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Characterization of molecular biological indicators to define stabilization of landfills

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Abstract−This study was assessed such that a molecular biological investigation in uncontrolled landfills can be comparably used for characterizing its stability in association with the conventional water quality parameters. At first, the microbiological diversity in two landfill sites (Cheonan and Wonju landfills in Korea) was identified by 16s rDNA cloning. It was also quantitatively investigated for denitrification enzyme coding genes (nitrite reductase; *nirS* and nitric oxide reductase; *cnorB*) and methane producing enzyme coding gene (methyl coenzyme M reductase; MCR) by real-time polymerase chain reaction. The copy numbers of *nirS* and *cnorB* in Cheonan were higher than those in Wonju, which was well reflected from the time difference after site closure, respectively. The greatest number of MCR, *nirS* and *cnorB* genes of C1 spot (Cheonan) verified that it was immediately bordering the sources, which was steeply decreased toward the outer boundary landfill. Cheonan landfill is more rapidly stabilized based on molecular biological indicator even if the present state of Wonju landfill is more stable based on BOD and COD concentrations. However, the comparison between the copy number of these genes and the conventional water quality monitoring parameters addressed that those of parameters have similar patterns, especially for the source or the boundary. It was concluded that the molecular biological parameters could be used for determining microbial stability toward down-gradient around the uncontrolled sites.

Key words: 16S rDNA, Landfill, Groundwater Monitoring, Real-time Polymerase Chain Reaction, Stabilization

INTRODUCTION

Unlined landfills in Korea, which were finally closed, have been numbered to 1170 sites [1]. Among them, only 232 sites have been legitimately managed such that the leachate was properly treated to meet the regulation standards. However, the rest of the sites have no suitable facilities to come up with those requirements. In other words, there were no interceptor trenches or storage and treatment plants properly placed to effectively treat leachate. Moreover, they were not equipped with any landfill gas collecting or treatment systems. This will surely contribute to contaminating soil or groundwater and damaging neighboring environments and furthermore limiting land use to be developed for future city planning.

Various conventional ways of physical-chemical investigations have been dealt with characterizing the practical condition of the landfill of concern. Conventional water quality characterization (Chemical oxygen demand; COD and Biochemical oxygen demand; BOD) or relative comparisons of waste components (Carbon/Nitrogen, cellulose/lignin and BOD/COD) are known as major indicators to distinguish the stabilization [2]. Repetitive isolation and culturing of microorganisms from the ecosystem have been implemented for most biological investigations, but it could lead to biased characterization coupled with solely cultivated microcosms. In general, bacteria that are strongly related to significantly functioning in an ecosystem are rather uncultivated, and the accuracy of the culture dependent method is quite low because only 1% of microorganisms could be cultured [3]. So the microbiological methods based on culturing may lead to false understanding of intrinsic characteristics of microbes and not to be representative of the concerned ecosystem.

Therefore, distinctively defining the diversity of microbes or enzymes catalytic reactions would be the most demanding issues for immediately characterizing the actual status of the ecosystem around the landfill. However, molecular biological indicators such as 16S rDNA or several specific genes related with biological reactions have been rarely used for distinguishing the stabilization in the landfill even though these are direct effect parameters. For that reason, we have attempted to assess the stability of two unsanitary landfills in Korea by employing molecular tools. Typical enzyme genes mainly catalyzing for denitrification and methane production in landfill were quantified with real-time PCR (polymerase chain reaction). On the basis of molecular biological technology, various representative bacteria were also characterized. In addition, conventional water quality parameters were compared with these enzyme coding genes, which will determine molecular biological parameters efficiently used for distinguishing intrinsic stabilization of landfill.

MATERIALS AND METHODS

1. Study Sites

Two unsanitary landfills (Cheonan and Wonju) in South Korea were chosen for this study, and Fig. 1 shows the location of sampling points of each site. Historical records for two sites are summarized as shown in Table 1. Cheonan landfill closed 3 years later than Wonju and the volume of waste disposed in Wonju landfill was 7.8 times more than that of Cheonan.

Groundwater samples were taken by employing a sterilized bailer (Cole-Parmer, USA) from 7 and 8 dedicated monitoring wells (i.e., 5 cm in diameter), Cheonan and Wonju, respectively, installed within or around 100 m toward down-gradient direction of groundwater

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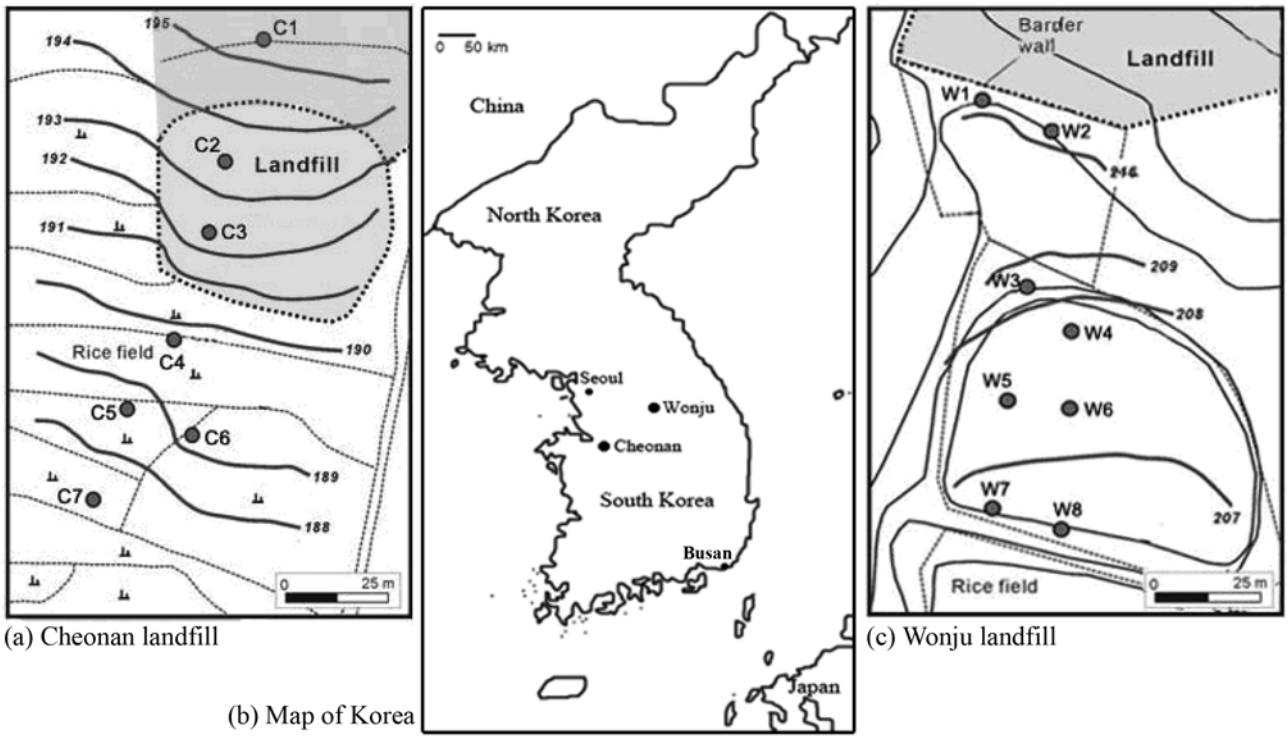

Fig. 1. Map of Korea and the locality on the monitoring wells in two landfills (7 and 8 monitoring wells, Cheonan; C1-C7 and Wonju; W1-W8).

Table 1. Historical surveys on two sites, Cheonan and Wonju landfills

	Cheonan landfill	Wonju landfill
Operation periods	1991-1998	1982-1995
Geography	Plain	Valley
Volume of waste disposed (m^3)	41,000	322,000
Area of waste disposed (m^2)	6,000	81,000
Type of waste	Domestic waste	Domestic waste

flow. The sample was placed into a 1 L sterilized amber jar and stored at 4 °C without any headspace. ORP (oxidation-reduction potential) of groundwater was analyzed on site using an ORP meter (YSI pH100 pH/ORP/Temp Instrument). Groundwater quality parameters such as NH_3-N, NO_3-N, TOC, BOD_5 and COD_{Cr} were analyzed by standard methods [4]. Samples were taken in March, June and September 2005.

2. 16S rDNA Cloning

A 200 ml of groundwater sample was centrifuged for 15 min at 3,000 rpm (HA-1000-3, Hanil Science), thereby the segregated concentrate of approximately 500 mg was bead-beaten for 5 sec at speed 5 by employing FastDNA®SPIN kit (Bio101 system, Q-Bio gene) and FastPrep® Instrument (Q-Bio gene), which eventually produced genomic DNA.

Thereafter, a polymerase chain reaction (PCR) was conducted for characterizing general species of microbes such that 16S rDNA was amplified by universal primer 27F and 1492R [5]. The amplified PCR products were then subject to electrophoresis for 30 min at 100 mV (Mupid-α, Japan) where the resultant 16S rDNA bands in gel were purified by Winzard® SV Gel and PCR Clean-Up System (Promega, USA). The refined 16S rDNA PCR products were ligated to pGEM-T easy vector (Promega, USA), which then transformed into competent cell (*Escherichia coli* XL1-blue) where it

Table 2. Sequences of primers for quantifying of specific bacteria used in this study

Species	Primer	Sequence (5'-3')	Size (bp)
Flavobacterium xinjiangense	Fxi-459F	ACG TGT GGA AGA TTG ACG G	468
	926 R	CCG TCA ATT CCT TTR AGT TT	
Rhodoferax ferrieducens	Rfe-454F	GGC CTG CCC TAA TAC GGT G	473
	926 R	CCG TCA ATT CCT TTR AGT TT	
Dechloromonas denitrificans	Dde-210F	GGC CTT GCG CTG ATT GTG	310
	519R	GWA TTA CCG CGG CKG CTG	
Ferribacterium limineticm	Fli-994F	CAT GTC CAG AAG CCC TTA GA	499
	1492R	GGT TAC CTT GTT ACG ACT T	

was selectively incubated in LB (Luria-Bertani) medium treated with Ampicillin, X-gal/IPTG (Promega, USA). The resultant vector was finally purified by Winzard® Plus Minipreps DNA Purification System (Promega, USA).

By using purified plasmid (i.e., vector), DNA sequences were characterized by 3100 Capillary DNA sequencer (Applied Biosystems); thereby the analyzed DNA sequences were comparably identified by 16S rDNA sequence recorded in the BLAST (Basic Local Alignment Search Tool) network service of NCBI (National Center for Biotechnology Information) web site. From this result, the dominant bacteria and their specific genes were selected so that specific primer sets (i.e., the sequences and length of base pair) were prepared as shown in Table 2. It was used for quantification of each gene.

3. Denitrification and Methane Production Enzyme Coding Genes

Denitrification and methane production are the main biological reactions of landfill sites [6] and their enzyme coding genes are known. Accordingly, these two reactions were selected to define the characteristics of the landfill.

There are several denitrification genes dominantly concerned with the denitrification process, where nitrite reductase reducing NO_2^- to NO in terms of *nirS* gene [7] and nitric oxide reductase reducing NO to N_2O in terms of *cnorB* gene were quantified by real-time PCR. The primers were prepared as proposed [8,9]. For methane producing enzyme coding gene, methyl coenzyme M reductase (MCR gene) finally catalyzed in the methane producing processes was quantified by the primer and PCR condition in the same manner as suggested [9].

4. Quantification of Genes by Real-time PCR

Real-time PCR was performed as the target DNA was amplified by iQ SYBR Green Supermix (2X, BioRad, USA). During the target DNA being amplified, the extent of fluorescence emitted from SYBR Green I dye selectively combined with DNA of double strands was obtained by iCyber iQ Real-Time Detection System (Bio-Rad, USA). Amplification was performed for 50 μL of reaction mixture by using pre-sterilized distilled water including 0.3 μL of genomic DNA, 200 μM of each primer and 25 μL of iQ SYBR Green Supermix (2×, Bio-Rad, USA). The standard curve of DNA concerned with specific type of gene was prepared for *nirS*, *cnorB*, MCR genes and bacterial specific genes where each PCR product was inserted into pGEM-T easy vector (Promega, USA), which was then cloned by a competent cell. The concentration of a plasmid vector for a specific type of gene was quantified by Spectrophotometer (HP-Agilent 8453), which then converted into the number of copy by Eq. (1).

$$\frac{\text{copies}}{\mu\text{L}} = \frac{\text{O.D.}_{260} \times 50 \frac{\text{ng}}{\mu\text{L}} \times (6.022 \times 10^{23})}{\text{template legth (bp)} \times 650 \times 10^{9}} \quad (1)$$

O.D._{260} is the optical density obtained at 260 nm and bp is the number of base pair.

RESULTS AND DISCUSSION

1. Characterization of Microbial Diversity by 16S rDNA Sequences in the Sites

From a total of 15 sampling points (Cheonan 7 points; C1-C7, Wonju 8 points; W1-W8), 518 microorganisms of 16S rDNA sequences were identified and compared with NCBI database (http://www.ncbi.nlm.nih.gov/). From Cheonan, 47.6% of microbes were exposed to as related species with more than 97% of phylogenetic relationships where 53.1% were uncultured microbes, while 46.9% were identified microbes. Identified microbes predominantly consisted of 53.1% of Proteobacteria as well as Actinobacteria, Flavobacteria, and Lactobacillales phyla. In Wonju landfill, 32.5% of microbes were related species with more than 97% of phylogenetic relationships showing that uncultured microbes were 85.2%, while identified microbes were 14.8%. Identified microbes were composed of 22.2% of Proteobacteria along with Actinobacteria and Flavobacteria phyla. From BLAST searching, approximately 59% did not have any phylogenetic relationships with DNA sequences of microorganisms in the database, which strongly supposed that those microbes may be categorized into unknowns of newly found uncultured species. Overall, Cheonan and Wonju sites have 78 and 95% of uncultured microbes predominantly proliferating, respectively.

Table 3 shows species of microorganisms which were found in monitoring wells of the study landfills and their NCBI accession number based on 16S rDNA sequences. The molecular analysis for identification of bacteria was conducted before quantification of specific genes for selection of microbial indicator. The following information was acquired in the genome project database of NCBI. *Rhodoferax ferrireducens* can couple the oxidation of organic molecules to the reduction of iron by using insoluble Fe(III) as an electron acceptor. And *Dechloromonas aromatica* strain RCB may be used for bioremediation as it can oxidize aromatic hydrocarbon compounds in the absence of oxygen by coupling it to nitrate reduction. *Pseudomonas fluorescens* can degrade multiple different pollutants and *Acidovorax* strans also have been identified that are able to degrade aromatic compounds.

2. Quantification of Bacterial Specific Genes

From the microbial diversity results, *Rhodoferax ferrieducens*, *Ferribacterium limneticum*, *Flavobacterium xinjiangense* and *Dechloromonas denitrificans* were selected as indicator species because these were the most common and seemed to be attributed to biological reaction in the landfill as their known roles. These microbial indicators revealed that the characteristics of the landfill mostly depended upon the distance from the down-gradient of the sources and the age of the closing date. Fig. 2 shows the result of the quantity of these bacteria according to the location of each monitoring well. It reveals the mean value of three samples of each of sampling time and the error bar shows standard deviation. The populations of *R. ferrieducens* and *F. limneticum* in Cheonan (C1-C7) landfill were much more than those in Wonju (W1-W8) landfill where these bacteria are known to be associated with Fe^{3+} reduction. In the meantime, *D. denitrificans*, a type of denitrifier, was at the higher extent observed at C1, C2, C3, W1 and W2, which are all seated near the source.

3. Quantification of Enzyme Coding Genes

The results of real-time PCR analysis using the specific primers on target enzyme are shown in Fig. 3 where *nirS* and *cnorB* genes, which are denitrification enzyme coding gene, and MCR gene, which is methane production enzyme coding gene, were decreasingly observed in the down-gradient directions. For *nirS* gene, the copy num-

Table 3. Microorganisms in monitoring wells and their NCBI accession number based on 16S rDNA sequences

Accession number	Organism	Detection well
AY651926	*Comamonadaceae bacterium MPsc*	C1, C5, C7, C8
X87150	*Lactosphaera pasteurii*	C2
AJ009471	*Uncultured SJA-63*	C1,C2,W1
AY050600	*Uncultured GOUTB17*	C6, W3
AF435948	*Rhodoferax ferrireducens*	C5, C7, W1
AY609198	*Rhodoferax antarcticus*	C2,C3,C6,W2
AF407391	*Uncultured RA9C8*	C2
AJ440986	*Antarctic bacterium R-7724*	C3,C5, C7,W4
AY050578	*Uncultured GOUTA6*	C7,W2, W6, W8
AY699582	*Uncultured PHE7d8*	C6,W4
AY955085	*Uncultured 5-1a*	W1,W8
CP000089	*Dechloromonas aromatica RCB*	C1,C3,C6
AJ318917	*Dechloromonas denitrificans*	C2, C3,C6, W1
AJ414655	*Methylobacter sp. SV96*	C4, C6, C8
U51104	*Denitrifying Fe-oxidizing bacteria*	C6
AF072920	*Uncultured H2O*	C1, C4, C5, C6,W8
AY218607	*Uncultured KD3-141*	C5,C7,W1
Y17060	*Ferribacterium limneticum*	C2
AB080645	*Sulfuricurvum kujiense*	C2,C3
AB240330	*Uncultured SRRT25*	C3,C7
AJ519665	*Uncultured Holophaga sp.*	C6
U41563	*Geothrix fermentans*	C5, C6, W8
AJ583090	*Pseudomonas fluorescens*	C7, C8, W2, W4, W7
AY904765	*Bradyrhizobium japonicum*	W4
AY167838	*Janthinobacterium agaricidamnosum*	W2, W4, W5, W6, W7, W8
Y18617	*Acidovorax sp. BSB421*	C7, C8, W6
AB021385	*Vogesella indigofera*	C5,C4, W7
AY622267	*Uncultured S-E105*	W5,W6,W8
AJ581999	*Pseudomonas lurida*	W5
AF433174	*Flavobacterium xinjiangense*	W3, W5
AJ544690	*Burkholderia fungorum*	W6

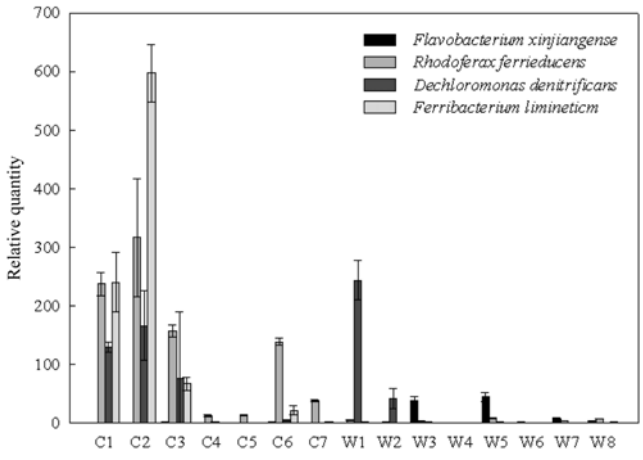

Fig. 2. Quantities of specific bacteria obtained from existing two landfills by real-time PCR.

bers of gene in Cheonan and Wonju were on an average of 192,800 and 26,799 copies/μL, respectively, showing 7 times greater in Cheonan. For *cnorB* gene, it was observed 1,596 and 371 copies/μL, respectively, showing 4 times higher in Cheonan. As a result of the comparison of copy numbers between *nirS* and *cnorB* genes, the number of *nirS* genes was two orders of magnitude higher than that of *cnorB* gene. It indicates that the reduction of NO to N_2O was more actively conducted than that of the reduction of N_2O to N_2 derived by *nirS* gene.

From Cheonan, the number of MCR genes was at the greatest extent of 1,310,000 copies/μL observed at C1, while it was less obtained at 101,000, 21,500 and 14,200 copies/μL, respectively, from C2, 3 and 4 in the down-gradient. On the contrast, for W1 and 2 from Wonju, close to the source, it was 5,410 and 7,280 copies/μL, respectively, which was relatively lower ranging from 1/20 to 1/2 compared to that in Cheonan. Toward down-gradient, the number of MCR genes has directionally decreased since 10 to 250 times higher number of genes was seen in the source of C1. It well reflected from turning a reduction state into re-aeration or transitional oxidation state through down-gradient direction since a nutrient-rich anaerobic environment has been overwhelmingly depleted near to the source.

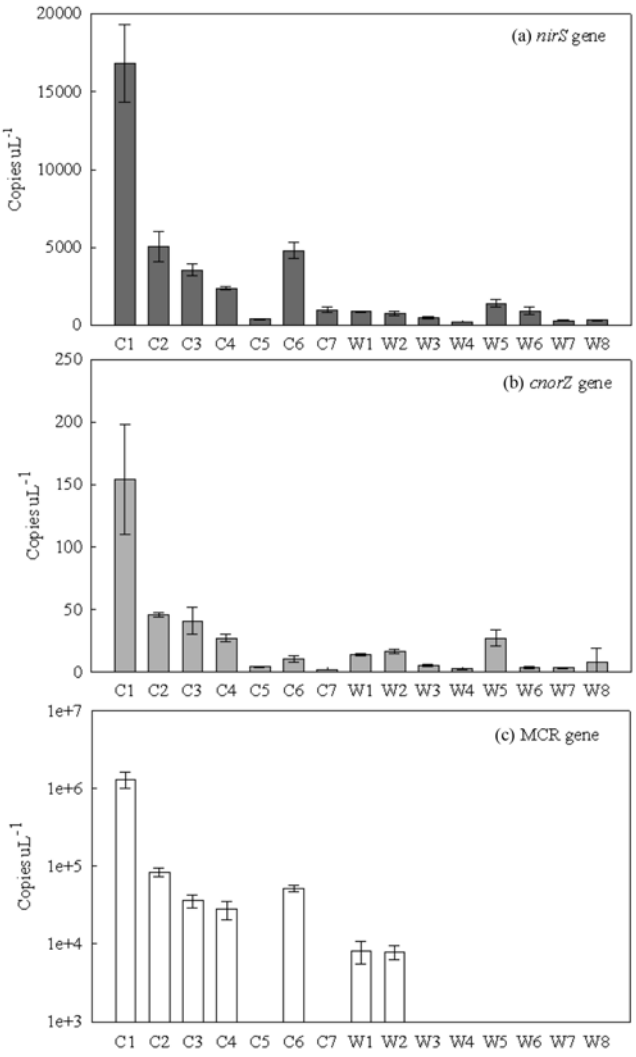

Fig. 3. Quantities of *nirS*, *cnorB* and MCR genes observed from two sites by real-time PCR.

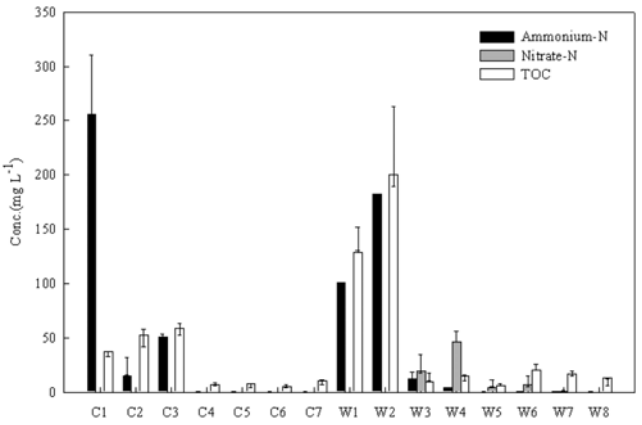

Fig. 4. Concentrations of ammonium nitrogen (NH_3-N), nitrate nitrogen (NO_3-N) and TOC from the groundwater samples in the two sites.

Consequently, methane production and denitrification could be more actively achieved in Cheonan landfill. That was well consistent with the patterns of variations of concentration for NH_3-N, NO_3-N and TOC (Fig. 4), except for W1 and W2 where they were highly observed due to persistent existence of aerobic state (Table 4). In other words, they were inversely correlated, which will not provide proper condition of denitrification and methane production [11]. Consequently, their anaerobic degradation will be retarded at that spot. This result is different from the results of conventional water quality parameters presented in Table 4. BOD and COD are used as conventional parameters for assessment of landfill leachate [12, 13]. Concentrations of BOD_5, COD_{Cr} and BOD/COD were higher in W1 and W2 than C1-C3 wells. It means Wonju landfill is in more stable condition than Cheonan landfill since these parameters are regarded as a landfill stabilization indicator. In conclusion, Wonju landfill is presently stable but the stabilization velocity is higher in Cheonan landfill.

Table 4. ORP range and average value with standard deviation of conventional water quality parameters of monitoring wells

Well	ORP (mV)	BOD_5 (mg/L)		COD_{Cr} (mg/L)		BOD/COD	
	Range	Average	STD	Average	STD	Average	STD
C1	−125~−84	58.4	17.66	283.3	113.39	0.25	0.183
C2	−108~−74	42.5	33.80	161.2	146.21	0.27	0.066
C3	−76~−65	39.7	19.22	83.7	35.68	0.47	0.032
C4	225~328	24.2	5.48	44.8	6.05	0.54	0.053
C5	189~313	17.7	2.04	42.0	8.43	0.44	0.122
C6	163~268	29.2	9.77	45.1	6.40	0.64	0.130
C7	107~209	9.2	1.47	21.4	3.65	0.43	0.012
W1	−21~71	17.2	5.04	78.6	9.90	0.22	0.055
W2	−34~278	21.8	4.39	113.0	24.57	0.19	0.011
W3	229~300	23.6	12.96	45.4	21.75	0.51	0.096
W4	235~308	54.7	59.41	167.6	162.74	0.59	0.480
W5	247~303	14.6	10.48	85.8	49.24	0.17	0.044
W6	264~305	30.1	21.13	65.3	14.51	0.48	0.312
W7	153~287	10.0	5.47	65.9	36.28	0.23	0.237
W8	156~346	11.4	7.12	77.5	23.17	0.14	0.053

CONCLUSIONS

In this study, the variation of microorganisms in the affected area around the landfill was characterized by molecular biological methods and compared with the conventional water quality parameters. Some of the species which were commonly detected from the most of sampling points were chosen and quantified. In addition, some of the enzyme genes, which are concerned mainly with denitrification and methane production, were characterized and quantified with real-time PCR. Methane production and denitrification enzyme coding genes more predominantly existed in Cheonan landfill. In spite of the fact that Cheonan landfill was younger than that of Wonju (Cheonan landfill was closed in 1998 compared to Wonju landfill being closed in 1995), the genes related with denitrification and methane production were at a greater extent observed in Cheonan landfill. The highly observed nutrients from W1 and W2 were inversely related with the level of anaerobic genes, which means that they will be retarded to be anaerobically degraded due to the presence of aerobic condition. However, this result is different with conventional stabilization parameters such as BOD/COD. Therefore, it could be concluded that Cheonan landfill is more rapidly stabilized than that of Wonju landfill even if the present state of Wonju landfill is more stable. It was thus decided that the molecular-based microbial analysis would play a critical role for distinguishing the extent of practical stabilization in the landfills.

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