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Bacterial Community Structure in Activated Sludge Reactors Treating Free or Metal-Complexed Cyanides

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Abstract The microbial activity and bacterial community structure of activated sludge reactors, which treated free cyanide (FC), zinc-complexed cyanide (ZC), or nickel-complexed cyanide (NC), were studied. The three reactors (designated as re-FC, re-ZC, and re-NC) were operated for 50 days with a stepwise decrease of hydraulic retention time. In the re-FC and re-ZC reactors, FC or ZC was almost completely removed, whereas approximately 80–87% of NC was removed in re-NC. This result might be attributed to the high toxicity of nickel released after degradation of NC. In the batch test, the sludges taken from re-FC and re-ZC completely degraded FC, ZC, and NC, whereas the sludge from re-NC degraded only NC. Although re-FC and re-ZC showed similar properties in regard to cyanide degradation, denaturing gradient gel electrophoresis (DGGE) analysis of the 16S rRNA gene of the bacterial communities in the three reactors showed that bacterial community was specifically acclimated to each reactor. We found several bacterial sequences in DGGE bands that showed high similarity to known cyanide-degrading bacteria such as *Klebsiella* spp., *Acidovorax* spp., and *Achromobacter xylosoxidans*. Floc-forming microorganism might also be one of the major microorganisms, since many sequences related to *Zoogloea*, *Microbacterium*, and phylum TM7 were detected in all the reactors.

Key words: Aerobic, cyanide, metal-complexed cyanide, DGGE, diversity

Cyanide-containing wastewater is a byproduct of metal-extracting, electroplating, metal-finishing, metal-hardening, and printed-circuit-board manufacturing [1]. Cyanide inhibits cytochrome oxidase in the respiratory electron transport chain via the formation of complexes with metal ions, which function as enzyme cofactors [2]. Because of the high toxicity of cyanide, current water pollution control laws in most countries require the complete removal of cyanide from wastewater effluent before discharge [3]. Cyanide in these wastewater creates heavy-metal complexes of varying stability and toxicity [3], resulting in difficulties in developing effective treatments.

Lately, scientific investigation of the biological treatment of cyanide-containing wastewater as an alternative to chemical processes is being increasingly undertaken. Biological treatment of cyanide degradation has been proven to be efficient and inexpensive [4]. However, most researchers have classically focused on free cyanide degradation, and research regarding the degradation of metal-complexed cyanide has been sparse at best [3, 5].

The rapid evolution of molecular ecological methods has facilitated the study of microbial structure analysis, without the bias inherent in cultivation [6–8]. We previously characterized the microbial diversity in an anaerobically enriched cyanide-degrading consortium by batch test using polymerase chain reaction (PCR)-amplification and cloning of 16S rRNA genes [9]. However, no studies have yet been reported regarding microbial activity and community dynamics in continuous aerobic reactors for treating free or metal-complexed cyanides.

In order to correlate the cyanide-degrading activity and the structure of the bacterial community in free or metal-complexed cyanide degrading reactors, we compared the activity of bioreactors for treating free cyanide (FC), zinc-complexed cyanide (ZC), or nickel-complexed cyanide (NC), and studied changes of the bacterial community in activated

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sludge using 16S rRNA gene PCR-amplification and denaturing gradient gel electrophoresis (DGGE).

MATERIALS AND METHODS

Bioreactor Set-Up and Operating Conditions

In order to aerobically degrade different types of cyanide, we operated three laboratory-scale activated sludge reactors, each of which was composed of a 3.0-l closed aeration tank and a 0.5-l settling tank. Activated sludge inoculated in the reactors was obtained from the main aerobic treatment tank of a municipal sewage treatment plant (Daejeon, Korea). Mixed liquor volatile suspended solids (MLVSS) in the reactor were maintained at 1,000 mg/l by discharging excess sludge. The artificial wastewater contained 1.25 g K_2HPO_4, 0.38 g KH_2PO_4, 0.2 g $NaSO_4$, 0.06 g $CaCl_2 \cdot 2H_2O$, 0.06 g $MgCl_2$, and 0.72 g glucose per liter. Three types of cyanide-containing wastewater were also produced by the addition of 2 mM FC, ZC, or NC to be used as the sole nitrogen source for the microorganisms. The metal-complexed cyanides were produced according to a previous protocol described [10]. Each type of wastewater flowed into one of three corresponding reactors (re-FC, re-ZC, and re-NC, respectively). The reactors were run in a room adjusted to 30°C with aeration rate of 1 l/min throughout the experimental period. In order to collect the volatized cyanide, the air discharged from the closed aeration tank was passed through 1 M NaOH solution. The hydraulic retention time (HRT) was decreased in a stepwise fashion, from 48 h to 6 h, by changing the influent flow rate. The effluent and sludges in the reactors were sampled periodically for analysis of cyanide concentration and bacterial communities.

Batch Test for Cyanide-Degrading Activity of Different Reactor Sludges

After operating the reactor, sludge was retrieved in order to perform activity tests for the degradation of different types of cyanide. The equivalent of 100 mg MLVSS from each sludge type was suspended in 250-ml flasks with 100 ml of artificial wastewater containing 2 mM each of FC, ZC, or NC. These flasks were then incubated aerobically in a shaking incubator set at 150 rpm and 30°C. Flasks containing no sludge were used as negative controls. All batch tests were done in duplicate.

Analytical Methods

For the analysis of substrates, the samples were centrifuged and the supernatants were stored at −20°C with one drop of 5 M NaOH until analyzed. The concentrations of FC and ZC were determined using cyanide electrode (Orion, Model 9606, Cambridge, Massachusetts, U.S.A.). Because of high stability, NC could not be determined using cyanide electrode. Therefore, we determined the concentration of NC with UV spectrophotometery, because NC has specific absorption peaks at 267 nm and 283 nm [10]. We calibrated the absorption and concentration of NC (for the standard, the product of SIGMA was used) and determined the concentration of NC in the linear range. MLVSS was determined by standard methods [11].

Bacterial Community Analysis Using 16S rRNA Gene PCR-DGGE

Bulk community DNA was extracted from 1.0 ml (equal to 1.0 mg of suspended solid) of sludges using a Fast DNA Spin Kit for Soil (Bio101, Vista, California, U.S.A.). The extracted DNA was then subjected to touchdown PCR, using primers 341F and 534R [12]. The primer 341F contained a 40 bp GC clamp. Amplification was performed in a thermal cycler (Perkin-Elmer 9600), as previously described [13]. PCR products were separated, using a D-Code System (Bio-Rad, Hercules, California, U.S.A.) and 1-mm-thick polyacrylamide gels containing 8% (w/v) acrylamide-bisacrylamide (37.5:1), 1× TAE buffer, and a denaturant gradient from 30% to 70% (v/v) [100% solution contained 7 M urea and 40% (v/v) formamide]. Electrophoresis was conducted in 1× TAE buffer at 60°C and constant voltage (60 V) for 14 h. The gels were stained with 1:10,000 (v/v) SYBR Green I and photographed using GelDoc2000 (Bio-Rad) equipped with MULTIANALYST software (Bio-Rad). The central portions of the DGGE bands were excised with a razor blade and soaked overnight in 50 µl of purified water. A portion of this (10 µl) was then removed and re-amplified, as described above. The re-amplified DNA fragments from the DGGE bands were either directly sequenced or cloned into the pGEM Easy-T vector system (PROMEGA, Madison, Wisconsin, U.S.A.) prior to sequencing. Sequences were checked for possible chimeras using the CHIMERA_CHECK program at the Ribosomal Database Project Web site (http://rdp8.cme.msu.edu). In order to determine the phylogenetic position of microorganisms detected in DGGE, the 16S rRNA gene sequences analyzed were compared with available database sequences via BLAST search, and the related sequences were obtained from GenBank. The 16S rRNA gene sequences of DGGE bands determined in this study were deposited in GenBank under accession numbers AY766329-AY766356.

The band patterns and intensities of the scanner gels were analyzed with the GelCompar software package (version 3.0, Applied Maths, Kortrijk, Belgium). After applying rolling disc background subtraction, an analysis of each lane, acquiring densitometric curves, was performed by the software. A band of DNA was identified if the band accounted for more than 1.0% of total lane intensity. A matrix was then constructed using this information, and was used to calculate a set of numerical values to describe the diversity of the bacterial communities. As a parameter for the structural diversity of the microbial community, the

Shannon index [14] of general diversity, H, was calculated with the formula, $H=-\Sigma P_i \cdot \ln(P_i)$, in which P_i is the importance probability of the bands in a track: H was calculated on the basis of the bands on the gel tracks, using the intensities of the bands as judged by peak heights in the densitometric curves. The importance probability, P_i, was calculated as $P_i=n_i/N$, in which n_i is the height of the ith peak, and N is the sum of all peak heights in the densitometric curve.

RESULTS

Performance of Bioreactors Removing Various Cyanides

Three reactors were operated for 50 days to degrade FC, ZC, or NC in wastewater. The cyanide loading rate of the three reactors was increased in a stepwise fashion by decreasing HRT from 48 h to 6 h. During operation of the reactors, the volatized cyanide concentration was under detection limit. Cyanide removal efficiency in the three reactors is illustrated in Fig. 1. FC and ZC were completely removed at HRT of 48 h. After reducing HRT to 24 h, the cyanide removal efficiency decreased to 75–83%, and then quickly recovered to 100% in re-FC and re-ZC. After further reduction of the HRT to 6 h, FC was completely removed, but the removal efficiency of ZC was reduced to 90% after 43 days. The removal efficiency of NC was about 60% at the beginning of reactor operation, reached 87% during the 24 HRT of 24 h, and persisted at about 80%, even when the HRT was reduced to 6 h.

Cyanide-Degrading Activity of Sludges from Different Bioreactors

After completion of reactor operation, sludges were retrieved from the three reactors and tested for cyanide degradation (Fig. 2). The sludges in re-FC and re-ZC efficiently degraded all three types of cyanide, whereas the sludge from re-NC efficiently degraded NC only. The initial (four hours) degradation rates of FC and ZC by sludges from re-FC and re-ZC were in the range of 0.24–0.28 mmol/h/g MLVSS, while those by sludge from re-NC

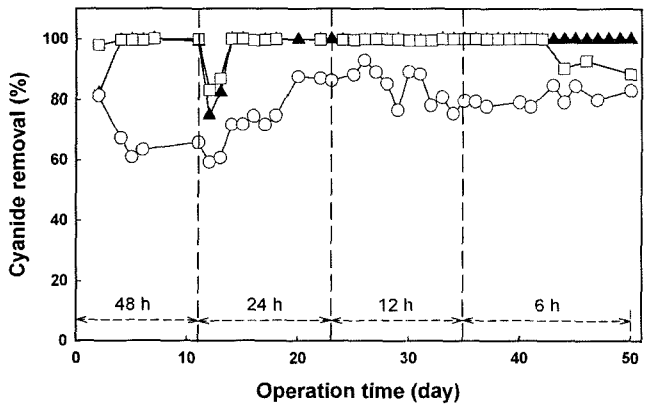

Fig. 1. Removal efficiency of free or metal-complexed cyanide during 50 days operation of activated sludge reactors with decrease of HRT. Arrows indicate the periods with different HRT. Three different reactors were used for treatment of FC (▲), ZC (□), and NC (○), respectively.

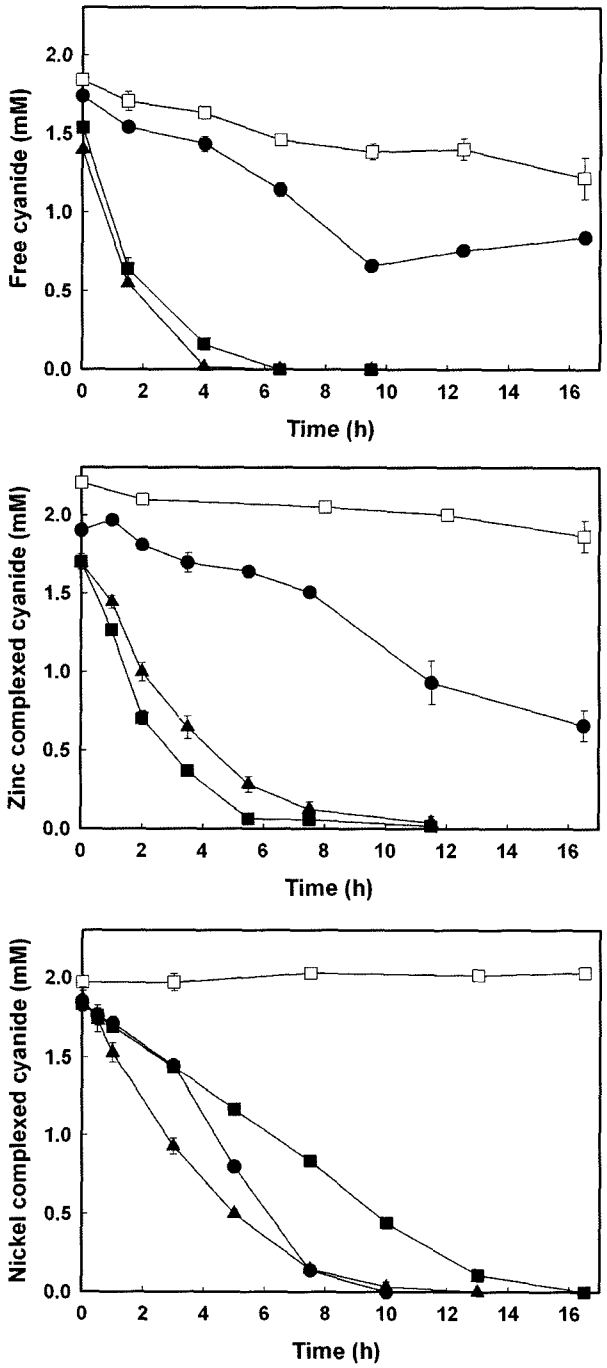

Fig. 2. Free and metal-complexed cyanide degradation by the sludges from three reactors (▲: re-FC; ■: re-ZC; ●: re-NC). The flask without added sludge (□) was used as the control. Vertical bars represent standard deviation.

were lower than 0.04 mmol/h/g MLVSS. NC was efficiently degraded by sludges from three different reactors (0.28, 0.13, and 0.13 mmol/h/g MLVSS in re-FC, re-ZC, and re-NC, respectively). In the control without the addition of sludge, more than 30% of FC disappeared, whereas the concentrations of ZC or NC did not change significantly (see control in Fig. 2). This is attributable to volatilization [15], and was considered in the calculation of the cyanide degradation rate. The absorbed cyanide was not detected by batch test. These results imply that different bacterial communities might have adapted to each type of cyanide during the operation of reactors.

Bacterial Community Analysis Using 16S rRNA Gene PCR-DGGE

In order to monitor changes in the microbial community during reactor operation, PCR-amplified 16S rRNA gene fragments were analyzed using DGGE (Fig. 3). DGGE profiles show changes occurring in the microbial population owing to stepwise decreases of HRT. The average band number per lane in each reactor used for diversity analysis was 19.7 (from 17 to 22) in re-FC, 18.8 (from 15 to 22) in re-ZC, and 19.7 (from 17 to 22) in re-NC. The number and thickness of bands observed in DGGE profiles provide an estimate of species richness. The Shannon index of diversity, H, from the DGGE band pattern of each sample was calculated in order to determine the diversity of the microbial community. Figure 4 shows changes of Shannon diversity index H in the three reactors, concurrently occurring with changes in HRT. The profile shows that, after initial decrease from shock of free or metal-complexed cyanides, the community diversity recovered slightly before a final decrease, which coincided with the highest cyanide loading levels, whereas the value of H in re-ZC and re-NC decreased from 2.94 (original sludge) to 2.61 and 2.37, respectively,

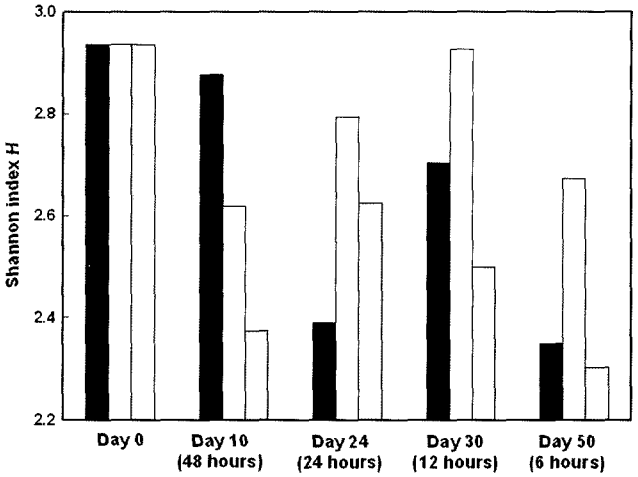

Fig. 4. Change of Shannon index values throughout reactor operation (■: re-FC; □: re-ZC; ▨: re-NC). Shannon index values (H) were calculated on the basis of the number and intensity of bands on the gel tracks. HRT is indicated in parenthesis.

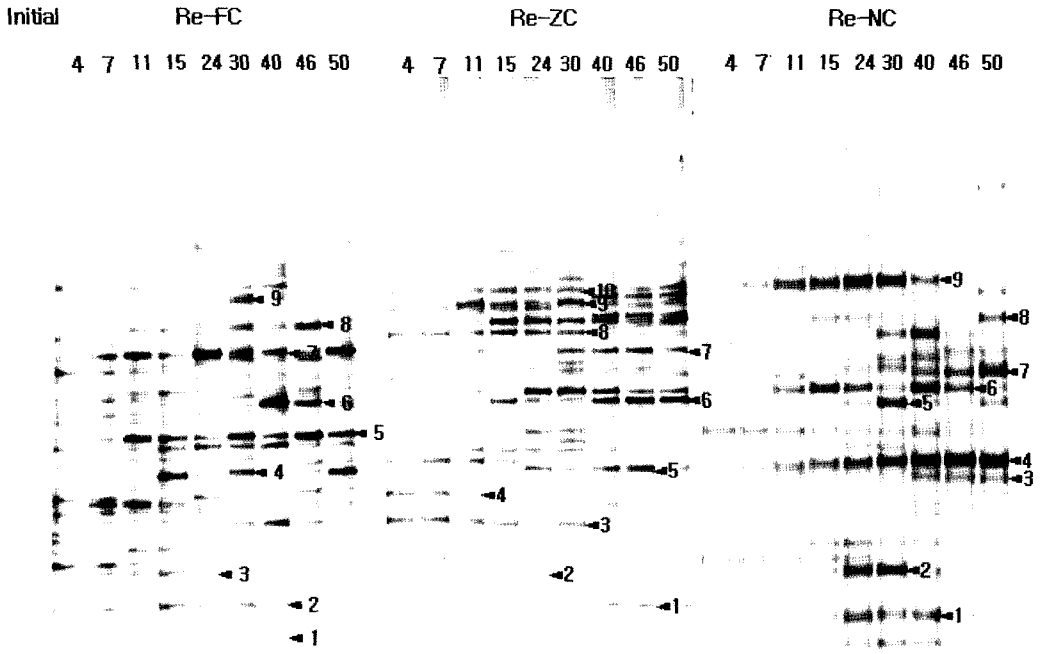

Fig. 3. DGGE profiles of 16S rRNA gene of bacterial communities from three reactors. Numbers on top of the lane indicate the date of sampling (day). Numbers on gels indicate the bands that were excised and sequenced, and correspond to Table 1.

at HRT of 48 h, H in re-FC decreased only slightly to 2.87. After the decrease of HRT from 48 h to 24 h, the values of H in re-ZC and re-NC increased slightly (2.80 and 2.63, respectively). At HRT of 12 h, the microbial diversity recovered slightly in re-FC. The final decrease of HRT to 6 h decreased H for all three reactors.

16S rRNA gene sequences of 28 major bands (9 bands in re-FC, 10 bands in re-ZC, and 9 bands in re-NC) were analyzed and compared with available database sequences via BLAST search (Table 1). Most of the sequences were found to be clustered in the Proteobacteria (17 bands) and Bacteroidetes (6 bands). The sequences of other bands were found to be clustered in the Actinobacteria (2 bands), phylum TM7 (2 bands), and Acidobacteria (1 band). Within the Proteobacteria, most of the sequences were clustered in the β-Proteobacteria, particularly in the order Burkholderiales (10 bands). In re-FC, bands F4, F5, and F7 were present throughout all operation periods and became prevalent as HRT was reduced. F1, F2, and F3 constituted minor bands, but were observed in nearly all of the periods. F6 became

Table 1. Sequence similarities of partial 16S rRNA clones from selected DGGE bands from three reactors.

Phylogenetic group	DGGE bands*	Sequence length (bp)	Phylogenetically related organism (Accession No.)	Percent of similarity
Alpha-proteobacteria				
	F3	130		100
Rhizobiales	Z2	130	*Zoogloea ramigera* ATCC19623 (X74915)	100
	N2	131		98
Beta-proteobacteria				
Burkholderiales				
Comamonadaceae	Z5	178	Acidovorax sp. BSB421 (Y18617)	100
	F7	163	Acidovorax sp. DSM649 (AJ420323)	100
	F6	130		98
	Z6	168	Acidovorax sp. Smarlab 133815 (AY093698)	99
	N5	164		98
	F5	163	*Pseudomonas saccharophila* DSM654 (AB021407)	100
	Z4	148	Uncultured *Leptothrix* clone AV011a (AF385528)	98
Alcaligenaceae	Z9	199	*Achromobacter* sp. MBRG4.31 (AJ508608)	99
Oxalobacteraceae	N9	173	*Herbaspirillum* sp. DA.1 (AJ430686)	98
	Z8	197	*Janthinobacterium* sp. N (AY044090)	100
Rhodocyclales				
	N6	173	*Zoogloea* sp. DHA-35 (AJ011506)	98
Uncultured beta-proteobacteria	N3	198	Uncultured beta-proteobacteria clone SBR1001 (AF204252)	97
	N4	171		95
Gamma-proteobacteria				
Enterobacteriales	N1	197	*Klebsiella* sp. ATCC13883 (AF130981)	98
Actinobacteria				
	F2	147	*Microbacterium* sp. Al-28 (AY437637)	100
	Z1	158		96
Acidobacteria				
	F1	149	Uncultured *Acidobacteria* clone C16.47WL (AF431421)	89
Bacteroidetes				
Flavobacteriales	Z3	193	*Algibacter* sp. KMM3914 (AY187690)	94
	Z7	168	*Chryseobacterium* sp. AU939 (AY043370)	98
Sphingobacteriales	F4	194	Uncultured *Pedobacter* clone AV100 (AF385531)	92
	F8	165	Uncultured *Haliscomenobacter* clone SBRT303 (AF368190)	96
	N7	168	Uncultured *Cytophaga* clone ESR5 (AF268289)	90
	N8	198		99
Candidate division TM7				
	F9	135	Uncultured TM7 phylum clone HD027 (AY349415)	96
	Z10	174		97

*The symbols F, Z, and N in front of the band number indicate the DGGE bands from re-FC, re-ZC, and re-NC, respectively.

prevalent on days 40 and 46 (HRT of 6 h). In re-ZC, the profile did not change significantly during days 15–30 (HRT of 24 h and 12 h) or days 40–50 (HRT of 6 h). The major bands, including Z3, Z4, and Z8, which were present during long HRT periods (HRT of 24 h and 48 h) persisted, but became minor after 30 days (HRT of 12 h). Z6, Z7, and Z9 became major bands after 40 days (HRT of 6 h) of operation. In re-NC, band N4, which constituted a minor community component during the early period, gradually became dominant after sustained reactor operation (with HRT decrease). N1, N2, N5, N6, and N9 became dominant between days 15 and 40. Finally, on days 40–50, new bands such as N7 and N8 became dominant.

Only a limited number of bands with higher than 98% similarity with each other was retrieved from all three reactor sludges. One contained F3, Z2, and N2, which are associated with *Zoogloea*, and the other contained F6, Z6, and N5, which are associated with *Acidovorax*. The sludges of re-FC and re-ZC had two sequences in common: One contained F2 and Z1, which were related to *Microbacterium*, and the other contained F9 and Z10, which were associated with the phylum TM7. These results demonstrate that bacterial community is significantly different among reactors, depending on the type of cyanide used.

DISCUSSION

Cyanide is a strong ligand capable of forming complexes with a variety of heavy metals even at low concentrations, and this complexed cyanide is quite difficult to treat. The toxicity of metal-complexed cyanide is lower than FC [16], and the toxicity of NC is lower than ZC [17]. In fact, *Pseudomonas fluorescens* grew well with NC and grew only a little with ZC, whereas it was able to grow on FC, but only in extremely low concentrations [10]. Since toxicity correlates with the dissociation constant of metal-complexed cyanide, the concentration of free cyanide (dissociated) should determine the toxicity [9]. However, this contrasts with the results of continuous reactor experiments [18]: In the continuous reactors used in this experiment, the efficiency of NC degradation was the lowest, compared with the degradation of FC and ZC. In general, cyanide in its metal-complexed form can be biodegraded to carbon dioxide and ammonia, hence releasing a free metal ion. Therefore, when the reactor treats metal-complexed cyanide efficiently, the cyanide concentration in the reactor is very low; however, the concentration of released metal ions consequently becomes very high, and this high concentration of free metal ions might be a key factor in the toxicity associated with continuous reactor systems. Under aerobic conditions, the toxicity of free nickel ions is higher than that of zinc [10, 19]. Therefore, the lowest efficiency of NC degradation would be due to higher toxicity of the free nickel ion. The stable degradation of NC, even with reduced HRT, also supports this explanation. The decrease of HRT increased cyanide loading without affecting the concentration of heavy metal in the reactors.

The sludge that adapted to each type of cyanide during reactor operation for 50 days was tested for its activity in the batch experiments. According to the speciation diagram [20], more than 50% of cyanide in the ZC form is present as a free ion, whereas most cyanide (more than 99%) is present in the metal-complexed form in NC at pH 7, because of different dissociation constant. This explains why re-FC and re-ZC showed similar activity profiles with different cyanide complexes. The lower degradation efficiency of FC and ZC by the sludge in re-NC is attributable to its higher degree of sensitivity to the toxicity of free cyanide. Accordingly, the toxicity of heavy metal and the stability of the metal-cyanide complex should be considered together for the prediction of metal-complexed cyanide degradation.

With improved understanding of the bacterial community present in cyanide-degrading reactors, we could be able to develop better systems for the treatment of various cyanides-containing wastewater. DGGE analysis makes it possible to rapidly compare bacterial communities. Furthermore, specific phylogenetic information can be derived from the bands excised from the gel [21]. The Shannon index, H, which is applicable to diversity analysis of complex microbial communities [22, 23], can also be calculated from the DGGE band profile. After the start of the reactor operation, the value of H in re-NC decreased rapidly. This low diversity in re-NC corresponds with the high toxicity of free nickel ion released from NC, as discussed above and shown in Fig. 1. In fact, NC degradation efficiency was decreased to 60% during this period, although those of FC and ZC were maintained at 100%. After the reduction of HRT to 24 h, the value of H in re-FC rapidly decreased. This is most likely due to the increase of the cyanide loading rate. Differences in diversity and changes in microbial populations in the three reactors indicate that each type of cyanide had a different effect on the microbial community. It is of interest to note that, although the degradation efficiency was maintained at a high level throughout the operation, the succession profile of microbial communities showed that change of microbial community was significant in each reactor.

From the sequences of major DGGE bands, we found several bacterial groups that are related with cyanide degradation. The sequence of the Z9 major band exhibited 99% identity with *Achromobacter xylosoxidans*, which was reported to effectively degrade free cyanide [24]. The sequence of N1 related to *Klebsiella*. Several *Klebsiella* strains were reported to have cyanide-degrading activity [25] in aerobic condition. The sequences related to *Acidovorax*, one of the main genera found in activated sludge [26],

were retrieved from all three reactors. Some of *Acidovorax* strains were reported to degrade various organic cyanides (nitriles) [27].

The sequences related to *Zoogloea* were retrieved from all three reactors, and *Zoogloea* has long been considered as typical activated sludge bacteria responsible for the formation of activated sludge flocs [28]. The other two groups of sequences related to phylum TM7 and genus *Microbacterium* were retrieved from re-FC and re-ZC. Approximately 15% of the filaments in the Eikelboom Type 0041 were related to the phylum TM7 [29]. Nonfilamentous bacteria also constitute a large fraction (65%) of the flocs of activated sludge [30], and the nonfilamentous bacteria related to genus *Microbacterium* were able to coaggregate with other bacteria [31]. These results indicate that a significant fraction of the bacterial community is associated with sludge flocculation in cyanide-treating reactors.

In this study, we compared the microbial activity and bacterial community structure of continuous reactors that treated three different types of cyanide, and observed that the metal-cyanide degrading property of sludge is more similar between re-FC and re-ZC, than between re-NC and the others. This could be due to the high toxicity of nickel ion released after degradation of nickel-chelated cyanide. DGGE experiment showed dynamic succession of the bacterial community throughout the operation period, although degradation efficiency was maintained at a stable level. Our study indicates that specific bacterial communities are enriched for the degradation of each type of cyanide, although re-FC and re-ZC showed similar activity properties with respect to cyanide degradation. Further studies are in need to identify the microorganisms playing key roles in different types of cyanide degradation, and their interaction within the bacterial community. For this, it is necessary to develop new molecular biological methods to detect genes involved in cyanide degradation and their expression.

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