A culture-independent approach for studying microbial diversity in aerobic granules

S. Yi, J.-H. Tay , A.M. Maszenan and S.T.-L. Tay

Environmental Engineering Research Centre, Division of Environmental and Water Resources Engineering, School of Civil and Structural Engineering, Nanyang Technological University, 50 Nanyang Avenue, Singapore 639798

Abstract This study reports the use of ribosomal-based molecular techniques to study the microbial diversity in aerobic granules. Aerobic granules at different growth stages (young, mature and old) were obtained from a laboratory scale sequential aerobic sludge blanket (SASB) bioreactor fed with glucose as the main source of carbon and energy. Deoxyribonucleic acid (DNA) was extracted from the young, mature and old granules. The polymerase chain reaction (PCR) was used to amplify the Eubacterial 16S ribosomal DNA (rDNA) and three clone libraries were constructed, corresponding to each of the three growth stages. The microbial diversity in each clone library was assessed by amplified ribosomal DNA restriction analysis (ARDRA). The results reveal that there was considerable diversity in each clone library and there were variations in microbial diversity among the three different clone libraries. This suggests a shift in the composition of the microbial communities. Microorganisms associated with 5 restriction fragment length polymorphism (RFLP) types (A, B, C, D and E) appear to play an important role in the development of aerobic granules.

Keywords Aerobic granules; ARDRA; 16S rDNA; microbial diversity; PCR-cloning

Water Science and Technology **Vol 47 No 1 pp 283–290** © IWA Publishing 2002

Introduction

During the early development of biological wastewater treatment systems, the "black box" philosophy was used to study these systems (Head *et al*., 1998). It is now widely accepted that a knowledge of the microbial community would assist in improving the design and performance of these systems (Cloete, 1997). In order to have a comprehensive understanding of the microbial community in wastewater treatment systems, studying the microbial diversity is the primary step (Amann and Kühl, 1998). However, defining the microbial diversity through the investigation of microbial populations in wastewater treatment systems, as well as in other natural environments, has been a long-standing challenge in microbial ecology (Amann *et al*., 1995; Pace, 1997; Amann and Kuhl, 1998).

Microbial populations present in wastewater treatment plants have been studied conventionally by culturing bacterial isolates (Snaidr *et al*., 1997; Vainio *et al*., 1997). These culture-dependent methods suffer from several limitations (Moyer *et al*., 1994; Amann *et al*., 1995; Head *et al*., 1998) and therefore are inadequate to represent the *in situ* diversity and ecophysiology for a meaningful analysis of community structure or specific organism functionality. In contrast, culture-independent techniques based on ribosomal ribonucleic acid (RNA) provide a more comprehensive, rapid and concise characterization of bacteria taxa present in discrete habitats (Amann *et al*., 1995). One such method is the PCR-cloning method which includes the following steps: 1) isolating native DNA from the natural community; 2) PCR amplification of small subunit (SSU) rRNA gene sequences with universal primers; 3) screening of clones for genetic variability and 4) using these detected variations to estimate genetic diversity and to select clones for subsequent sequencing to determine phylogenic affiliation. For screening SSU rDNA clone libraries, the amplified restriction rDNA analysis (ARDRA) of 16S rRNA genes has become an effective strategy to identify

putative operational taxonomic units (OTUs) in various microbial communities such as those from hydrothermal vent systems (Moyer *et al*., 1994); subsurface soil (Chandler *et al*., 1997); marine sediment (Urakawa *et al*., 1999), contaminated aquifer (Dojka *et al*., 1998) and also in activated sludge wastewater treatment plants (Gich *et al*., 2000).

In this study, the constituent populations in the aerobic granules were defined in terms of operational taxonomic units (OTUs). The relative abundance of individual clones within each OTU was also assessed. The aerobic granules in this study were from a laboratory-scale sequential aerobic sludge blanket (SASB) bioreactor. The SASB process offers several advantages over conventional aerobic wastewater treatment systems such as high biomass settling ability and retention, high degrading capability and high volumetric conversion capacity (Beun *et al*., 1999; Peng *et al*., 1999). Granules obtained in this study were fed by artificial wastewater containing glucose and peptone. Four different growth stages were observed associating with aerobic granules in the bioreactor. They are young, mature, old, and disintegrated stages. This study is the first report on the microbial community in this novel system based on construction of clone libraries for young, mature and old granules and estimation of microbial diversity within each clone library by performing ARDRA.

Materials and methods

Sample collection

Aerobic granules were collected from a laboratory-scale sequential aerobic sludge blanket (SASB) bioreactor. The effective volume of the reactor is 2.4 litres. Seed sludge was obtained from a wastewater treatment plant and inoculated into the reactor. A synthetic wastewater consisting of glucose, peptone and meat extract was used as the substrate for the reactor. The composition of feed is as listed in Table 1. Young and mature granules were collected 14 days after granules first appeared, and old granules were collected 7 days later. After collection, granule samples were gently washed three times with 1 × phosphate-buffered saline (PBS [0.13 mol l^{-1} NaCl, 10 mmol l^{-1} sodium phosphate, pH 7.2]). All samples were used immediately or stored at –20°C in 1 × PBS.

Genomic DNA extraction

Nucleic acids were extracted from two 200–300 mg (wet weight) aliquots each of young, mature and old aerobic granules. Direct DNA extractions were performed after cell disruption by a modification of the method described by Tay *et al*. (1998). All extracted DNAs were purified by DNA Clean System (Promega, USA). The concentration and purity of the genomic DNA was assessed by absorbance measurements and by agarose gel electrophoresis. The effectiveness of the cell lysis procedure was confirmed by microscopic examination of samples taken before and after lysis treatment.

Table 1 Synthetic wastewater composition

Components	Concentration (mg/L)	Components	Concentration (mg/L)
COD	2000	Na_2S^*	45
Glucose	1400	H_3BO_3	0.05
Peptone	400	$ZnCl_2$	0.05
Meat extract	250	$CuCl_2$	0.03
NH_4Cl	200	$MnSO_4{\cdot}H_2O$	0.05
K_2HPO_4	45	$(NH_4)_6{\cdot}Mo_7O_{24}{\cdot}4H_2O$	0.05
$CaCl_2{\cdot}2H_2O$	30	$AlCl_3$	0.05
$MgSO_4{\cdot}7H_2O$	25	$CoCl2{\cdot}H_2O$	0.05
$FeSO_4{\cdot}7H_2O$	20	$NiCl_2$	0.05

Amplification of 16S rRNA genes from genomic DNA

Bacterial 16S rDNA was amplified with forward primer Eubac27F (5′-AGAGTTTGATCMTGGCTCAG-3′) and reverse primer Universal 1492R1 (5′-TACGGYTACCTTGTTACGACTT-3′) (Tay *et al*., 1998). PCR amplification was carried out with a thermal cycler (Mastercycler, Eppendorf, Germany) under the following conditions: 94°C for 3 min; denaturation at 94°C for 1.5 min, primer annealing at 60°C for 1.5 min, extension at 72°C for 1.5 min for a total of 30 cycles. Reaction mixtures (final volume, 100 µl) contained 100 ng purified template DNA, 50 mmol l^{-1} KCl, 10 mmol l^{-1} Tris.HCl pH 8.3, 2.5 mmol l^{-1} $MgCl_2$, 5% (w/v) acetamide, 0.05% NP40, 200 µmol l^{-1} each deoxynucleoside triphosphate, 0.2 µmol l^{-1} each oligonucleotide primer, and 2.5 U of Taq polymerase (Promega, USA). The triplicate amplification products were purified using the Wizard PCR system (Promega, USA) and visualized by electrophoresis through a 2% agarose gel (Promega, USA).

Construction of 16S rRNA gene clone libraries

The 16S rDNA clone libraries of young, mature and old granules were generated by ligation of the purified 16S rDNAs into the pGEM-T vector (Promega, USA). Ligation, transformation into *Escherichia coli* JM109 competent cells, and blue/white screening were performed according to the manufacturer's instructions. For amplifying of the cloned 16S rDNA, plasmid DNAs were isolated and purified from recombinants with Wizard *Plus* MiniPreps DNA Purification System (Promega, USA). The composition of the reaction mixtures and PCR conditions of plasmid DNA amplification were as described above.

Amplified rDNA restriction analysis (ARDRA)

Following purification, the amplified 16S rDNA were checked on 2% agarose gels. Only full-length inserts (≈1500 bp) were selected for restriction enzyme digestion to define restriction fragment length polymorphism (RFLP) patterns. Aliquots (≈ 0.5 µg) of 16S rDNA were digested with restriction endonuclease *Cfo*I (Promega, USA) under the conditions specified by the manufacturer. The resulting fragments were separated by gel electrophoresis in 3% metaphor agarose gels (FMC, Rockland, Maine). To determine the similarities of bacterial populations present in three clone libraries, comparisons of the RFLP types within and among each clone library were performed using GelCompar II (version 1.5) software (Applied Maths, Belgium). Binary coefficient Dice was applied to calculate the similarity of cloned 16S rDNA fragments, and the Unweighted Pair Group Method using Arithmetic averages (UPGMA) was used to construct the dendrogram.

Calculation of community diversity

Various diversity indices were used to compare the bacterial communities associated with the three clone libraries. Species richness, which represents the total number of species or operational taxonomic units (OTUs), was calculated by rarefaction (Cho and Kim, 2000) with the online Rarefaction Calculator (*http://gause.biology.ualberta.ca/jbrzusto/rarefact.html*). Bacterial diversity was calculated on the basis of RFLP types by using the Shannon-Weaver index (H), Pielou's evenness index (e), Simpson's dominance index (c), and equitability (J). The estimated percentages of coverage (Dang and Lovell, 2000) for the different libraries were calculated as follows: [1-(n/N)] ×100, where n is the number of unique clones detected in a subsample (library) of size N.

Results and discussion

Morphology study

The sampled granules were categorized into four growth stages (Figure 1). Young granules

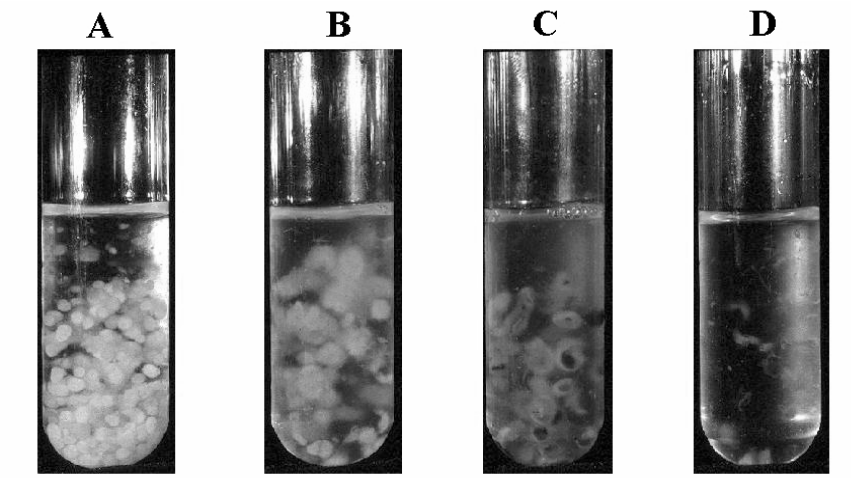

Figure 1 Four stages of aerobic granules from SASB bioreactor: a) young granules; b) mature granules; c) old granules with black cores; and d) disintegrated granules

are less than 2 mm in diameter appearing light yellow and have smooth surfaces (a). Mature granules are greater than 2 mm in diameter and have fluffy edges (b). Old granules contain a black core (c). Finally, the old granules disintegrate into fluffy flocs (d).

The observation of the different growth stages of aerobic granules implied that the development of aerobic granules might go through a sequence of specific, but poorly understood chemical and biological processes. The investigation on tracking the microbial population dynamics from young to old granules would provide important information to identify the key organisms in aerobic granules development. This information would in turn provide useful physiological indicators for the purpose of monitoring and controlling this novel wastewater treatment process.

ARDRA

A total of 144 clones containing the full-length inserts (≈1,500 bp), were digested with restriction enzyme *Cfo*I, which has been shown to be particularly effective at defining operational taxonomic units (OTUs) (Moyer *et al*., 1996; Chandler *et al*., 1997). *Cfo*I digestion of full-length inserts resulted in 2–6 easily resolved bands, which were used for RFLP cluster analysis (data not shown). The results of 16S rDNA clone library and ARDRA were summarized in Table 2. A total of 56 different RFLP types (OTUs), were identified from the three clone libraries. 21 RFLP types were detected from 45 clones from the young granules, 27 types were identified from 52 clones from mature granules, and 23 types were recognized from 47 clones from old granules. The distribution of clones in each clone library, both in RFLP types and relative abundance is plotted in Figure 2.

Diversity indices

The number of RFLP types (richness) and the frequency distribution of the RFLP types (evenness) in each of the clone libraries were evaluated by using a variety of standard diversity indices and results were summarized in Table 3. Since the libraries differed in size, estimated RFLP type richness [E(S)] was calculated by rarefaction for smaller sample sizes (40 clones) to allow standardized comparisons. The estimated value of richness in the mature clone library was much higher than that in the young and old libraries. With the exception of Simpson's dominance index as shown in Table 3, the mature and old clone libraries had higher values on diversity indices such as richness, the Shannon-Weaver diversity index, evenness, and equitability than the young clone libraries. The young clone

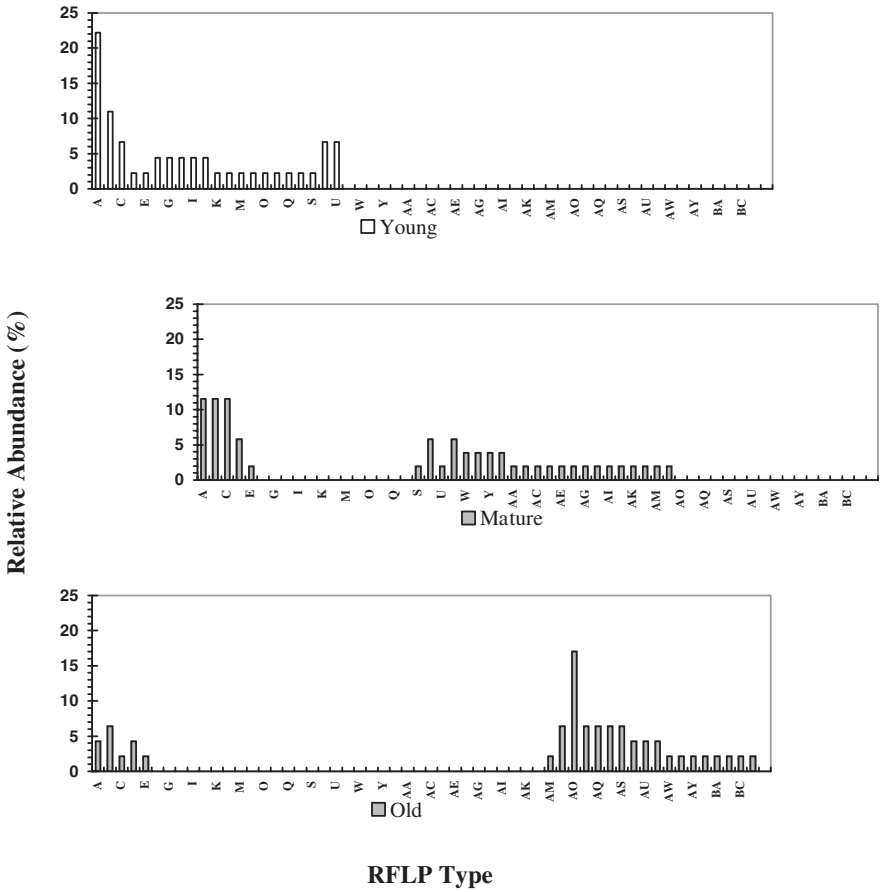

Figure 2 Distribution and relative abundance of 16S rDNA clones from different growth stages of aerobic granules

library contained a few RFLP types. Overall, the calculated diversity index values showed that the bacterial communities of mature and old granules were more diverse than that of the young granules. The values of coverage (Table 2) were also used to approximate the probability that all species present in a given sample were represented at least once in the library (Dang and Lovell, 2000). These three libraries had percent coverage values ranging from 68.6 to 77.1, which indicated that the microbial communities present in this study contained substantial diversity.

Microbial community analysis

The results of ARDRA revealed that the development of aerobic granules were dynamic and involved an assemblage of organisms. Shifts in bacteria community were observed in the three clone libraries (Figure 2). For example, 46 RFLP types were detected that were

Table 2 Summary of 16S rDNA clones and RFLP types recovered from aerobic granule samples

Growth stages	Full-length clones	RFLP types	Unique clones	Coverage %
Young	45	21	11	75.6
Mature	52	27	17	67.3
Old	47	23	11	76.6
Total	144	56		

Table 3 Diversity indices based on *Cfo* I RFLP patterns in 16S rDNA clone libraries from young, mature and old granules

Parameter	Young	Mature	Old
RFLP type richness E(S)[a]	19.7	22.8	21.2
Shannon-Weaver diversity (H)[b]	2.742395	3.034237	2.927977
Evenness (E)[c]	0.900764	0.920627	0.933817
Simpson's dominance (c)[d]	0.09037	0.06213	0.067451
Equitability (J)[e]	0.72042	0.767919	0.760484

[a] E(S)) was calculated by rarefaction for a standardized sample size of 40 clones for each clone library.
[b] H was calculated as follows: $H = -\sum(p_i)(\ln p_i)$, where p_i is the proportion for each RFLP pattern.
[c] Evenness (E) was calculated from H as follows: E= H/ln S, where S is the total number of RFLP patterns in the each clone library.
[d] c was calculated as follows: $c = \sum(p_i)^2$.
[e] J was calculated as follows: J = H/Hmax, where $Hmax = Log_2 X$ where X is the total number of clones in each library.

unique to each clone library (13 in young granules, 17 in mature granules, and 16 in old granules). Different RFLP types were also observed to dominate different clone libraries. In young granules, RFLP types A, B, C, T, and U were the most frequently occurring, but were not uniformly abundant throughout the different growth stages. In mature granules, the most commonly occurring RFLP types were A, B, C, D, T, and V. RFLP types B, AN, AO, AP, AQ, and AR dominated the old granules.

Several RFLP types were also found in more than one clone library. Eight RFLP types (A, B, C, D, E, S, T, and U) from the young granules were also found in the mature granules. Seven types (A, B, C, D, E, AM, and AN) from the mature granules were also found in the old granules. Five RFLP types, A, B, C, D, and E, appeared in all three libraries, which suggests that these 5 RFLP types may have important roles in the development of aerobic granules. The relative abundance of RFLP type A decreased from young to mature to old granules. On the other hand, the relative abundance of RFLP types B, C and D increased slightly from young to mature granules and decreased significantly in old granules. The relative abundance of RFLP type E did not change from young to mature to old granules. This finding is important since the changes in relative abundance may reflect the onset of granule lysis.

Estimating community structure and diversity at the DNA level is an invaluable tool for microbial ecology, but this strategy also has its potential problems and limitations (von Wintzingerode *et al.*, 1997; Head *et al.*, 1998). As a consequence, the clone libraries should not be viewed as quantitative representations of microbial abundance in the original community. Nevertheless, some researchers (Farrelly *et al.*, 1995; Suzuki and Giovannoni, 1996) have demonstrated that changes on the composition of clone libraries can signal temporal (or spatial) variations within identical environmental matrices and therefore should be representative of qualitative changes in the microbial community. Results obtained in this study show significant differences in clone abundance at different growth stages of aerobic granules, which probably reflect relative changes in abundance of that gene or organism in the original community.

In general, ARDRA is an effective strategy to provide a general view of how the clone libraries differ and to identify important OTUs for further analysis (Moyer *et al.*, 1994; Chandler *et al.*, 1997; Urakawa *et al.*, 1999; Gich *et al.*, 2000). In this study, ARDRA was chosen as an initial measure of genetic diversity within each clone library and the response of the microbial community during granule development. Community changes in bacterial composition and relative abundance would be due to interactions among different groups of bacteria and the micro-niches in which they reside. Community changes are therefore a

consequence of the natural phenomenon of physiological adaptation by bacteria to the surrounding environment and their mutual interactions.

Conclusion

56 RFLP types (OTUs) were identified based on the results of cluster analysis. Diversity indices revealed that bacterial communities of mature and old granules were more diverse than in young granules. Shifts in microbial populations were also confirmed by ARDRA. Such changes were attributed to physiological adaptation of bacteria during aerobic granulation process. Microorganisms associated with 5 OTUs (A, B, C, D and E) appeared in all three clone libraries at different growth stages, which suggests that these bacteria may have an important role in the development of aerobic granules. The genetic relatedness among the populations identified here will be assessed by phylogenetic analysis in a future study.

References

Amann, R and Kühl, M. (1998). *In situ* methods for assessment of microorganisms and their activities. *Current Opinion in Microbiology*, **1**, 352–358.

Amann, R, Ludwig, W. and Schleifer, K.-H. (1995). Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiological Reviews*, **59**, 143–169.

Beun, J.J., Hendriks, A., van Loosdrecht, M.C.M., Morgenroth, E., Wilderer, P.A. and Heihnen, J.J. (1999). Aerobic granulation in a sequencing batch reactor. *Wat. Res.*, **33**, 2283–2290.

Chandler, D.P., Brockman, F.J. and Fredrickson, J.K. (1997). Use of 16S rDNA clone libraries to study changes in a microbial community resulting from ex situ perturbation of a subsurface sediment. *FEMS Microbiol. Rev.*, **20**, 217–230.

Cho, J.C. and Kim, S.J. (2000). Increase in bacterial community diversity in subsurface aquifers receiving livestock wastewater input. *Appl. Envir. Microbiol.*, **66**, 956–965.

Cloete, T.E. (1997). Introduction In: *Microbial Community Analysis: the Key to the Design of Biological Wastewater Treatment Systems*, T.E. Cloete and N.Y.O. Muyima (ed.), IAWQ. London, England.

Dang, H. and Lovell, C.R. (2000). Bacterial primary colonization and early succession on surfaces in marine water as determined by amplified rRNA gene restriction analysis and sequence analysis of 16S rRNA genes. *Appl. Environ. Microbiol.*, **66**, 467–475.

Dojka, M.A., Hugenholtz, P., Haack, S.K. and Pace, N.R. (1998). Microbial diversity in a hydrocarbon- and chlorinated-solvent-contaminated aquifer undergoing intrinsic bioremediation. *Appl. Envir. Microbiol.*, **64**, 3869–3877.

Farrelly, V., Rainey, F.A. and Stackebrandt, E. (1995). Effect of genome size and rrn gene copy number on PCR amplification of 16S rRNA genes from a mixture of bacterial species. *Appl Environ Microbiol.*, **61**, 2798–2801.

Gich, F.B., Amer, E., Figueras, J.B., Abella, C.A., Balaguer, M.D. and Poch, M. (2000). Assessment of microbial community structure changes by amplified ribosomal DNA restriction analysis (ARDRA). *Int Microbiol.*, **3**, 103–106.

Head, I.M., Saunders, J.R. and Pickup, R.W. (1998). Microbial evolution, diversity, and ecology: A decade of ribosomal RNA analysis of uncultivated microorganisms. *Microbiol. Ecol.*, **35**, 1–21.

Moyer, C.L., Dobbs, F.C. and Karl, D.M. (1994). Estimation of diversity and community structure through restriction fragment length polymorphism distribution analysis of bacterial 16S rRNA genes from a microbial mat at an active, hydrothermal vent system, Loihi Seamount, Hawaii. *Appl. Environ. Microbiol.*, **60**, 871–879.

Moyer, C.L., Tiedje, J.M., Dobbs, F.C. and Karl, D.M. (1996). A computer-simulated restriction fragment length polymorphism analysis of bacterial small-subunit rRNA genes: efficacy of selected tetrameric restriction enzymes for studies of microbial diversity in nature. *Appl. Environ. Microbiol.*, **62**, 2501–2507.

Pace, N.R. (1997). A molecular view of microbial diversity and the biosphere. *Science*, **276**, 734–740.

Peng, D.C., Bernet, N., Delgenes, J.-P. and Moletta, R. (1999). Aerobic granular sludge – a case report. *Wat. Res.*, **33**, 890–893.

Snaidr, J., Amann, R., Huber, I., Ludwig, W. and Schleifer, K.H. (1997). Phylogenetic analysis and in situ identification of bacteria in activated sludge. *Appl. Environ. Microbiol.*, **63**, 2884–2896.

Suzuki, M.T. and Giovannoni, S.J. (1996). Bias caused by template annealing in the amplification of mixtures of 16S rRNA genes by PCR. *Appl. Environ. Microbiol.*, **62**, 625–630.

Tay, S.T.-L., Hemond, H.F., Polz, M.F., Cavanaugh, C.M., Dejesus, I. and Krumholz, L.R. (1998). Two New Mycobacterium Strains and Their Role in Toluene Degradation in a Contaminated Stream. *Appl. and Environ. Microbiol.*, **64**(5), 1715–1720.

Urakawa, H., Kita-Tsukamoto, K. and Ohwada, K. (1999). Microbial diversity in marine sediments from Sagmi bay and Tokyo bay, Japan, as determined by 16S rRNA gene analysis. *Microbiology*, **145**, 3305–3315.

Vainio, E.J., Moilanen, A., Koivula, T.T., Bamford, D.H. and Hantula, J. (1997). Comparison of partial 16S rRNA gene sequences obtained from activated sludge bacteria. *Appl. Microbiol. Biotechnol.*, **48**, 73–79.

von Wintzingerode, F., Gobel, U.B. and Stackebrandt, E. (1997). Determination of microbial diversity in environmental samples: pitfalls of PCR-based rRNA analysis. FEMS *Microbiol Rev.*, **21**, 213–229.