

Change in the Bacterial Community of Natural River Biofilm during Biodegradation of Aniline-Derived Compounds Determined by Denaturing Gradient Gel Electrophoresis

Ruben Araya, Nobuyasu Yamaguchi, Katsuji Tani, and Masao Nasu*

Environmental Science and Microbiology, Graduate School of Pharmaceutical Sciences, Osaka University, 1–6, Yamada-oka, Suita, Osaka 565–0871, Japan

(Received May 26, 2003; Accepted July 5, 2003)

The biodegradation capacity of planktonic cells and biofilms obtained from a natural river located in an industrial area was compared through the use of a river die-away biodegradation test. The change in the bacterial community during the biodegradation process was also investigated using denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rDNA fragments and multidimensional scaling (MDS) analysis of banding patterns in DGGE gel. The bacterial community structure of biofilm formed on ceramic slides submerged in natural river water were similar to those formed on natural stones in the river bed, that is, ceramic is a more suitable material for forming natural biofilm than glass or polycarbonate. Biodegradation of aniline-derived compounds revealed that aniline was easily biodegraded by both planktonic and biofilm communities. *N*-methylaniline, a difficult compound to biodegrade, could not be biodegraded by planktonic bacterial cells, although biofilm communities could biodegrade this compound within 7 days. The banding pattern in DGGE gel showed that one dominant bacterium appeared during biodegradation of aniline and the bacterial community was disturbed by the addition of chemicals, although bacteria formed a relatively stable community after biodegradation was completed. DGGE of PCR-amplified 16S rDNA fragments and MDS analysis of banding patterns in DGGE gel are available for monitoring bacterial populations in the biodegradation test system. These results should be useful to improve the river die-away biodegradation test.

Key words — biodegradation, biofilm, river die-away test, freshwater environment, bacterial community structure, denaturing gradient gel electrophoresis

INTRODUCTION

The spread of pollutants in aquatic ecosystems, as the product of accidental spills attributable to anthropogenic activities, represents a real risk for ecosystems as well as for human beings. It is now well established that most of the heterotrophic microbial activity in rivers resides in sessile bacteria or biofilms,^{1,2)} which play an important role in the degradation of natural and artificial substances.³⁾ Their contribution to river self-purification is poorly understood, however, in contrast to our knowledge of marine and freshwater lake microbiology.⁴⁾ Investigation of the biodegradation capacity of river biofilm

communities is indispensable in assessment of the impact of natural biodegradation on cleaning strategies in river ecosystems.

The current interest in the fate of xenobiotic compounds and their effects on the ecosystem, including aquatic environments, has resulted in the development of a variety of screening tests to determine the biodegradability of xenobiotics.^{5–7)} However, the described river die-away tests, which evaluate the biodegradation of chemical compounds, do not consider the effect of biofilms on biodegradation, because most tests are performed routinely using several microbial sources, such as running water, sewage, activated sludge, and soil.^{8,9)}

The development of new molecular methods applicable to microbial ecology has made it possible to study the effects of chemical contamination on the structure of microbial communities without the bias introduced by culture-dependent methods.^{10–13)}

*To whom correspondence should be addressed: Environmental Science and Microbiology, Graduate School of Pharmaceutical Sciences, Osaka University, 1–6, Yamada-oka, Suita, Osaka 565–0871, Japan. Tel.: +81-6-6879-8170; Fax: +81-6-6879-8174; E-mail: nasu@phs.osaka-u.ac.jp

Denaturant gradient gel electrophoresis (DGGE),¹⁴⁾ which separates similarly sized DNA fragments with different sequences generated by PCR amplification, in conjunction with statistical tools such as the diversity index¹⁵⁾ and multidimensional scaling (MDS) analysis,¹⁶⁾ has already been applied successfully to the examination of structural diversity in microbial communities.^{17,18)}

In this study, a river die-away test, known as a TOC-HANDAI test,⁷⁾ was applied to river microcosms specifically to compare the biodegradation capacity between biofilms and free-living (planktonic) bacterial cells. Chemical compounds, such as aniline and *N*-methylaniline, were investigated for biodegradation due to their broad use in the manufacture of pesticides, dyes, plastics, and pharmaceutical products. Additionally, biofilm and planktonic microbial communities with and without chemical addition were monitored during the study period using MDS analysis from generated DGGE profiles, with the purpose of evaluating microbial community changes during the biodegradation process.

MATERIALS AND METHODS

Sampling Site — Biofilms and planktonic bacterial cells were collected from Juhachijo (34°44'91"N, 135°29'81"E) in the Kanzaki River located in an industrial area of Osaka, Japan, during October and November 2001. This river is regarded as a eutrophic system due to the high level of total organic carbon exceeding 10 mg/l.¹⁹⁾

Artificial River Water — Contents of artificial river water (ARW) are shown in Table 1. A diluted solution used for biochemical oxygen demand (BOD) measurement was used as ARW. Since ARW becomes cloudy when it is autoclaved, only deionized water was autoclaved and the four stock solutions were sterilized by filtration (MILLEX-GS, 0.22- μ m pore size; Millipore).

Preparation of Natural Biofilm — The bacterial community structures of biofilms, grown at Juhachijo for 1 week on natural stones (collected in the Kanzaki River) and on polycarbonate, glass, and ceramic slides, were compared by DGGE analysis to determine the most representative surface for biofilm formation. All stones and slides were cleaned, H₂O₂ treated (to prevent contamination by carbon from detergent), and sterilized (180°C for 2 hr in a dry oven) before being submerged in the river water.

Table 1. Stock Solution of Artificial River Water

1. Buffer (pH 7) ^{a)}	K ₂ HPO ₄	21.8 g
	KH ₂ PO ₄	8.5 g
	Na ₂ HPO ₄ ·12H ₂ O	44.6 g
	NH ₄ Cl	1.7 g
	Deionized water	1 l
2. MgSO ₄ solution ^{a)}	MgSO ₄ ·7H ₂ O	22.5 g/l
3. CaCl ₂ solution ^{a)}	CaCl ₂	27.5 g/l
4. FeCl ₃ solution ^{a)}	FeCl ₃ ·6H ₂ O	0.25 g/l

^{a)} Each stock solution was dissolved in deionized water (0.1%) for use as artificial river water.

Preparation of Microbial Source for Microcosms

— River biofilms formed on ceramic slides and planktonic cells were collected and stored under aseptic conditions in sterile plastic tubes (50 ml) and Duran Shott bottles (500 ml), respectively. The samples were refrigerated and transported to the laboratory within 1 hr of collection for the study. Bacterial inocula from biofilms and planktonic cells were prepared as follows: ceramic slides with biofilm were washed carefully with sterilized deionized water (filtered through a cellulose acetate filter [0.2- μ m pore size; ADVANTEC, Tokyo, Japan] and autoclaved) to remove free cells from the surface and then distributed in the microcosms. Planktonic bacterial cells from surface water were prefiltered through a polypropylene filter (10- μ m pore size; Millipore) and concentrated with a filter trap on polycarbonate filters (diameter, 47 mm; 0.2- μ m pore size; ADVANTEC), and then removed with 1 min of vortexing and resuspended in ARW. The recovery rates of bacterial cells from polycarbonate filters were confirmed by epifluorescent microscopy with DAPI staining (total direct counting)²⁰⁾ and the rates were greater than 90% using this procedure. Microbial number in the microcosms was adjusted to 10⁶ cells/ml at the beginning of the experiments to compare biodegradation capacity between planktonic and biofilm bacterial cells with the same biomass.

River Microcosms — The experimental procedure is shown in Fig. 1. The microcosms consisted of glass sterile beakers (volume: 3 l, previously treated with H₂O₂). Each microcosm was filled with 2 l of ARW. Test systems consisted of ARW plus microbial source (biofilms or planktonic bacterial cells) and test chemicals (aniline or *N*-methylaniline; Nacalai Tesque, Kyoto, Japan) as a sole carbon source with a final concentration of 20 mg/l. Con-

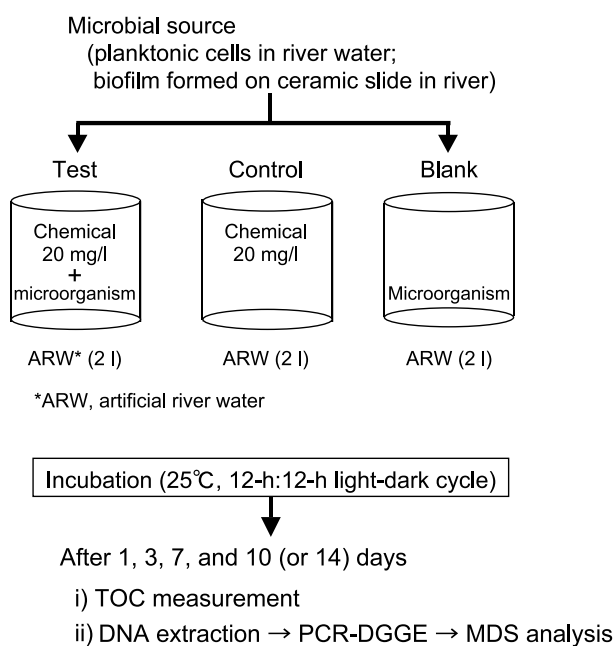


Fig. 1. Experimental Procedure Based on the River Die-Away Test

control systems to evaluate natural biodegradation of chemicals in the absence of a microbial component were composed of a test chemical at a final concentration of 20 mg/l. Blank systems designed to evaluate changes in the population of microbial source without chemical addition were comprised of ARW and microbial source. All the microcosms were covered by aluminum foil and performed in duplicate in an ambient-controlled laboratory at 25°C, with shaking at 40 rpm and illuminated by fluorescent lamps providing illumination on a 12 : 12-hr light:dark period.

After 1, 3, 7, and 10 (or 14) days, samples were obtained to estimate the biodegradation and monitor community changes by PCR-DGGE as described below. To confirm the reproducibility of the results, the experiment was performed twice, during November and December 2001.

Biodegradation was evaluated in triplicate subsamples of each test and control system, following the TOC-HANDAI method⁷⁾ using a TOC-5000 analyzer (Shimadzu). Biodegradability was calculated as described in the modified OECD Screening Test Guidelines.⁹⁾

DNA Extraction and Purification — To extract DNA from planktonic cells, ARW from the systems was filtered with sterile polycarbonate filters (0.2- μ m pore size; ADVANTEC) to concentrate bacterial cells. To extract DNA from biofilm-forming

cells, biofilms were removed from the slides or natural stones with a sterile razor blade and sonicated for 2 min at 125 W, 400 kHz with a JUS-S01 bathsonicator (JEOL, Tokyo, Japan). To remove the majority of the remaining cells, the slides or stones were washed several times with sterile deionized water and finally concentrated in sterile plastic tubes at a final volume of 30 ml. The bacterial suspensions were filtered through polycarbonate filters like planktonic cells. Bacterial DNA for DGGE analysis was extracted using the method of Tsai and Olson,²¹⁾ which uses freeze and thaw treatments for cell lysis. The RNA molecules in the crude DNA extracts were removed by incubation with heat-treated pancreatic RNase A (final concentration: 0.2 μ g/ μ l) for 2 hr at 37°C. RNA-free DNA was then purified for PCR amplification with an Elutip-d column (Schleicher & Schuell, Keene, NH, U.S.A.) attached to a Schleider & Schuell NA010/27 prefilter (0.45- μ m pore size cellulose acetate). DNA was recovered from the column as suggested by the manufacturer.

Primers, PCR Amplification, and DGGE Analysis — 16S rDNA fragments were amplified using EUB f933 (5'-GC-clamp-GCACAAGCGG-TGGAGCATGTGG-3') and EUB r1387 (5'-GCCCGGGAACGTATTCACCG-3') primers, which are specific for universally conserved bacterial 16S rDNA sequences.¹⁷⁾ A 40-bp GC-rich sequence (GC-clamp; 5'-CGCCCCGCGCGCGCGGGCGGGCGG-GGCGGGGGCACGGGGGG) was attached to the 5' end of primer EUB f933 to prevent complete melting of the DNA fragments during DGGE analysis.²²⁾ PCR was carried out using AmpliTaq Gold (Applied Biosystem) reagents in 50 μ l of PCR reaction mixture with 3 mM of MgCl₂ and 20 pmol of each primer. A hot-start PCR was performed at 95°C for 9 min and a touchdown PCR was performed as follows: the annealing temperature was initially set at 65°C and was then decreased by 0.5°C every cycle until 55°C, followed by primer extension at 72°C for 3 min. Then 15 additional cycles were carried out at 55°C for 1 min (primer annealing) followed by denaturing at 94°C for 1 min, and primer extension was performed at 72°C for 3 min. The final extension step was 7 min at 72°C.

DGGE analysis was performed as described previously.¹⁷⁾ The electrophoresis was run at 55°C for 12 hr at 100 V. After electrophoresis, the gels were stained for 20 min with SYBR Gold nucleic acid gel stain (Molecular Probes). DGGE gels were scanned with a FluorImager (Molecular Dynamics) for digitalization using a 488-nm argon laser. Images were

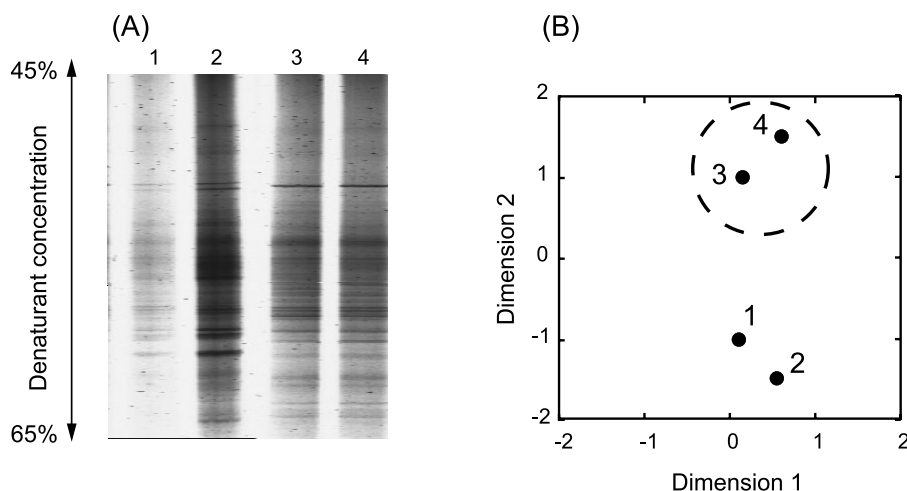


Fig. 2. Bacterial Community Structure in Biofilm Formed on (1) Polycarbonate, (2) Glass, (3) Ceramic, and (4) Natural Stones on the River Bed

DGGE profile (A) and two-dimensional plots of MDS analysis (B). 100% denaturant contains 7 M urea and 40% formamide.

analyzed using Image QuaNT (ver. 4-2-J) to generate a densitometric profile. The bands were detected when the relative peak height to total peak height exceeded 1%. To avoid the effect of nonreproducible PCR biases, parallel PCR amplifications from the same sample were compared, obtaining identical DGGE profiles.

DGGE banding patterns were analyzed by MDS analysis¹⁶⁾ as described previously¹⁷⁾ to assess changes in the genetic diversity of bacterial communities over time. MDS is a mathematical technique that generates a spatial configuration map of data points (MDS map). This MDS map shows every band pattern as one plot, and relative changes in community structure can be visualized and interpreted as the distance among the plots, that is, distances between points reflect the relationships of individual data. For this purpose, the presence and absence of DGGE bands and their overall intensity profiles were recorded in a binary matrix, which then was analyzed using SPSS 10.0 J (SPSS Japan) using a stress value of < 0.1.

RESULTS AND DISCUSSION

Preparation of Natural Biofilm

The microbial source used in river microcosms should be similar to the microbial population in the natural river environment. In this study, planktonic cells or biofilm were used as microbial sources, and the procedure for preparation of biofilm was first

determined. The bacterial community structure of biofilms grown under *in situ* conditions on ceramic, glass, and polycarbonate slides was compared with that of biofilms obtained from natural stone controls *via* MDS analysis (Fig. 2B) from DGGE profiles (Fig. 2A). Recovery rates from the surfaces were confirmed by total direct counting, and more than 90% of the bacterial cells were removed from the surfaces of slides or stones. The number of bands observed in the DGGE profile provides an estimate of species richness, and the relative intensity of each band provides a rough estimate of the relative abundance of each species. MDS was applied to the DGGE banding pattern to illustrate the similarity of all possible pairs of each gel track. The positive and negative values displayed along the X and Y axes of the figure are simply for plotting purposes. The favorable conditions of ceramic slides for the simulation of natural biofilm formation were reflected in the similarities of DGGE profiles between biofilms grown on ceramic slides and natural stones. The utility of ceramic as an artificial substrate for the study of biofilm bacterial populations in river microcosms clearly exceeds that of other substances employed in ecologic studies on biofilm bacterial communities.²³⁾ Thus ceramic slides were used in the following experiments on natural biofilm formation.

Biodegradation of Aniline and *N*-Methylaniline by Planktonic Cells and Biofilm in a River

The biodegradability of aniline and *N*-methylaniline by planktonic and biofilm bacterial

populations is shown in Fig. 3. Biodegradation curves for corresponding experimental rounds of duplicate microcosms were both similar and reproducible. The reproducibility of the results was also confirmed by experiments performed two times in November and December 2001.

The biodegradation of aniline showed that both planktonic and biofilm bacterial communities initiated mineralization of this chemical compound after 1 day. The maximum biodegradation rate was observed between days 1 and day 3, with 80% biodegradation. After this period, the biodegradation curve did not show any further significant change until day 10 (Fig. 3A). *N*-methyl aniline was not biodegraded by planktonic bacterial cells during the study period (10 days). Biofilm bacterial communities biodegraded *N*-methyl aniline after day 3 and achieved greater than 80% biodegradation during 7 days of incubation (Fig. 3B). The easy biodegradation of aniline observed in this study by planktonic and biofilm bacterial communities is in accordance with results obtained for free-living cells.⁷⁾ Similarly, the inability to degrade *N*-methyl aniline seen in this study is in agreement with that previously observed in planktonic cells from polluted and unpolluted rivers.⁷⁾ In contrast, bacterial communities associated with biofilms can biodegrade *N*-methyl aniline, as shown in Fig. 3B. This may be explained by the complex assemblages of biofilm-related microbial communities in comparison with planktonic cells.³⁾ In some cases, although a complete pathway for the biodegradation of a particular substrate may not exist in a single organism, partial and complementary pathway segments may exist in communities of organisms. Bacterial cells associated with biofilms appear to degrade target substrates through such cometabolic processes in which different microorganisms contribute to the metabolic pathway.²⁴⁾ This phenomenon has been described during the biodegradation of polychlorinated biphenyls (PCBs), where microorganisms usually metabolize only one aromatic ring and accumulate the others as the corresponding chlorobenzoates.^{25,26)}

MDS Analysis of DGGE Banding Patterns

Changes in the bacterial community in biofilm during the biodegradation of aniline were investigated by DGGE of PCR-amplified 16S rDNA fragments (Fig. 4A) and MDS analysis (Fig. 4B). A single band indicated by the signpost in Fig. 4A (band 1) appeared in the test system (with chemical) after day 3 and it was the most intense band in

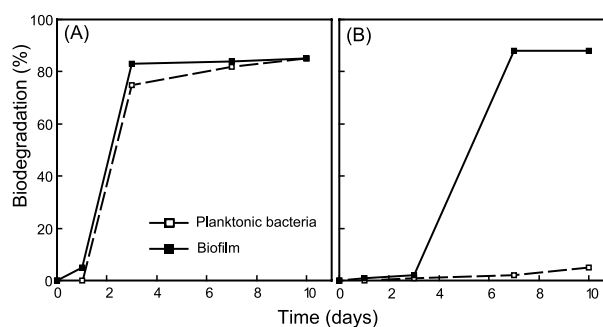


Fig. 3. Biodegradation of Aniline (A) and *N*-Methylaniline (B) by Planktonic Bacteria (□) and Biofilm (■). Data represent means of triplicate samples.

all cases. The relative intensity of this band decreased after day 3. This band did not appear in the blank system (without chemical). As shown in Fig. 3A, the biodegradation of aniline by biofilm started on day 1. The maximum biodegradation rate was observed between day 1 and day 3. After day 3, the biodegradation curve did not show any further significant change. Thus the dominant bacterium appearing after day 3 may be an aniline degrader. We previously investigated the changes in bacterial number during aniline biodegradation using culture-dependent methods and found that the aniline degrader increased when the biodegradation rate was maximum.²⁷⁾ Our results indicate that the bacterial community from the Kanzaki River, especially biofilm, can adapt well to aniline-derived compounds. DGGE also enables us to obtain sequence data on dominant species from individual bands,^{17,28)} that is, bacteria that contribute to the biodegradation of test chemicals can be identified by sequencing the PCR fragment excised from DGGE gel.

By comparing the biodegradation curve (Fig. 3A) and results of MDS analysis (Fig. 4B), the bacterial community was disturbed by the addition of aniline, and bacteria in biofilm formed a relatively stable community after biodegradation was completed. Without chemical addition (blank system), the bacterial community changed continuously and did not form a stable structure through the study period. MDS analysis of DGGE banding patterns provided an interpretable picture of the changes that occurred during biodegradation processes.

This study illustrates the important contribution of biofilm communities to the river die-away test, due to their high abundance, physiological activity,²⁹⁾ and capacity to biodegrade chemical compounds, and hence the significant participation of these cells

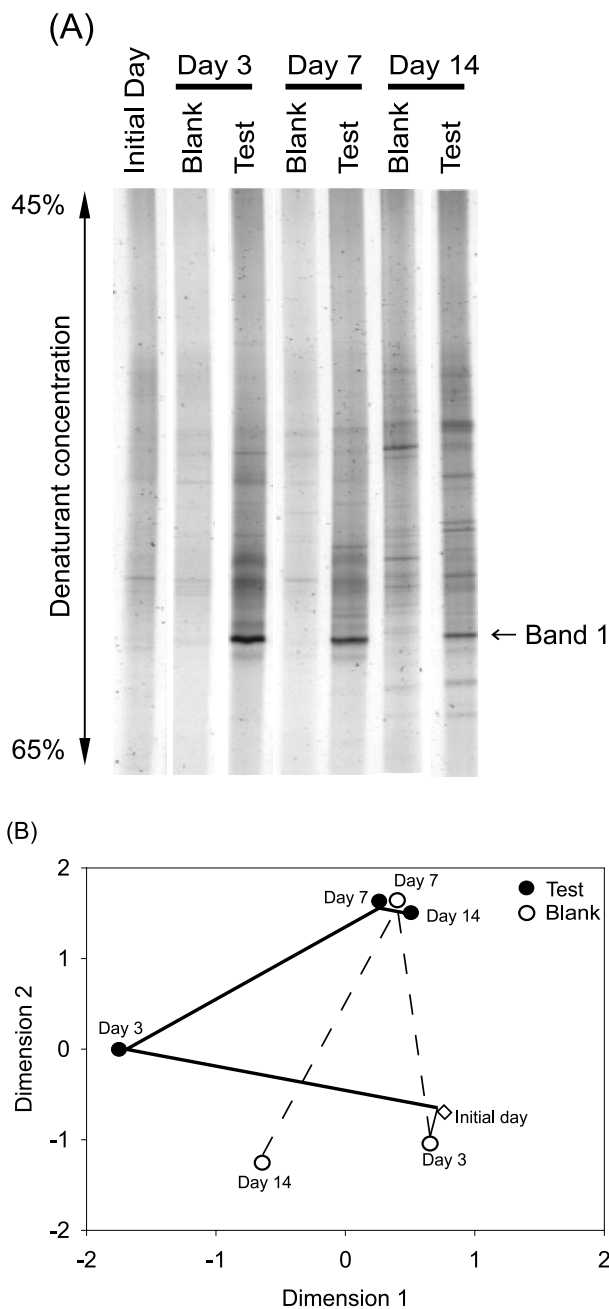


Fig. 4. Change in Bacterial Community Structure in Biofilm during Biodegradation of Aniline

DGGE profile (A) and two-dimensional plots of MDS analysis (B). Band 1, specific band appearing during biodegradation. 100% denaturant contains 7 M urea and 40% formamide.

in the biodegradation capacity of river ecosystems. Biofilm should be used in river die-away tests as a microbial source, and bacterial populations in this test system could be monitored by MDS analysis following PCR-DGGE, which enables us to understand the dynamics of degraders in natural river environments.

Acknowledgements This study was supported by the Science and Technology Agency, Japan (Promotion System for Intellectual Infrastructure of Research and Development, under special Coordination Funds for Promoting Science and Technology).

REFERENCES

- 1) Costerton, J. W., Irvin, R. T. and Cheng, K. J. (1981) The bacterial glycocalyx in nature and disease. *Annu. Rev. Microbiol.*, **35**, 299–324.
- 2) Lock, M. A., Wallace, R. R., Costerton, J. W., Ventullo, R. M. and Charlton, S. E. (1984) River epilithon: toward a structural-functional model. *Oikos*, **42**, 10–22.
- 3) Costerton, J. W., Cheng, K. J., Geesey, G. G., Ladd, T. I., Nickel, J. C., Dasgupta, M. and Marrie, T. J. (1987) Bacterial biofilms in nature and disease. *Annu. Rev. Microbiol.*, **41**, 435–464.
- 4) Left, L. (1994) Stream bacterial ecology: A neglected field? *ASM News*, **60**, 135–138.
- 5) Cripe, C. R., Walker, W. W., Pritchard, P. H. and Bourquin, A. W. (1987) A shake-flask test for estimation of biodegradability of toxic organic substances in the aquatic environment. *Ecotoxicol. Environ. Saf.*, **14**, 239–251.
- 6) Lee, C., Russel, N. J. and White, G. F. (1995) Modelling the kinetics of biodegradation of anionic surfactants by biofilm bacteria from polluted riverine sites: a comparison of five classes of surfactant at three sites. *Water Res.*, **29**, 2491–2499.
- 7) Yamaguchi, N., Ohmori, H., Welikala, N. and Nasu, M. (1997) Biodegradation of chemical compounds in a newly developed modified river die-away test. *Jpn. J. Toxicol. Environ. Health*, **43**, 209–214.
- 8) Organization for Economic Cooperation and Development (OECD). (1981) Environment Directorate. In *OECD Guidelines for Testing of Chemicals* (OECD Environmental Directorate, Ed.), OECD Publications, Paris, p. 301C.
- 9) Organization for Economic Cooperation and Development (OECD). (1981) Environment Directorate. In *OECD Guidelines for Testing of Chemicals* (OECD Environmental Directorate, Ed.), OECD Publications, Paris, p. 301E.
- 10) Wolfaardt, G. M., Lawrence, J. R., Robarts, R. D. and Caldwell, D. E. (1995) Bioaccumulation of the herbicide Diclofop in extracellular polymers and its utilization by a biofilm community during starvation. *Appl. Environ. Microbiol.*, **61**, 152–158.
- 11) Engelen, B., Meinken, K., von Wintzingerode, F., Heuer, H., Malkomes, H. P. and Backhaus, H. (1998)

- Monitoring impact of a pesticide treatment on bacterial soil communities by metabolic and genetic fingerprinting in addition to conventional testing procedures. *Appl. Environ. Microbiol.*, **64**, 2814–2821.
- 12) Wu, Q., Sowers, K. R. and May, H. D. (2000) Establishment of a polychlorinated biphenyl-dechlorinating microbial consortium, specific for doubly flanked chlorines, in a defined, sediment-free medium. *Appl. Environ. Microbiol.*, **66**, 49–53.
 - 13) Gieseke, A., Purkhold, U., Wagner, M., Amann, R. and Schramm, A. (2001) Community structure and activity dynamics of nitrifying bacteria in a phosphate-removing biofilm. *Appl. Environ. Microbiol.*, **67**, 1351–1362.
 - 14) Muyzer, G., de Waal, E. C. and Uitterlinden, A. G. (1993) Profiling of complex microbial populations by denaturant gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.*, **59**, 695–700.
 - 15) Eichner, C. A., Erb, R. W., Timmis, K. N. and Döbler, I. W. (1999) Thermal gradient gel electrophoresis analysis of bioprotection from pollutants shocks in the activated sludge microbial community. *Appl. Environ. Microbiol.*, **65**, 102–109.
 - 16) Van Hannen, E. J., Veninga, M., Bloem, J., Gons, H. J. and Laanbroek, H. J. (1999) Genetic changes in the bacterial community structure associated protistan grazers. *Arch Hydrobiol.*, **145**, 25–38.
 - 17) Iwamoto, T., Tani, K., Nakamura, K., Suzuki, Y., Kitagawa, M., Eguchi, M. and Nasu, M. (2000) Monitoring impact of in situ biostimulation treatment on groundwater bacterial community by DGGE. *FEMS Microbiol. Ecol.*, **32**, 129–141.
 - 18) Schäfer, H., Bernard, L., Courties, C., Lebaron, P., Servais, P., Pukall, R., Stackebrandt, E., Troussellier, M., Guindulain, T., Vives-Rego, J. and Muyzer, G. (2001) Microbial community dynamics in Mediterranean nutrient-enriched seawater mesocosms: changes in the genetic diversity of bacterial populations. *FEMS Microbiol. Ecol.*, **34**, 243–253.
 - 19) Kenzaka, T., Yamaguchi, N., Prapagdee, B., Mikami, E. and Nasu, M. (2001) Bacterial community composition and activity in urban rivers in Thailand and Malaysia. *J. Health Sci.*, **47**, 353–361.
 - 20) Hobbie, J. E., Daley, R. J. and Jasper, S. (1977) Use of nucleopore filters for counting bacteria by fluorescence microscopy. *Appl. Environ. Microbiol.*, **33**, 1225–1228.
 - 21) Tsai, Y. L. and Olson, B. H. (1991) Rapid method for direct extraction of DNA from soil and sediments. *Appl. Environ. Microbiol.*, **57**, 1070–1074.
 - 22) Myers, R. M., Fischer, S. G., Lerman, L. S. and Maniatis, T. (1985) Nearly all single base substitutions in DNA fragments joined to a GC-clamp can be detected by denaturing gradient gel electrophoresis. *Nucleic Acids Res.*, **13**, 3131–3145.
 - 23) Cunliffe, D., Smart, C. A. and Vulfson, E. N. (2000) Bacterial adhesion at synthetic surfaces. *Appl. Environ. Microbiol.*, **65**, 4995–5002.
 - 24) Kenneth, N. T. and Diemar, H. P. (1999) Bacteria designed for bioremediation. *Trends Biotechnol.*, **17**, 200–204.
 - 25) Sondossi, M., Sylvestre, M. and Ahmad, D. (1992) Effects of chlorobenzoate transportation on the *Pseudomonas testosteroni* biphenyl and chlorobiphenyl degradation pathway. *Appl. Environ. Microbiol.*, **58**, 485–495.
 - 26) Stratford, J., Wright, M. A., Reineke, W., Mokross, H., Havel, J., Knowles, C. J. and Robinson, G. K. (1996) Influence of chlorobenzoates on the utilisation of chlorobiphenyls and chlorobenzoate mixtures by chlorobiphenyl/chlorobenzoate-mineralising hybrid bacterial strains. *Arch. Microbiol.*, **165**, 213–218.
 - 27) Tani, K., Masuhara, M., Welikala, N., Yamaguchi, N. and Nasu, M. (1998) Change in bacterial community during biodegradation of aniline. *J. Appl. Microbiol.*, **84**, 859–864.
 - 28) Kawai, M., Matsutera, E., Kanda, H., Yamaguchi, N., Tani, K. and Nasu, M. (2002) 16S ribosomal DNA-based analysis of bacterial diversity in purified water used in pharmaceutical manufacturing processes by PCR and denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.*, **68**, 699–704.
 - 29) Araya, R., Tani, K., Takagi, T., Yamaguchi, N. and Nasu, M. (2002) Bacterial activity and community composition in stream water and biofilm from an urban river determined by fluorescent in situ hybridization and DGGE analysis. *FEMS Microbiol. Ecol.*, **43**, 111–119.