

Denitrification Activity of a Poly-Hydroxybutyrate (PHB) Embedded Soil-Column Reactor and Genomic Study of the Bacterial Community

HU JIN¹, YASUNORI KAWAGOSHI², AYA FUJIMOTO², SHIGERU MARUYAMA²,
TEIJI URAKAMI³, and KENJI FURUKAWA²

¹Graduate School of Science and Technology, Kumamoto University

²Department of Civil Engineering and Architecture, Faculty of Engineering, Kumamoto University
/ 2-39-1 Kurokami, Kumamoto 860-8555, Japan

³Biochemical Division, Mitsubishi Gas Chemical Company, Inc.
/ 2-5-2, Marunouti, Tiyoda-ku, Tokyo 100-8324, Japan

Abstract

Denitrification activity behavior and bacterial community structure in a poly- β -hydroxybutyrate (PHB) embedded soil-column reactor were investigated. The influent containing nitrate ($\text{NO}_3\text{-N}$) was applied in a downward flow. The $\text{NO}_3\text{-N}$ concentration rapidly decreased only in the middle part of the column where PHB was embedded, which indicated that the denitrification activity in the reactor was related to PHB. Influent $\text{NO}_3\text{-N}$ was effectively removed in the reactor following 60 days of continuous operation. The total number of bacteria in the lower and middle parts of the column decreased after 30 days, and then increased again after 60 days; however, the number in the upper part continuously decreased with time. The number of PHB utilizing denitrifiers increased in both the middle and lower parts with time, contributing to denitrification activity in the reactor. From the result of PCR and denaturing gradient gel electrophoresis (PCR-DGGE), the presence of two denitrifying and a few other bacteria were determined, and which suggested of *Ralstonia* related species played an important role in the denitrification capability of the reactor.

Key words: denitrification, biodegradable plastic, poly-hydroxybutyrate, DGGE, bacterial community

INTRODUCTION

Groundwater pollution caused by $\text{NO}_3\text{-N}$ and nitrite ($\text{NO}_2\text{-N}$) has become a serious problem in recent years. The high percentage (5~6 %) of groundwater sources contaminated with $\text{NO}_3\text{-N}$ and $\text{NO}_2\text{-N}$ has exceeded the Japanese environmental quality standard from 5 years of 1997 to 2001^{1,2)}. There are several reasons for the increases of $\text{NO}_3\text{-N}$ and $\text{NO}_2\text{-N}$ concentrations in groundwater, e.g., fertilization for agriculture, excrements of domestic animal, and infiltration of nitrogen containing wastewater^{3,4)}. Ground-

water pollution with NO_3 is mostly caused by non-point source pollution, thus, it is quite important to develop cost effective onsite groundwater remediation. The application of onsite bioremediation techniques, for which a carbon source must be added to the polluted groundwater for nitrate reducing bacteria (denitrifiers) to remove nitrogen, is considered as a promising method for this purpose^{1,5)}.

In order to utilize denitrifiers for $\text{NO}_3\text{-N}$ removal, addition of an electron donor is required because most of them are heterotrophs. Methanol is the most

commonly used electron donor for denitrifiers in conventional wastewater treatment processes; however, methanol (a liquid) is directly added onsite, there is a possibility of secondary pollution by unused product or metabolites. In addition, the cost of methanol can also be a decisive factor⁶⁻⁹. Accordingly, the use of a solid electron donor that gradually dissolves into the groundwater is of interest. Tanaka^{1,4} proposed the use of reduced sulfur compounds as an electron donor for denitrification; however, the oxidation of sulfur results in an increase of sulfate, which if anaerobic conditions occur, yields hydrogen sulfide and resulting odor problems.

We considered the use of poly- β -hydroxybutyrate (PHB) which is a biodegradable plastic as a carbon source and an electron donor for denitrifier. The effectiveness of PHB as an electron donor for groundwater denitrification in a soil-column reactor was investigated using a soil-column reactor^{9,10} and the relationship between the numbers of bacteria (including denitrifiers) and $\text{NO}_3\text{-N}$ removal was studied. In addition, there are only a few studies on denitrifiers which can utilize PHB as an electron donor^{11,12}, and no report about bacterial community concerned with denitrification process using biodegradable plastic like PHB. We therefore determined the composition of the bacterial community which contributed to denitrification in the PHB embedded soil-column reactor by PCR-DGGE analysis.

MATERIALS AND METHODS

PHB embedded soil-column reactor The PHB embedded soil-column reactor is shown in Fig.1. The reactor column (length:1000 mm, ID:150 mm, inner volume: 15l) was made of polyvinyl chloride. Masa-soil (grussoil) collected in Kumamoto Prefecture was packed in the reactor. The soil was packed from the bottom of the column up to 270 mm (lower part), and then 171g of PHB pellets were packed with soil up to 310 mm from the top (middle part) and finally the soil was packed again to the top (upper part). Sampling holes were made at each part of the column for taking effluent and soil samples. The synthetic groundwater (140 mg/l

K_2HPO_4 , 20 mg/l KH_2PO_4 , 6 mg/l NaCl, 2.8 mg/l KCl, 5.1 mg/l $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 4.1 mg/l $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ containing 108.2 mg/l KNO_3 (nitrogen concentration: 15 mg/l) was applied down-flow mode at 3 l/day and hydraulic retention time (HRT) was controlled at 6 days). The reactor was operated at room temperature (20 ~30 °C).

Analytical method Effluent and soil samples were taken from the each sampling hole at set intervals for analyses. $\text{NO}_3\text{-N}$ and $\text{NO}_2\text{-N}$ concentrations were measured according to Japanese Standard Methods for Water Analysis¹³. Total organic carbon concentration (TOC) in effluents was measured by using TOC-5050A (Shimadzu, Kyoto) after filtration with 0.45 μm pore size membrane filter. Total number of bacteria adherent to the soil were enumerated by direct microscopic counts after staining bacterial cells with DAPI and the number of denitrifiers were determined by the MPN method using Giltay medium with PHB powder as a carbon source^{14,15}.

DNA Extraction from soil sample Two methods were used for DNA extraction from the soil. One was by using a commercial product (kit) for DNA extraction from soil (UltraClean Soil DNA Isolation Kit; MO BIO

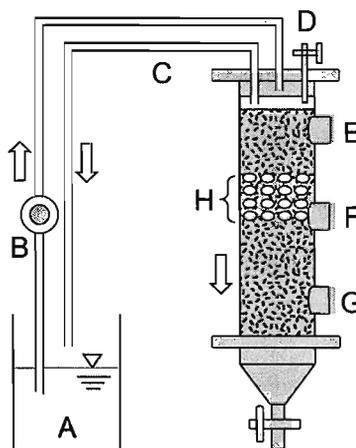


Fig. 1 Schematic diagram of the PHB embedded soil-column reactor (A, Influent tank; B, Pump; C, Overflow Pipe; D, Exhaust pipe; E, Sampling hole (upper); F, Sampling hole (middle); G, Sampling hole (bottom); H, PHB pellets)

Laboratories, Inc., California, USA) and another was the method developed by Sei *et al.*¹⁶⁾. The commercial kit method was performed according to the manufacturer's protocol, and the extracted DNA was provided for PCR (DNA sample 1). The another method was performed as follows: 0.5mg of soil was put in a 2 ml microtube, then 490 μ l of 1.0 % SDS-Extraction buffer (250mM Tris-HCl, 50mM EDTA, 125mM NaCl, pH 8.0) and 10 μ l of 10 mg/ml proteinase K were added, and kept at 37 °C for 2 hours with gentle shaking. The sample was sonicated by ultrasonicator (Model JS-150T, NISSEI, Tokyo) and purified by the phenol-chloroform extraction method. The nucleic acid was recovered by the ethanol precipitation method and dissolved in TE buffer for PCR (DNA sample 2).

PCR-DGGE analysis The amplification of DNA by PCR was performed by using a thermal cycler PC-708-02 (ASTECC, Fukuoka) as follows. The PCR cocktail without template was prepared with 10 μ l of Taq- & Go ready mix (Qbiogene inc., California, USA), 1 μ l of 25 μ M universal primer solution for 16S rDNA of bacteria (forward: GM-5F with GC-clamp; 5'-CGCCCCGCCGCGCGGGCGGGCGGGGCGGGGGCACGGGGG GCCTACGGGAGGCAGCAG-3'; reverse: DS 907R: 5'-CCCCGTCAATTCCTTTGAGTTT-3')¹⁷⁾, and 36 μ l of sterilized purified water. PCR was performed by "hot-start"¹⁸⁾ and "touch-down"¹⁹⁾ methods. The cocktail was set in the thermal cycler and DNA was denatured at 95 °C for 5 min. The temperature was then lowered to 80 °C. The 2 μ l of template DNA (extracted DNA) solution was added and PCR was restarted. The temperatures condition were set as follows: 65 °C for first annealing reaction (1 min), 72 °C for elongation (3 min) and 95 °C for denaturing again. Subsequently the PCR cycle was repeated with the annealing temperature being lowered to 55 °C in 1.0 °C increments (19 cycles) and continued 10 cycles at 55 °C for annealing. The amplified DNA (approximately 600 bps) was verified by agarose gel electrophoresis prior to DGGE. The DGGE gel was made by SJ-1060GF gradient gel maker (ATTO, Tokyo) with a 20-

60 % denaturing gradient. Electrophoresis was performed by using the AE-6290E system (ATTO) at 200 V for 6 hours with reference to literature and manufacturer's protocol²⁰⁾. The gel was put into a SYBR Green -I solution for 30 min to stain DNA bands. The DNA bands were confirmed by using the EM-20E UV transilluminator (UVP Inc., California, USA) and cut out using a plastic pipette tip. The each DNA band was put into a PCR tube and amplified again. PCR was performed with the same chemicals and primers as above, and temperature conditions were as follows: 95 °C (1 min) - 55 °C (1 min) - 72 °C (3 min) \times 35 cycles. Each PCR product was purified by Ultra Clean PCR Clean-up Kit (MO BIO Laboratories, Inc.). The DNA sequencing was performed by using the DTCS Quick Start Kit and CEQ8000 (Beckman Coulter Inc., California, USA) according to the manufacturer's protocol. The sequence of each DNA band was compared with the FASTA DNA database²¹⁾. The sequence alignment and genomic analyses were performed by using the ClustalW software²²⁾.

RESULTS AND DISCUSSION

Changes in NO₃-N concentration with time

Fig.2 shows the time courses of NO₃-N concentrations at each sampling position (lower, middle, and upper) of the column. After 30 days, NO₂-N concentrations were less than 0.01 mg/l in all parts (sampling holes) of column. NO₃-N concentrations in the upper part decreased only from 15.0 mg/l to 13.1 mg/l (13% removal), while the concentrations in the middle part decreased

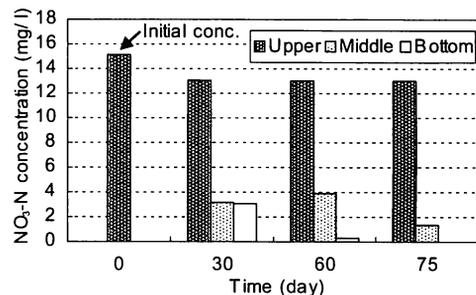


Fig. 2 Time courses of NO₃-N concentrations in each part of the reactor

to 3.16 mg/l (79% removal). In addition, the removal of $\text{NO}_3\text{-N}$ in the upper part was only 14 % even after 60 days, in comparison to 74 % in the middle part and 98 % in the lower part. Finally, $\text{NO}_3\text{-N}$ was completely removed and the TOC in effluent was around 3 mg/l at lower part after 75 days. These results indicated that the embedded PHB was degraded and used as an electron donor for indigenous denitrifiers in the soil, and the denitrification activity increased with time in the middle part. Additionally, it is considered that PHB was dissolved and flowed down, which promoted the growth of denitrifiers in the soil resulting in complete removal of $\text{NO}_3\text{-N}$ in the lower part.

Enumeration of total bacteria and PHB utilizing denitrifiers

The time courses of total bacteria at each part of the reactor is shown in Fig. 3. The total number of the bacteria was 1.2×10^{10} cells/g of wet-soil at the beginning of the study. In the upper part of the column, the total number decreased continuously with time to 5.2×10^9 cells/g of wet-soil over 75 days. On the other hand, the number in the middle part decreased to 9.2×10^8 over only 30 days, and then increased to 7.1×10^9 cells/g of wet-soil after 60 days. The numbers in the lower part also showed similar results to that of the middle part, *i.e.*, the total number decreased at first and then increased. As described above, it was assumed that the embedded PHB was degraded and served as a carbon source and an electron donor, which resulted in bacterial growth in the middle and lower parts.

However, biodegradation of solid compounds like a PHB generally require a lag time for acclimation by the bacteria; thus, it was considered that the transient decrease of total bacteria after 30 days was due to a lag time. Conversely, the number in the upper part decreased very little with time and was not significantly different from the initial value even after 75 days, even though no carbon source was provided. The reason of this phenomenon was uncertain, but it was presumed that DAPI stain method counted not only active cells but also inactive cells. The numbers of total bacteria in the reactor was almost same as that reported by Murasawa *et al.*²³.

The time course of the number of denitrifiers that can utilize PHB as an electron donor in each part is shown in Fig. 4. The number of PHB utilizing denitrifiers was quite low at the beginning; however, it increased with time in the middle and lower parts. Meanwhile, there was no increase in the upper part during the experimental period. The number in the lower part increased about 100 times and reached 3.2×10^6 cells/g wet-soil following 75 days. This value was almost the same as that of the number of denitrifiers in an agricultural soil reported by others²⁴, which suggests that the PHB utilizing denitrifiers grew well in the PHB embedded soil-column reactor. The increase in the number of the denitrifiers in the middle and lower parts with time was associated with the decrease of $\text{NO}_3\text{-N}$ concentrations, which confirmed the $\text{NO}_3\text{-N}$ removal ability of this PHB embedded soil-

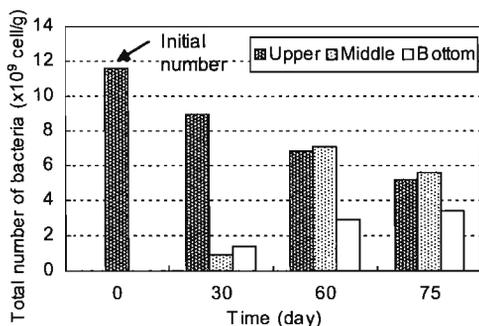


Fig. 3 Time courses of total bacteria in each part of the reactor

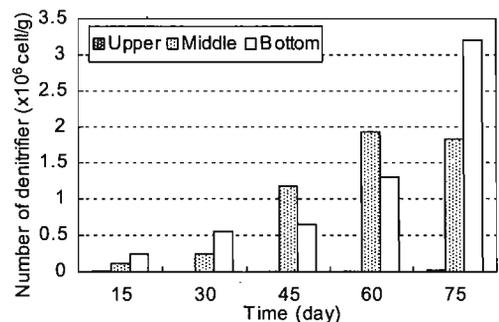


Fig. 4 Time courses of the number of denitrifiers in each part of the reactor

column reactor.

Bacterial community analysis by PCR-DGGE In this study, two methods, *i.e.*, a commercial kit method and the method developed by Sei *et al.*¹⁶⁾, were used for the extraction of DNA from soil samples in order to examine their effects on subsequent PCR-DGGE analyses. The DNA was extracted from the soil in the middle part (after 75 days operation) by both methods and amplified using PCR (as described above). The results of agarose gel electrophoresis and DGGE are shown in Figs. 5 and 6, respectively. There was no significant difference between the results on the agarose gel electrophoresis, while a little difference was evident in the DGGE band patterns. As shown in Fig. 6, the number of DNA bands in lane 1 (DNA sample 1) was more than in lane 2 (DNA sample 2); however, the DNA band No.4 in lane 2 was clear compared with that in lane 1.

The sequences of DNA bands on DGGE were determined and compared with those in the FASTA database. The sequences of 5 DNA bands on DGGE (No.1~No.5, Fig. 6) had high similarities (over 93 %) to those of known bacteria. The known bacterial species whose 16S rDNA sequences had the highest similarities are shown in Table 1. The sequence of band No.1 had a 100 % similarity to that of *Ralstonia pickettii*, which is a facultative anaerobic denitrifying bacterium. The sequence of band No.3 had the highest similarity (93.3 %) to that of *Dechloromonas* sp. SIUL, also known to have denitrification activity. In addition, it was quite interesting to find a DNA band with a high similarity (98.6 %) to *Nitrospira* sp. (band No.5), which is a common ammonium oxidizer. It was puzzling why such an aerobic nitrifier was

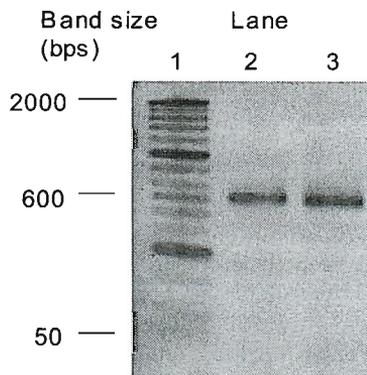


Fig. 5 Results of Agarose gel electrophoresis of PCR products (Lane 1, molecular size marker; Lane 2, DNA sample 1; Lane3, DNA sample 2)

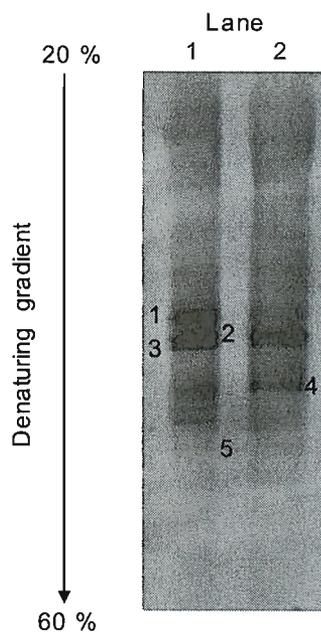


Fig. 6 DGGE profile of PCR products (Lane 1, DNA sample 1; Lane 2, DNA sample 2)

Table 1 List of bacteria closely related to the DNA sequence of bands on DGGE

Band No.	Closest relative (accession number)	% Similarity
1	<i>Ralstonia pickettii</i> (AY741342)	100
2	<i>Ideonella</i> sp. B508-1(AB049105)	93.8
3	<i>Dechloromonas</i> sp. SIUL (AF170356)	93.3
4	<i>Aquabacterium</i> sp. Aqua2 (AF089858)	99.3
5	<i>Nitrospira</i> sp. (Y14637)	98.6

coexistent in the PHB embedded soil-column reactor. From these result, it was evident that denitrifiers such as *Ralstonia* sp. and *Dechloromonas* sp. contributed to denitrification in the reactor; furthermore, it was evident that the contribution of the bacteria corresponding to band No.1 was quite high because *Ralstonia* sp. is well known not only as denitrifier in groundwater²⁵⁾ but also as the PHB degradable bacteria which have poly(3-hydroxybutyrate) depolymerase^{11, 12)}. We are currently working on isolation of unique denitrifiers that can utilize PHB in this reactor.

SUMMARY

The denitrification activity and the composition of the bacterial community in a PHB embedded soil-column reactor were studied, and the following results were obtained:

- 1) NO₃-N was significantly removed in the middle part of the reactor where PHB was embedded following 60 days of operation, which indicated that PHB was utilized as an electron donor for denitrifier in this reactor.
- 2) The total number of bacteria in the middle and lower parts of the reactor initially decreased after the first 30 days, and then increased again following 60 days of operation. Conversely, the number of PHB utilizing denitrifiers in the middle and lower parts of the column gradually increased with time. These results suggested that PHB utilizing denitrifier grew well and contributed to the denitrification capability in the reactor.
- 3) Two kinds of denitrifiers were identified from the results of PCR-DGGE analyses, from which it was suggested that bacteria closely related to the well known denitrifier *Ralstonia* sp. contributed to denitrification capability in the reactor.

REFERENCES

- 1) Tase, N.: Present situation and research on nitrate concentration in ground water, *J. Japan Soc. Water Environ.*, 26, 546-550 (2003)
- 2) Maruyama, M.: Measures of ministry of the environment for nitrate and nitrite contaminated groundwater, *J. Japan Soc. Water Environ.*, 26, 567-571 (2003)
- 3) Koori, N.: Sustainable agriculture and the measures against water pollution derived from nitrate nitrogen, *J. Japan Soc. Water Environ.*, 26, 551-556 (2003)
- 4) Tanaka, Y.: Present situation and future trends of wastewater treatment technology for animal farming, *J. Japan Soc. Water Environ.*, 26, 557-562 (2003)
- 5) Ii, H.: Countermeasure of contaminated groundwater by nitrate nitrogen, *J. Japan Soc. Water Environ.*, 26, 563-566 (2003)
- 6) Akunna, J. C., Bizeau, C., and Moletta, R.: Nitrate and nitrite reductions with anaerobic sludge using various carbon sources: Glucose, glycerol, acetic acid, lactic acid and methanol, *Water Res.*, 27, 1303-1312 (1993)
- 7) Kim, Y.-S., Nakano, K., Lee, T.-J., Kanchanatawee, S., and Matsumura, M.: On-site nitrate removal of groundwater by an immobilized psychrophilic denitrifier using soluble starch as a carbon source, *J. Biosci. Bioeng.*, 93, 303-308 (2002)
- 8) Grabinska-Noniewska, A., Slomczynski, T., and Kanska, Z.: Denitrification studies with glycerol as a carbon source, *Water Res.*, 19, 1471-1477 (1985)
- 9) Jin, H., Urakami, T., and Furukawa, K.: Denitrification of groundwater using biodegradable plastic poly-β-hydroxybutyrate, *Japan. J. Water Treat. Biol.*, 39, 183-188 (2003)
- 10) Jin, H., Morita, M., Kawagoshi, Y., Maruyama, S., Urakami, T., and Furukawa, K.: Comparison of availability of various biodegradable plastics as H-donor for denitrification, *Japan. J. Water Treat. Biol.*, 40, 45-52 (2004)
- 11) Sei, K., Nakao, M., Mori, K., Ike, M., Kohno, T., and Fujita, M.: Design of PCR primers and a gene probe for extensive detection of poly(3-hydroxybutyrate) (PHB)-degrading bacteria possessing fibronectin type III linker type-PHB depolymerases, *Appl. Microbiol. Biotechnol.*, 55, 801-6 (2001)
- 12) Saito, T., Suzuki, K., Yamamoto, J., Fukui,

- T., Miwa, K., Tomita, K., Nakanishi, S., Odani, S., Suzuki, J., and Ishikawa, K.:** Cloning, nucleotide sequence, and expression in *Escherichia coli* of the gene for poly (3-hydroxybutyrate) depolymerase from *Alcaligenes faecalis*, *J. Bacteriol.*, 171, 184-9 (1989)
- 13) **JWWA:** *Japan Water Works Association method 2001*, Japan Water Works Association, Tokyo, (2001)
- 14) **Alexandre, M.:** *Denitrifying bacteria. In Methods of soil analysis, part 2*, American Society of Agronomy Inc., Wisconsin, (1965)
- 15) **Nishio, T.:** *Dojo biseibutu jikken-hou*, Yokendo, Tokyo, (1997)
- 16) **Sei, K., Asano, K.-I., Tateishi, N., Mori, K., Ike, M., Kohno, T., and Fujita, M.:** Development of Simple Methods of DNA Extraction from Environmental Samples for Monitoring Microbial Community Based on PCR, *Japan. J. Water Treat. Biol.*, 36, 194-204 (2000)
- 17) **Weisburg, W. G., Barns, S. M., Pelletier, D. A., and Lane, D. J.:** 16S Ribosomal DNA Amplification for Phylogenetic Study, *J. Bacteriol.*, 173, 697-703 (1991)
- 18) **Chou, Q., Russell, M., Birch, D., Raymond, J., and Bloch, W.:** Prevention of pre-PCR mis-priming and primer dimerization improves low-copy-number amplifications, *Nucl. Acids Res.*, 20, 1717-1723 (1992)
- 19) **Don, R., Cox, P., Wainwright, B., Baker, K., and Mattick, J.:** 'Touchdown' PCR to circumvent spurious priming during gene amplification, *Nucl. Acids Res.*, 19, 4008- (1991)
- 20) **Ishii, K., Nakagawa, T., and Fukui, M.:** Application of Denaturing Gradient Gel Electrophoresis approach to microbial ecology, *Microbes and Environ.*, 15, 59-73 (2000)
- 21) **Pearson, W. R., and Lipman, D. J.:** Improved tools for biological sequence comparison, *Proc. Natl. Acad. Sci. USA*, 85, 2444-2448 (1988)
- 22) **Thompson, J. D., Higgins, D. G., and Gibson, T. J.:** CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice, *Nucl. Acids Res.*, 22, 4673-4680 (1994)
- 23) **Murasawa, K., Osamura, K., Morisaki, H., Sano, A., Terada, T., and Tainaka, Y.:** Direct denitrification of a canal using fatty acid compounds as hydrogen donors, *J. Japan Soc. Water Environ.*, 25, 737-742 (2002)
- 24) **Iwata, S., Oka, T., Kida, T., and Suzuki, K.:** *Tuti no Kankyo-ken*, Fuji Techno-system, Tokyo, (2002)
- 25) **Park, J., Kukor, J. J., and Abriola, L. M.:** Characterization of the adaptive response to trichloroethylene-mediated stresses in *Ralstonia pickettii* PKO1, *Appl. Environ. Microbiol.*, 68, 5231-40 (2002)

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