and 30 μg/ml chloramphenicol was added to repress bacterial growth. Bacteria were cultivated at 37°C for 48 h and fungi were cultivated 30°C for 48 h.

2.4. Community structure by cultivation independent method

It is well known that the cultivation and quantification of ammonia- and nitrite-oxidizing bacteria are difficult and time- and labor-consuming[6]. For that reason, we examined the dynamics of ammonia- and nitrite-oxidizing bacteria in the sludge by the fluorescence *in situ* hybridization (FISH) technique with their bacteria-specific 16S rDNA-targeted probes (Table 1)[10~13]. For the in situ hybridization, after washing the sludge samples three times with two volumes of phosphate-buffered saline (PBS, pH 7.2), they were immediately fixed with 4% (w/v) paraformaldehyde in PBS for 3 h at 4°C. The samples were subsequently washed twice with PBS, and add to 1 μl on silane coated slide glass. Then, they were dehydrated with series of 50, 80, and 100% (v/v) ethanol solutions. The dried samples were used in the FISH experiments. FISH with 16 rRNA targeted probes was done on slides according to modified protocols[14]. The samples were hybridized with 9 μl of hybridization solution containing 50 ng of probe and then incubated for 90 min at 46°C in an equilibrated humid chamber. The probes were removed from the slides with washing solution and the slides were immersed in 50 ml of a 48°C pre-warmed washing solution and incubated for 30 min. After *in situ* hybridization with the EUB probe, the sludge was stained with 4',6-diamidino-2-phenylindole (DAPI, 0.33 μg/ml in H_2O) for 5 min. The fluorescent-labeled probes used in this research are listed in Table 2[15]. The probes were labeled at their 5'-ends with either Cy 5 (EUB, Nso190) or rhodamin green (EUB, Nb1000). For dual staining, a ammonia- or nitrite- oxidizing bacteria-specific probe was employed for the first hybridization and, after washing, followed by a second hybridization with the EUB probe[14,16]. Images were obtained by epifluorescence microscopy (Axioskop 2 plus, Zeiss, Germany) using a Neoflur lens. Quantification of probe-targeted bacteria to the area of all bacteria was determined by an area-based method from ten images[11,17]. The gained values were averaged with the other data, after discarding the highest and lowest values.

2.5. Statistical Analysis

We used the single linear regression analysis program to determine the correlation coefficient between temperature in the basin and other parameters, such as the number of bacteria and fungi per g MLSS of dry weight and the ratio of ammonia- and nitrite- oxidizing bacteria to entire bacteria. The correlation could be divided into 3 parts: strong correlation ($| r | \geq$

Table 2. Physico-chemical conditions of the aeration basin on the sampling day

Month	Temp. (°C)	pH	MLSS (mg/L)	SVI
Apr.[04]	18.3±0.1	6.6±0.1	1,600±60	301-324
May	21.2±0.1	6.4±0.1	1,250±0	200
Jun.	22.8±0.1	6.5±0.1	1,200±50	152-168
Jul.	27.3±0.1	6.6±0.1	1,120±20	351-364
Aug.	24.7±0.1	6.7±0.1	1,040±10	167-170
Sep	22.3±0.1	6.7±0.1	1,050±30	241-254
Oct.	19.6±0.1	6.8±0.1	1,500±60	353-382
Nov.	15.7±0.1	7.0±0.1	2,720±0	276
Dec.	15.1±0.1	6.6±0.1	2,000±0	325
Jan.[05]	9.6±0.1	6.9±0.1	2,100±60	241-255

Table 1. The rRNA targeted oligonucleotide probes for FISH

Probe name	Nucleotide sequence (5'?3')	Target position[a]	Specificity
EUB 338	GCTGCCTCCCGTAGGAGT	16S, 338-355	All bacteria
Nso 190	CGATCCCCTGCTTTTCTCC	16S, 190-208	Ammonia-oxidizers of β-subclass of proteobacter
Nb 1000	TGCGACCGGTCATGG	16S, 1000-1014	*Nitrobacter* spp. of α-subclass of proteobacter

[a]16S rRNA position according to *Escherichia coli* numbering

0.75), intermediate correlation ($0.25 < |r| < 0.75$) and weak correlation ($|r| \leq 0.25$)[18].

3. Results and Discussion

By the 2003 year data reported from the S WWTP, the WWTP was operated under a year-average of BOD 95.8 mg/L, total-nitrogen of 26.2 mg/L in the influent, and a year-average of BOD 8.5 mg/L and total-nitrogen of 14.5 mg/L in the effluent.

The mixed liquid in the aeration basin on the sampling day was in the temperature range of 9.6~27.3°C, pH range of 6.4~7.0, MLSS range of 1,040~2,720 mg/L and the SV_{30} range of 17.5~75% (Table 2). The numbers of microbes contained in the mixed liquid were 3.1×10^6~1.5×10^8 colony-forming units (CFU) bacteria per g of dry weight of MLSS and 1.1×10^3 ~1.1×10^5 CFU fungi per g dry weight of MLSS (Fig. 1). The number of bacterial cells per g dry MLSS was higher when the temperature in the basin was high than when the temperature in the basin was low. This suggests that the bacterial activities for degrading organic materials were controlled, not only by the concentration of MLSS which is one of main parameters in operating general municipal WWTPs but also by the number of viable bacteria per g of dry MLSS. The number of bacteria observed in our results is agreed well with the data reported by Kampfer[12]. Concerning that bacterial cells per g dry MLSS, they were more when the temperature in the basin was high than when the temperature in the basin was low. This suggests that the bacterial activities for degrading organic materials were controlled, not only by the concentration of MLSS which is one of main parameters in operating but also by the number of viable bacteria per g of dry MLSS. The number of bacteria was approximately 10^2~10^4 times higher than that for fungi. It thus appears that the main degraders of organic materials in wastewater are heterotrophic bacteria, and not fungi as shown in Fig. 1. The SVI was even higher than the generally recommended value (≤150) of managing activated sludge in municipal WWTPs[19]. However, no problem was noted in the quality of the effluent. This indicates that activated sludge is tolerant and doesn't pose a problem with respect to poor settle-ability with high SVI values in operating plants.

The proportion of eubacteria in the complete biomass was more than 80%, indicating that the sludge was largely composed of eubacteria with less amounts of eucaryotic cells. The average ratio of ammonia-oxidizing bacteria and nitrite-oxidizing bacteria were 7.0~9.8% and 3.3~6.2% of the entire eubacteria population, respectively (Fig. 2). These results are in agreement with the results of Daims *et al* that ammonia-oxidizers occupy a higher percentage of the total bacteria than nitrite-oxidizers[11]. The ratio of ammonia-oxidizing bacteria to eubacteria was higher at a high temperature than at a low temperature of the basin, and nitrite-oxidizing bacteria showed a different tendency than the ammonia-oxidizing bacteria group as shown in Fig. 2. However, the ratio to eubacteria remained relatively constant, in spite of a period of relatively low temperature in the basin. It would expected that

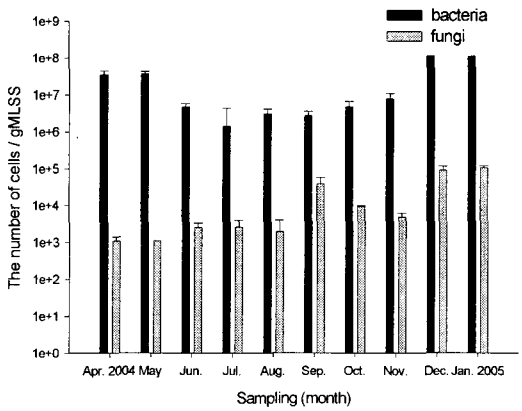

Fig. 1. The distribution of the number of bacteria and fungi per g of dry weight of MLSS on each month.

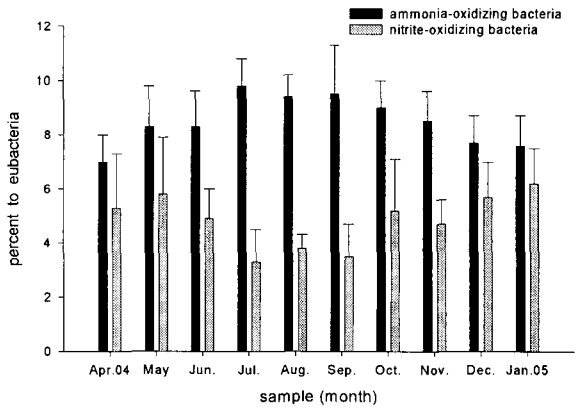

Fig. 2. The distribution of the ammonia- and nitrite-oxidizing bacteria group to eubacteria of S municipal WWTP Sludge.

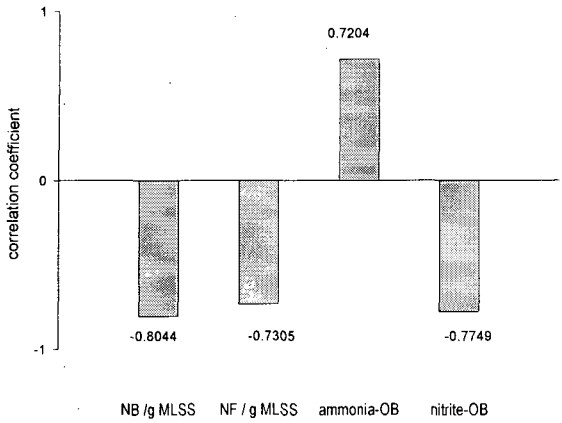

Fig. 3. The correlation coefficient values for several biological indexes to water temperature.
NB : numbers of bacterial cells, NF : numbers of fungal cells, OB : oxidizing bacteria

nitrification would occur or at least co-exist throughout the year in the sludge of many municipal WWTP with influents that contain sufficient nitrogen sources. Therefore, the sludge itself could be a candidate as a good inoculum for developing WWTP that would efficiently support nitrification. By a simple correlation analysis, the number of bacteria and fungi per g of dry weight of MLSS and nitrite-oxidizing bacteria showed a negative correlation coefficient while ammonia-oxidizing bacteria displayed a positive correlation coefficient with the temperatures of the basin (Fig. 3). These results can be explained that the activities of ammonia-oxidizing bacteria were higher at high temperatures than at low temperature in the basin whereas the activities of others including them of nitrite-oxidizing bacteria were lower at high temperature than at low temperature in the basin.

4. Conclusion

From the biological investigation of sludge of a municipal WWTP operating by standard activated sludge process, we gained following results. The numbers of microbes contained in the mixed liquid were 3.1×10^{6} ~1.5×10^{8} colony-forming units (CFU) bacteria per g of dry weight of MLSS and 1.1×10^{3}~1.1×10^{5} CFU fungi per g dry weight of MLSS. The proportion of eubacteria in the complete biomass was more than 80%, indicating that the sludge was largely composed of eubacteria with less amounts of eucaryotic cells. The average ratio of ammonia-oxidizing bacteria and nitrite-oxidizing bacteria were 7.0~9.8% and 3.3~6.2% of the entire eubacteria population, respectively. The ratio of ammonia-oxidizing bacteria to eubacteria was higher at high temperature than at low temperature of the basin, and nitrite-oxidizing bacteria showed a different tendency than the ammonia-oxidizing bacteria group. By a simple correlation analysis, the number of bacteria and fungi per g of dry weight of MLSS and nitrite-oxidizing bacteria showed a negative correlation coefficient while ammonia-oxidizing bacteria displayed a positive correlation coefficient with the temperatures of the basin.

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References

1) Dignae, M. F., P. Glnestet, D. Rybacki, A. Bruchet, V. Urbain and P. Scribe, 2000, Fate of wastewater organic pollution duringactivated sludge treatment: nature of residual organic matter, Wat. Res., 34, 4185-4194.
2) Bond, P. L., P. Hugenoltz, J. Keller and L. L. Blackall, 1995, Bacterial community structures of phosphate-removing and non-phosphate-removing activated sludges from sequencing batch reactors, Appl. Environ. Microbiol., 61, 1910-1916.
3) Cervantes, F. J., D. A. Rosa and J. Gemez, 2001, Nitrogen removal from wastewater at low C/N ratios with ammonium and acetate as electron donors, Bioresource Technol., 79, 165-170.
4) Mitsuyo, H., M. Kamamoto, M. Yani and M. Shoda, 2001, Comparison of the biological NH3 removal characteristics among four inorganic packing materials, J. Biosci. Biotechnol., 91, 428-430.
5) Yoshiteru, A. T. M., T. Okamoto, S. Tsuneda, A. Hirata, A. Kitayama and T. Nagamune, 2000, Microbial ecology of nitrifying bacteria in wastewater treatment process examined by fluorescence in situ hyridization, J. Biosci. Bioeng., 90, 234-240.
6) Ulrike, P., A. Pommerening-Roser, S. Juretschko, M. C. Schmid, H. P. Koops and M. Wagner, 2000, Phylogeny of all recognized species of ammonia

oxidizers based on comparative 16S rRNA and amoA sequence analysis: implications for molecular diversity surveys, Appl. Environ. Microbiol., 66, 5368-5382.

7) Laborotaries, D., 1984, Difco Manual 10th (ed), Difco, U.S.A., 690pp.
8) Egli, K., A. J. B. Zehnder, M. Wagner and J. R. Meer, 2003, Community analysis of ammonia and nitrite oxidizer during start-up of nitrification reactors, Appl. Environ. Microbiol., 69, 3213-3222.
9) APHA, AWWA, WPCF, 1992, Standards methods for the examination of water and wastewater, 8th ed. American Public Health Association, Washington D.C., U.S.A.
10) Amann, R. and W. Ludwig, 2000, Ribosomal RNA-targeted nucleic acid probes for studies in microbial ecology, FEMS Microbiol. Rev., 24, 555-565.
11) Daims, H., U. Purkhold, L. Bjerrum, E. Arnold, P. A. Wilderer, and M. Wagner, 2001, Nitrification in sequencing biofilm batch reactors: lessons from molecular approachs, Wat. Sci. Technol., 43, 9-18.
12) Kampfer, P., P. Kampfer, R. Erhart, C. Beimfohr, J. Bohringer, M. Wagner and R. Amann, 1996, Characterization of bacterial communities from activated sludge, culture-dependent numerical identification versus in situ identification using group- and genus-specific rRNA-targeted oligonucleotide probes, Microbioal Ecol., 32, 101-121.
13) Morris, C. E., M. Bardin, O. Berge, P. Frey-Klett, N. Fromin, H. Girardin, M. H. Guinebretiere, P. Lebaron, J. M. Thiery and M. Troussellier, 2002, Microbial biodiversity: approaches to experimental design and hypothesis testing in primary scientific literature from 1975 to 1999, Microbiol. Mol. Biol. Rev., 66, 592-616.
14) Ahn, Y. H. and S. H. Park, 2003, Microbial and physicochemical monitoring of granular sludge during start-up of thermophilic UASB reactor, J. Microbiol. Biotechnol., 13, 378-384.
15) Mobarry, B. K., M. Wagner, V. Urbain, B. E. Rittmann and D. A. Stahl, 1996, Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria, Appl. Environ. Microbiol., 62, 2156-2162.
16) Hicks, R., R. Amann and D. Stahl, 1992, Dual staining of natural bacterioplankton with 4',6- diamidino-2-phenylindol and fluorescent oligonuleotide probes targeting kingdom-level 16S rRNA sequences, Appl. Environ. Microbiol., 58, 2158-2163.
17) Lee, H. W., S. Y. Lee, J. W. Lee, J. B. Park, E. S. Choi and Y. K. Park, 2002, Molecular characterization of microbial community in nitrate-removing activated sludge, FEMS Microbiol. Ecol., 41, 85-94.
18) Berthouex, P. M. and L. C. Brown, 1994, Statistics for environmental engineers, Lewis Publishers, U.S.A., 273-284pp.
19) Fernando, M. S. and D. G. Allen, 2003, Effects of temperature conditions on aerobic biological treatment of wastewater, Wat. Res., 37, 3590-3601.