Symbiosis, 37 (2004) 335–344
Balaban, Philadelphia/Rehovot

Community Profiles of Bacterial Endosymbionts in Four Species of *Caulerpa*

LAURA DELBRIDGE[1,2], JOANNE COULBURN[1], WAYNE FAGERBERG[3], and LOUIS S. TISA[1*]
[1]*Department of Microbiology, University of New Hampshire, 46 College Rd., Durham, NH 03824-2617, USA, Tel. +1-603-862-2442, Fax. +1-603-862-2621, Emails. joanne.coulburn@unh.edu and LST@hypatia.unh.edu;*
[2]*Present address: Department of Microbiology, University of Michigan, Ann Arbor, MI 48109, USA, Email. lauradel@umich.edu;*
[3]*Department of Plant Biology, University of New Hampshire, Durham, NH 03824, USA, Email. wrf@cisunix.unh.edu*

Received October 28, 2003; Accepted March 4, 2004

Abstract

Caulerpa is a tropical marine coenocytic green alga that coexists with bacteria. The exact relationship between these bacteria and the plant has not been established. The bacteria may be associated with the plant surface or exist as endosymbionts. Electron micrographs revealed the presence of at least three different bacterial morphologies within the plant cell. Four *Caulerpa* species were examined for the existence of bacteria by the use of a culture-independent approach. A protocol was developed to extract plant cytoplasm that was free of contaminating surface materials. DNA was extracted from the cytoplasmic fraction, washed plant pieces, and resultant wash solution. Denaturing gradient gel electrophoresis (DGGE) of PCR-amplified 16S rDNA was used as a molecular fingerprinting technique to profile the bacterial community. Individual DGGE bands representing

Presented at the 4th International Symbiosis Congress, August 17–23, 2003, Halifax, Canada

*The author to whom correspondence should be sent.

unique bacteria were excised from the gel and sequenced to identify these organisms. Samples from four plant species were examined to determine if all *Caulerpa* species coexisted with same or different bacterial species. Preliminary results indicate that *Caulerpa* cytoplasm contains endosymbionts and the bacterial strains within individual *Caulerpa* species differed.

Keywords: Coenocytic green alga, endosymbionts

1. Introduction

Caulerpa is a coenocytic (single celled, multinucleate) algae, that is found in tropical marine environments. These algae can reach over 2 m in length and have 5 morphologically distinct regions: a horizontal stolon-like stipe; petiole-like stalks which emerge upward from the stipe; erect blades; downward growing rhizoids which emerge from the stipe; and distinct growing tips (Jacobs, 1964, 1994).

Many coenocytic green algae harbor endosymbiotic bacteria, and several researchers have observed bacteria inside *Caulerpa* species (Dawes and Lohr, 1978; Liddle, 1998; Meusnier et al., 2001). The relationship of these bacteria to *Caulerpa* and their role in the algal life-cycle are unknown. They may form, for example, mutualistic, neutral, or pathogenic/parasitic interactions. One hypothesis is that these endosymbiotic bacteria are beneficial to *Caulerpa* because of their nitrogen fixation capabilities. Alternatively, they may offer protection via the production of various toxic compounds (Chisholm et al., 1996). Although a number of species of *Caulerpa* have been observed to contain bacteria it is not known if all *Caulerpa* species contain the same bacterial communities or if each *Caulerpa* species has a unique community profile. Alternatively, habitat may determine the microbial community found within *Caulerpa*.

Meusnier et al. (2001) identified 5 lines of bacteria associated with *C. taxifolia* living in the Mediterranean. Their study focused on all of the bacteria associated with the algae including epiphytic and endosymbiotic bacteria. In that study, plant material was macerated and the DNA extracted. Unfortunately, this methodology probably introduces bacterial contamination from the surface of the plant.

The purpose of the present study was two-fold. First, we were interested in developing a method of extracting plant cytoplasm to investigate the community profile of endosymbiotic bacteria by molecular finger-printing techniques. Second, we wanted to test whether these community profiles were related to individual *Caulerpa* species or determined by habitat. For this

study, the bacterial community profiles for four strains of *Caulerpa* (*C. prolifera, C. cupressoides, C. sertularioides* and *C. mexicana*) from Tampa Bay, FL were determined by the use of denaturing gradient gel electrophoresis (DGGE).

2. Materials and Methods

Organism and samples

Samples of *Caulerpa mexicana, C. sertulariodes, C. prolifera* and *C. cupressoides* were collected from the northern part of the Tampa Bay complex in Florida. The plant species were segregated and maintained in individual aquarium tanks containing artificial seawater (Instant Ocean™) with $NaNO_3$ (1 ml of 1.0 M stock/gallon of seawater) and KPO_3 (1 ml of 0.05 M stock/gallon of seawater) refreshed on a weekly basis. The plants were incubated at 22°C for at least 3 weeks prior to sampling. The tanks were illuminated with 100 W fluorescent bulbs on a 15 hr light/ 9 hr dark cycle.

Electron microscopy

Plants were first double wounded (pinched with a forceps to induce a wound plug) and cuts were made between the adjacent wound plugs. The resulting pieces, 5 mm in length, were placed in vials and fixed for 0.5, 1 or 2 h in 1% OsO_4/2% FA/3.6% GTA combination fixative suspended in 0.1 M cacodylate buffer with 32 ppt salt water solution. The fixative and buffer solutions were kept in an ice bath for the duration of the fixation procedure. After fixation, the samples were washed four times with 0.1 M cacodylate buffer. The samples were dehydrated by incubating in a graded acetone series then transferred to a solution of acidified dimethoxypropane (DMP™) for 20 h to remove all bound water. Tissues were then rinsed 3 times in 100% acetone for 15 min per rinse, infiltrated and embedded in Mollenhauer's resin mixture II (Dawes, 1988). Silver sections were made and specimens were observed with a JOEL 100s transmission electron microscope at 80 kV accelerating voltage.

Extraction of plant cytoplasm

Briefly, the plants were removed from the aquarium tanks and washed with sterile dH_2O. After the plants were surface sterilized with 70% ethanol, they were placed on a watchglass. During the cutting process, the plant was pinched for 10 sec with sterile tweezers on either side of the cut site to induce a wound reaction and then cut with a sterile razor blade. The plant cytoplasm was

collected by the use of a syringe and a 21-gauge needle, suspended in 500 µl of sterile dH_2O and processed for DNA extraction as described below. Several methods were tested to remove the cytoplasm from the wounded plant including the use of an applicator stick, a wire loop, and "squeezing" the plant.

For comparative purposes, plant washings and unwashed plant samples were also examined. Plant washings were collected by filtration. Plant pieces from washed and unwashed plants were also sampled and DNA was extracted by the UltraClean™ Plant DNA Extraction kit as described below. For each plant species, 2 or 3 plants were used for sampling.

DNA extraction and PCR amplification

Genomic DNA (gDNA) was extracted from the filtered samples and plant pieces with UltraClean™ Water DNA Isolation Kits (Mo Bio Laboratories, Inc., CA, USA) and the UltraClean™ Plant DNA Extraction Kit (Mo Bio Laboratories, Inc., CA, USA) according to the manufacturer's recommendations. The extracted gDNA samples were concentrated and further purified using QIAEX II Kit (Qiagen, CA, USA). For large volumes of DNA eluate from the UltraClean™ Water DNA Isolation Kit, the extracted gDNA was concentrated by adding 0.7 (V/V) isopropanol and 0.1 (V/V) 3 M Na-acetate (pH 5.2) and centrifuging at 16,000 x g for 15 min at 4°C. After the DNA pellet was washed once with cold 70% ethanol and air-dried for 15 min, the gDNA was resuspended in 200 µl of elution buffer (100 mM Tris-HCl, pH 8.0) and further purified as above.

The V3 region of the 16S rRNA gene was amplified by PCR with 338F and 907R primers (Lane et al., 1985; Amann et al., 1990) using 2.5 ng of DNA template. A GC-clamp (Muyzer et al., 1998) was attached to the 5' end of the forward primer, 338F for DGGE analysis. The PCR was performed in triplicate with 25 µl reaction volumes using 0.5 µM of each primer and Qiagen HotStarTaq Master Mix (Qiagen, CA, USA).

Thermal cycling parameters were as follows: i) an activation step at 95°C for 15 min was performed to activate HotstartTaq DNA polymerase, ii) DNA denaturation at 94°C for 2.5 min was followed by 11 cycles of denaturation at 94°C for 30 s, primer annealing at 56°C for 45 s, and primer extension at 72°C for 1 min, iii) 11 cycles of denaturation at 94°C for 30 s, primer annealing at 56°C for 1 min and primer extension at 72°C for 90 s followed, iv) finally 14 cycles of denaturation at 94°C for 30 s, primer annealing at 56°C for 75 s , primer extension at 72°C for 2 min 15 s and an extension step of 7.5 min completed the procedure. PCR products were pooled and purified using QIAquick PCR Purification Kit (Qiagen, CA, USA).

Denaturing gradient gel electrophoresis (DGGE)

DGGE was performed with the Bio-Rad Dcode™ Universal Detection System. Samples were loaded on an 8% (w/v) acrylamide gel with a denaturing gradient ranging from 40% to 60%. Gels were run in 1x TAE buffer for 16 h at 70 V and were stained for 20 min in 1x TAE buffer (Sambrook et al., 1989) containing ethidium bromide (50 µg/ml). Gels were destained for 30 min in 1x TAE buffer and photographed on an UV transilluminator (FVSTI-88). The resulting banding patterns were analyzed by the use of Quantity One program (Bio-Rad Laboratories, CA, USA). A dendogram was created from the cluster analysis using the unweighted paired group method with arithmetic averages (UPGAMA).

Sequencing and phylogenetic analyses

DGGE bands were excised from the gels using sterile razor blades, and purified with QIAEX II Gel Extraction Kit (Qiagen, CA, USA). For further purification, the extracted bands were re-amplified and re-run on a second DGGE gel as before. After a final band extraction and purification step (as described above), the samples were re-amplified by PCR with the 338F and 907R primers but without the GC clamp. The PCR products were separated on a 2% agarose gel, purified with the QIAquick PCR Purification Kit (Qiagen, CA, USA) and were used as a template for DNA sequencing reactions with DYEnamic ET terminator cycle sequencing Kit (Amershan Pharmacia Biotech) in the ABI PRISM 377 sequencer (Perkin Elmer). The 338F primer was used for these sequencing reactions. The sequences were compared with 16S rRNA genes available from the GeneBank and EMBL database using of the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/; Alyschul et al., 1997). Partial sequences of 16S rDNA from the excised DGGE bands have been deposited in GenBank under separate accession numbers (AY555445 to AY555452).

3. Results

Ultrastructural studies

Four *Caulerpa* sp. (*C. prolifera, C. cupressoides, C. sertularioides* and *C. mexicana*) were examined. Each species had different morphological characteristics, determined primarily by the "leaf-like" or blade structure (Dawes, 1967). Electron micrographs show the presence of bacteria inside the plants (Figs. 1a–d). Three different types of bacteria were observed inside *Caulerpa* determined by morphology. Bacteria were most frequently associated

with the vacuole, but occasionally they were found in cell wall like material inside the cell (Fig. 1c). Some bacteria appeared to have structures reminiscent of photosynthetic membranes or inclusions.

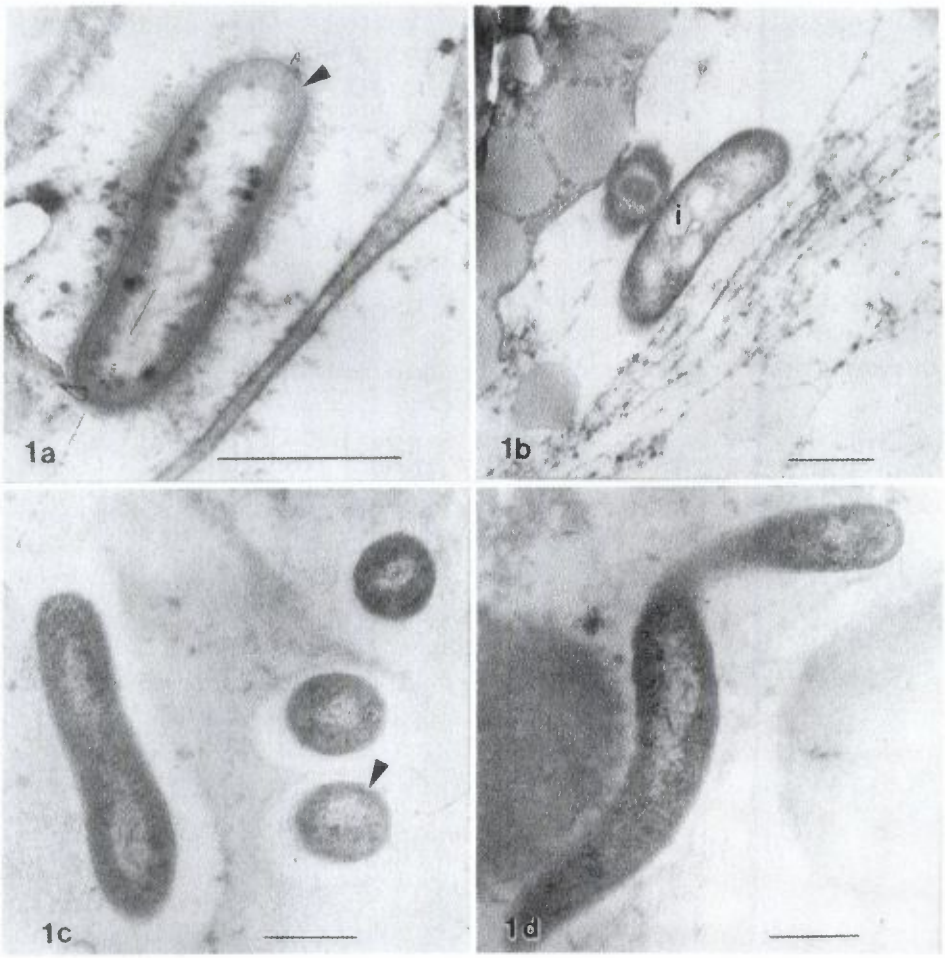

Figure 1. Electron micrographs of *Caulerpa cupressoides* show the presence of three different prokaryotic morphological types. Panel a, morphological type 1 (magnification 51,000×); Panel b, morphological type 2 (magnification 24,000×) [Note inclusions (i)]; Panel c, morphological type 3 (magnification 27,000×), [Note: possible photosynthetic membrane (arrow) and cell wall like material surrounding the bacterium]; Panel d, morphological type 3 (magnification 27,000×). Bar = 0.5 μm.

Community profiling

A protocol was developed to extract plant cytoplasm that was free of contaminating surface materials. DNA was extracted from the cytoplasmic fraction, washed plant pieces and the wash solution from the washed plant pieces. A culture-independent approach, DGGE molecular fingerprinting, was used to determine the diversity of prokaryotic microbial communities found associated with *Caulerpa*. Fig. 2 shows DGGE fingerprinting profiles of PCR-amplified 16S rDNA from the Tampa Bay samples. The community profiles from the cytoplasm samples had fewer numbers of DGGE bands than those obtained with tissues pieces and the wash rinse samples. The large numbers of DGGE bands recovered from plant tissues and wash rinse samples confirm a high level of microbial diversity on the plant surface.

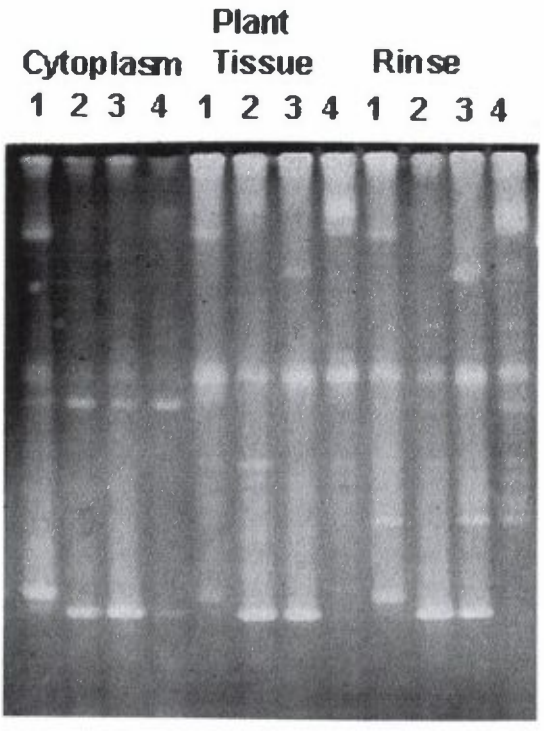

Figure 2. The effect of sampling method on the PCR-DGGE bacterial community profiles of *Caulerpa* samples obtained from Tampa Bay. The profiles of extracted cytoplasm, plant tissue and the rinse from the plants are shown. Lanes: (1) *C. sertularioides*, (2) *C. cupressoides*, (3) *C. mexicana*, and (4) *C. prolifera*.

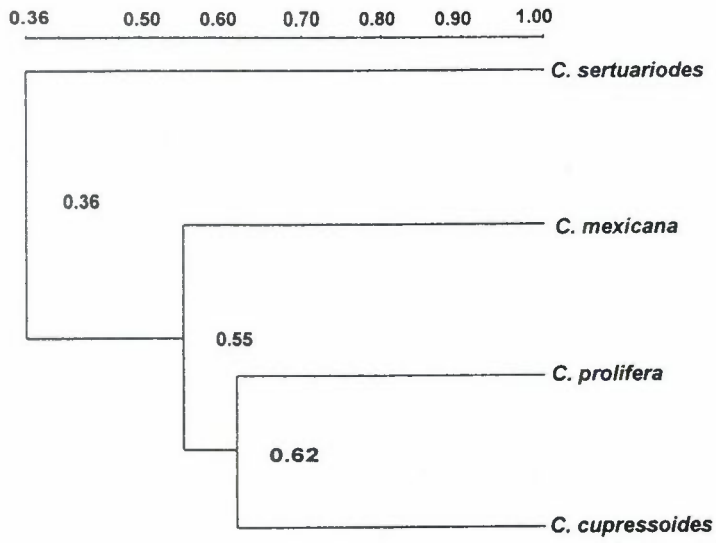

Figure 3. Cluster analysis of the DGGE banding patterns of the *Caulerpa* cytoplasm samples obtained from Tampa Bay based on the position of bands using UPGAMA. A similarity coefficient of >0.70 indicated that the samples are similar while those values <0.70 indicated that the samples are very different (Röling et al., 2001). A lower value indicates a greater difference between the samples.

Although the samples from the cytoplasm had a lower number of DGGE bands, the profiles obtained for each *Caulerpa* sp. were different. The banding patterns of the cytoplasm of each *Caulerpa* sp. were analyzed by UPGAMA to determine the similarity of the samples (Fig. 3). The banding patterns of all of the samples were significantly different indicating that the bacterial communities present were unique to each plant species.

Several DGGE bands were excised, purified and sequenced to identify the closest phylogenetic group. Partial sequencing of the V3 region of the 16S rRNA gene (500 bp on average) of 8 DGGE bands confirmed the differences in the phylotypes recovered from the cytoplasm of the *Caulerpa* sp. (Table 1). The highest degrees of similarity were found for the sequence of Rhodobacteraceae (99% sequence identity), *Rhodovulum iodosum* (96% sequence identity), *Rhodovulum sulfidophilum* (95% sequence identity), and *Ruegeria* (96% sequence identity). Since these bacteria are members of the *Rhodobacter*, a group of anoxygenic photosynthetic bacteria, these results confirm the presence of photosynthetic membranes observed in the electron micrographs (Fig. 1). Several of the sequences had a high degree of commonality to those reported by Meusnier et al. (2001) including band A12 with uncultured *Rhodobacter* group bacterium CtaxTah-22.

Table 1. Phylogenetic affiliations of 8 DGGE bands according to similarity searches with their 16S rDNA sequences (BLAST; Altschul et al., 1997). The percent identity, Blast bit score and E values obtained by the BLAST program are presented.

Sample-DGGE band	Similarity (%)	BLAST score	E value	Closest phylogenetic affiliation (accession number)
***C. sertularioides* cytoplasm**				
A3	428/447 (95%)	745	0.0	*Rhodovulum sulfidophilum* (D16430)
A4	404/415 (96%)	735	0.0	*Rhodovulum iodosum* (Y15011)
A12	374/387 (96%)	664	0.0	Uncultured *Ruegeria* CtaxMed-21 (AF259606)
E1	266/299 (88%)	388	5e-83	*Olavius loisae* endosymbiont 3 (AF104474)
E2	316/362 (87%)	363	2e-97	*Rhodospirillum photometricum* (AF487434.1)
***C. mexicana* cytoplasm**				
A5	375/413 (90%)	511	e-142	*Tistrella mobilis* (AB071665.1)
A6	308/333 (92%)	462	e-127	*Olavius loisae* endosymbionts 3 (AF104474)
***C. cupressoides* cytoplasm**				
A7	410/414 (99%)	783	0.0	*Rhodobacteraceae* bacterium R11 (AF539789)

4. Discussion

Each of the four plant species examined had a unique DGGE profile (Figs. 2 and 3), confirming that in this study endosymbiotic bacterial communities formed were plant host- and not habitat-specific. Electron microscopy (Fig. 1) showed the presence bacteria inside *Caulerpa* confirming the findings of previous studies (Dawes and Lohr, 1978; Liddle, 1998; Meusnier et al., 2001). Analysis of the sequences for several DGGE bands (Table 1) supports the conclusion that these are photosynthetically capable bacteria.

Acknowledgements

This research was supported by UNH Vice President for Sponsored Research Discretionary Funds and College of Life Science and Agriculture. We thank C.

Dawes (University of South Florida, Tampa, FL) for the Tampa Bay samples and for help with background information. We also thank Ellen Lavoie (UNH) for her technical assistance.

REFERENCES

Altschul, S.F., Madden, T.L., Schäffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Research* **25**: 3389–3402.

Amann, R.I., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., and Stahl, D.A. 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Applied Environmental Microbiology* **56**: 1919–1925.

Chisholm, J.R.M., Dauga, C., Ageron, E., Grimont, P.A.D, and Jaubert, J.M. 1996. "Roots" in mixotrophic algae. *Nature* **381**: 382.

Dawes, C.J. 1967. *Marine Algae in the Vicinity of Tampa Bay, Florida.* University of South Florida Press. Tampa, Fla. pp. 105.

Dawes, C.J. 1988. *Introduction to Biological Electron Microscopy: Theory and Techniques.* Ladd Research Industries. Burlington, Vt. 315 pp.

Dawes, C.J. and Lohr, C.A. 1978. Cytoplasmic organization and endosymbiotic bacteria in the growing points of *Caulerpa prolifera. Revue Algologique* **13**: 309–314.

Jacobs, W.P. 1964. Rhizoid-production and regeneration of *Caulerpa prolifera. Pubbl Staz Zool Napoli* **34**: 185–196.

Jacobs, W.P. 1994. *Caulerpa. Scientific American* **271**: 100–105.

Lane, D.J., Pace, B., Olson, G.J., Stahl, D.A., Sogin, M.L., and Pace, N.R. 1985. Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proceeding of the National Academy for Science USA* **82**: 6955–6959.

Liddle, L. B., Carvalho, N., and Ameinesz, A. 1998. Use of immunofluorescence microscopy to compare small nuclei in two populations of *Caulerpa taxifolia* (Caulerpales, Chlorophyta). *Phycologia* **37**: 310–312.

Meusnier, I., Olsen, J.L., Stam, W.T., Destombe, C., and Valero, M. 2001. Phylogenetic analysis of *Caulerpa taxifolia* (Chlorophyta) and of its associated bacterial microflora provide clues to the origin of the Mediterranean introduction. *Molecular Ecology* **10**: 931–946.

Muyzer, G., Hottentrager, S., Teske, A., and Wawer, C. 1998. Denaturing gradient gel electrophoresis of PCR-amplified 16S rDNA-A new molecular approach to analyze the genetic diversity of mixed microbial communities. *Molecular Microbial Ecology Manual* **3.4.4**: 1–23.

Sambrook, J., Fritch, E.F., and Maniatis, T. 1989. *Molecular Cloning: A Laboratory Manual,* 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Röling, W.F.M., van Breukelen, B.M., Braster, M., Lin, B., and van Verseveld, H.W. 2001. Relationships between microbial community structure and hydrochemistry in a landfield leachate-polluted aquifier. *Applied Environmental Microbiology* **67**: 4619–4629.