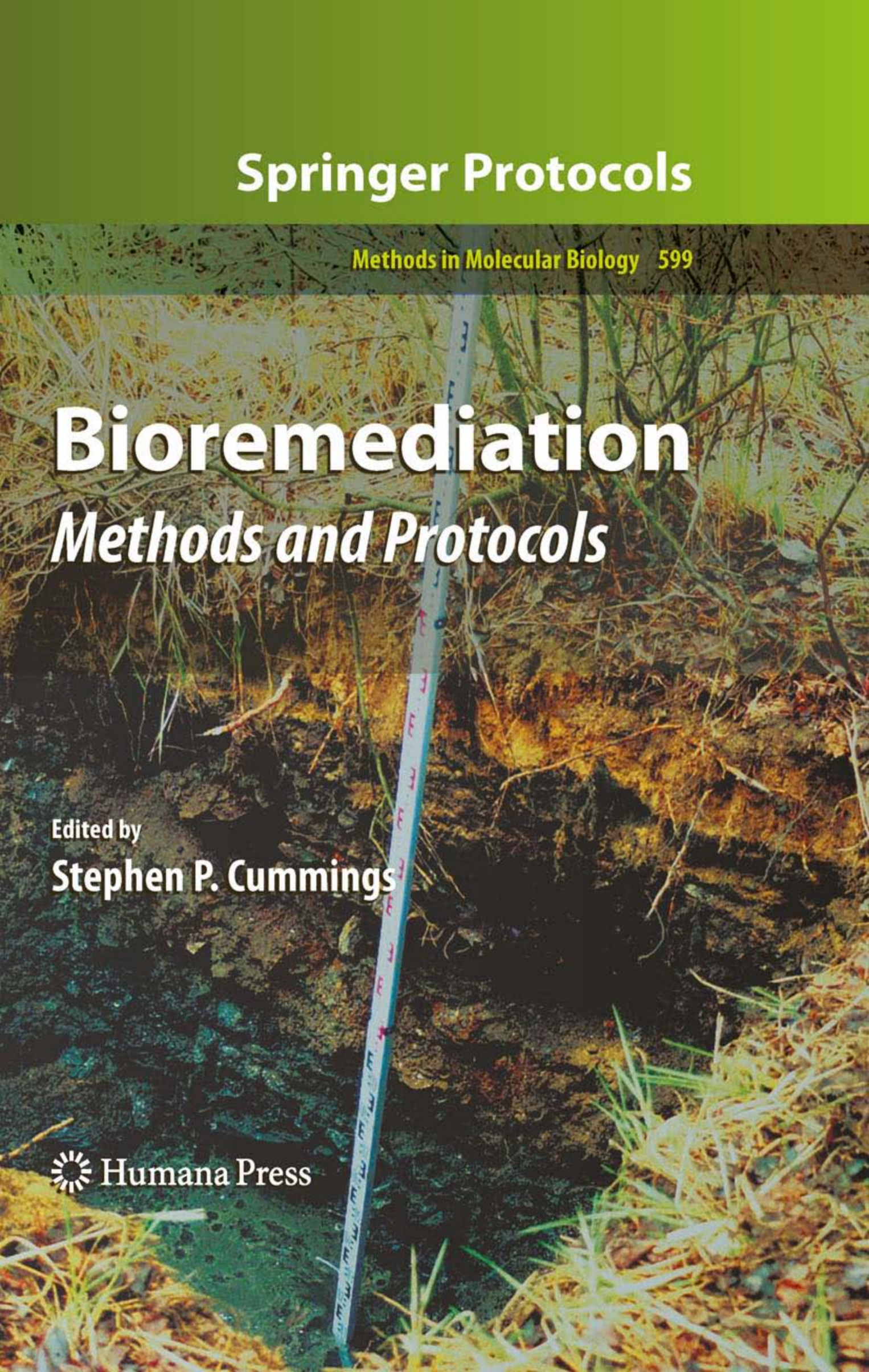
Springer Protocols

Methods in Molecular Biology 599

Bioremediation

Methods and Protocols



Edited by

Stephen P. Cummings

Humana Press

METHODS IN MOLECULAR BIOLOGY™

Series Editor
John M. Walker
School of Life Sciences
University of Hertfordshire
Hatfield, Hertfordshire, AL10 9AB, UK

For other titles published in this series, go to
www.springer.com/series/7651

Bioremediation

Methods and Protocols

Edited by

Stephen P. Cummings

University of Northumbria, Newcastle-upon-Tyne, UK

Humana Press

Editor
Stephen P. Cummings
School of Applied Sciences
Div. Biomedical Sciences
Northumbria University
Ellison Place
Newcastle upon Tyne
Ellison Bldg.
UK NE1 8ST
stephen.cummings@northumbria.ac.uk

ISSN 1064-3745 e-ISSN 1940-6029
ISBN 978-1-60761-438-8 e-ISBN 978-1-60761-439-5
DOI 10.1007/978-1-60761-439-5

Library of Congress Control Number: 2009938190

Printed on acid-free paper

springer.com

Preface

Anthropogenic activity has led to significant degradation of the environment through the discharge of pollutants. Enormous volumes of wastewater are generated daily by a range of industrial, agricultural, and domestic activities. Much is treated prior to release; however, accidental spillage can lead to significant pollution incidents. In addition, the legacy of industrial, agricultural, or military activity has resulted in long-term contamination problems in soil and sediments.

A key issue in treating contaminated soil and water is dealing with the diversity of potentially toxic pollutants, including heavy metals, polyaromatic hydrocarbons (PAH), cyanides, and radioactive material, often contained within the same waste. Frequently, such chronically contaminated material is treated using stringent chemical methods or, for solid waste, physical processes such as encapsulation. Whilst effective, such processes are often expensive, environmentally damaging in their turn, and contain rather than remove contaminated material. Bioremediation offers a viable alternative to these methods by harnessing the degradative potential of biological systems.

Bioremediation has a long history. The exploitation of microbial activity to successfully degrade organic pollutants in soil using land-farming techniques was reported in the early 1970s. In situ treatment protocols appeared in the literature shortly afterwards, typically in the treatment of hydrocarbon-contaminated groundwater by the injection of air and nutrients or the application of lime and fertilizer to treat to surface contamination with oil derivatives. However, subsequent events, particularly the decline of heavy industry and the end of the cold war, led to large areas of land on which industrial or military activity was conducted becoming available for redevelopment. In these areas, the anthropogenic pollution had occurred over decades and involved an array of both organic and non-organic contaminants. In order to effectively treat these large areas of land in which the extent and type of pollution was often poorly defined and heterogeneous, a range of new techniques were developed that relied on multidisciplinary approaches to define the problem, treat the pollutants, and effectively monitor the consequences of these activities.

Devising a bioremediation strategy requires an understanding of the interaction of the particular pollutant, or mixture, with the environment into which it is released, both in the short and long term. Often this is dependent on the mobility or solubility of the particular compound, its response to the physicochemical conditions in which it finds itself, and the interaction between it and the biological activity found within the environment being remediated. Many studies have observed that there are temporal changes affecting the fate of toxic compounds within the environment. In some cases, these can be exploited as part of the remediation process, while in others they result in long-term issues with the persistence of compounds. Therefore, in tandem with monitoring and managing the biological aspects of bioremediation, there is a requirement to characterize the fate of the compounds of interest in the environment to inform the choice of the bioremediation strategy to employ.

The application of molecular techniques into bioremediation protocols to add value to the process will require a number of current issues to be resolved. In practical terms,

the utility of these techniques for the removal of pollutants in the field requires that rates of degradation can be quantified. Moreover, techniques that are used to reduce pollutants to levels acceptable to legislative requirements will need to be validated to the satisfaction of the regulatory bodies.

The challenge for both the researcher and practitioner is to more accurately model the complex network of relationships between the pollutant, its physical and chemical environment, and the biological systems that are involved in the degradation or remediation activity. To achieve this will require the imaginative and ambitious application of multidisciplinary techniques that will enable more predictable removal of a pollutant from any given environment. In this volume, we explore a range of such techniques, including the contribution that recent advances in molecular biology can make to this ongoing work. In addition, the volume addresses some of the broader issues such as the effect of the environment in determining the availability and fate of organic and inorganic compounds and how choices around the most appropriate bioremediation process can be arrived at, as well as complementary techniques that support the effective deployment and monitoring of a bioremediation approach.

In assembling this volume, I am indebted to the authors of each chapter for offering their expert insight into how we might effectively apply the techniques described to enhance our work to deal with environmental contamination. Each chapter amply demonstrates their expertise and will, I am sure, lead to the adoption of many of these techniques and approaches in laboratories around the world.

Stephen P. Cummings

Contents

Contributors

Mar Babín • *Laboratory for Cell Toxicity, Department of the Environment, INIA, Madrid, Spain*

Stephan Bathe • *Institut für Ingenieurbiologie und Biotechnologie des Abwassers, Universität Karlsruhe, Karlsruhe, Germany*

Katarina Björklöf • *Finnish Environment Institute, Helsinki, Finland*

Anuj K. Chandel • *Celestial Labs Ltd., Hyderabad, India*

Yin Chen • *Department of Biological Sciences, The University of Warwick, Coventry, UK*

Agnieszka Cydzik-Kwiatkowska • *Department of Environmental Biotechnology, University of Warmia and Mazury in Olsztyn, Olsztyn, Słoneczna, Poland*

John Richard Dean • *Biomolecular and Biomedical Research Centre, School of Applied Sciences, Northumbria University, Newcastle upon Tyne, UK*

Elizabeth E. Diplock • *Institute of Biological and Environmental Sciences, University of Aberdeen, Aberdeen, UK; Remedios Ltd., Aberdeen, UK*

María Dolores Fernández • *Laboratory for Soil Ecotoxicology, Department of the Environment, INIA, Madrid, Spain*

Martina Hausner • *Department of Chemistry and Biology, Ryerson University, Toronto, ON, Canada*

Kirsten S. Jørgensen • *Finnish Environment Institute, Helsinki, Finland*

Dimitrios G. Karpouzas • *Department of Biochemistry & Biotechnology, University of Thessaly, Larisa, Greece*

Kenneth S. Killham • *Institute of Biological and Environmental Sciences, University of Aberdeen, Aberdeen, UK; Remedios Ltd., Aberdeen, UK*

Caroline Kragelund • *Department of Biotechnology, Chemistry and Environmental Engineering, Aalborg University, Aalborg, Denmark*

Raymond L. Legge • *Department of Chemical Engineering, University of Waterloo, Waterloo, ON, Canada*

Dave P. Mardlin • *Remedios Ltd., Aberdeen, UK*

Károly Márialigeti • *Department of Microbiology, Eötvös Loránd University, Budapest, Hungary*

Laura McAllister • *Lancaster Environment Centre, Lancaster University, Lancaster, UK*

J. Colin Murrell • *Department of Biological Sciences, The University of Warwick, Coventry, UK*

Jeppe Lund Nielsen • *Department of Biotechnology, Chemistry and Environmental Engineering, Aalborg University, Aalborg, Denmark*

Per Halkjær Nielsen • *Department of Biotechnology, Chemistry and Environmental Engineering, Aalborg University, Aalborg, Denmark*

Graeme I. Paton • *Institute of Biological and Environmental Sciences, University of Aberdeen, Aberdeen, UK; Remedios Ltd., Aberdeen, UK*

Alfredo Perez de Mora • *Helmholtz Zentrum München, Institute of Soil Ecology, Department for Terrestrial Ecogenetics, Neuherberg, Germany*

Sára Révész • *Department of Microbiology, Eötvös Loránd University, Budapest, Hungary*
Jani M. Salminen • *Finnish Environment Institute, Helsinki, Finland*
Michael Schloter • *Helmholtz Zentrum München, Institute of Soil Ecology, Department for Terrestrial Ecogenetics, Neuherberg, Germany*
Stephan Schulz • *Helmholtz Zentrum München, Institute of Soil Ecology, Department for Terrestrial Ecogenetics, Neuherberg, Germany*
Kirk T. Semple • *Lancaster Environment Centre, Lancaster University, Lancaster, UK*
Brajesh K. Singh • *The Macaulay Institute, Aberdeen, UK*
Om V. Singh • *Division of Biological and Health Science, University of Pittsburgh-Bradford, Bradford, PA, USA*
Rita Sipos • *Department of Microbiology, Eötvös Loránd University, Budapest, Hungary*
Anna Székely • *Department of Microbiology, Eötvös Loránd University, Budapest, Hungary*
José Vicente Tarazona • *Laboratory for Ecotoxicology, Department of the Environment, INIA, Madrid, Spain*
Sonia M. Tiquia • *Department of Natural Sciences, University of Michigan, Dearborn, MI, USA*
Jyotsna Vohra • *Department of Biological Sciences, The University of Warwick, Coventry, UK*
Kela P. Weber • *Department of Chemical Engineering, University of Waterloo, Waterloo, ON, Canada*
Irena Wojnowska-Baryła • *Department of Environmental Biotechnology, University of Warmia and Mazury in Olsztyn, Olsztyn, Słoneczna, Poland*
Magdalena Zielińska • *Department of Environmental Biotechnology, University of Warmia and Mazury in Olsztyn, Olsztyn, Słoneczna, Poland*

Chapter 1

Measurement of Bioaccessibility of Organic Pollutants in Soil

Laura McAllister and Kirk T. Semple

Abstract

The quantification of organic contaminant bioaccessibility in soils and sediments is essential for the risk assessment and remediation of contaminated land. Within this framework, practitioners require standardised protocols. Cyclodextrins are a group of macrocyclic compounds that can form inclusion complexes with organic xenobiotics. This occurrence can be exploited to measure the labile/rapidly desorbable compound fraction, which correlates with microbial degradation. We present a rapid and easily reproducible HPCD shake extraction technique that has been experimentally demonstrated to directly predict microbial availability and degradation in soil. This method can provide practitioners with both an indication of bioremediation end-points and may be valuable in the risk assessment of contaminated land.

Key words: Bioaccessibility, hydroxypropyl-β-cyclodextrin (HPCD), organic contaminants, non-exhaustive extraction techniques.

1. Introduction

The identification and quantification of organic contaminants in soils have been traditionally achieved using exhaustive chemical extraction techniques (1). However, experimental evidence has clearly demonstrated that such measurements are not representative of contaminant availability to soil and sediment biota (2,3). Sequestration processes, including sorption to soil solid phases and diffusion into micro- and nanopores with increased soil–contaminant contact time, have been shown to reduce the availability of contaminants to a given organism (3). Therefore, the quantification of "total" concentrations bears little relevance

S.P. Cummings (ed.), *Bioremediation*, Methods in Molecular Biology 599,
DOI 10.1007/978-1-60761-439-5_1,

for the assessment of contaminated land, in terms of both potential risk and viability of bioremediation (3, 4).

Bioassays can provide a direct measurement of bioaccessibility; however, these techniques can be expensive, time-consuming and lack in precision (5). As a result, a number of non-exhaustive extraction techniques have been proposed to measure bioaccessibility, and biodegradation in particular (5, 6, 7), by mimicking microbe–contaminant interactions (**Fig.1.1**). The development of such a method would provide practitioners with an indication of bioremediation end-points and risk to potential receptors (3).

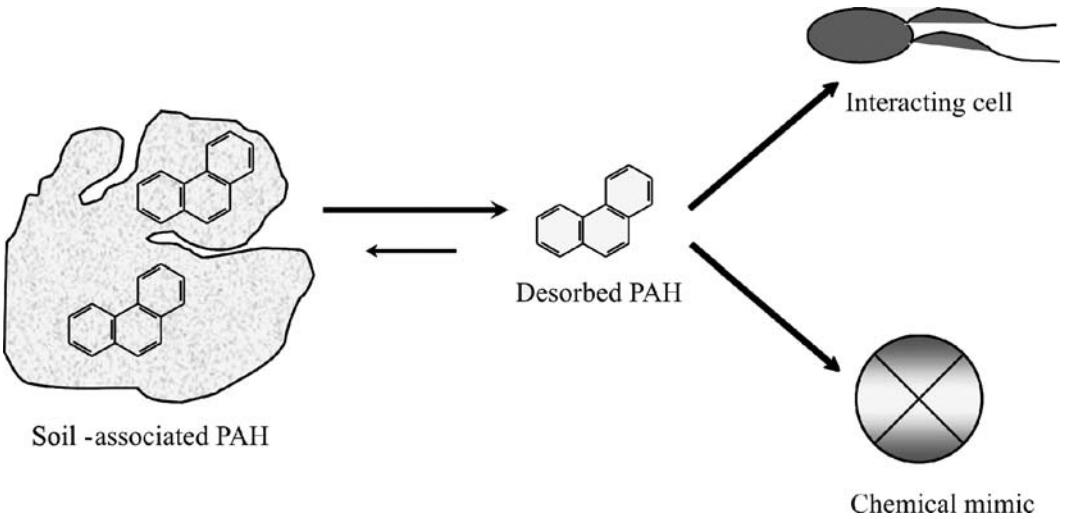


Fig. 1.1. Theoretical mechanisms for the biodegradation of phenanthrene and a putative chemical mimic allowing the determination of biodegradation end-points. Reproduced from Semple et al. (2003) with permission from Blackwell Publishing.

One technique that has generated considerable scientific interest is the use of cyclodextrin solutions to predict microbial bioaccessibility. Cyclodextrins are a group of macrocyclic oligosaccharides, also known as cycloamyloses, cyclomaltoses and Schardinger dextrins (8). Three major types are known: α, β and γ cyclodextrin, which are composed of 6, 7 and 8 α-1,4-glycoside bonds (glycosidic/glucopyranose units), respectively. Their physicochemical properties are such that each molecule possesses a hydrophilic exterior and hydrophobic interior cavity. As a result, cyclodextrins in solution can form inclusion complexes with an extensive range of hydrophobic organic compounds, including polycyclic aromatic hydrocarbons (PAHs) (9). It is believed that contaminant mass transfer processes, most notably desorption to the aqueous phase (where substrate uptake is more extensive), govern microbial availability (10, 11). As cyclodextrin extraction is also dependent upon contaminant transfer, it thereby mimics biodegradative processes (12). **Figure 1.2**

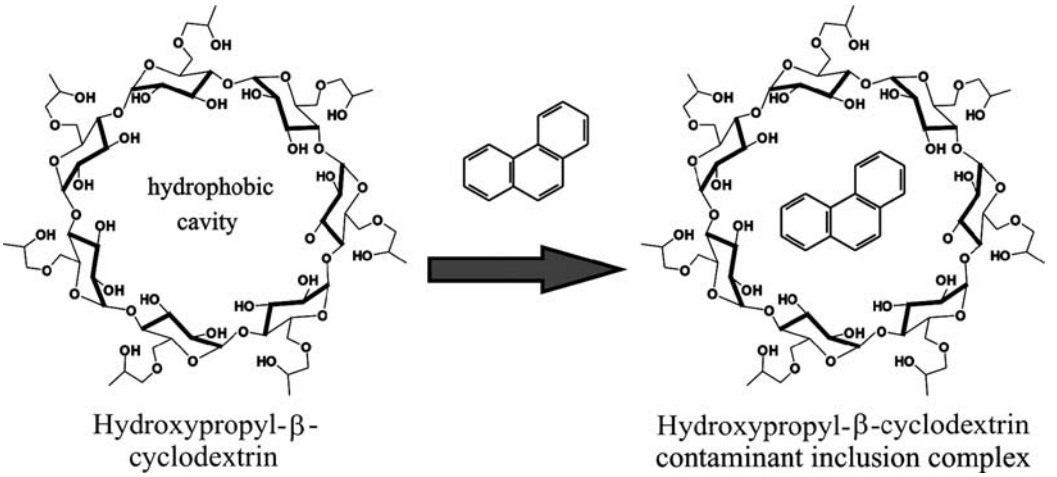


Fig. 1.2. Molecular structure of hydroxypropyl-β-cyclodextrin (HPCD) and diagrammatic representation of HPCD–contaminant inclusion complex. Reproduced from Reid et al. (2004) with permission from Allen Press Publishing Services.

conceptualises the interaction between the cyclodextrin molecule and phenanthrene.

The laboratory protocol was pioneered by Reid et al. (13) using a hydroxypropyl-β-cyclodextrin (HPCD) solution with soils artificially contaminated with PAHs. The fractions of phenanthrene extracted (after known ageing periods) were correlated with the corresponding microbially mineralised fractions, yielding not only a strong relationship ($r^2 = 0.964$) but a slope that approximated to 1 (0.997, i.e. a direct prediction). Further, this compared favourably to correlations between biodegradation and dichloromethane (0.648), butan-1-ol (0.614) and extractions (**Fig.1.3**).

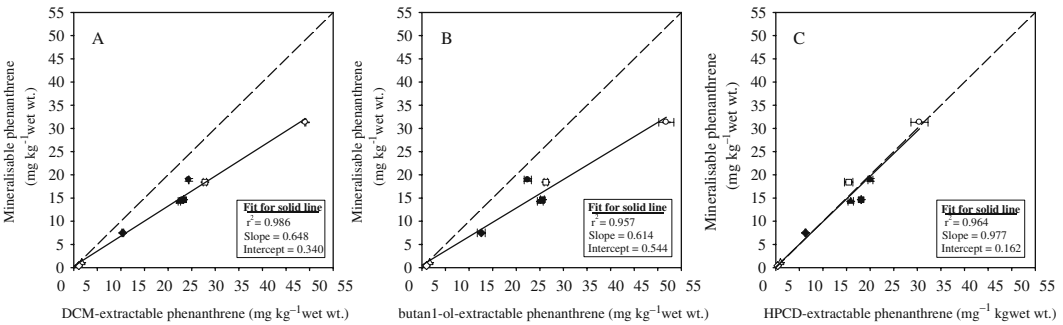


Fig. 1.3. Correlation of phenanthrene mineralisation (as determined using catabolically active microorganisms) with (A) Soxtex-DCM-extractable phenanthrene; (B) butan-1-ol-extractable phenanthrene; and (C) HPCD-extractable phenanthrene. Assessments were made after 1 (●), 42 (■), 84 (▲), and 322 days (◇) for soil systems originally spiked with 25 mg/kgwet weight (*black*) and 50 mg/kgwet weight (*white*). The dashed lines on each plot are of a 1:1 slope. Reproduced from Reid et al. (13) with permission from the American Chemical Society.

Subsequently, this procedure has been studied and validated extensively. In artificially spiked systems, 1:1 relationships between extractability and biodegradability have been established in a wide variety of dissimilar soils (14, 15, 16), in the presence of multi/co-contaminants (1, 17), cable-insulating oil (18) and transformer oil (19). Good predictability of biodegradation has also been established in field-contaminated sediments (20, 21). Further, one extraction has been found to be sufficient to determine the biodegradable fraction in both field-contaminated (1) and artificially spiked soils (12).

It has also been successfully tested with aged and field-contaminated soils (1, 12, 15, 18, 19, 22, 23, 24, 25); all investigations have produced direct 1:1 relationships between extractability and microbial degradation. To date, extractions have been predominantly tested with β-cyclodextrin, with only some studies investigating α-cyclodextrins (23, 25), presenting opportunities for further research. The rapid and easily reproducible technique described here involves the addition of a HPCD solution to a sample of contaminated soil, shaking, centrifuging and finally either sampling the supernatant or quantifying the residual concentrations in the soil (**Fig.1.4**). The procedure provided for spiking soils uses phenanthrene for ease of explanation; however, this can be substituted for other chemicals/chemical mixtures. Protocols are provided for artificially spiked and field-contaminated soils. The procedure of quantifying microbial biodegradation, to verify the HPCD extraction, is also described using both microbial inocula and indigenous soil microbes.

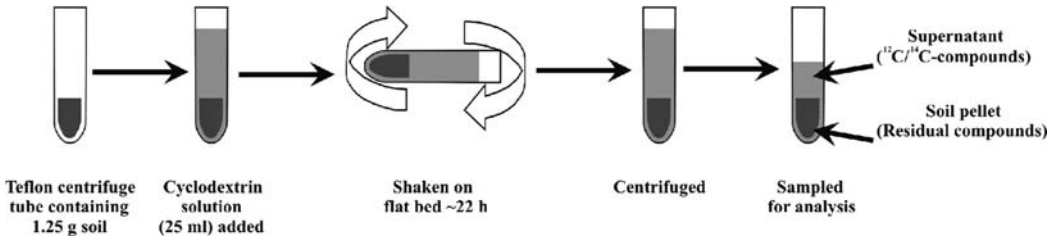


Fig. 1.4. Schematic diagram illustrating the HPCD extraction protocol.

2. Materials

2.1. Phenanthrene Standards

1. Non-labelled phenanthrene and its radiolabelled analogue [9-^{14}C] phenanthrene (radiochemical purity >98%) (Sigma Aldrich Co. Ltd., U.K).

2. Carrier solvent for soil spiking: acetone (*see* **Note 1**). A maximum ratio of 1:20 solvent:soil is observed.
3. Phenanthrene standard for degraders: *N*,*N* -dimethyl- formamide (DMF). ^{12}C-phenanthrene standard is added at a ratio of 100 g/L DMF.

2.2. Sub-culturing of Phenanthrene Degraders

1. To prepare 1 L of minimal basal salts: add to 1 L of d H_2O, 0.3 g NaCl, 0.6 g $(NH_4)_2SO_4$, 0.6 g KNO_3, 0.25 g KH_2PO_4, 0.75 g K_2HPO_4, 0.15 g $MgSO_4{\cdot}7H_2O$ and 1mL trace element solution (as described below). Prior to use, the solution must be autoclaved at 121°C on a 20 min cycle (*see* **Note 2**). The final solution should be stored in a fridge.
2. Trace element solution: To 250 mL d H_2O, add 5 μg $LiCl(LiBO_2)$, 20 μg $CaSO_4{\cdot}5H_2O$, 25 μg $ZnSO_4{\cdot}7H2O$, 25 μg $Al_2(SO_4)_3{\cdot}16H_2O$, 25 μg $NiCl{\cdot}6H_2O(CoNO_3)$, 25 μg $CoSO_4{\cdot}7H_2O(CoNO_3)$, 7.5 μg KBr, 7.5 μg KI, 150 μg $MnCl_2{\cdot}2H_2O$, 10 μg $SnCl_2{\cdot}2H_2O$ and $FeSO_4{\cdot}7H_2O$. The final solution should be stored in a fridge.
3. Phenanthrene standard for degraders (*see* **Section 2.1.3**).

2.3. HPCD Shake Extraction

1. For each sample extraction, 2 g of hydroxypropyl-β-cyclodextrin (HPCD) (Aldrich Chemical Co., UK) is mixed well into 25 mL of deionised water (d H_2O) to give an optimal concentration of 50 mM (13).

2.4. Sample Oxidisation

1. Organic-based combustion-aiding solution (maybe put ingredients, i.e. chemical formula or something) (Combustaid) (Canberra Packard, UK).
2. To conduct a Spec check (to assess instrument trapping efficiency): 40 μL Spec check standard is dissolved in 960 μL ethanol (EtOH).

2.5. Respirometry

1. Sodium hydroxide (NaOH) (BDH Laboratory Supplies, UK). A 1 M solution is made up with d H_2O.
2. Ultima Gold liquid scintillation cocktail (Canberra Packard, UK).

2.6. DCM–Soxtec Extraction and Analysis

1. To dry soil before extraction: anhydrous sodium sulphate (Na_2SO_4) in a powdered or granular form (VWR International, Lutterworth, UK). Prior to use, dry in an oven at 450°C for 6 h.
2. Dichloromethane (DCM) (Sigma Aldrich, UK).
3. To remove sulphur from DCM extracts: copper granules, hydrochloric acid (HCl) (both from VWR International, Lutterworth, UK), methanol and hexane.

4. Sorbents: 923 silica and F-20 alumina. Prior to use, the sorbents need to be activated by baking ~12 h in a furnace at 120°C (silica) and 450°C (alumina). Once cooled, sorbents are deactivated by shaking in 5% (silica) and 1% (alumina) d H_2O (by weight of sorbent). Deactivated sorbents should be used within 1 week (16).
5. To quantify compound losses during extraction and cleanup: 50 ng (per extraction) of deuterated surrogates of each contaminant known to be present in the soil.

3. Methods

The HPCD shake extraction procedure was refined by Reid et al. (13). Their research found that maximum phenanthrene extraction was achieved with a HPCD concentration of ~50–60 mM. Further, extraction efficiency plateaued after 6 h, although at least 20 h is recommended to ensure complete extraction. The optimised technique was then tested, firstly using different PAHs over a range of concentrations, which indicated that extraction efficiencies were consistent and reproducible. The extent of ^{14}C-phenanthrene extracted from dissimilar soil types varied as a function of soil characteristics; for example, less was extracted from soils with higher organic matter (OM). Finally, the fraction extracted was observed to decrease as soil–phenanthrene contact time (1–322 days) progressed, thus reflecting that extractability was influenced by ageing.

The most effective method of quantifying chemical extractability varies upon the nature of contamination. If soils are artificially spiked with ^{14}C-PAHs, sampling the HPCD supernatant itself is the most straightforward technique, as ^{14}C-labelled chemicals are easily quantified using liquid scintillation counting (LSC). In field-contaminated soils, residual PAHs post-HPCD extraction, as determined by DCM extraction, are compared with residual concentrations after biodegradation, also as determined by DCM extraction. It is essential that the necessary health and safety measures assessments are conducted for using hazardous substances, including radioactive compounds, solvents, and PAHs, as well as carrying out appropriate disposal methods.

Thorough and effective cleaning of equipment is essential prior to use. Standard cleaning procedure used in this experiment requires a Decon90® soak (24 h), a thorough rinse/1 h soak in normal water, a d H_2O soak (3 h) and an acetone rinse. In addition, each extraction/bioassay should be carried out in triplicate, including controls/analytical blanks.

3.1. Soil Preparation

1. Soil is passed through a 2 mm sieve (to remove large debris) and air dried for 24 h.
2. The moisture content is determined by drying 3 ~2 g soil samples at 105°C for 24 h in porcelain crucibles. After drying, samples are cooled in a desiccator for 1–6 h and re-weighed to calculate moisture loss.
3. Re-hydrate soil back to its original field moisture content with d H_2O in a large glass bowl.

3.2. Preparation of Bacterial Degraders for Inoculated Soil

1. Methods for culturing an inoculum may vary; however, a final concentration of ca. 10^7 cells/g soil is recommended in each bioassay/respirometer (15).
2. To sub-culture (from previously inoculated MBS): conical flasks (1 L) with 600 mL of autoclaved MBS solution are sealed with a cotton wool bung and tin foil (600 mL produces a final concentration of 50 mL; 2 × 600 mL = 100 mL concentrate, 3 × 600 mL = 150 mL concentrate, etc.).
3. Use an autoclaved (as described in **Section 2.4.1**) pipette tip (5 mL) to add 10 mL of previously inoculated MBS containing degraders per 100 mL MBS (i.e. 60 mL for 600 mL MBS). Cold phenanthrene standard is added at 10 μL per 100 mL MBS. Flasks are incubated at 22 ± 2°C for 4 days on a rotary shaker at 100 rpm.
4. Degraders are harvested after 4 days (late exponential phase growth). The following instructions assume the use of a Beckman J-6 M centrifuge (Beckman Coulter, USA). Sub-cultured degraders are centrifuged in 500 mL Beckman centrifuge tubes (~45% of capacity; *see* **Note 2**) at 3600 × *g*, 15°C for 30 min. At this point, the supernatant is disposed and fresh autoclaved MBS is added to re-suspend the cell pellet.
5. Step 3 is repeated to remove any residual phenanthrene. After the second centrifugation, dispose off the supernatant again and re-suspend the cell pellet with ~1 mL of MBS. Transfer the concentrate into a conical flask (250 mL), rinsing centrifuge tubes with MBS to remove all degrading cells. For every 600 mL of MBS-degrading solution, re-suspend with 50 mL of fresh MBS.

3.3. Studies Involving ^{14}C-Spiked Soils

3.3.1. Soil Spiking

1. Both labelled and non-labelled phenanthrene are added to the soil to provide both radioactivity and mass. Whilst both may vary depending on the nature of the experiment, the level of radioactivity is typically ~83.3 Bq/g of soil required and mass is 50 mg/kg soil.

2. ^{12}C-Phenanthrene and ^{14}C-phenanthrene are added into a vial with acetone. Standards are stored in the freezer until use (*see* **Note 3**).
3. Standard radioactivity is quantified (in triplicate) by pipetting 3 μL of prepared standard into 20 mL of scintillation fluid in an economy glass vial. Samples are then counted using LSC (Tri Carb 2300 TR liquid scintillation counter; Canberra Packard, Belgium) (*see* **Note 4**). Standard calibration and quench correction techniques must be applied.
4. The "bolus" methodology is employed for soil spiking. Approximately one fourth of the soil is spiked initially and blended (1–3 min). Soil is left in a fume hood for a period of 1–2 h to allow solvent venting/evaporation. Remaining soil is added in approximately three separate portions and blended to ensure good homogeneity (*see* **Note 5**).
5. Where experiments are investigating the effects of ageing upon bioaccessibility, spiked soils are stored for the required aging period in sealed amber glass jars in the darkness at $20 \pm 2^{\circ}$C.
6. After spiking, soils that are to be inoculated should be promptly sterilised to eradicate indigenous microbial activity using γ-irradiation (32.2 KGy).

3.3.2. Determining HPCD Extractable Fraction of ^{14}C-Spiked Soil

1. One Teflon^{TM} centrifuge tube (35 mL) is required for each individual extraction (individual tube weights must be recorded prior to soil addition). Soil (1.25 ± 0.1 g) is weighed into a tube with 25 mL of HPCD solution.
2. Tubes are sealed, placed horizontally on an orbital shaker (Janke and Kunkel, IKA-Labortechnik KS 250, supplied by Fisher Scientific) and shaken at 100 rpm for 20–22 h at room temperature.
3. Weigh all tubes and compare with weights recorded previously. If significant differences are observed, add d H_2O to balance.
4. Tubes are then centrifuged (Centaur 2 centrifuge; Measuring scientific Equipment, Beckenham, UK) for 1 h at $3600 \times g$.
5. Once finished, 6 mL of each supernatant is sampled and added to 14 mL Goldstar scintillation cocktail in economy glass vials (20 mL). Place caps on all vials and shake manually.
6. Quantification of ^{14}C-activity is performed by LSC (as aforementioned in **Section 3.3.1**, Step 3).

7. Discard remaining supernatant fluid (tubes must then be weighed again) and remove the soil pellets from the tubes. Weigh approximately 1 ± 0.1 g soil into a cellulose cone.
8. Add 200 μL of Combustaid and sample oxidise (*see* **Note 6**). These instructions assume the use of a Canberra Packard model 307 sample oxidiser.
9. Put 2× high-performance vials into holder (one each for the sample and waste), put soil pellets into the basket and burn each for ~3 min (*see* **Note 7**). Put a lid on the vial, shake and store overnight before scintillation counting (as aforementioned in **Section 3.3.1**, Step 3).
10. Regular spec checks should be conducted to verify the trapping efficiency of the instrument and analysed using LSC. To determine background measurements, 200 μL Combustaid is burnt. To quantify the standard, 200 μL Combustaid is burnt, followed by 50 μL of spec check. The recovery efficiency is carried out by adding 50 μL of spec check to 200 μL Combustaid and sample oxidised. The memory after this is then measured by burning 200 μL Combustaid. All checks are carried out in triplicate; recovery found should be ~96–99%. Sample oxidations are then calibrated by spec check data.

3.3.3. Bioassays of ^{14}C-Spiked Soil

1. For inoculated soil, each microcosm consists of 10 ± 0.1 g of each soil treatment, 5 mL of enriched inoculum and 25 mL of autoclaved MBS. Non-inoculated soil includes 10 ± 0.1 g of each soil treatment with 30 mL of MBS (*see* **Note 8**).
2. Microcosms are performed in respirometers, as designed by Reid et al.(26).Respirometers consist of an Erlenmeyer flask with a Teflon^{TM}-lined screw top lid that has been drilled through the centre. Through this, a stainless steel rod is placed and secured on either side with a washer and a nut. A fine stainless steel crocodile clip is attached to a section of rod on the inside of the lid.
3. A glass scintillation vial (7 mL) containing 1 mL sodium hydroxide solution (NaOH) to trap $^{14}CO_2$ evolution is suspended in each flask through attachment to the crocodile clip.
4. Respirometers are shaken horizontally on an orbital shaker at 100 rpm, 20 ± 2°C for the required length of time.
5. Respirometers are periodically sampled at desired time intervals (typically at 12 or 24 h intervals). The lid of the Erlenmeyer flask is removed and the CO_2 trap quickly replaced with a fresh vial. Wipe the removed vial with an acetone-moistened tissue, add 6 mL of liquid scintillation fluid and

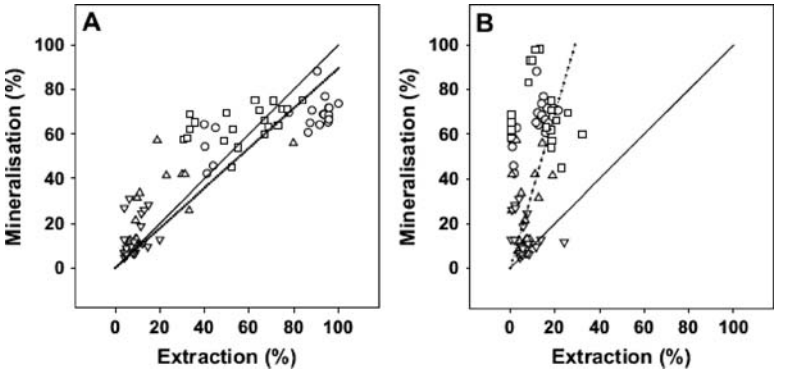


Fig. 1.5. Relationship between fractions of ^{14}C-phenanthrene extracted from four dissimilar soils using: (A) HPCD extraction; (B) water-only extraction, and the fraction mineralised in biodegradation assays after 1 day (○), 20 days (□), 50 days (△), 100 days (▽) contact times. *Solid* and *dotted lines* represent a 1:1 relationship and regression lines, respectively. Reproduced from Allan et al. 2006(16) with permission from Elsevier.

cap. Count radioactivity using LSC as aforementioned in **Section 3.3.1**, Step 3.

6. Final extents of microbial mineralisation can be correlated with HPCD extracted fractions to verify the technique (an example is provided in **Fig 1.5**).

3.4. Studies Involving Field-Contaminated Soils

3.4.1. Determining Total Extractable Concentrations of Field-Contaminated Soil

1. Samples of untreated soil are first exhaustively extracted to determine the total extractable contaminant fraction. In this example, dichloromethane (DCM)–soxtec extraction is used. These instructions assume the use of Foss Tecator Soxtec (model 2055, Avanti).
2. Approximately 1.5–2.5 g of soil is ground with ~8 g anhydrous sodium sulphate. Quantitatively transfer into Whatman cellulose extraction thimbles.
3. Add 50 ng of deuterated surrogate compounds of each contaminant known to be present in the soil. Extract for 18 h with 270 mL DCM.
4. Following extraction, supernatants require preparation before gas chromatography-mass spectrometry (GC-MS) analysis. Copper granules are added to HCl in a conical flask for 5 min, stirring occasionally with a glass rod. The HCl is decanted and granules rinsed with water, methanol, DCM and hexane. Activated granules are stored under hexane and should be used within ~1 h.
5. Activated copper granules are added to all DCM extracts. Reduce extracts to 1 mL by rotary evaporation and leave to stand for 1 h. If the granules are black after standing, add more granules until they appear shiny.

6. Using Pasteur pipettes, quantitatively transfer samples with multiple DCM rinses and reduce in volume to 1 mL.
7. The cleanup columns are prepared by wet-packing glass chromatography columns with 5 g of deactivated alumina followed by 10 g of deactivated silica (both in DCM) and finally sodium sulphate. Columns are rinsed with DCM and then exchanged into pentane.
8. Quantitatively add samples to the columns and elute using 100 mL of DCM:penatane (1:1). Reduce samples to 1 mL and exchange into 0.5 mL iso-octane before quantitative transfer into gas chromatography vials (2 mL).
9. Samples are analysed for contaminants by gas chromatography with a mass selection detector (Hewlett-Packard 5890 Series II gas chromatograph equipped with a 7673 autosampler, 5972 mass selection detector (MSD), supplied by Agilent Technologies, Cheadle, UK) and an HP-5 column (5% phenyl methylsiloxane, 30 m length × 0.25 mm i.d., film thickness of 0.25 μm) (Metlab, Hawarden, UK).
10. The GC temperature programme is 70°C for 2 min, ramped at 20°C/min to 100°C, and finally at 6°C/min to 300°C (held for 3 min). The MSD is maintained at 280°C.
11. Data are collected and quantified using HP Chemstation software; quantification is conducted with the external calibration method.

3.4.2. Determining HPCD Extractable Fractions of Field-Contaminated Soil

1. The HPCD extraction is carried out above up until and including Step 2. At this point, the supernatant is discarded and the soil shaken for 10 s with 25 mL of d H_2O. Vials are then centrifuged and the supernatant discarded again.
2. A sample of the soil is DCM–soxtec extracted (with an extra 15 g sodium sulphate) and analysed for contaminants with GC-MS (as described above)

3.4.3. Bioassays of Field-Contaminated Soil

1. Indigenous microbial degradation assays are set up by adding 30 mL of autoclaved MBS to 10 ± 0.1 g of soil in modified loosely capped Schott bottles (15).
2. The bottles are incubated on an orbital shaker at 100 rpm and 20 ± 2°C for ~6 weeks.
3. Inoculated bioassays are conducted as above, with the exception of adding 1 mL of enriched catabolic inoculum and 29 mL of MBS to the microcosms (soils require sterilisation as described in **Section 3.3.1**, Step 6).
4. After a specified assay time, soils are recovered by centrifugation and a ~1.5 g portion is DCM–soxtec extracted and analysed for contaminants with GC-MS (as described above).

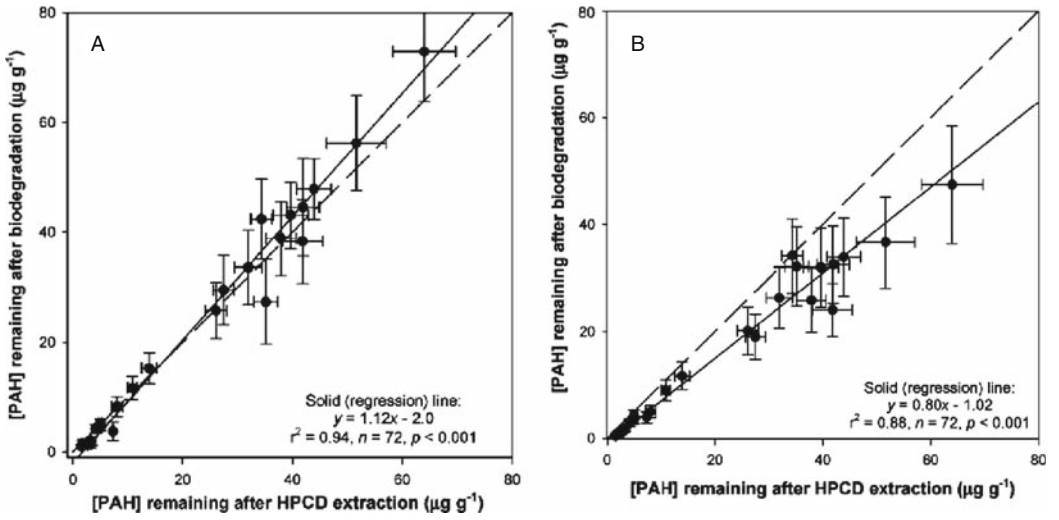


Fig. 1.6. Linear regression correlating total PAH (naphthalene, biphenyl and flourene, phenanthrene and pyrene) concentrations following degradation by (**A**) an enriched inoculum and (**B**) indigenous microflora in 6 week bioassays and the HPCD predicted end-point ($n = 3$) of soil from a former coke plant. The *dashed line* represents the $y = x$ correlation between predicted and observed concentration end-points. Error bars represent 1 standard error of the mean. Reproduced from Doick et al. (2005) (19) with permission from the American Chemical Society.

5. Residual contaminant concentrations after biodegradation can be correlated with residual concentrations after HPCD extraction to verify chemical extractability (an example is provided in **Fig 1.6**).

4. Notes

1. Acetone is a suitable carrier solvent for phenanthrene, although another solvent, e.g. toluene, may be required to dissolve highly hydrophobic contaminants.
2. MBS should not be autoclaved more than once; otherwise, the compounds will precipitate out of solution and the microbial degraders will not grow properly.
3. If centrifuge tubes are filled >45%, they will collapse at full speed.
4. Standards can also be stored in the fridge, although freezing is more effective in limiting volatilisation.
5. Storing vials in the darkness at room temperature for 24 h prior to scintillation counting limits chemiluminescence.
6. Alternatively, soils can be spiked and re-hydrated (**Section 3.1.3**) in a single step at this point, rather than carrying out both separately.

7. If sample oxidisation cannot take place on the same day, put soil into cellulose cones, wrap in tin foil and freeze. Must be fully defrosted (~30 min) before sample oxidised.
8. The burn time can be adjusted depending on the soil wetness/soil moisture content. For example, a soil with a moisture content of ~50% may require 5 min burn time.
9. It is recommended that in each respirometer, the slurry system has a soil:liquid ratio of 1:3 (27) for homogeneous distribution of ^{14}C compounds (28) and a rapid and reproducible determination of bioaccessibility (27).

References

1. Doick, K. J., Clasper, P. J., Urmann, K., and Semple, K. T. (2006) Further validation of the HPCD-technique for the evaluation of PAH microbial availability in soil. *Environ. Pollut.* **144**, 345–354.
2. Kelsey, J. W., Kottler, B. D., and Alexander, M. (1997) Selective chemical extraction to predict bioavailability of soil-aged organic chemicals. *Environ. Sci. Technol.* **31**, 214–217.
3. Alexander, M. (2000) Ageing, bioavailability and overestimation of risk from environmental pollutants. *Environ. Sci. Technol.* **34**, 4259–4262.
4. Semple, K. T., Doick, K. J., Jones, K. C., Burauel, P., Craven, A., and Harms, H. (2004) Defining bioavailability and bioaccessibility of contaminated soil and sediment is complicated. *Environ. Sci. Technol.* **38**, 228A–231A.
5. Liste, H.-H., and Alexander, M. (2002) Butanol extraction to predict bioavailability of PAHs in soil. *Chemosphere* **46**, 1011–1017.
6. Cuypers, C., Grotenhuis, T., Joziasse, J., and Rulkens, W. (2002) Rapid persulphate oxidation predicts PAH bioavailability in soils and sediments. *Environ. Sci. Technol.* **34**, 2057–2063.
7. Hawthorne, S. B., and Grabanski, C. B. (2000) Correlating selective supercritical fluid extraction with bioremediation behaviour of PAHs in a field treatment plot. *Environ. Sci. Technol.* **34**,4103–4110.
8. Szejtli, J. (1998) Introduction and general overview of cyclodextrin chemistry. *Chem. Rev.* **98**, 1743–1753.
9. Del Valle, E. M. M. (2004) Cyclodextrins and their uses: a review. *Process Biochem.* **39**, 1033–1046.
10. Bosma, T. N. P., Middeldorp, P. J. M., Schraa, G., and Zehnder, A. J. B. (1997) Mass transfer limitation of biotransformation: Quantifying bioavailability. *Environ. Sci. Technol.* **31**, 248–252.
11. Cornelissen, G., Van Noort, P. C. M., and Govers, H. J. A. (1998) Mechanism of slow desorption of organic compounds from sediments: A study using model sorbents. *Environ. Sci. Technol.* **32**, 3124–3131.
12. Rhodes, A. H., Dew, N. M., and Semple, K. T. (2008) Relationship between cyclodextrin extraction and biodegradation of phenanthrene in soil. *Environ. Toxicol. Chem.* **27**, 1488–1495.
13. Reid, B. J. Stokes, J. D. Jones, K.C., and Semple, K. T. (2000) Nonexhaustive cyclodextrin-based extraction technique for the evaluation of PAH bioavailability. *Environ. Sci. Technol.* **34**, 3174–3179.
14. Patterson, C. J., Semple, K. T., and Paton, G. I. (2004) Non-exhaustive extraction techniques (NEETs) for the prediction of naphthalene mineralization in soil. *FEMS Microbiol. Lett.* **241**, 215–220.
15. Hickman, Z. A., and Reid, B. J. (2005) Towards a more appropriate water-based extraction for the assessment of organic contaminant availability. *Environ. Pollut.* **138**,299–306.
16. Allan, I. J., Semple, K. T., Hare, R., and Reid, B. J. (2006) Prediction of mono- and polycyclic aromatic hydrocarbon degradation in spiked soils using cyclodextrin extraction. *Environ. Pollut.* **144**, 562–571.
17. Hickman, Z. A., Swindell, A. L., Allan, I. J., Rhodes, A. H., Hare, R., Semple, K. T. and Reid, B. J. (2008) Assessing biodegradation potential of PAHs in complex multi-contaminant matrices. *Environ. Pollut.* doi:10.1016/j.envpol.2008.04.022.
18. Dew, N. M., Paton, G. I., and Semple, K. T. (2005) Prediction of phenyldodecane biodegradation in cable insulating

oil-spiked soil using selected extraction techniques. *Environ. Pollut.* **138**, 316–323.
19. Doick, K. J., Dew, N. M., and Semple, K. T. (2005) Linking catabolism to cyclodextrin extractability: Determination of the microbial availability of PAHs in soil. *Environ. Sci. Technol.* **39**, 8858–8864.
20. Cuypers, C., Clemens, R., Grotenhuis, T., and Rulkins, W. (2001) Prediction of petroleum hydrocarbon bioavailability in contaminated sediments and soils. *Soil Sediment. Contam.* **19**, 459–482.
21. Cuypers, C., Clemens, R., Grotenhuis, T., and Rulkins, W. (2002) The estimation of PAH bioavailability in contaminated sediments using hydroxypropyl-β-cyclodextrin and Triton X-100 extraction techniques. *Chemosphere* **46**, 1235–1245.
22. Stokes, J. D., Wilkinson, A., Reid, B. J., Jones, K. C., and Semple, K. T. (2005) Prediction of polycyclic aromatic hydrocarbon biodegradation in contaminated soils using an aqueous hydroxypropyl-β-cyclodextrin extraction technique. *Environ. Toxicol. Chem.* **24**, 1325–1330.
23. Papadopoulos, A., Paton, G. I., Reid, B. J., and Semple, K. T. (2007) Prediction of PAH biodegradation in field contaminated soils using a cyclodextrin extraction technique. *Jl Environ. Monit.* **9**, 516–522.
24. Stroud, J. L., Paton, G. I., and Semple, K. T. (2008) Predicting the biodegradation of target hydrocarbons in the presence of mixed contaminants in soil. *Chemosphere* doi:10.1016/j.chemosphere.2008.09.071.
25. Stroud, J. L., Paton, G. I., and Semple, K. T. (2008) Linking chemical extraction to microbial degradation of ^{14}C-hexadecane in soil. *Environ Pollut.* doi:10.1016/j.envpol.2008.01.018.
26. Reid, B. J., MacLeod, C. J. A., Lee, P. H., Morriss, A. W. J., Stokes, J. D., and Semple, K. T. (2001) A simple ^{14}C-respirometric method for assessing microbial catabolic potential and contaminant bioavailability. *FEMS Microbiol. Lett.* **196**, 141–146.
27. Doick, K. J., and Semple, K. T. (2003) The effect of soil : water ratios on the mineralization of phenanthrene: LNAPL mixtures in soil. *FEMS Microbiol. Lett.* **220**, 23–33.
28. Carmichael, L. M., and Pfaender, F. K. (1997) Polynuclear aromatic hydrocarbon metabolism in soils: relationship to soil characteristics and pre-exposure. *Environ. Toxicol. Chem.* **16**, 666–675.

Chapter 2

Heavy Metal Bioavailability and Bioaccessibility in Soil

John Richard Dean

Abstract

This chapter considers the use of a variety of approaches to assess either the bioavailability or the bioaccessibility of metals in soil. The bioavailability of metals from soils is considered with respect to a series of single-extraction methods, including the use of ethylenediaminetetraacetic acid (EDTA), acetic acid, diethylenetriaminepentaacetic acid (DTPA), ammonium nitrate, calcium chloride and sodium nitrate. Then, a procedure for the recovery of metals using a three-stage sequential extraction protocol is described. Two alternate approaches for assessing the environmental health risk to humans by undertaking in vitro gastrointestinal extraction (also known as the physiologically based extraction test, PBET) are considered. Finally, two acid digestion protocols that allow the pseudo-total metal content of samples to be assessed are provided.

In all cases details of how the different approaches can be performed are provided, including the specific reagents required (and their preparation), details of the different extraction and acid digestion protocols to be followed and suitable analytical details to allow the measurement of metals by inductively coupled plasma mass spectrometry (ICP-MS) with/without a collision/reaction cell. A detailed Notes section provides experimental details to guide the reader through some of the practical aspects of the procedures. Finally, some experimental results are provided as evidence of the suitability of the approaches described including single-extraction data, using EDTA and acetic acid, for metals in CRM BCR 700. In addition, in vitro gastrointestinal extraction data are provided for metals in CRM SRM 1570A (spinach leaves). The influence of time on the intestinal fluid phase on the recovery of metals in CRM SRM 1570A (spinach leaves) and CRM INCT-TL-1 (tea leaves) is investigated, as well as the repeatability in terms of recovery of metals from soil over a 3-week period by in vitro gastrointestinal extraction.

Key words: Single-extraction methods, sequential extraction method, physiologically based extraction test (PBET), in vitro gastrointestinal extraction, inductively coupled plasma mass spectrometry (ICP-MS).

1. Introduction

The release of metals from soil is normally accomplished using heat and concentrated acids (in a process termed acid digestion)

S.P. Cummings (ed.), *Bioremediation*, Methods in Molecular Biology 599,
DOI 10.1007/978-1-60761-439-5_2, © Humana Press, a part of Springer Science+Business Media, LLC 2010

(1). The aim of this approach is to destroy the soil matrix releasing metals into solution. In reality, depending upon the choice of acid (or acid combination) this may or may not be possible, but the approach is nevertheless used to determine the metal (pseudo)total in the soil matrix. Approaches to assess the metal bioavailability and bioaccessibility are available (2). In the case of metal bioavailability, the approaches are based on the use of selective chemical extractants to liberate the metals from the soil matrix by overcoming specific interactions. These approaches are based on single- or sequential extraction methods, which were originally developed by the Standard, Measurements and Testing Programme (SM & T – formerly BCR) of the European Union (3–5). Single-extraction methods are based on the use of ethylenediaminetetraacetic acid (EDTA), acetic acid or diethylenetriaminepentaacetic acid (DTPA) as well as some other reagents, whereas the sequential extraction method uses specific reagents to assess the exchangeable, reducible and oxidisable fractions of metals in soil. In the case of metal (oral) bioaccessibility, the approach is based on the use of reagents that seek to mimic the human digestive system (2). This method is often described as either in vitro (simulated) gastrointestinal extraction or the physiologically based extraction test (PBET). In each case the use of specific extraction scenarios to provide an estimation of the environmental risk to humans and plants from heavy metal contaminated soil is done.

2. Materials

2.1. Extraction Reagents for Single-Extraction Methods

1. 50 mM ethylenediaminetetraaceticacid (EDTA): In a fume cupboard add 146 +/− 0.05 g of EDTA (free acid) to 800 +/− 20 mL of distilled water (*see* **Note 1**). To aid dissolution of EDTA, stir in 130 +/− 5 mL of saturated ammonia solution (prepared by bubbling ammonia gas into distilled water). Continue to add the ammonia solution until all the EDTA has dissolved. The resultant solution should be filtered, if necessary, through a filter paper of porosity 1.4–2.0 μm into a pre-cleaned 10 L polyethylene bottle and then diluted to 9.0 +/− 0.5 L with distilled water. Adjust the pH to 7.00 +/− 0.05 by addition of a few drops of either ammonia or concentrated hydrochloric acid, as appropriate. The solution should then be made up to 10 L with distilled water to obtain an EDTA solution of 50 mM. Analyse a sample of each fresh batch of EDTA solution for its metal impurity content (*see* **Notes 2 and 3**).

2. 0.43 M acetic acid: In a fume cupboard add 250 +/− 2 mL of glacial acetic acid (AnalaR or similar) to approximately 5 L of distilled water in a pre-cleaned 10 L polyethylene bottle and make up to 10 L with distilled water. Analyse a sample of each fresh batch of acetic acid solution for its metal impurity content (*see* **Notes 2 and 3**).
3. 5 mM diethylenetriaminepentaacetic acid (DTPA): In a fume cupboard dissolve 149.2 g triethanolamine (0.01 M), 19.67 g DTPA (5 mM) and 14.7 g calcium chloride in approximately 200 mL distilled water. Allow the DTPA to dissolve and then dilute to 9 L. Adjust the pH to 7.3 +/− 0.5 with concentrated HCl while stirring and then dilute to 10 L in distilled water. Analyse a sample of each fresh batch of DTPA solution for its metal impurity content (*see* **Notes 2 and 3**).
4. 1 M ammonium nitrate (NH_4NO_3): In a fume cupboard dissolve 80.04 g of NH_4NO_3 in water, then make up to 1 L with water. Analyse a sample of each fresh batch of NH_4NO_3 solution for its metal impurity content (*see* **Notes 2 and 3**).
5. 0.01 M calcium chloride: In a fume cupboard dissolve 1.470 g of $CaCl_2 2H_2O$ in water, then make up to 1 L with water. Verify that the Ca concentration is 400 +/− 10 mg/L by EDTA titration. Analyse a sample of each fresh batch of $CaCl_2$ solution for its metal impurity content (*see* **Notes 2 and 3**).
6. 0.1 M sodium nitrate ($NaNO_3$): In a fume cupboard dissolve 8.50 g of $NaNO_3$ in water, then make up to 1 L with water. Analyse a sample of each fresh batch of $NaNO_3$ solution for its metal impurity content (*see* **Notes 2, 3 and 4**).

2.2. Extraction Reagents for Sequential Extraction Method

1. Solution A: 0.11 M acetic acid. Add in a fume cupboard 25 +/− 0.1 mL of glacial acetic acid to approximately 0.5 L of water in a 1 L polyethylene bottle and make up to 1 L with water. Take 250 mL of this solution (acetic acid 0.43 M) and dilute to 1 L with water to obtain an acetic acid solution of 0.11 M. Analyse a sample of each fresh batch of solution A for its metal impurity content (*see* **Note 2**).
2. Solution B: 0.5 M hydroxylamine hydrochloride or hydroxyammonium chloride. Dissolve 34.75 g of hydroxylamine hydrochloride in 400 mL of water. Transfer to a 1 L volumetric flask and add 25 mL of 2 M HNO_3 (prepared by weighing from a concentration solution) (the pH should be 1.5). Make up to 1 L with water. Prepare this solution on the same day as the extraction is carried out. Analyse a sample of each fresh batch of solution B for its metal impurity content (*see* **Note 2**).

3. Solution C: (8.8 M hydrogen peroxide (300 mg/g). Use H_2O_2 as supplied by the manufacturer, i.e. acid-stabilized to pH 2–3. Analyse a sample of each fresh batch of solution C for its metal impurity content (*see* **Note 2**).
4. Solution D: (1 M ammonium acetate). Dissolve 77.08 g of ammonium acetate in 800 mL of water. Adjust to pH 2 +/− 0.1 with concentrated HNO_3 and make up to 1 L with water. Analyse a sample of each fresh batch of solution D for its metal impurity content (*see* **Note 2**).

2.3. Extraction Reagents for In vitro Gastrointestinal Extraction: Approach 1

1. Gastric solution: 1.25 g pepsin (1 Anson unit/g lactose as diluents), 0.5 g sodium malate, 0.5 g sodium citrate, 420 μL lactic acid and 500 μL acetic acid made up to 1 L with water, adjusted to pH 2.5 with concentrated HCl.
2. Intestinal solution: 52.5 mg bile salts (bovine) and 15 mg pancreatin (pig) added into the sample−gastric solution mixture and the pH adjusted to pH 7.0 with saturated $NaHCO_3$.

2.4. Extraction Reagents for In vitro Gastrointestinal Extraction: Approach 2

2.4.1. Simulated Saliva Fluid

1. First add 145 mg of α-amylase (bacillus species), 50.0 mg mucin and 15.0 mg uric acid to a 2 L HDPE screw-top bottle.
2. Separately add 896 mg of KCl, 888 mg NaH_2PO_4, 200 mg KSCN, 570 mg Na_2SO_4, 298 mg NaCl and 1.80 mL of 1.0 M HCl into a 500 mL volume container and make up to the mark with water (inorganic saliva components).
3. In a second 500 mL volume container, add 200 mg urea and make up to the mark with water (organic saliva components).
4. Then, simultaneously pour 500 mL of inorganic and 500 mL of organic saliva components into the 2 L HDPE screw-top bottle.
5. Shake the entire contents of the screw-top bottle thoroughly.
6. Measure the pH of this solution (gastric-simulated fluid). The pH should be 6.5 ± 0.5. If necessary, adjust the pH by adding either 1.0 M NaOH or 37% HCl.

2.4.2. Simulated Gastric Fluid

1. First add 1000 mg of bovine serum albumin, 3000 mg mucin and 1000 mg pepsin to a 2 L HDPE screw-top bottle.

2. Separately add 824 mg of KCl, 266 mg NaH_2PO_4, 400 mg $CaCl_2$, 306 mg NH_4Cl, 2752 mg NaCl and 8.30 mL of 37% HCl into a 500 mL volume container and make up to the mark with water (inorganic gastric components).
3. In a second 500 mL volume container, add 650 mg glucose, 20.0 mg glucuronic acid, 85.0 mg urea and 330 mg glucosaminehydrochloride and make up to the mark with water (organic gastric components).
4. Then, simultaneously pour 500 mL of inorganic and 500 mL of organic components into the 2 L HDPE screw-top bottle.
5. Shake the entire contents of the screw-top bottle thoroughly.
6. Measure the pH of this solution (gastric-simulated fluid). The pH should be within the range 0.9–1.0. If necessary, adjust the pH to this range (0.9–1.0) by adding either 1.0 M NaOH or 37% HCl.
7. Check that the combination of mixed saliva fluid (1 mL) and gastric fluid (1.5 mL) is in the pH 1.2–1.4. If the combined mixture is not within this range, it is necessary to adjust the pH of the gastric fluid by adding either 1.0 M NaOH or 37% HCl.
8. Re-check that the combination of mixed saliva fluid (1 mL) and gastric fluid (1.5 mL) is in the pH 1.2–1.4.

2.4.3. Simulated Duodenal Fluid

1. First add 200 mg of $CaCl_2$, 1000 mg bovine serum albumin, 3000 mg pancreatin and 500 mg lipase to a 2 L HDPE screw-top bottle.
2. Separately add 564 mg of KCl, 80 mg KH_2PO_4, 50.0 mg $MgCl_2$, 5607 mg $NaHCO_3$, 7012 mg NaCl and 180 μL of 37% HCl into a 500 mL volume container and make up to the mark with water (inorganic duodenal components).
3. In a second 500 mL volume container, add 100 mg urea and make up to the mark with water (organic duodenal components).
4. Then, simultaneously pour 500 mL of inorganic and 500 mL of organic duodenal components into the 2 L HDPE screw-top bottle.
5. Shake the entire contents of the screw-top bottle thoroughly.
6. Measure the pH of this solution (simulated duodenal fluid). The pH should be within the range 7.4 ± 0.2. If necessary, adjust the pH of the duodenal fluid by adding either 1.0 M NaOH or 37% HCl.

2.4.4. Simulated Bile Fluid

1. First add 222 mg of $CaCl_2$, 1800 mg bovine serum albumin and 6000 mg bile to a 2 L HDPE screw-top bottle.
2. Separately add 376 mg of KCl, 5785 mg $NaHCO_3$, 5259 mg NaCl and 180 μL of 37% HCl into a 500 mL volume container and make up to the mark with water (inorganic bile components).
3. Into a second 500 mL volume container, add 250 mg urea and make up to the mark with water (organic bile components).
4. Then, simultaneously pour 500 mL of inorganic and 500 mL of organic bile components into the 2 L HDPE screw-top bottle.
5. Shake the entire contents of the screw-top bottle thoroughly.
6. Allow the solution to stand for approximately 1 h, at room temperature, to allow for complete dissolution of solid reagents.
7. Measure the pH of this solution (simulated bile fluid). The pH should be within the range 8.0 ± 0.2. If necessary, adjust the pH of the duodenal fluid by adding either 1.0 M NaOH or 37% HCl.
8. Check that the combination of saliva fluid (1.0 mL), gastric fluid (1.5 mL), 3.0 mL duodenal fluid and 1.0 mL bile fluid is in the pH 6.3 ± 0.5. If the combined mixture is not within this range, it is necessary to adjust the pH of the duodenal fluid by adding either 1.0 M NaOH or 37% HCl.
9. Re-check that the combination of saliva fluid (1.0 mL), gastric fluid (1.5 mL), 3.0 mL duodenal fluid and 1.0 mL bile fluid is in the pH 6.3 ± 0.5.

2.5. Instrumentation for Metal Analysis

1. All metal measurements were made using an inductively coupled plasma mass spectrometer (ICP-MS, XSeries II, Thermo Electron Corporation, Cheshire, UK).
2. A multi-element standard is used for K, Ca, Mg, Na, Cr, Mn, Fe, Ni, Cu, Zn, Mo, Cd and Pb and internal standard solutions for Sc, In and Tb (SPEXCertiPrep, Middlesex, UK).

3. Methods

3.1. Chemical-Selective Extraction for Single-Extraction Methods

Chemical-selective extractions of the soil are carried out in order to assess the metal bioavailability. The main procedures identified for the extraction of metals using single-extraction methods are based on the use of ethylenediaminetetraacetic acid, acetic acid or

diethylenetriaminepentaacetic acid. However, other reagents are also used and include the use of ammonium nitrate, calcium chloride and sodium nitrate. The extraction protocols, using EDTA, CH_3COOH and $CaCl_2$, used are based on those developed by the Standard, Measurements and Testing Program (formerly BCR) of the European Community (3–5) and subsequently re-evaluated for EDTA and acetic acid (6).

3.1.1. Ethylenediaminetetraacetic Acid Extraction

1. 2 g of soil sample is weighed into a 50 mL Sarstedt extraction tube and 20 mL of 0.05 M EDTA (pH 7.0) is added (*see* **Note 5**).
2. The mixture is shaken in an end-over-end shaker at 30 rpm for 1 h at ambient temperature (20 ± 2°C) (*see* **Note 6**).
3. Then centrifuge the mixture for 10 min at 3000*g*.
4. Remove the supernatant with a pipette and store in a polyethylene bottle at 4°C.
5. Prior to analysis, re-homogenise the sample by manually shaking for 5 min.
6. Analyse by ICP-MS (*see* **Notes 10 and 11**).

Example results for the EDTA extraction of nine elements from a certified reference material (BCR 700) are shown in**Table2.1**.

Table 2.1
Example results for selected single-extraction protocols

	EDTA extraction		CH_3COOH extraction	
Element	CRM organic-rich soil (BCR 700) (mg/kg)	Concentrations (mg/kg) Mean ± SD, *n* = 6	CRM organic-rich soil (BCR 700) (mg/kg)	Concentrations (mg/kg) Mean ± SD, *n* = 6
Cr	10.1 ± 0.9	9.2 ± 0.2	19.0 ± 1.1	20.5 ± 0.7
Mn	na	146 ± 6	na	266 ± 19
Fe	na	1224 ± 95	na	33.0 ± 1.8
Ni	53.2 ± 2.8	51.5 ± 1.0	99.0 ± 5.1	102.8 ± 2.6
Cu	89.4 ± 2.8	91.9 ± 1.3	36.3 ± 1.6	37.3 ± 2.6
Zn	510 ± 17	455 ± 5	719 ± 24	715.7 ± 55.5
Mo	na	1.10 ± 0.08	na	0.06 ± 0.01
Cd	65.2 ± 3.5	65.7 ± 5.1	67.5 ± 2.8	67.1 ± 2.5
Pb	103 ± 5	101.9 ± 0.9	4.85 ± 0.38	4.82 ± 0.44

na = not available

3.1.2. Acetic Acid Extraction

1. 1 g of soil sample is weighed into a 50 mL Sarstedt extraction tube and 40 mL of 0.43 M CH_3COOH is added (*see* **Note 5**).
2. The mixture is shaken in an end-over-end shaker at 30 rpm for 16 h at ambient temperature (20 ± 2°C) (*see* **Note 6**).
3. Then centrifuge the mixture for 10 min at 3000*g*.
4. Remove the supernatant with a pipette and store in a polyethylene bottle at 4°C.
5. Prior to analysis, re-homogenise the sample by manually shaking for 5 min.
6. Analyse by ICP-MS (*see* **Notes 10 and 11**).

   Example results for the acetic acid extraction of nine elements from a certified reference material (BCR 700) are shown in **Table 2.1**.

3.1.3. Diethylenetriaminepentaacetic Acid Extraction

1. 2 g of soil sample is weighed into a 50 mL Sarstedt extraction tube and 4 mL of 0.005 M DTPA is added (*see* **Note 5**).
2. The mixture is shaken in an end-over-end shaker at 30 rpm for 2 h at ambient temperature (20 ± 2°C) (*see* **Note 6**).
3. Then centrifuge the mixture for 10 min at 3000*g*.
4. Remove the supernatant with a pipette and store in a polyethylene bottle at 4°C.
5. Prior to analysis, re-homogenise the sample by manually shaking for 5 min.
6. Analyse by ICP-MS (*see* **Notes 10 and 11**).

3.1.4. Calcium Chloride Extraction

1. 2 g of soil sample is weighed into a 50 mL Sarstedt extraction tube and 20 mL of 0.01 M $CaCl_2$ is added (*see* **Note 5**).
2. The mixture is shaken in an end-over-end shaker at 30 rpm for 3 h at ambient temperature (20 ± 2°C) (*see* **Note 6**).
3. Decant 12 mL into a centrifuge tube and centrifuge for 10 min at 3000*g*.
4. Analyse extracts immediately by ICP-MS (*see* **Notes 10 and 11**).

3.1.5. Ammonium Nitrate

1. 2 g of soil sample is weighed into a 50 mL Sarstedt extraction tube and 5 mL of 1.0 M NH_4NO_3 is added (*see* **Note 5**).
2. The mixture is shaken in an end-over-end shaker at 50–60 rpm for 2 h at ambient temperature (20 ± 2°C) (*see* **Note 6**).
3. Then, pass the supernatant through an acid-washed filter paper into a 50 mL polyethylene bottle (discard the first

5 mL of the filtrate). Stabilise by adding 1 mL of concentrated HNO_3.

4. If solids remain, centrifuge or filter through a 0.45 μm membrane filter.
5. Analyse extracts immediately by ICP-MS (*see* **Notes 10 and 11**).

3.1.6. Sodium Nitrate Extraction

1. 2 g of soil sample is weighed into a 50 mL Sarstedt extraction tube and 5 mL of 0.1 M $NaNO_3$ is added (*see* **Note 5**).
2. The mixture is shaken in an end-over-end shaker at 120 rpm for 2 h at ambient temperature (20 ± 2°C) (*see* **Note 6**).
3. Then centrifuge the mixture for 10 min at 4000*g*.
4. Remove the supernatant with a syringe and filter through a 0.45 μm membrane filter into a 50 mL polyethylene bottle. Add 2 mL of concentrated HNO_3 to a 50 mL volumetric flask and make up to volume with the filtered extract.
5. Analyse extracts immediately by ICP-MS (*see* **Notes 10 and 11**).

3.2. Chemical-Selective Extraction for Sequential Extraction Method

The procedure adopted for the sequential extraction of metals from soil/sediments is based on three distinct stages (6). In stage 1 (exchangeable fraction), the metals released are representative of those that are the most bioavailable (and hence most mobile). They include those metals that are weakly absorbed on the sediment/soil surface by relatively weak electrostatic interaction, metals that can be released by ion exchange processes or metals that can be co-precipitated with carbonates present in many sediments/soils. Any changes in the ionic composition, influencing adsorption–desorption reactions, or lowering of pH could cause mobilisation of metals from this fraction. In stage 2 (reducible fraction), the metals bound to iron/manganese oxides are identified; they are therefore unstable under reduction conditions. Changes in the redox potential (E_h) could induce the dissolution of these oxides, leading to their release from the soil/sediment. Finally, in stage 3 (oxidisable fraction), those metals bound to organic matter within the sediment/soil matrix are released into solution. The residual fraction is then acid-digested (*see* **Section 6**).

3.2.1. Stage 1 Extraction

1. 1 g of soil sample is weighed into a 80–100 mL PTFE centrifuge tube and 40 mL of acetic acid (0.11 M) – Solution A – is added (*see* **Note 5**).

2. The mixture is shaken in an end-over-end shaker at 30 rpm for 16 h at ambient temperature (22 ± 5°C) (*see* **Notes 6 and 7**).
3. Centrifuge at 3000*g* for 20 min.
4. Remove the supernatant with a pipette and store in a polyethylene bottle at 4°C.
5. Analyse extracts by ICP-MS (*see* **Notes 10 and 11**).
6. Wash the residue with 20 mL of water by shaking for 15 min.
7. Centrifuge the residue for 20 min at 3000*g* and discard the supernatant. Take care not to lose any of the solid residue.
8. Break the "cake" formed during centrifugation prior to stage 2.

3.2.2. Stage 2 Extraction

1. Add 40 mL of hydroxylammonium chloride (0.1 M, adjusted to pH 2 with nitric acid) – Solution B – to the residue from stage 1.
2. The mixture is shaken in an end-over-end shaker at 30 rpm for 16 h at ambient temperature (22 ± 5°C) (*see* **Note 6**).
3. Centrifuge at 3000*g* for 20 min.
4. Remove the supernatant with a pipette and store in a polyethylene bottle at 4°C.
5. Analyse extracts by ICP-MS (*see* **Notes 10 and 11**).
6. Wash the residue with 20 mL of water by shaking for 15 min.
7. Centrifuge the residue for 20 min at 3000*g* and discard the supernatant. Take care not to lose any of the solid residue.
8. Break the "cake" formed during centrifugation prior to stage 3.

3.2.3. Stage 3 Extraction

1. Add carefully, to avoid losses due to any violent reaction, 10 mL of hydrogen peroxide (8.8 M) – Solution C – to the residue from stage 2.
2. Allow the sample to digest for 1 h with occasional manual stirring. Ensure the container is covered with a watch glass (or similar).
3. Continue the digestion by heating the sample to 85 ± 2°C, with occasional manual stirring for the first 30 min, for 1 h in a water bath or similar.
4. Reduce the volume of liquid to 2–3 mL by further heating, after removal of the watch glass.
5. Add a further 10 mL of hydrogen peroxide (Solution C) and heat to 85 ± 2°C for 1 h in a water bath (with occasional manual stirring for the first 30 min).

6. Remove the watch glass and reduce the volume of liquid to approximately 1 mL by further heating.
7. Add 50 mL of ammonium acetate (1.0 M) – Solution D – to the cooled, moist residue.
8. The mixture is shaken in an end-over-end shaker at 30 rpm for 16 h at ambient temperature (20 ± 5°C).
9. Centrifuge at 3000*g* for 20 min.
10. Remove the supernatant with a pipette and store in a polyethylene bottle at 4°C.
11. Analyse extracts by ICP-MS (*see* **Notes 10 and 11**).

3.3. Physiologically Based Extraction Test or In vitro Gastrointestinal Extraction

In vitro gastrointestinal extraction consists of two sequential processes, a gastric and an intestinal digestion, each one carried out employing simulated human conditions (enzymes, pH and temperature) (2). Several distinct approaches for performing in vitro gastrointestinal extraction are available (7, 8); however, two are considered in this chapter.

3.3.1. Approach 1: Gastric Extraction

1. 0.3 g (accurately weighed) of sample is placed into a 50 mL screw-cap Sarstedt tube and treated with 30 mL of gastric juice.
2. The mixture is then shaken at 100 rpm in a thermostatic water bath maintained at 37°C.
3. After 1 h, the solution is centrifuged at 3000 rpm for 10 min and a 5 mL aliquot is removed and filtered through 0.45 μm filter disk.
4. The extracts are analysed by ICP-MS (*see* **Notes 10 and 11**).
5. 5.0 mL of the original gastric solution is then backflushed through the filter into the sample tube to retain the original solid:solution ratio, i.e. 0.3:30 g/mL.

3.3.2. Approach 1: Intestinal Extraction

1. Intestinal juice (52.5 mg bile salts and 15 mg pancreatin) is added into the sample tube and the mixture is adjusted to pH 7.0 with saturated $NaHCO_3$.
2. The sample is shaken at 100 rpm in a thermostatic water bath maintained at 37°C for a further 2 h.
3. A 5.0 mL aliquot is removed and filtered and analysed by ICP-MS.
4. After an additional 2 h, a second 5.0 mL extract aliquot is removed, filtered and analysed by ICP-MS (*see* **Notes 10 and 11**).
5. The second intestinal aliquot is used to check that the small intestinal equilibrium has been reached (9).

Example results for the in vitro gastrointestinal extraction of nine elements from two certified reference materials (INCT-TL-1 and SRM 1570a) are shown in **Table 2.2**. Data indicating that the additional 2 h equilibration period (*see* **Section 3.3.2**, Step 4) had no significance at the 95% confidence interval are shown in **Table 2.3** for the two certified reference materials. The repeatability of the in vitro gastrointestinal extraction for the recovery of eight elements from a contaminated soil digest on three separate occasions is shown in **Table 2.4**.

3.3.3. Approach 2: "Stomach" Extraction

1. 0.6 g (accurately weighed) of sample is placed into a 50 mL screw-cap Sarstedt tube and treated with 9 mL of simulated saliva fluid (*see* **Note 12**).
2. With the screw cap closed, manually shake the soil–fluid mixture.
3. After 5–15 min, add 13.5 mL of simulated gastric fluid.
4. The mixture is then shaken on an end-over-end shaker maintained at 37 ± 2°C.
5. After 1 h check the pH of the soil suspensions; the pH should be 1.2–1.7 (*see* **Note 13**).
6. The solution is centrifuged at 3000 rpm for 5 min and a 1.0 mL aliquot of supernatant is removed.
7. To the supernatant add 9.0 mL of 0.1 M HNO_3.
8. The sample is then stored at <8°C prior to analysis by ICP-MS (*see* **Notes 10 and 11**).

3.3.4. Approach 2: "Stomach + Intestine" Extraction

1. 0.6 g (accurately weighed) of sample is placed into a 50 mL screw-cap Sarstedt tube and treated with 9 mL of simulated saliva fluid (*see* **Note 12**).
2. With the screw cap closed, manually shake the soil–fluid mixture.
3. After 5–15 min, add 13.5 mL of simulated gastric fluid (*see* **Note 12**).
4. The mixture is then shaken on an end-over-end shaker maintained at 37 ± 2°C.
5. After 1 h check the pH of the soil suspensions; the pH should be 1.2–1.7 (*see* **Note 13**).
6. Then, add 27.0 mL of simulated duodenal fluid and 9.0 mL of simulated bile fluid (*see* **Note 12**).
7. With the screw cap closed, manually shake the soil–fluid mixture.
8. Adjust the pH of the resultant suspension to 6.3 ± 0.5 by the drop-wise addition of 37% HCl, 1 M or 10 M NaOH, as required.

Table 2.2
Example results for in vitro gastrointestinal extraction using approach 1
(A)

Element	Certified values of tea leaves (INCT-TL-1) (mg/kg)	Concentration (mg/kg)							
		Gastric stage		Intestinal stage		Residual stage		Σ Total stages	
	Mean ± SD	Mean ± SD ($n = 3$)	%	Mean ± SD ($n = 3$)	%	Mean ± SD ($n = 3$)	%	Mean ± SD ($n = 3$)	
Cr	1.91 ± 0.22	0.67 ± 0.13	32.57	0.73 ± 0.09	35.73	0.65 ± 0.09	31.70	2.04 ± 0.11	
Mn	1570 ± 110	998 ± 298	58	356 ± 231	21	360 ± 32	21	1714 ± 105	
Fe	(432)	1 ± 1	0.2	6 ± 2	1.5	429 ± 46	98.3	437 ± 43	
Ni	6.12 ± 0.52	2.68 ± 0.57	39.82	2.43 ± 0.24	36.05	1.63 ± 0.46	24.13	6.74 ± 0.43	
Cu	20.4 ± 1.5	3.7 ± 1.0	17.3	7.2 ± 0.5	33.3	10.7 ± 1.1	49.5	21.7 ± 0.4	
Zn	34.7 ± 2.7	17.0 ± 2.8	40.8	10.9 ± 1.3	26.2	13.7 ± 2.6	32.9	41.7 ± 4.9	
Mo	Na	0.005 ± 0.003	6.13	0.024 ± 0.005	27.20	0.058 ± 0.002	66.67	0.087 ± 0.003	
Cd	0.030 ± 0.004	0.016 ± 0.013	41.69	0.004 ± 0.003	9.91	0.018 ± 0.020	48.40	0.038 ± 0.012	
Pb	1.78 ± 0.24	0.13 ± 0.02	7.45	0.20 ± 0.02	11.51	1.40 ± 0.01	81.04	1.73 ± 0.05	

(continued)

Table 2.2 (continued)
(B)

Element	Certified values of spinach leaves (SRM 1570a) (mg/kg)	Concentration (mg/kg)						Σ Total stages
		Gastric stage		Intestinal stage		Residual stage		
	Mean ± SD	Mean ± SD ($n = 3$)	%	Mean ± SD ($n = 3$)	%	Mean ± SD ($n = 3$)	%	Mean ± SD ($n = 3$)
Cr	na	0.15 ± 0.02	9.64	0.29 ± 0.07	18.54	1.11 ± 0.07	71.82	1.54 ± 0.08
Mn	75.9 ± 1.9	39.0 ± 0.6	47.0	31.0 ± 4.9	37.3	13.0 ± 2.0	15.7	83.0 ± 4.0
Fe	NA	38 ± 3	20.2	63 ± 3	33.3	88 ± 5	46.5	189 ± 6
Ni	2.14 ± 0.10	0.87 ± 0.06	42.13	0.73 ± 0.09	35.53	0.46 ± 0.18	22.33	2.06 ± 0.20
Cu	12.2 ± 0.6	6.4 ± 0.1	44.7	5.7 ± 0.4	40.4	2.1 ± 0.5	14.9	14.2 ± 0.4
Zn	82 ± 3	52 ± 2	57.1	30 ± 1	32.2	10 ± 0.4	10.7	92 ± 3
Mo	na	0.206 ± 0.041	37.07	0.312 ± 0.052	55.99	0.039 ± 0.011	6.94	0.557 ± 0.086
Cd	2.89 ± 0.07	1.02 ± 0.19	37.44	0.64 ± 0.11	23.42	1.07 ± 0.08	39.14	2.73 ± 0.37
Pb	na	0.120 ± 0.068	30.68	0.110 ± 0.075	28.15	0.161 ± 0.083	41.17	0.392 ± 0.076

na = not available

Table 2.3
Example results for the extraction equilibrium of the intestinal fluid phase

	Bioaccessible metals (mg/kg) – tea leaves (INCT-TL-1)						Bioaccessible metals (mg/kg) – spinach leaves (SRM 1570a)					
	Intestinal stage IIA		Intestinal stage IIB				Intestinal stage IIA		Intestinal stage IIB			
Element	Mean ($n = 3$)	SD	Mean ($n = 3$)	SD	*t*-stat	*P*-value	Mean ($n = 3$)	SD	Mean ($n = 3$)	SD	*t*-stat	*P*-value
Cr	0.730	0.093	0.760	0.100	–0.512	0.660	0.286	0.073	0.302	0.098	–0.794	0.511
Mn	356.020	230.635	324.751	201.412	1.737	0.225	30.972	4.873	30.086	3.757	0.946	0.444
Fe	6.415	1.901	5.990	1.912	5.899*	0.028*	62.857	3.005	59.936	1.364	2.752	0.111
Ni	2.429	0.236	2.208	0.101	1.247	0.339	0.733	0.091	0.720	0.117	0.873	0.475
Cu	7.212	0.465	7.147	0.985	0.162	0.886	5.741	0.431	5.923	0.601	–0.773	0.520
Zn	10.934	1.264	10.832	1.304	0.191	0.866	28.621	1.011	28.731	2.432	–0.109	0.923
Mo	0.024	0.005	0.020	0.003	3.417	0.076	0.312	0.052	0.278	0.059	3.687	0.066
Cd	0.004	0.003	0.003	0.001	1.153	0.368	0.639	0.115	0.603	0.131	2.705	0.114
Pb	0.199	0.024	0.215	0.038	–1.019	0.415	0.110	0.075	0.115	0.078	–1.982	0.186

Note: *t*-critical (two-tail) is 4.303 and *P*-values are reported at 5% significance level.
*1% significance level giving *t*-critical = 9.925.
Intestinal stage IIA refers to **Section 3.3.2**, Step 1–3, while intestinal stage IIB refers to **Section 3.3.2**, Step 4.

Table 2.4
Example results for in vitro gastrointestinal extraction using approach 1
(a) Data from week 1

Element	Aqua regia digest of soil (mg/kg)	Concentration (mg/kg)						
		Gastric stage		Intestinal stage		Residual stage		Σ Total stages
	Mean ± SD	Mean ± SD ($n = 3$)	%	Mean ± SD ($n = 3$)	%	Mean ± SD ($n = 3$)	%	Mean ± SD ($n = 3$)
Cr	130.2 ± 4.8	7.46 ± 0.15	5.2	9.89 ± 0.32	6.8	127.0 ± 12.9	88.0	144.3
Mn	4980 ± 207	2269 ± 99	46.2	1349 ± 104	27.5	1293 ± 23	26.3	4911
Ni	69.1 ± 3.3	7.80 ± 0.22	11.9	5.80 ± 0.21	8.9	51.8 ± 1.5	79.2	65.4
Cu	25.0 ± 2.8	1.78 ± 0.03	8.9	5.25 ± 0.09	26.5	12.8 ± 0.3	64.6	19.8
Zn	133.4 ± 3.8	19.8 ± 1.4	14.2	4.86 ± 0.11	3.5	114.6 ± 4.7	82.3	139.2
Mo	4.2 ± 0.4	0.43 ± 0.01	12.3	0.62 ± 0.01	17.7	2.45 ± 0.02	70.0	3.5
Cd	0.91 ± 0.02	0.14 ± 0.00	17.1	0.04 ± 0.01	4.9	0.64 ± 0.01	78.0	0.82
Pb	59.8 ± 0.4	0.67 ± 0.06	1.6	0.50 ± 0.03	1.2	41.8 ± 0.8	97.2	43.0

(continued)

Table 2.4 (continued)
(b) Data from week 2

Element	Aqua regia digest of soil (mg/kg)	Concentration (mg/kg)						Σ Total stages
		Gastric stage		Intestinal stage		Residual stage		
	Mean ± SD	Mean ± SD ($n = 3$)	%	Mean ± SD ($n = 3$)	%	Mean ± SD ($n = 3$)	%	Mean ± SD ($n = 3$)
Cr	130.2 ± 4.8	6.31 ± 0.13	4.5	10.78 ± 0.28	7.7	122.9 ± 8.9	87.8	139.9
Mn	4980 ± 207	1554.3 ± 50.2	29.6	802.0 ± 18.2	15.2	2900 ± 70	55.2	5256
Ni	69.1 ± 3.3	6.20 ± 0.40	11.1	7.59 ± 0.37	13.7	41.8 ± 3.1	75.2	55.6
Cu	25.0 ± 2.8	1.62 ± 0.05	6.3	6.00 ± 0.21	23.2	18.2 ± 1.1	70.5	25.8
Zn	133.4 ± 3.8	24.4 ± 4.9	19.0	4.44 ± 0.23	3.5	99.6 ± 3.6	77.5	128.4
Mo	4.2 ± 0.4	0.51 ± 0.01	10.5	0.76 ± 0.03	15.6	3.59 ± 0.06	73.9	4.9
Cd	0.91 ± 0.02	0.13 ± 0.01	14.3	0.06 ± 0.06	6.6	0.72 ± 0.01	79.1	0.9
Pb	59.8 ± 0.4	0.29 ± 0.03	0.5	0.62 ± 0.06	1.1	55.7 ± 1.5	98.4	56.6

(continued)

Table 2.4 (continued)
(c) Data from week 3

Element	Aqua regia digest of soil (mg/kg)	Concentration (mg/kg)						
		Gastric stage		Intestinal stage		Residual stage		Σ Total stages
	Mean ± SD	Mean ± SD ($n = 3$)	%	Mean ± SD ($n = 3$)	%	Mean ± SD ($n = 3$)	%	Mean ± SD ($n = 3$)
Cr	130.2 ± 4.8	8.05 ± 0.43	7.6	9.62 ± 0.57	9.1	88.3 ± 8.5	83.3	106
Mn	4980 ± 207	1591.7 ± 34.5	30.8	606.6 ± 14.2	11.8	2963 ± 101	57.4	5161
Ni	69.1 ± 3.3	6.64 ± 0.30	11.7	3.64 ± 0.14	6.4	46.5 ± 2.2	81.9	56.8
Cu	25.0 ± 2.8	1.38 ± 0.10	5.8	2.60 ± 0.14	10.8	20.0 ± 1.3	83.4	24.0
Zn	133.4 ± 3.8	18.83 ± 0.83	13.0	1.90 ± 0.27	1.3	124.6 ± 2.3	85.7	145.3
Mo	4.2 ± 0.4	0.60 ± 0.03	13.2	0.62 ± 0.01	13.6	3.32 ± 0.18	73.2	4.5
Cd	0.91 ± 0.02	0.24 ± 0.01	32.9	0.04 ± 0.00	5.5	0.45 ± 0.01	61.6	0.73
Pb	59.8 ± 0.4	0.33 ± 0.03	0.6	0.88 ± 0.06	1.5	58.8 ± 6.1	98.0	60.0

9. The mixture is then shaken on an end-over-end shaker maintained at 37 ± 2°C for 4 h.
10. Remove the soil suspension.
11. Measure (and record) the pH of the soil suspension; pH should be 6.3 ± 0.5.
12. The soil suspension is then centrifuged at 3000 rpm for 5 min and a 1.0 mL aliquot of supernatant is removed.
13. To the supernatant is added 9.0 mL of 0.1 M HNO_3.
14. The sample is then stored at <8°C prior to analysis by ICP-MS (*see* **Notes 10 and 11**).

3.4. Method: Soil Digestion Procedure

An acid digestion procedure is used to provide pseudo-total metal analysis.

3.4.1. Acid Digestion Procedure

1. Approximately 1 g of soil sample is accurately weighed into a digestion tube (250 mL volume).
2. Add 0.5–1.0 mL of water to obtain a slurry.
3. Then add, while mixing, 7 mL of 12.0 M HCl, followed by 2.3 mL of 15.8 M HNO_3 (drop by drop, if necessary to reduce foaming) (***see Note 8***).
4. Add 15 mL of 0.5 M HNO_3 to the reaction vessel and connect to a water-cooled reflux condenser.
5. Allow to stand for 16 h at room temperature to allow slow oxidation of the organic matter of the soil.
6. Raise the temperature of the reaction mixture until reflux conditions are achieved and maintain for 2 h.
7. Allow to cool slowly to room temperature.
8. Rinse the contents of the condenser into the reaction vessel with 10 mL of 0.5 M HNO_3.
9. Quantitatively transfer the contents of the reaction vessel to a 100 mL volumetric flask. Rinse the vessel with 0.5 M HNO_3 and transfer as well. Make up to the mark with water, stopper and shake.
10. Allow the undissolved matter to settle and then analyse the supernatant solution by ICP-MS (*see* **Notes 10 and 11**).

3.4.2. Alternate Acid Digestion Procedure

1. Approximately 1 g of soil sample is accurately weighed into a digestion tube and 10 mL of 1:1 v/v concentrated HNO_3:water is added.
2. The mixture is then heated at 95°C on a heating block for 15 min without boiling.

3. After cooling at room temperature for 5 min, 5 mL concentrated HNO_3 is added and the sample is heated at 95°C for 30 min.
4. An additional 5 mL of concentrated HNO_3 is added until no brown fumes are given off.
5. Evaporate the solution to <5 mL.
6. After cooling, 2 mL of water and 3 mL of 30% H_2O_2 are added and heated (<120°C) until effervescence subsides and the solution cools. Additional H_2O_2 is added until effervescence ceased (but add no more than 10 mL H_2O_2). This stage is continued for 2 h.
7. Evaporate the solution to <5 mL.
8. After cooling, add 10 mL of concentrated HCl and heat (<120°C) for 15 min.
9. After cooling, filter the sample through a Whatman No. 41 filter paper into a 100 mL volumetric flask, and then make up to the mark with water.
10. Analyse by ICP-MS (*see* **Notes 10 and 11**).

3.5. Method: Sample Analysis by ICP-MS

ICP-MS measurement conditions are optimised daily using the built-in PlasmaLab software procedure. Samples of the soil extracts/digests are analysed by ICP-MS using an external calibration technique. Sc, In and Tb internal standards (10 μg/L) are added to all samples, blanks and standard solutions. A blank is analysed with each analytical batch (*see* **Note 9**).

3.5.1. ICP-MS Operating Conditions: Standard Mode

1. In standard mode the following elements can be analysed: >90 amu
2. Forward power, 1400 W; coolant gas flow, 13.0 L/min; auxiliary gas flow, 0.90 L/min; nebuliser gas flow, 0.80 L/min; quadrupole bias, −1.0 V; hexapole bias, 0.0 V; dwell time per isotope, 10 ms.

3.5.2. ICP-MS Operating Conditions: Collision/Reaction Cell Mode

1. In collision/reaction cell mode the following elements can be analysed: <90 amu
2. Forward power, 1400 W; coolant gas flow, 13.0 L/min; auxiliary gas flow, 0.90 L/min; nebulizer gas flow, 0.80 L/min; collision cell gas, 4.50 L/min of 7% H_2/93% He; quadrupole bias, −14.0 V; hexapole bias, −15.0 V; dwell time per isotope, 10 ms.

4. Notes

1. Unless otherwise stated, all solutions should be prepared in water that has a resistivity of 18.2 MΩ × cm. This standard is referred to in the text as "water".
2. All laboratory ware should be made of borosilicate glass, polypropylene, polyethylene or PTFE, except for the centrifuge tubes, which should be made of borosilicate glass or PTFE.
3. All vessels in contact with samples or reagents should be cleaned in HNO_3 (4 mol/L) for at least 30 min, then rinsed with distilled water, cleaned with 0.05 mol/L EDTA and rinsed again with distilled water. Alternatively clean all vessels by immersing in HNO_3 (4 mol/L) overnight and then rinse two to three times with water.
4. When extracting with sodium nitrate ($NaNO_3$), it is necessary to correct the results for the difference in final volume, i.e. 2 mL of HNO_3 was added to 48 mL of extract to give a final volume of 50 mL.
5. When using sequential extraction methods for the analysis of sediment or soil samples, a separate sub-sample should be dried (in a layer of approximately 1 mm depth) in an oven at 105 +/− 2°C for 2–3 h, transferred to a desiccator and allowed to cool prior to weighing.
6. Ensure that the sample, i.e. sediment/soil, does not form a "cake" during the extraction procedure. If a cake is formed, either adjust the shaking speed to ensure that the suspension is maintained or mechanically break the solid "cake" with a pre-cleaned glass rod. It is important that the sample remain in complete suspension during the extraction process.
7. In sequential extraction the mechanical shaker, preferably of the end-over-end type, should be operated at a speed of 30 +/− 10 rpm and a temperature of 22 +/− 5°C. All samples should be centrifuged at 3000*g* for 20 min.
8. The combination of 12.0 mol/L HCl and 15.8 mol/L HNO_3 in a volume ratio of 3:1, respectively, is known as aqua regia.
9. Calibration solutions for ICP-MS should be prepared with the appropriate extraction solution, i.e. use matrix-matched calibration solutions.
10. It is important to prepare a sample blank for every batch of extractions, i.e. prepare a container with no sediment/soil,

but treated in the same manner as though it contained the sample.

11. It is recommended for ICP-MS that all extracts be filtered (0.45 μm) prior to analysis.
12. Simulated gastrointestinal fluids are stored at room temperature overnight prior to use. Prior to their use for bioaccessibility studies, the fluids need to be heated to 37°C at least 2 h before their use on the day following their preparation.
13. If the pH of a sample suspension is not within the guideline of 1.2–1.7, the sample should be discarded and subsamples re-extracted. Before re-extracting, however, add an additional amount of 37% HCl (up to a maximum of 1.0 mL).

Acknowledgements The author would like to thank Dr Marisa Intawongse for evaluation of some of the methods described. Also, the technical assistance of Mr. Gary Askwith is acknowledged.

References

1. Dean, J.R. (2003) *Methods for Environmental Trace Analysis*, John Wiley and sons Ltd., Chichester.
2. Dean, J.R. (2007) *Bioavailability, Bioaccessibility and Mobility of Environmental Contaminants*, John Wiley and sons Ltd., Chichester.
3. Quevauviller, Ph., (1998) Operationally defined extraction procedures for soil and sediment analysis I. Standardization. *Trends Anal. Chem.* **17**, 289–298.
4. Quevauviller, Ph. (1998) Operationally defined extraction procedures for soil and sediment analysis: II. Certified reference materials. *Trends Anal. Chem.* **17**, 632–642.
5. Quevauviller, Ph. (2002) Operationally-defined extraction procedures for soil and sediment analysis. Part 3: New CRMs for trace-element extractable contents. *Trends Anal. Chem.* **21**, 774–785.
6. Rauret, G., Lopez-Sanchez, J.F., Sahuquillo, A., Barahona, E., Lachica, M., Ure, A.M. et al. (2000) Application of a modified BCR sequential extraction (three-step) procedure for the determination of extractable trace metal contents in a sewage sludge amended soil reference material (CRM 483), complemented by a three-year stability study of acetic acid and EDTA extractable metal content *J. Environ. Monit.* **2**, 228–233.
7. Oomen, A.G., Hack, A., Minekus, M., Zeijdner, E., Schoeters, G., Verstraete, W. et al. (2002) Comparison of five in vitro digestion models to study the bioaccessibility of soil contaminants. *Environ. Sci. Technol.* **36**, 3326–3334.
8. Intawongse, M. and Dean, J.R. (2006)*In vitro* testing for assessing oral bioaccessibility of trace metals from soil and food samples. *Trends Anal. Chem.* **25**, 876–886.
9. Wragg, J. and Cave, M.R. (2002) *In vitro Methods for the Measurement of the Oral Bioaccessibility of Selected Metals and Metalloids in Soils: A Critical Review*. Environmental Agency, Bristol, UK.

Chapter 3

Addressing PCR Biases in Environmental Microbiology Studies

Rita Sipos, Anna Székely, Sára Révész, and Károly Márialigeti

Abstract

Each step of a molecular environmental microbiology study is prone to errors, though the qualitative and quantitative biases of PCR amplification could result in the most serious biases. One has to be aware of this fact, and well-characterized PCR biases have to be avoided by using target-optimized PCR protocols. The most important tasks are primer and thermal profile optimization. We have shown that primer mismatches, even in the case of universal primers, can cause almost complete missing of common taxa from clone libraries, for example. Similarly high annealing temperatures can drastically distort community composition of the sample in the PCR product. Strategies of primer selection and PCR thermal profile design are discussed in detail.

Key words: PCR bias, environmental microbiology, PCR optimization, PCR primer selection, PCR thermal protocol.

1. Introduction

PCR-based microbial community analyses are widely used in microbial ecology and, for most environments, they give a more realistic picture about community structure than classical techniques based on cultivation. Over the past decades, this technique has helped to identify, taxonomically, microorganisms that have never been cultured. In environmental microbiology, a routine molecular analysis starts with the thorough sampling of the material to be investigated (soil, water, sediment, etc.). Subsequently, isolation and purification of nucleic acids is followed by the corner-stone of the technique: a nucleic acid amplification

S.P. Cummings (ed.), *Bioremediation*, Methods in Molecular Biology 599,
DOI 10.1007/978-1-60761-439-5_3,

step using PCR, with primers binding to conserved regions of specific genes containing phylogenetic or functional information. The template for the PCR amplification is a mixture of homologous genes; therefore, the objective is to produce adequate amounts of DNA from each taxon present in the sample from which specific taxa can be distinguished qualitatively and quantitatively. The analysis of the heterogeneous PCR products is carried out by cloning and sequence analysis or by different fingerprinting techniques, such as denaturing gradient gel electrophoresis (DGGE), single-strand conformation polymorphism (SSCP), or terminal restriction fragment length polymorphism (T-RFLP).

During the introduction of PCR-based analysis of rRNA genes into environmental microbiology, acquiring sufficient amount of specific PCR product seemed to be the ultimate goal of the studies. However, with an increasing interest in understanding the community function, both the qualitative and the quantitative compositions of microbial communities have become important. Thus, emphasis has been put on the possible distorting effects of sample preparation and PCR conditions on the original gene ratios of the investigated samples. This is particularly important in bioremediation studies, where detecting the presence of a given taxon may not be informative, whereas knowledge of the activity and quantity of that particular taxon is crucial for in situ intervention and remediation.

Although each step in the molecular approach can introduce errors, the qualitative and quantitative biases experienced during the PCR amplification step may result in the most serious distortions of the original composition of the microbial community investigated. The operation of the PCR technique is based on an exponential increase in the amount of original templates with each amplification cycle, thus the negative effect of bias occurring during the first cycles of the reaction could be magnified, leading to false conclusions about the initial composition of the microbial community. Moreover, any errors during the preceding steps of sample preparation (e.g., sampling and nucleic acid isolation) would also be multiplied by the PCR technique. Subsequent analyses (clone library or fingerprinting techniques) carry over these biases, questioning the implicit assumption that abundant bacteria in environmental samples are represented by dominant clones in 16S/18S rDNA libraries or strong bands in molecular fingerprinting patterns (1).

For these reasons, the "multi-template" or "mixed-template" PCR is the most crucial step in the procedure of microbial community analysis. The fundamental problem of "multi-template" PCR arises from the fact that this technique – as with most of the other techniques applied in microbial ecology – was derived from the field of diagnostic microbiology, and was therefore, developed for specialized detection purposes. Originally, PCR was devel-

oped to multiply one specific DNA fragment. Similarly, DGGE was used for mutation detection and the method was optimized for a single organism and usually a single DNA template. Neither technique was developed and optimized for complex template systems of such diverse environmental templates as soil, sediment, or sludge microbiota (2). While differences in cell lysis efficiencies may be overcome by the combination of different nucleic acid isolation techniques (3), the quantitative aspect of the multi-template PCR amplification step is poorly understood for complex genomic DNA templates.

2. Materials

Any PCR reaction is made up of the following components: the template DNA, forward and reverse primers, DNA polymerase enzyme, and all the chemicals required for its proper functioning (dNTPs, Mg^{2+}, buffer, additives if any). There are several commercially available enzymes, buffers, and kits that can be chosen according to the type of experiment; however, it is very important to use the same brand and lot of chemicals, consumables, and types of instrument throughout the entire investigation, ensuring the consistency of the results for correct conclusions. The ultimate specificity of a given PCR setup is determined by the template and the primer system together, as their complementarity is the underlying principle of the reaction.

2.1. Template

The quality and concentration of environmental DNA templates can significantly affect the outcome of multi-template PCR.

2.1.1. Template Concentration

For standard mono-template PCR, the recommended amount of DNA template is around 1 ng in 50 μL reaction mixture (a concentration of 0.02 ng/μL). In multi-template environmental PCRs, higher amounts of DNA template are used; nevertheless, a DNA concentration of maximum 1 ng/μL is suggested because too high amounts of DNA template could increase the yield of nonspecific PCR products (4). To achieve this DNA concentration, measurement of the isolated DNA is recommended. The problem of too high concentrations can easily be solved by diluting the original DNA solutions. Amplification should also be attempted with lower template DNA concentrations. However, if little or no PCR product is detected, the following solutions can be considered: (1) repeating the DNA isolation step in the hope of higher DNA yields; (2) concentrating the DNA template by co-purification of various parallel isolated DNA; (3) conducting nested PCR (see below).

The following example illustrates how to concentrate DNA templates in an environmental sample that yields no more than 1 ng/μL DNA. Combining the results of 10 parallel DNA isolation procedures, each with a volume of 100 μL, will lead to 1 mL of crude DNA solution with 1 ng/μL concentration. If you concentrate your 1 mL DNA solution with a silica matrix-based purification system generating a 30% loss of DNA, and elute your DNA in 50 μL, you will get a DNA template with 14 ng/μL concentration, resulting in a 14-fold concentration. The same procedure can be applied to PCR products if downstream applications (e.g., DGGE) require a more concentrated sample.

2.1.2. Template Quality

The quality of DNA templates is a common reason for environmental PCR failure. In order to avoid this, special care must be taken when performing nucleic acid extractions. Isolation of pure DNA from soil samples has been a challenge because of the complex and heterogeneous nature of the soil matrix and the inhibition of biochemical reactions by co-extracted substances (5-8). It is of primary importance to take into consideration the chemical characteristics (pH, clay content, organic matter content, and composition) of a given soil sample prior to DNA extraction, as the choice of method greatly affects both DNA yield and purity, influencing subsequent PCR performance (9). Most common contaminants include humic acids, polysaccharides, and urea, which exhibit similar solubility properties to DNA. Humic impurities from soil are resistant to separation from DNA because of their similar chemical properties (10). They are not completely removed during classical extraction protocols, such as phenol–chloroform, detergent or protease treatments, remaining as contaminants in the final DNA preparations. Furthermore, highly organic soils containing humic acids with phenolic groups covalently bind to DNA or proteins.

Prior to PCR amplification, isolated DNA should be quality checked on an agarose gel. Observing a smear is the usual sign of degraded or sheared DNA, which can lead to poor PCR performance and nonspecific products. In this case, repeated DNA isolation, with special care against nicking and shearing, and/or purification on a size-selecting system is recommended. Bluish clouds on the lower part of the agarose gel indicate the presence of humic substances that inhibit DNA polymerases (11).

Several purification methods have also been evaluated to remove humic acids from soil DNA. Some of these include the use of hexadecyltrimethylammonium bromide (CTAB), caesium chloride density gradients, polyvinylpolypyrrolidone (PVPP), various gel filtration resins, and ion exchange and size-exclusion chromatography (12). However, many inhibitors cannot be detected and identified; therefore, their specific elimination is not possible. Repurification and/or dilution (5- to 100-fold) or

repeated DNA isolation are generally proper solutions for inhibition problems.

2.2. Primer

The most essential factor affecting a successful PCR amplification is the choice of PCR primers. Choosing the appropriate primer for a given experiment in microbial ecology depends on the purpose of the investigation: whether it is the survey of the genetic diversity of total microbial communities, the detection of individual taxa of interest, or the determination of the relative abundance and activity of certain groups of microbes. The intended use ultimately determines whether primers for broad or narrow phylogenetic spectrum are required, and whether protein-coding genes or structural genes, like ribosomal genes, are targeted.

2.2.1. Broad Spectrum or "Universal" Primers

In the case of targeting to reveal the prokaryotic diversity of a sample of previously unknown composition, the most common choice is to target the gene coding the small ribosomal subunit (16/18S rRNA). However, designing the perfect "universal" primer pair is not possible (13). In the case of bacterial 16S rRNA sequences, the completely conserved regions make up more than 10% of the entire 16S rRNA sequence; however, the longest running segment is merely 10 bases long. Moreover, these completely conserved nucleotide positions are usually not adjacent to each other and in most cases these stretches are less than 3 bases long, making it impossible to design a primer of sufficient length that would match all bacterial sequences 100% (14) (**Table 3.1**). The "universal" primers were originally designed on database information available at the time of "invention," and they are rarely updated in light of the new 16S rRNA sequence information available. Some of the "truly" universal primers – or "sequencing primers," as they are called in laboratory slang such as 27F or 1492R (15), should not be taken for granted since a large portion of database sequences is missing parts at either end of the whole 16S sequence, making them less reliable when checking complementarity between primer and target sequences during primer selection.

The bacterial 63F forward primer was designed by Marchesi et al. (16) as part of a new 16S RNA bacterial "universal" primer set, which, despite having bias against certain groups of bacteria, demonstrated better PCR amplification performance from cultures and environmental samples than other "universal" primers. This primer became widely used in microbial ecology studies, but successful PCR amplification with 63F from mono-cultured bacteria was not always realized when using complex environmental samples as templates, resulting in completely missing taxa from clone libraries (17). The phenomenon was most striking when an investigation was carried out with culture-dependent and culture-independent methods in parallel, and the dominant

Table 3.1
Widely used 16S rRNA primers (16, 40, 48) and their specificity measured by the percent ratio of the number of sequences that matched (allowing 0 or 2 mismatches) and the number of all good-quality sequences for the given taxon (domain, phylum) deposited in the RDP database as of September 2008. Percent of matching sequences below 10% are highlighted black, between 10% and 50% are gray, and >50% is not highlighted

Primer	Mismatches	Bacteria (579 640)	Proteobacteria (214 950)	Firmicutes (149 767)	Bacteroidetes (66 677)	Actinobacteria (49 833)	Acidobacteria (21 222)	Cyanobacteria (17 507)	Verrucomicrobia (6 066)	Planctomycetes (5 475)	Chloroflexi (4 754)
63F	0	6.7	16	0.22	5.7	0.46	0.56	0.85	0.02	0.00	0.06
	2	29	39	21	56	3.4	25	8.2	0.18	0.02	6.7
341F	0	73	74	83	81	78	72	55	0.69	0.47	29
	2	80	77	89	84	82	81	58	79	72	65
519R	0	70	71	77	81	66	70	58	68	19	12
	2	79	76	86	84	78	79	79	75	78	74
968F	0	34	27	59	0.97	50	24	39	55	4	10
	2	56	55	64	59	54	28	46	58	50	67
1387R	0	23	26	18	23	32	13	30	15	28	17
	2	39	41	42	35	40	17	32	36	31	34
1401R	0	23	26	18	24	33	13	30	12		18
	2	40	41	43	35	42	19	32	38	31	36
Uncultured sequences		77	71	81	91	51	99	75	98	97	98

isolates (*Bacillus* spp.) were completely missing from the clone library, most likely due to the numerous mismatches against the genus (18). The problematic amplification of *Firmicutes* due to primer mismatches with the 63F primer from multi-template PCR is described in detail later in this section. This example shows how difficult the case of "universal primers" is, as with the onset of newly discovered taxonomical groups within the prokaryotes, and the wider range of microbial ecology applications, the previously termed "universal" primers may turn out to be preferential and might even fall back to be tagged as "taxon-specific."

When the aim is to reveal the total diversity of a given sample, the best approach would be to attack the community total genomic DNA with several "broad-spectrum" primers at the same time. Once the general composition of a microbial community is determined, key groups of interest can be targeted for further, more detailed analysis with "taxon-specific primers."

The use of several core housekeeping genes, such as those coding for the RNA polymerase (*rpoB*), DNA recombinase (*recA*), or the GTP-binding protein (*lepA*), have been suggested as alternative or additional universal phylogenetic markers (19). Case et al. (20) compared the phylogenetic resolution of *rpoB* with that of the 16S rRNA gene. The results were comparable

at all taxonomic levels; furthermore, *rpoB* provided better resolution at species and subspecies levels. An additional advantage is that *rpoB* is a single-copy gene, therefore, free of the bias resulting from different copy numbers encountered with the 16S rRNA-based investigations (21). The major obstacle of using housekeeping genes or any other protein coding gene is the saturation of all third codon positions, which makes it even more difficult to design primers, especially universal ones. Among the disadvantages of housekeeping genes is the lower concentration of target mRNAs in cells, which makes them less suitable for analysis like FISH (20).

2.2.2. Narrow Spectrum or Taxon/Functional Group-Specific Primers

Phylogenetic analyses make use of 16S rRNA primers targeting defined groups within the domain Bacteria (often used in conjunction with broad-spectrum "universal" primers, e.g., different types of Proteobacteria, Actinobacteria, Acidobacteria (22–24), or the Archea (25)). These group-specific 16S rDNA primers are used for inferring phylogenetic relationship among different taxa; however, discrimination of closely related species, e.g., within a given genus, is often impaired. As an example, in the case of investigating the diversity of the beta-subclass of ammonia-oxidizing bacteria (AOB), due to the limited resolution of several 16S rDNA primers (26) the use of protein-encoding genes for certain functions of AOB (e.g., *amo* A, *amo* B genes) should be a better choice. As with all protein-coding genes, usually degenerate primers are utilized.

Often the primary objective of an experiment is the investigation of a certain biological function, and the gene which is responsible for that function might well be identified in distantly related groups; this could happen when the genes are evolutionarily ancient or as a result of horizontal gene transfer (27). Such functional analyses include the examination of biodegradation of certain compounds or key enzymatic transformations in the biogeochemical cycling of elements; in these cases, the protein-encoding genes for the given functions are also applied (biogeochemical cycling: *mcr* A, *dsr* A, *pmo* A, *nir* S, *nir* K, *nos* Z (27); biodegradation: *bph* A, *cpr* A, *cat* A). However, one must be very careful when employing previously described primers from the existing literature, as primer selectivity could give false results, thus primers must be re-evaluated in the context of the given experiment (28).

2.3. Primer Selection

2.3.1. General Aspects

There is no golden rule for finding the perfect primer for an experimental setup; one must always balance between specificity and efficiency or yield of the PCR product. Several publications have been released on the general attributes for primer selection for a given PCR that have to be met in order to achieve a successful PCR with a high yield. These include optimal primer size (18–28

mer oligonucleotide), melting temperature (T_m), difference of melting temperatures of primers from both directions (maximum of 5°C), G/C content (40–60%), specific binding at the 3′ end of primers, avoidance of secondary structures produced by inter- or intramolecular interactions (primer–dimer formation and hairpin structures), primer concentration, etc. (29). Several computer programs aiding the primer designing process are also available. The type of post-PCR analysis (DGGE, T-RFLP, SSCP, clone library construction) also help to select from possible primer pair alternatives, as the limitations and resolution of the applied techniques confine the original search. For example, in the case of DGGE, the maximum size of PCR amplicons that can be successfully resolved with electrophoretic separation is approximately 500–600 bp long, which greatly affects primer selection.

2.3.2. Multi-template PCR

The above-mentioned features are practical considerations for almost any kind of PCR amplification strategy. However, when dealing with environmental samples in a multi-template PCR setting, one must concentrate, most of all, on avoiding biases, such as preferential amplification due to primer selectivity in the case of total community analyses, or the production of unwanted amplicons owing to inadequate selectivity of the primers when aiming at the detection of a specific group. Primer design is the most important element of conducting an unbiased PCR amplification (21, 30). Primers perfectly binding to all targeted templates should be selected to avoid biases. Nonetheless, achieving this theoretical criterion is impossible due to the lack of appropriate conserved regions with identical sequence in every known and yet-to-be-known target; therefore, primers usually contain mismatches.

A mismatch position within a primer is a nucleotide position where the sequence of the target and the primer are not complementary. Although mismatch sites naturally reduce PCR specificity and increase the possibility of mispriming and nonspecific amplification, their incorporation into primer design is inevitable in many cases. This is particularly true for the so-called universal 16S rRNA primers. The task is to find a compromise between the desired "universal complementarity" and specificity. Several studies concluded that mismatches within primers greatly affect the outcome of a multi-template PCR. Moreover, different factors were determined (number, position and types of mismatches), which significantly affected the exact degree of mismatch impact on the product-to-template ratios of multi-template PCR; these are summarized below:

1. The presence of mismatch sites within the primer causes preferential amplification of the perfectly matching targets, as demonstrated in **Fig. 3.1**(21).

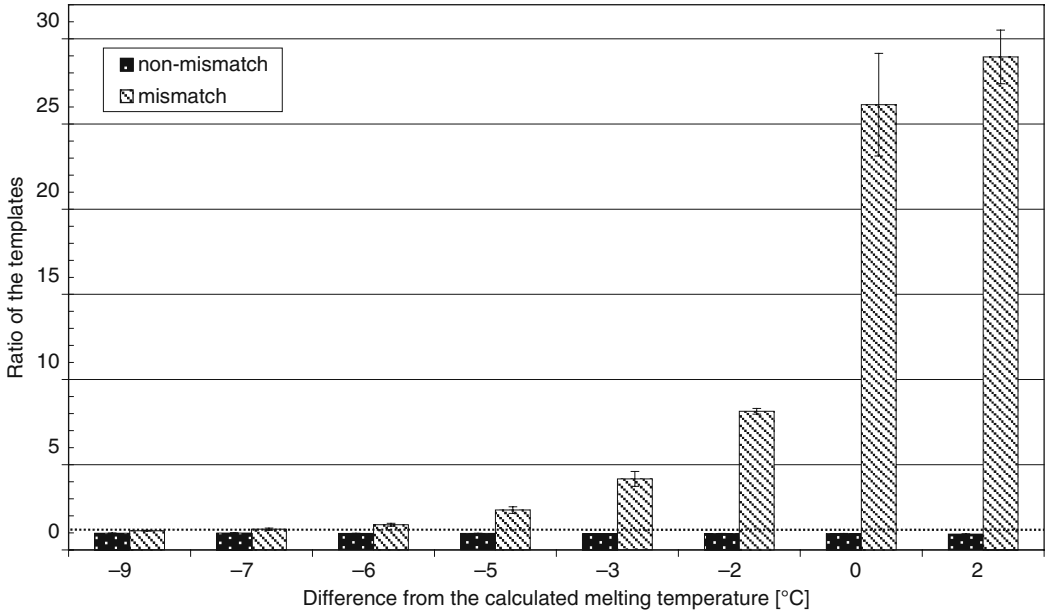


Fig. 3.1. The presence of mismatch sites within the primer causes preferential amplification. Applying mixed DNA templates of strains in a 1:1 concentration ratio. Strains containing no mismatches were preferentially amplified over the ones showing three mismatches with this primer (mismatch setup), while the perfectly matching primer amplified all templates without bias (non-mismatch setup). The relationship between the applied annealing temperature (expressed here as the difference from the calculated melting temperature of the primer) and the deviation from the theoretical PCR amplicon ratios changed almost exponentially.

2. The position of the mismatches are crucial for amplification, as when the mismatch is closer to the 3′ end of the primer, the drop in PCR amplification efficiency is more pronounced. A single mismatch at the 3′ end of a primer can inhibit PCR amplification altogether (30). Furthermore, it makes a difference on which primer the mismatch is positioned (31).
3. The number of mismatches greatly influences PCR amplification efficiency, as the number of mismatches per sequence is negatively correlated with amplification efficiency (32, 33).
4. Lowering the annealing temperature helps to compensate for the weak PCR amplification that is obviously caused by the mismatch effect (21, 34).

Intentional utilization of the mismatch effect is worthwhile when the objective of the experiment is detecting low-frequency species of great economic or physiological importance. The design of a detection system with deliberate utilization of preferential amplification could result in a lower detection limit of the given taxon, thus making it a useful approach when the absence or presence of a certain group or initial estimation is needed.

Alternative solutions to the use of mismatches:

1. A PCR primer sequence is called degenerate if some of its positions have several possible nucleotide bases. A degenerate primer is an equimolar mixture of a number of primers. One solution to increase the amplification spectrum of primers is to introduce degenerate nucleotide positions, but some studies report biased template-to-product ratios (35) and too high a degree of degeneracy could lead to amplification of non-target sequences. Also when applying degenerate DGGE primers in microbial community fingerprinting analysis, a single sequence produces multiple bands, overestimating the "true" diversity (30).
2. Another option is entering deoxyinosines at mismatch positions instead of increasing the degeneracy of the primer and thereby extending the detectable diversity in environmental samples (36).
3. In order to reduce PCR amplification bias due to primer selectivity, one could perform several parallel PCR reactions with different, slightly modified primers and, thereafter, merge these reaction mixtures prior to further genetic analysis (14).

Steps of primer selection

1. Choose TARGET (protein-coding genes or structural genes) and RANGE (broad = "universal" and narrow = "taxon-specific")
2. Check the scientific literature for existing primers where available, and make your choice again from the accessible primer selection according to the purpose of the investigation, general primer criteria (length, T_m, number of allowed mismatches) and post-PCR analysis type.
3. Even when the reference article demonstrates good applicability of the given primer, always verify the specificity of the primers *in silico* against the currently available database information. ProbeCheck provides the latest platform for oligonucleotide probe and primer specificity evaluation against several databases of phylogenetic and functional marker genes including RDP II, Greengenes, SILVA (official database of ARB), and FGPR sequence collections (37). Other widely used programs for 16S rRNA primer coverage estimation include the "probe match" function of the RDP-II (http://rdp.cme.msu.edu (38)), or the ARB-PMO-"probe match online" as part of the ARB program package (http://pmo.arb-home.de (39)). The ARB package has the advantage of supplementing sequence information with 3D 16S rRNA structure; although it was originally designed for ribosomal RNA data, it can also be used with any kind of nucleic acid or amino acid sequence.

4. Even the very best of *in silico* evaluation of primer selectivity does not substitute for proper empirical evaluation against the desired target sequence (37).

If already existing primers are not available or do not meet certain key primer criteria set forth by the objective of the given investigation, one needs to design new primers.

1. Collect available sequences from databases as well as your own sequences for which the primer would be targeted. Align the different sequences using, e.g., ClustalW, to help identify similarities among different sequences. Saved alignment sessions could further serve as the basis for a magnitude of primer design programs (Primrose, PROBEmer, Primer3, GeneFisher, Primer Premier, etc.). Some of the programs offer the possibility to check non-target sequences alongside the target sequences for planning the suitable oligo. Generation of consensus sequences is followed by the selection of possible primers, which from here on depends on the user-specified criteria (length, number of degenerate nucleotides and maximum number of mismatch sites, self-homology, melting temperature, etc.). The offered oligonucleotides should be re-checked for the user-defined requirements.
2. A newly designed primer is primarily judged upon its phylogenetic coverage; thus always test the designed primer's specificity *in silico*, screening against databases to see how well it matches target sequences and excludes nontarget sequences. However, empirical testing with type strains and mixed-template or environmental genomic DNA templates is almost as relevant as the theoretical evaluation. Always use a negative as well as a positive control sample when checking the amplification efficiency. When testing the newly designed primer on environmental samples, determine both whether amplification has taken place and the composition of the PCR product by cloning and sequencing (40).

2.4. Enzyme and Accessories

Thermostable DNA polymerase enzyme is the key to PCR reaction, since it catalyzes the incorporation of dNTPs into DNA during amplification. The enzyme needs Mg^{2+} cation (0.5–5 mM), appropriate buffer, and dNTPs (20–200 μM each) for proper functioning. There are several commercially available DNA polymerases supplied with different buffer systems and, in some cases, built-in Mg^{2+} ion concentration (already optimized for the enzyme) for ready-to-use applications. However, these parameters must be optimized for each experiment individually, as the success of PCR amplification is highly dependent on the composition of the buffer system (41).

Standard PCR amplifications use *Taq* DNA polymerases with $5' \rightarrow 3'$ exonuclease activity, thus no proofreading function. Several thermostable DNA polymerases possess $3' \rightarrow 5'$ exonuclease-dependent proofreading activity (*Pfu*, *Vent*, *Deep Vent*, *Pwo*), which make them an excellent choice for high-fidelity amplifications desired for cloning, mutation analysis, and long PCR. For improved specificity, "hot start" versions of DNA polymerases (*PfuTurbo* Hotstart, HotMaster *Taq*, HotStar*Taq*, FastStart *Taq*) have been developed by reversible inactivation or addition of a blocking agent as an alternative for performing a "hot start" at the beginning of a PCR protocol.

The ultimate goal of a PCR is to achieve maximum product yield within the shortest time. Many studies demonstrated that the amplification efficiency of PCR is strongly affected by the type of DNA polymerase used (42-44). Because of the exponential nature of PCR, even a small difference in amplification efficiency could produce big variations in the amounts of PCR products. In order to obtain higher PCR yields, mixtures or blends of DNA polymerases, mixing non-proofreading and proofreading enzymes, have been applied (45). A smaller amount of proofreading enzyme has shown to improve the performance of the non-proofreading *Taq*, making it suitable for long PCR amplifications and providing enhanced yields.

The spectrum of DNA polymerases is very wide and one must make a choice in agreement with the objective of the investigation and according to the applied post-PCR applications, yet trying to obtain the highest PCR yield. Below are some examples where the selection of the appropriate DNA polymerase enzyme is of great importance.

1. High-fidelity and long PCR applications should apply proofreading polymerases or DNA polymerase blends. Difference in replication efficiencies exists among different DNA polymerases; pH and buffer composition exert an effect on the error rates of the different enzymes. Fidelity analyses proved that DNA polymerase mixtures showed error rates less than that of *Taq* polymerase but several fold higher than the error rate of *Pfu* alone (45).
2. The difference in the amplification efficiency of the various DNA polymerase enzymes has to be considered when long amplicons (>1–2 kb) or a GC-rich target is to be amplified. When performing standard PCR with short amplicons of low GC content, the difference of PCR yield resulting from the choice of polymerase is not so pronounced (43).
3. The thermostable DNA polymerase is exposed to a variety of inhibitors originating from either the sample itself or the sample preparation. In the case of soil samples, *Taq* polymerase exhibited high inhibitory susceptibility for

humic acids; the effect was strongly dependent on the type of the *Taq* enzyme used (46). Polymerase type-dependent PCR inhibition was also observed with environmental samples containing blood, feces, meat, or various cations (Ca^{2+}, Mg^{2+}, Na^{+}, or K^{+}) at different concentrations (42).

Purposeful enzyme selection is not always possible, as the composition of targeted microbial communities is often unknown. It is, therefore, crucial to maintain standardized conditions throughout an experiment: using only one type of DNA polymerase during an investigation, even creating larger stocks of the enzyme by pooling several kits together. Also the operation of a single thermocycler instrument with standardized thermal protocols is suggested for the comparison of intra-laboratory data (44).

3. Methods

3.1. Thermal Profile

Following the initial denaturation step, a general PCR is made up of several cycles (20–40) and each cycle contains two to three discrete temperature steps. The most commonly applied protocols are three-step PCRs containing sections of denaturation, primer annealing, and primer extension with varying incubation times. The basic optimization possibilities of the temperature steps of a typical PCR can be found in laboratory manuals (41); here we highlight aspects of PCR thermal profile optimization that could influence existing PCR biases.

Initial denaturation is usually performed at 92–95°C for 2–5 min to guarantee complete separation of the DNA strands. The length and temperature of this step are determined by the type and length of the template DNA:

1. In the case of genomic DNA templates and templates with high GC content or environmental templates of unknown composition, higher temperatures and longer denaturation are applied (e.g., 95°C for 5 min). However, care should be taken to preserve the activity of the polymerase enzyme used (e.g., applying a "hot-start" protocol (41)).
2. Shorter initial denaturation time and lower temperature can be used for nested-PCR application, or in cases where known taxa of low G+C content are targeted.

The *primer-annealing step* is of great importance as the specificity of the PCR reaction is highly dependent on the chosen primer-annealing temperature. The choice of PCR primers determines the applied annealing temperature, and this has to be optimized empirically. An ideal primer-annealing temperature is 5°C

below the calculated T_m of the primers. If the temperature is too high, little or no amplified product will form, whereas too low annealing temperature increases the possibility of nonspecific priming, resulting in spurious by-products. Although the efficiency of PCR can also be increased by modifying the chemical constitution of the buffer (PCR enhancers, co-solvents), primer, and Mg^{2+} concentration, the annealing temperature is the easiest PCR parameter to control and modify. Where mispriming by either primer occurs (internal or external to the target sequence), a relatively high annealing temperature or the application of touch-down protocol is suggested (47), exploiting the exponential nature of the PCR reaction. A higher temperature is applied in the first cycle for increased specificity of primer–template hybridization, then during the additional rounds the annealing temperature is continuously decreased, generally by 0.5–1.0°C with each cycle, to reach a touch-down temperature (5–10°C lower) at which additional cycles complete the PCR. The highly specific annealing during the initial rounds of the reaction ensures that PCR products of the desired length dominate in the later cycles. The touch-down PCR protocol is widespread in microbial ecology studies, since this modification of the thermal profile improves PCR efficiency for successful downstream applications. The first application of the DGGE technique in microbial ecology as a molecular fingerprinting method was described by Muyzer et al. (48) and has been cited by an incredible 2341 times as of September 2008. The authors applied a touch-down protocol for V3 16S rRNA primers for cultures and environmental samples and became the laboratory paradigm for DGGE analysis of complex environmental samples.

Later studies investigating the relationship of PCR bias and annealing temperature have found, however, that higher annealing temperatures increase preferential amplification while decreasing the complexity of community patterns (17, 49). When the effect of three primer mismatches was examined using predefined DNA template ratios of a model community, an almost exponential increase in the template-to-product ratios was observed in favor of the template containing no mismatches with the applied primer (**Fig. 3.1**, (21)). The degree of primer–template mismatches of different primers also greatly influenced DGGE community patterns, and the application of a touch-down PCR furthermore intensified the uneven amplification of a mixture of 16S rDNA fragments (30).

1. In agreement with these findings, the application of a touch-down protocol in microbial community analyses is not recommended where mismatches are expected, unless specificity is highly crucial and discarding spurious by-products is not otherwise possible (increasing Mg^{2+} concentration, addition of co-solvents, etc.).

2. In order to capture the total microbial diversity of an environmental sample, the lowest possible annealing temperature should be used, and the specificity of the obtained PCR product should be checked by agarose gel electrophoresis. Gradient PCR should always be the central point of PCR optimization for a given sample; and even if a thermal gradient cycler instrument is not available, several parallel PCRs at different annealing temperatures should be performed to select the optimal annealing temperature providing PCR products of appropriate size and quantity.

The temperature of the *primer extension* step is determined by the optimum temperature of the polymerase enzyme used, while the length of extension is affected by both the type of enzyme and the length of the target PCR amplicon. The commonly used temperature for *Taq* DNA polymerase is 72°C, and a 1 min extension time is well suited for the amplification of products shorter than 2 kb in length (rate of 60 nucleotides per second at 70°C). Using a model community template, a significant decrease in the number of chimeric PCR artifacts has been demonstrated by increasing the length of primer extension from 20 s to 4 min (4).

A *final elongation* step of 5–15 min is usually included at the end of the PCR program to ensure that all amplicons are fully extended; any deviation from this interval is strongly dependent on post-PCR analysis. An extra 30 min final extension protocol is described by Janse et al. in DGGE community profiling to remove artifactual double bands. A possible explanation for this is that certain secondary products are formed due to prematurely halted elongation, and these could be disrupted by longer incubation at a relatively high temperature (50).

Longer final extension times should also be applied for PCRs preceding "TA cloning." Cloning and sequencing of PCR-amplified sequences is the most widespread practice for the identification of microbial species. Among several methods developed for the cloning of PCR-amplified DNA molecules, "TA cloning," which makes use of 3′-A overhangs generated by nonproofreading DNA polymerase enzymes (*Taq*, *Tfl*, and *Tth*), is more efficient than blunt-end cloning. In order to make sure that the PCR products are fully extended and 3′ adenylated, a prolonged final extension of 7–30 min is recommended.

3.2. Cycle Numbers

According to the basic concept of PCR amplification, the number of product molecules doubles in every cycle, resulting in a theoretical achievable number of products of 2^n, where n stands for the number of cycles. This means around 10^3-fold amplification in 10, 10^6-fold in 20, 10^9-fold in 30, and 10^{12}-fold in 40 cycles. Therefore, if time is not limited, many choose to execute as many cycles as possible. However, PCR amplification is

not unlimited. Usually after reaching a product concentration of 10^{-8} M, the exponential amplification phase is replaced by the attenuating plateau phase (51). The concentration of 10^{-8} M is equivalent to approximately 10^{11}–10^{12} product molecules in 50–100 μL, meaning that 40 cycles would be needed only when starting with less than 10 copies of the template. Even considering that PCR amplification is generally less efficient than theory would suggest, the use of more than 30 cycles is in most cases unnecessary.

On the other hand, using too many cycles is not only a waste of time but can have an effect on some of the PCR biases. Earlier studies considered the use of excess cycles in multi-template PCR as the main reason of the distorted evaluation of species composition in molecular microbial ecology (52). However, subsequent studies showed that the effect of different cycle numbers is of minor importance (53, 54) or demonstrated that the unfavorable effect of primer mismatches, causing the underestimation of certain templates, is attenuated in the successive cycles (**Fig. 3.2**) (21).

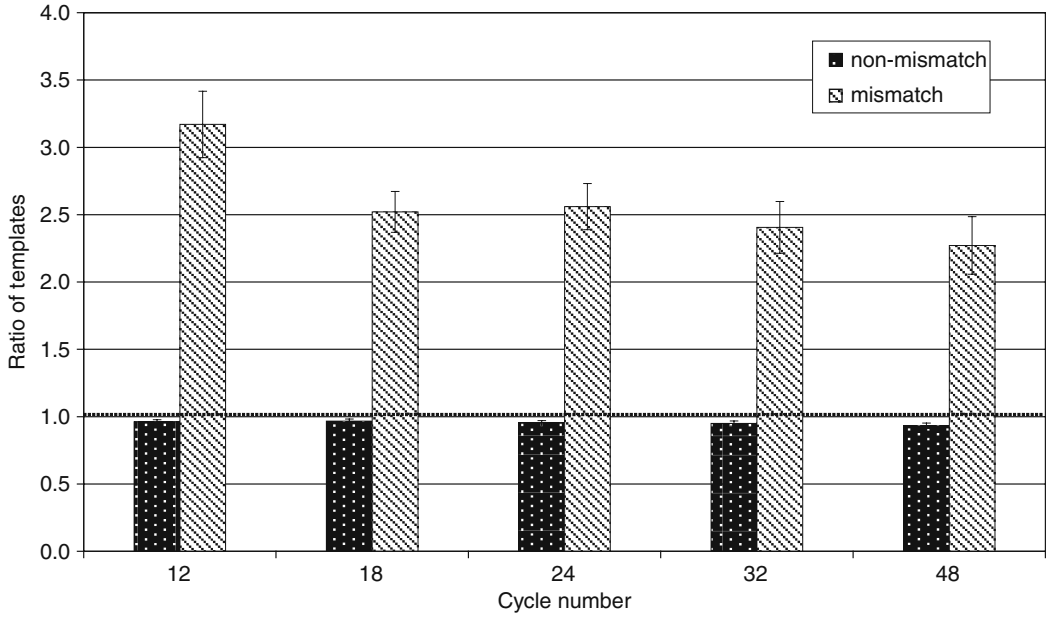


Fig. 3.2. The effect of cycle number on the deviation from the theoretical PCR amplicon ratios using mixed PCR product templates (1:1 concentration ratio). At each cycle number the presence of preferential amplification due to primer mismatches was obvious but was not significantly affected by cycle numbers.

Nevertheless, the probability of the formation of chimeras and heteroduplexes grows as the number of cycles increases. The theoretical reason of this phenomenon is that the heterologous products of the multi-template PCR are flanked by homologous sequences like the primer-binding sites. The cross-hybridization of the products could happen through these homol-

ogous sections. In later cycles, the concentration of the PCR products can reach a sufficiently high concentration for these kinds of hybridization to compete with the formation of the primer–template duplex (55). Significant growth in the number of chimeras has been proved by increasing the number of cycles from 20 to 30 in a model community study (4).

Another cycle number–dependent bias is the production of the so-called stutter or shadow amplicons well known in microsatellite analyses. In these cases several similar-sized bands can be detected instead of the one expected. The reason of the excess bands is the change in the number of repeating units of the microsatellites due to insertion–deletion mutations caused by the slippage of DNA polymerase during strand extension. Slippage happens in the primer–template complex by the formation of loops in either the primer or the template strand. The size of stutter bands differs from the original amplicon length by the multiples of size of the repeats of the microsatellite (e.g., in the case of tetranucleotide repeats, the stutter bands will be 4, 8, 12, 16, etc. shorter or longer than the expected band). The number of stutter bands increases with the number of PCR cycles and deletions are more likely to happen, accordingly shorter stutter products are more numerous (56). Strand slippage can occur during the amplification of any repetitive sequence of even single-nucleotide repeats. In genes with a functional secondary structure like ribosomal genes, the frequency of repeats and palindrome sequences is higher than in protein-coding genes. Therefore, the formation of loops facilitating slippage is possible, and stutter products will appear with the increasing number of cycles. This bias has to be considered especially when planning length-dependent post-PCR analyses like T-RFLP, ARISA, or LH-PCR (**Fig. 3.3**).

Recommended cycle number:

1. Start the optimization of your experiment with 25–30 cycles.
2. If the only aim of your PCR is to detect a gene (e.g., detection of *Dehalococcoides* spp. from a TCE-contaminated site) or to quantify it (real-time qPCR), you can go up to 40 cycles.
3. Try to use as few cycles as possible if any downstream protocol including sequence analyses (e.g., cloning) and/or length-based fingerprinting (e.g., T-RFLP) is planned.

3.3. Nested PCR

Nested or double-PCR is defined as a protocol consisting of two consecutive PCRs with two different primer pairs that are used for a single locus (57). First, a standard PCR reaction is performed with an external primer set. In the second PCR step, the nested primers bind within the sequence of the first PCR product, hence the second PCR generates a shorter product than the first

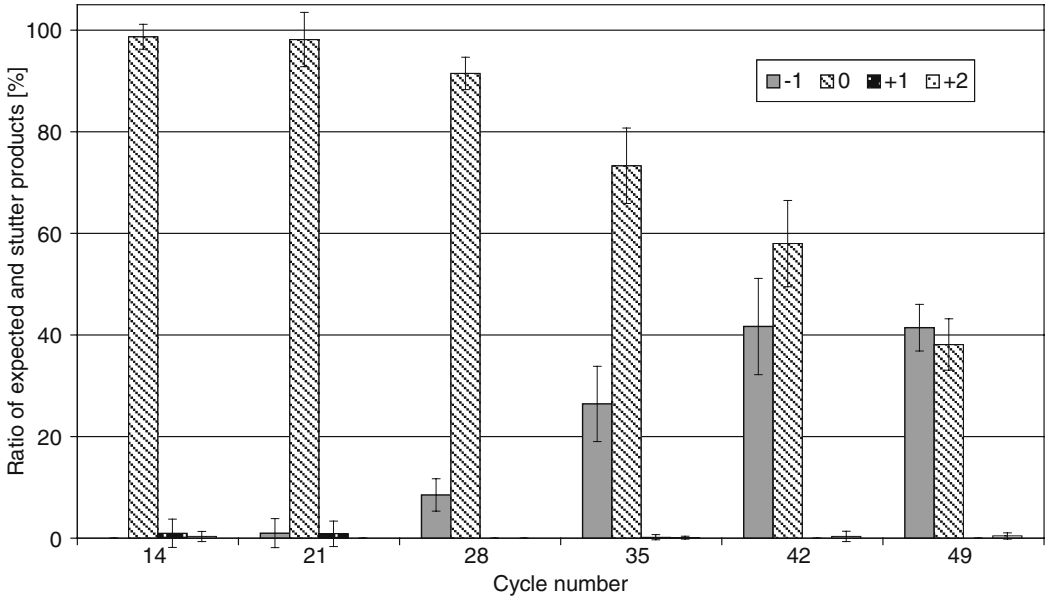


Fig. 3.3. Evaluation of cycle-dependent production of stutter terminal fragments (T-RFs). The dominant environmental clone of a wastewater sample was submitted to T-RFLP analysis after PCR amplification with different number of cycles. The diagram is the result of five parallel experiments: for each cycle number the expected-size T-RF (0) and the few bases shorter (–1) or longer (+1, +2) stutter T-RFs are displayed. The same stutter fragments could be detected in the original environmental T-RFLP pattern.

one. Sometimes, even a third PCR round is executed in order to detect certain microbial groups present in low numbers in complex microbial communities (58). Seminested PCR is conducted when one of the oligos of the first primer pair is used in combination with a third internal primer in the second PCR round (59).

The two sets of primers can have the same (60, 61) or different specificity. When the two PCR rounds differ in the specificity of the applied primer pairs, two setups are possible: using a universal primer pair in the first step and a group-specific primer set in the second (62), or in reverse order applying taxon-specific primers first and universal ones afterward (63, 64).

Nested PCR has several advantages.

1. It increases the specificity of the amplification, because if unspecific amplicons are produced in the first PCR step due to erroneous primer annealing, the probability that they would also be amplified in the second round with a second pair of primers is very low.
2. It improves sensitivity by increasing the yield of PCR products and by detecting species that are present in lower numbers (65). The enhanced detection capability may be attributed to the amplification of ample amounts of DNA even from the groups presented in low numbers during the

first PCR round, and can also be due to the dilution of inhibitory substances present in the DNA samples (58).

3. It allows the comparison of different microbial groups by the same DGGE/TGGE analysis, when the first taxon-specific PCR step is followed by a second PCR with internal "universal primers." In this case, intergel comparisons are easy to make, since the length of the different PCR amplicons is the same and can be separated under the same DGGE/TGGE conditions, requiring only one DGGE/TGGE electrophoretic optimization step (23).

Nevertheless, nested PCR should be evaluated with caution as the use of two successive PCR reactions can introduce even greater bias due to preferential amplification. However, according to several studies (23, 58, 61), the increased bias of preferential amplification by nested PCR may be overestimated. When using a less sensitive method such as DGGE to compare the fingerprints of the PCR products, the patterns obtained from nested PCRs are usually almost identical with the ones generated by direct PCR. Only minor variations in the relative band intensity could be observed, suggesting that the nested PCR approach did not result in changes in the dominant members of the given microbial community.

In conclusion, nested PCR is an appropriate choice when (1) our goal is the detection of a less abundant group or (2) we are planning to use a post-PCR protocol demanding a higher PCR yield, and direct PCR approach resulted in an insufficient amount of PCR product.

References

1. 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.
2. Fischer, S. G., and Lerman, L. S. (1983) DNA fragments differing by single base-pair substitutions are separated in denaturing gradient gels: correspondence with melting theory. *Proc. Natl. Acad. Sci. USA* **80**, 1579–1583.
3. Frostegard, A., Courtois, S., Ramisse, V., Clerc, S., Bernillon, D., Le Gall, F., et al. (1999) Quantification of bias related to the extraction of DNA directly from soils. *Appl. Environ. Microbiol.* **65**, 5409–5420.
4. Qiu, X. Y., Wu, L. Y., Huang, H. S., McDonel, P. E., Palumbo, A. V., Tiedje, J. M. and Zhou, J. Z. (2001) Evaluation of PCR-generated chimeras: Mutations, and heteroduplexes with 16S rRNA gene-based cloning. *Appl. Environ. Microbiol.* **67**, 880–887.
5. Porteous, L. A., and Armstrong, J. L. (1993) A simple mini-method to extract DNA directly from soil for use with polymerase chain reaction amplification. *Curr. Microbiol.* **27**, 115–118.
6. Steffan, R. J., and Atlas, R. M. (1991) Polymerase chain reaction: applications in environmental microbiology. *Annu. Rev. Microbiol.* **45**, 137–161.
7. Young, C. C., Burghoff, R. L., Keim, L. G., Minak-Bernero, V., Lute, J. R., and Hinton, S. M. (1993) Polyvinylpyrrolidone-agarose gel electrophoresis purification of polymerase chain reaction-amplifiable DNA from soils. *Appl. Environ. Microbiol.* **59**, 1972–1974.
8. Zhou, J., Bruns, M. A., and Tiedje, J. M. (1996) DNA recovery from soils of diverse

composition. *Appl. Environ. Microbiol.* **62**, 316–322.
9. Sagova-Mareckova, M., Cermak, L., Novotna, J., Plhackova, K., Forstova, J., and Kopecky, J. (2008) Innovative methods for soil DNA purification tested in soils with widely differing characteristics. *Appl. Environ. Microbiol.* **74**, 2902–2907.
10. Saano, A., and Lindström, K. (1995) Small scale extraction of DNA from soil with spun column cleanup, in *Molecular Microbial Ecology Manual* (Akkermans, A. D. L., van Elsas, J. D., and DeBruijn, F. J. ed.), Kluwer, Dordrecht, pp. 1–6.
11. Lakay, F. M., Botha, A., and Prior, B. A. (2007) Comparative analysis of environmental DNA extraction and purification methods from different humic acid-rich soils. *J. Appl. Microbiol.* **102**, 265–273.
12. Robe, P., Nalin, R., Capellano, C., Vogel, T. M., and Simonet, P. (2003) Extraction of DNA from soil. *Eur. J. Soil Biol.* **39**, 183–190.
13. Forney, L. J., Zhou, X., and Brown, C. J. (2004) Molecular microbial ecology: land of the one-eyed king. *Curr. Opin. Microbiol.* **7**, 210–220.
14. Baker, G. C., Smith, J. J., and Cowan, D. A. (2003) Review and re-analysis of domain-specific 16S primers. *J. Microbiol. Methods* **55**, 5541–555.
15. Lane, D. J. (1991) 16S/23S rRNA sequencing, in *Nucleic Acid Techniques in Bacterial Systematics* (Stackebrandt, E., and Goodfellow, M. ed.), Wiley, London, pp. 115–147.
16. Marchesi, J. R., Sato, T., Weightman, A. J., Martin, T. A., Fry, J. C., Hiom, S. J., et al. (1998) Design and evaluation of useful bacterium-specific PCR primers that amplify genes coding for bacterial 16S rRNA. *Appl. Environ. Microbiol.* **64**, 795–799.
17. Hongoh, Y., Yuzawa, H., Ohkuma, M., and Kudo, T. (2003) Evaluation of primers and PCR conditions for the analysis of 16S rRNA genes from a natural environment. *FEMS Microbiol. Lett.* **221**, 299–304.
18. Ellis, R. J., Morgan, P., Weightman, A. J., and Fry, J. C. (2003) Cultivation-dependent and -independent approaches for determining bacterial diversity in heavy-metal-contaminated soil. *Appl. Environ. Microbiol.* **69**, 3223–3230.
19. Santos, S. R., and Ochman, H. (2004) Identification and phylogenetic sorting of bacterial lineages with universally conserved genes and proteins. *Environ. Microbiol.* **6**, 754–759.
20. Case, R. J., Boucher, Y., Dahllof, I., Holmstrom, C., Doolittle, W. F., and Kjelleberg, S. (2007) Use of 16S rRNA and rpoB genes as molecular markers for microbial ecology studies. *Appl. Environ. Microbiol.* **73**, 278–288.
21. Sipos, R., Székely, A. J., Palatinszky, M., Révész, S., Márialigeti, K., and Nikolausz, M. (2007) Effect of primer mismatch, annealing temperature and PCR cycle number on 16S rRNA gene-targeting bacterial community analysis. *FEMS Microbiol. Ecol.* **60**, 341–350.
22. Blackwood, C. B., Oaks, A., and Buyer, J. S. (2005) Phylum- and class-specific PCR primers for general microbial community analysis. *Appl. Environ. Microbiol.* **71**, 6193–6198.
23. Boon, N., De Windt, W., Verstraete, W., and Top, E. M. (2002) Evaluation of nested PCR-DGGE (denaturing gradient gel electrophoresis) with group-specific 16S rRNA primers for the analysis of bacterial communities from different wastewater treatment plants. *FEMS Microbiol. Ecol.* **39**, 101–112.
24. Fierer, N., Jackson, J. A., Vilgalys, R., and Jackson, R. B. (2005) Assessment of soil microbial community structure by use of taxon-specific quantitative PCR assays. *Appl. Environ. Microbiol.* **71**, 4117–4120.
25. Baker, G. C., and Cowan, D. A. (2004) 16S rDNA primers and the unbiased assessment of thermophile diversity. *Biochem. Soc. Trans.* **32**, 218–221.
26. Purkhold, U., Pommerening-Roser, A., Juretschko, S., Schmid, M. C., Koops, H. P., and Wagner, M. (2000) Phylogeny of all recognized species of ammonia oxidizers based on comparative 16S rRNA and amoA sequence analysis: implications for molecular diversity surveys. *Appl. Environ. Microbiol.* **66**, 5368–5382.
27. Zak, D. R., Blackwood, C. B., and Waldrop, M. P. (2006) A molecular dawn for biogeochemistry. *Trends Ecol. Evol.* **21**, 288–295.
28. Táncsics, A., Szoboszlay, S., Kriszt, B., Kukolya, J., Baka, E., Márialigeti, K., and Révész, S. (2008) Applicability of the functional gene catechol 1,2-dioxygenase as a biomarker in the detection of BTEX-degrading Rhodococcus species. *J. Appl. Microbiol.* 105, 1026–1033.
29. Hyndman, D. L., and Mitsuhashi, M. (2003) PCR Primer Design, in *PCR Protocols* (Bartlett, J. M. S., and Stirling, D. ed.), Humana, Totowa, NJ, pp. 89–99.
30. Ahn, J. H., Kim, M. C., Shin, H. C., Choi, M. K., Yoon, S. S., Kim, T., et al. (2006) Improvement of PCR amplification bias for community structure analysis of soil bacteria

by denaturing gradient gel electrophoresis. *J. Microbiol. Biotechnol.* **16**, 1561–1569.
31. Bru, D., Martin-Laurent, F., and Philippot, L. (2008) Quantification of the detrimental effect of a single primer-template mismatch by real-time PCR using the 16S rRNA gene as an example. *Appl. Environ. Microbiol.* **74**, 1660–1663.
32. Okano, Y., Hristova, K. R., Leutenegger, C. M., Jackson, L. E., Denison, R. F., Gebreyesus, B., et al. (2004) Application of real-time PCR to study effects of ammonium on population size of ammonia-oxidizing bacteria in soil. *Appl. Environ. Microbiol.* **70**, 1008–1016.
33. Teske, A., and Sorensen, K. B. (2008) Uncultured archaea in deep marine subsurface sediments: have we caught them all? *ISME J.* **2**, 3–18.
34. Sekido, T., Bodelier, P. L., Shoji, T., Suwa, Y., and Laanbroek, H. J. (2008) Limitations of the use of group-specific primers in real-time PCR as appear from quantitative analyses of closely related ammonia-oxidising species. *Water Res.* **42**, 1093–1101.
35. Polz, M. F., and Cavanaugh, C. M. (1998) Bias in template-to-product ratios in multitemplate PCR. *Appl. Environ. Microbiol.* **64**, 3724–3730.
36. Watanabe, K., Kodama, Y., and Harayama, S. (2001) Design and evaluation of PCR primers to amplify bacterial 16S ribosomal DNA fragments used for community fingerprinting. *J. Microbiol. Methods* **44**, 253–262.
37. Loy, A., Arnold, R., Tischler, P., Rattei, T., Wagner, M., and Horn, M. (2008) probeCheck - a central resource for evaluating oligonucleotide probe coverage and specificity. *Environ. Microbiol.* **10**, 2844–2848.
38. Cole, J. R., Chai, B., Farris, R. J., Wang, Q., Kulam, S. A., McGarrell, D. M., et al. (2005) The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. *Nucleic Acids Res.* **33**, D294–296.
39. Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., et al. (2004) ARB: a software environment for sequence data. *Nucleic Acids Res.* **32**, 1363–1371.
40. Brons, J. K., and van Elsas, J. D. (2008) Analysis of bacterial communities in soil by use of denaturing gradient gel electrophoresis and clone libraries, as influenced by different reverse primers. *Appl. Environ. Microbiol.* **74**, 2717–2727.
41. Grunenwald, H. (2003) Optimization of Polymerase Chain Reactions, in *PCR Protocols* (Bartlett, J. M. S., and Stirling, D. ed.), Humana, Totowa, NJ, pp. 89–99.
42. Abu Al-Soud, W., and Radstrom, P. (1998) Capacity of nine thermostable DNA polymerases to mediate DNA amplification in the presence of PCR-inhibiting samples. *Appl. Environ. Microbiol.* **64**, 3748–3753.
43. Arezi, B., Xing, W., Sorge, J. A., and Hogrefe, H. H. (2003) Amplification efficiency of thermostable DNA polymerases. *Anal. Biochem.* **321**, 226–235.
44. Tyler, K. D., Wang, G., Tyler, S. D. and Johnson, W. M. (1997) Factors affecting reliability and reproducibility of amplification-based DNA fingerprinting of representative bacterial pathogens. *J. Clin. Microbiol.* **35**, 339–346.
45. Cline, J., Braman, J. C., and Hogrefe, H. H. (1996) PCR fidelity of pfu DNA polymerase and other thermostable DNA polymerases. *Nucleic Acids Res.* **24**, 3546–3551.
46. Tebbe, C. C., and Vahjen, W. (1993) Interference of humic acids and DNA extracted directly from soil in detection and transformation of recombinant DNA from bacteria and a yeast. *Appl. Environ. Microbiol.* **59**, 2657–2665.
47. Don, R. H., Cox, P. T., Wainwright, B. J., Baker, K., and Mattick, J. S. (1991) 'Touchdown' PCR to circumvent spurious priming during gene amplification. *Nucleic Acids Res.* **19**, 4008.
48. Muyzer, G., de Waal, E. C., and Uitterlinden, A. G. (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* **59**, 695–700.
49. Ishii, K., and Fukui, M. (2001) Optimization of annealing temperature to reduce bias caused by a primer mismatch in multitemplate PCR. *Appl. Environ. Microbiol.* **67**, 3753–3755.
50. Janse, I., Bok, J., and Zwart, G. (2004) A simple remedy against artifactual double bands in denaturing gradient gel electrophoresis. *J. Microbiol. Methods* **57**, 279–281.
51. Kainz, P. (2000) The PCR plateau phase – towards an understanding of its limitations. *Biochim. Biophys. Acta* **1494**, 23–27.
52. 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.

53. Lueders, T., and Friedrich, M. W. (2003) Evaluation of PCR amplification bias by terminal restriction fragment length polymorphism analysis of small-subunit rRNA and mcrA genes by using defined template mixtures of methanogenic pure cultures and soil DNA extracts. *Appl. Environ. Microbiol.* **69**, 320–326.
54. Acinas, S. G., Sarma-Rupavtarm, R., Klepac-Ceraj, V., and Polz, M. F. (2005) PCR-induced sequence artifacts and bias: Insights from comparison of two 16S rRNA clone libraries constructed from the same sample. *Appl. Environ. Microbiol.* **71**, 8966–8969.
55. Jensen, M. A., and Straus, N. (1993) Effect of PCR conditions on the formation of heteroduplex and single-stranded-DNA products in the amplification of bacterial ribosomal DNA spacer regions. *PCR-Methods Appl.* **3**, 186–194.
56. Shinde, D., Lai, Y., Sun, F., and Arnheim, N. (2003) Taq DNA polymerase slippage mutation rates measured by PCR and quasi-likelihood analysis: (CA/GT)n and (A/T)n microsatellites. *Nucleic Acids Res.* **31**, 974–980.
57. Haqqi, T. M., Sarkar, G., David, C. S., and Sommer, S. S. (1988) Specific amplification with PCR of a refractory segment of genomic DNA. *Nucleic Acids Res.* **16**, 11844.
58. Dar, S. A., Kuenen, J. G., and Muyzer, G. (2005) Nested PCR-denaturing gradient gel electrophoresis approach to determine the diversity of sulfate-reducing bacteria in complex microbial communities. *Appl. Environ. Microbiol.* **71**, 2325–2330.
59. Oros-Sichler, M., Gomes, N. C., Neuber, G., and Smalla, K. (2006) A new semi-nested PCR protocol to amplify large 18S rRNA gene fragments for PCR-DGGE analysis of soil fungal communities. *J. Microbiol. Meth.* **65**, 63–75.
60. el Fantroussi, S., Mahillon, J., Naveau, H., and Agathos, S. N. (1997) Introduction of anaerobic dechlorinating bacteria into soil slurry microcosms and nested-PCR monitoring. *Appl. Environ. Microbiol.* **63**, 806–811.
61. Nikolausz, M., Sipos, R., Révész, S., Székely, A., and Márialigeti, K. (2005) Observation of bias associated with re-amplification of DNA isolated from denaturing gradient gels. *FEMS Microbiol. Lett.* **244**, 385–390.
62. Levesque, M. J., La Boissiere, S., Thomas, J. C., Beaudet, R., and Villemur, R. (1997) Rapid method for detecting *Desulfitobacterium frappieri* strain PCP-1 in soil by the polymerase chain reaction. *Appl. Microbiol. Biotechnol.* **47**, 719–725.
63. DeJournett, T. D., Arnold, W. A., and LaPara, T. M. (2007) The characterization and quantification of methanotrophic bacterial populations in constructed wetland sediments using PCR targeting 16S rRNA gene fragments. *Appl. Soil Ecol.* **35**, 648–659.
64. Temmerman, R., Masco, L., Vanhoutte, T., Huys, G., and Swings, J. (2003) Development and validation of a nested-PCR-denaturing gradient gel electrophoresis method for taxonomic characterization of bifidobacterial communities. *Appl. Environ. Microbiol.* **69**, 6380–6385.
65. Phillips, C. J., Harris, D., Dollhopf, S. L., Gross, K. L., Prosser, J. I., and Paul, E. A. (2000) Effects of agronomic treatments on structure and function of ammonia-oxidizing communities. *Appl. Environ. Microbiol.* **66**, 5410–5418.

Chapter 4

MPN- and Real-Time-Based PCR Methods for the Quantification of Alkane Monooxygenase Homologous Genes (*alkB*) in Environmental Samples

Alfredo Pérez-de-Mora, Stephan Schulz, and Michael Schloter

Abstract

Hydrocarbons are major contaminants of soil ecosystems as a result of uncontrolled oil spills and wastes disposal into the environment. Ecological risk assessment and remediation of affected sites is often constrained due to lack of suitable prognostic and diagnostic tools that provide information of abiotic–biotic interactions occurring between contaminants and biological targets. Therefore, the identification and quantification of genes involved in the degradation of hydrocarbons may play a crucial role for evaluating the natural attenuation potential of contaminated sites and the development of successful bioremediation strategies. Besides other gene clusters, the *alk* operon has been identified as a major player for alkane degradation in different soils. An oxygenase gene (*alkB*) codes for the initial step of the degradation of aliphatic alkanes under aerobic conditions. In this work, we present an MPN- and a real-time PCR method for the quantification of the bacterial gene *alkB* (coding for rubredoxin-dependent alkane monooxygenase) in environmental samples. Both approaches enable a rapid culture-independent screening of the *alkB* gene in the environment, which can be used to assess the intrinsic natural attenuation potential of a site or to follow up the on-going progress of bioremediation assays.

Key words: Most probable number-PCR, real time PCR, alkane monooxygenase homologous genes.

1. Introduction

Aliphatic *n*-alkanes are saturated hydrocarbons present in crude and refined oils. Although *n*-alkanes can be produced by plants and microorganisms (e.g., waxes) (1,2), they are mainly released into the environment by seepage from reservoirs or through anthropogenic activities related to the extraction, transportation,

S.P. Cummings (ed.), *Bioremediation*, Methods in Molecular Biology 599,
DOI 10.1007/978-1-60761-439-5_4,

further processing, handling, and disposal of oil (3). Some algae that degrade *n*-alkanes have been described; however, bacteria and fungi are the most important groups capable of using long-chain *n*-alkanes as a carbon and energy source (4). Bacterial degradation of *n*-alkanes is possible under aerobic and anaerobic conditions, although the former allows much faster rates of transformation (5).

During the past decades, research related to alkane degradation has focused on the identification and characterization of enzymes involved in the initial step of aerobic bacterial catabolic pathways. Recently, two unrelated classes of enzymes for long-chain *n*-alkane oxidation have been proposed (6): (1) Cytochrome-P450-related enzymes in both yeasts and bacteria, e.g., bacterial CYP153 enzymes, and (2) bacterial particulate alkane hydroxylases (pAHs). The latter class of integral membrane non-heme di-iron monooxygenases of the *alkB*-type catalyzes the terminal or subterminal oxidation of *n*-alkanes to primary or secondary alcohols, which is the initial step in the aerobic degradation of *n*-alkanes (3). This enzyme allows a wide range of Proteobacteria and Actinomycetales to grow on *n*-alkanes with carbon chain lengths from C5 to C16.

Most studies dealing with the detection of *alkB* in microbial communities to date are based on PCR methods using cultured isolates (7, 8, 9). Due to the high diversity of *alkB* sequences in bacteria from different taxonomic groups, analysis of environmental samples required the use of multiple primer and probe sets targeting the respective groups. In order to allow a specific and sensitive detection of *alkB* genes in environmental samples without discriminating any of the known bacterial groups harboring this gene, a PCR hybridization method was described recently (10). Based on this initial work, we present here an improved MPN- and a real-time PCR methods, which allow rapid quantification of *alkB* gene in numerous environmental samples. Furthermore, the amplified products can be directly used for assessing community diversity using fingerprinting or cloning techniques.

2. Materials

2.1. Cell Lysis and Nucleic Acid Extraction

1. FastDNA SPIN Kit for soil (MP Biomedicals, Heidelberg, Germany)
2. PreCellys24 homogeniser (Peqlab Biotechnologie GmbH, Erlangen, Germany)
3. PreCellys ceramic kit 1.4 mm (Peqlab Biotechnologie GmbH, Erlangen, Germany)

2.2. MPN-PCR Amplification of alkB

1. Autoclaved MiliQ water (*see* **Note 1**).
2. 3% bovine serum albumin solution. After sterilization by filtration (22 μm) store in aliquots at −20°C (*see* **Note 1**).
3. dNTPs (Fermentas GmbH, St. Leon-Rot, Germany)
4. Taq polymerase (5 U/μL) (Invitrogen, Carlsbad, CA, USA).
5. 10× buffer provided with Taq polymerase.
6. $MgCl_2$ (50 mM) provided with Taq polymerase or self prepared (in this case *see* **Note 1**).
7. Degenerate PCR primer (10) (Metabion, Martinsried, Germany)
8. alkB-forward (5′AAY ACI GCI CAY GAR CTI GGI CAY AA 3′)
9. alkB-reverse (5′ GCR TGR TGR TCI GAR TGI CGY TG 3′)
10. Template DNA (*see* **Note 2**). Dilutions of template DNA should be prepared directly before use (*see* **Note 3**). We recommend the use of freshly prepared dilutions for every new PCR reaction.
11. Thermocycler (T3 Thermocycler, Biometra, Goettingen, Germany)

2.3. Real-Time PCR Amplification of alkB

1. 3% bovine serum albumin solution. After sterilization by filtration store in aliquots at −20°C. Use filters with 22 μm pore size (*see* **Note 1**).
2. $MgCl_2$ solution (50 mM) (*see* **Note 1**).
3. Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA). Store Master Mix in single-use aliquots at 28°C for short-term storage (*see* **Note 4**).
4. Degenerated PCR primer (10) (Metabion, Martinsried, Germany) (*see* **Note 5**):
   alkB-forward (5′AAY ACI GCI CAY GAR CTI GGI CAY AA 3′)
   alkB-reverse (5′ GCR TGR TGR TCI GAR TGI CGY TG 3′)
   Store aliquots of appropriate dilution at −20°C (*see* **Note 3**).
5. Template DNA (*see* **Note 2**). Dilutions of template DNA should be prepared directly before use (*see* **Note 3**). Use freshly prepared dilutions for every new PCR reaction.
6. Thermo-Fast® 96 detection plate (Thermo Scientific)
7. MicroAmp™ optical adhesive film (Applied Biosystems, Foster City, CA, USA)

8. Domed 12 cap strips (Thermo Scientific)
9. Cooled centrifuge for detection plates (e.g., Omnifuge 2.0 RS, Heraeus Sepatech Osterode, Germany)
10. A repeater-step pipette (e.g., Distriman®, Gilson Inc., Middleton, WI, USA)
11. 7300 real-time PCR system (Applied Biosystems, Foster City, CA, USA)

2.4. Agarose Gel Electrophoresis

1. Agarose for gel electrophoresis is dissolved in 1× TAE buffer to a final concentration of 2% and should be prepared before use.
2. TAE buffer: 40 mM Tris-base, 1 mM disodium ethylendiamintetraacetate dihydrate dissolved in water pH 8.0 (*see* **Note 1**).
3. Loading dye 6× (Fermentas **GmbH**,St. Leon-Rot, Germany)
4. GeneRuler™ 100 bp or 1 kb DNA ladder (Fermentas GmbH, St. Leon-Rot, Germany)
5. PerfectBlue Gelsystem Mini L (Peqlab Biotechnologie GmbH, Erlangen, Germany) with Power Supply Power Pac 300 (Biorad, Hercules, CA, USA)
6. Gel documentation system

3. Methods

In this work we present two quantitative PCR methods to study the relative abundance of the bacterial *alkB* gene in environmental samples. The first one is based on an MPN PCR approach, while the second one is a real-time PCR procedure. Both methods were tested using a wide range of samples. These included different types of soil: forest and agricultural, low (<1%) and high (>5%) organic C content, noncontaminated (<100 mg total hydrocarbons/kg soil dry weight) and contaminated with hydrocarbons (5000 mg total hydrocarbons/kg soil dry weight), sand and loam texture. In addition, plant residual material was evaluated (pea and maize). For a more detailed discussion on the advantages and disadvantages of quantitative PCR procedures, we refer to (11). A brief introduction is given below.

The MPN PCR approach is based on the combination of PCR with the statistical MPN (most probable number) procedure developed by McCrady (12) and later improved by other authors (13). This method consists of serial dilutions of DNA/cDNA samples until extinction and replicated PCR reactions for every

dilution. The result of each reaction is scored positive or negative after gel electrophoresis analysis. The number of gene copies is calculated using the MPN statistics.

The real-time PCR technique is based on the detection of fluorescence signals emitted due to the synthesis of PCR amplicons by Taq polymerase (2). In the present method, amplicon synthesis is monitored using SYBR® Green, a fluorescent dye that binds to double-stranded DNA. In contrast to other PCR methods, quantification using real-time PCR is a non-end-point measurement and based on a continuous increase of a fluorescence signal, which is measured after each cycle.

3.1. Cell Lysis and Nucleic Acid Extraction

1. To ensure good homogeneity of samples, these may be additionally ground in liquid N until a fine powder is obtained.
2. Weigh 0.5 g of ground sample (fresh weight) into a lysis tube containing ceramic balls.
3. Add the corresponding buffers (*see* **Note 2**).
4. Lyse cells in a homogenizator (30 s at 5.5 m/s)
5. Proceed with the extraction protocol as indicated by the author's or the manufacturer's instructions.
6. Depending on the protocol, used nucleic acids may be either resuspended or eluted with water (*see* **Note 1**).

3.2. Quantification Using MPN-PCR

1. The extracts obtained in the previous section are used for the MPN PCR approach. Prepare a succession of 1/10 serial dilutions of the sample of interest (*see* **Notes 1** and **6**). At least three replicates of each dilution are required.
2. Prepare a PCR reaction mix (23 μl) following this scheme:

Reagents	Volume (μL)	Final concentration
10× buffer	2.5	1×
$MgCl_2$ (50 mM)	1	2 mM
dNTPs (2 mM)	1.25	0.1 mM
Fwd primer (4 μM)	0.625	0.1 μM
Rev primer (4 μM)	0.625	0.1 μM
BSA (3%)	0.5	0.06%
Taq polymerase (5 U/μL)	0.25	0.01 U/μL
Water	16.25	

3. We suggest that a Master Mix is prepared for the desired number of samples.

Stage	Time	Temperature (°C)	Touchdown	Cycles
Stage 1				
Hotstart	10 min	95	(*see* **Note 7**)	×1
Stage 2				
Denature	45 s	95		×5
Anneal	1 min	62	−1°C/cycle	
Extend	45 s	72	(*see* **Note 8**)	
Stage 3				
Denature	45 s	95		×30
Anneal	1 min	57		
Extend	45 s	72		
Stage 4				
Final extension	10 min	72		×1
Pause	–	4		

4. Load 23 μL of reaction mix in each reaction tube.
5. Add 2 μL of template. Store on ice until you are done with all samples. Vortex briefly and centrifuge.
6. Run the following PCR program:
7. When the PCR is completed, vortex the samples briefly for 2 s and centrifuge for 5 s) using a microcentrifuge (14000 × *g*).
8. Carry out an agarose gel electrophoresis (2% agarose). A total of 15 μL PCR product and 4 μL of loading dye should be sufficient for detection of the amplified product. Let the samples run for 1 h at 120 V. At least 100 bp DNA ladder should be included on one of the gel lanes.
9. Following gel electrophoresis, visualize amplicons on the gel with ethidium bromide. In general, a 10–15 min bath is enough if the bath has been recently prepared or has not been used too many times.
10. Detect amplified products with a gel documentation system – the size of the band of interest is approximately 550 bp.
11. Identify the three sets of tubes/reactions that show dilution of the gene to "extinction."
12. Note the number of positive and negative scores resulting from each reaction (e.g., 3 positives for the first dilution, 2 for the second, and 0 for the last).

13. Use an MPN-Table or MPN-calculator (*see* **Note 9**) to compute the most probable number of gene copies in 1 μL of the original dilution.
14. Since the amount of nucleic acids in the original extract is known (nondiluted sample), the final result can be expressed as the number of gene copies/mg of DNA or the number of gene copies/g of soil material (dry weight).

3.3. Quantification Using Real-Time PCR

1. Make 1/10 serial dilutions for the standard (*see* **Notes 3, 10, 11, and 12**). For samples we recommend that 1 in 2 or 1 in 3 serial dilutions are performed to find out the dilution that gives the highest copy number for that sample following PCR reaction (*see* **Notes 3, 11, and 13**).
2. Prepare a PCR reaction mix (23 μl) following this scheme (*see* **Note 14**):

Reagents	Volume (μL)	Final concentration
$MgCl_2$ (50 mM)	1	2 mM
Foward primer (4 μM)	0.625	0.1 μM
Reverse primer (4 μM)	0.625	0.1 μM
BSA (3%)	0.5	0.06%
SyberGreen Master Mix	12.5	
Water	7.75	

3. Prepare a master mix for the desired number of samples.
4. Load 23 μL of reaction mix in each reaction well of the plate (*see* **Note 15**).
5. Cover the negative controls with cap strips to prevent contamination.
6. Add 2 μl of the template DNA to each reaction well of the plate following your working scheme. The final volume of each reaction is 25 μL.
7. When you are done with the templates, add 2 μL of water to template-free reaction wells. These will act as negative controls. Use at least three wells for this purpose.
8. Seal the PCR plate with the MicroAmp™ optical adhesive film. Remove air bubbles by pushing the film to the plate tightly.
9. Spin down the plate for 30 s and load the qPCR system.
10. Run the following PCR program:
11. Values can be calculated based on the Ct value.

Stage	Time	Temperature (°C)	Touchdown	Cycles
Stage 1				
Hotstart	10 min	95	(*see* **Note 7**)	×1
Stage 2				
Denature	45 s	95		×5
Anneal	1 min	62	−1°C/cycle	
Extend	45 s	72	(*see* **Note 8**)	
Stage 3				
Denature	45 s	95		×40
Anneal	1 min	57		
Extend	45 s	72		
Data Collection	30 s	78	(*see* **Note 16**)	
Stage 4 (termination)				
Denature	15 s	95		×1
Anneal	30 s	60	(*see* **Note 17**)	
Extend	15 s	95		

12. To ensure that the quantified amplicons possess the right size, run an agarose gel as described in the MPN PCR approach.
13. As for MPN PCR the final result can be expressed either as the number of gene copies/mg of DNA or the number of gene copies/g of soil material (dry weight).

4. Notes

1. All solutions should be prepared in water treated with 0.1% diethylpyrocarbonate (DEPC) (Sigma-Aldrich, Munich, Germany). This standard is referred to as "water" in this text.
2. For extraction and isolation of nucleic acids, follow the instructions proposed by the authors of the method or the manufacturer's instructions in those cases where different kit systems are used. When measuring the concentration of the isolated nucleic acids, the ratio of sample absorbance at 260 and 280 nm (260/280) should be in the range of 1.8–2.2 for "pure" nucleic acids. A ratio appreciably lower

might be indicative for the presence of co-purified contaminants such as humic acids or phenol.

3. Make all dilutions with water.
4. The SYBR® Green 1 Dye in the master mix is light-sensitive and should be kept in the dark. See manufacturer's instructions for long-term storage and safety.
5. We inserted inosine where the bases were fourfold degenerated to reduce the degeneracy and improve binding of the primers to template.
6. For samples with low copy numbers, a succession of serial dilutions up to 10^4 might be sufficient. Dilutions up to 10^5 or 10^6 may be necessary for samples with high copy numbers.
7. The hotstart stage allows specific annealing reactions to occur and prevents nonspecific annealing events. With a lack of nonspecific hybridization of primers to template or to one another, the resulting amplified DNA bands are cleaner.
8. Due to the degeneracy of the primers and hence differing melting temperature ranges, a touchdown PCR step greatly improves annealing to the template.
9. MPN-tables are available in numerous books, e.g., (15). Instead, you can download a free MPN-calculator from the Internet. Both MPN-tables and calculators should provide the upper and lower limit of the 95% confidence interval.
10. We found copy numbers of the standard from $10^8/\mu l$ to $10^2/\mu l$ to be appropriate for this protocol.
11. When drops are located on the lid of the reaction tube, spin down the tubes. This helps to reduce the danger of cross-contaminations when opening the tubes.
12. We used a 550 bp long fragment of the *alkB* gene of *Pseudomanos putida* Gpo1 cloned into pCR 2.1-Topo vector (Invitrogen, Carlsbad, CA, USA) as the external standard. After plasmid isolation the DNA concentration was measured and the number of copies of the fragment calculated. Alkane monooxygenase genes from other strains may also be used as well.
13. In general, dilutions higher than 1/100 are not necessary for environmental samples.
14. We have used the 7300 Real-Time PCR System (Applied Biosystems, Foster City, CA, USA) and optimized the qPCR-protocol for this system. When other systems are used, other optimization steps might be necessary. Please follow the manufacturer's instructions for each system.

15. A repeater-step pipette may reduce pipetting errors when processing a large number of samples.
16. Due to high primer degeneracy primer-dimers can occur during the annealing step. These will give a positive signal using the Sybr Green detection system. To avoid this, an additional data collection step at 78°C is included to ensure that no primer-dimers are detected, since the latter have a denaturation temperature below 78°C.
17. During this step the dissociation curve is recorded, allowing the detection of unspecific PCR products.

References

1. Bird, C.W., and Lynch, J.M. (1974) Formation of hydrocarbons by microorganisms. *Chem. Soc. Rev.* 3, 309–328.
2. Taylor, S.E., and Calvin, M. (1987) Hydrocarbons from plants: biosynthesis and utilization. *Comm. Agric. Food Chem.* 1, 1–26.
3. Martin dos Santos, V.A.P., Yakimov, M.M., Timmis, K.N., and Golyshin, P.N. (2008) Genomic Insights into oil biodegradation in marine systems. In Díaz. E. (ed.) *Microbial Biodegradation. Genomics and Molecular Biology.* Caister Academic Press, Norfolk, UK, pp. 219–268.
4. van Beilen, J.B., Li, Z., Duetz, W.A., Smits, T.H., and Witholt, B. (2003) Diversity of alkane hydroxylase systems in the environment. *Oil Gas Sci Technol* 58, 427–440.
5. Widdel, F., and Rabus, R. (2001) Anaerobic biodegradation of saturated and aromatic hydrocarbons. *Curr. Opin. Biotechnol.* 12, 259–276.
6. van Beilen, J.B., and Funhoff, E.G. (2007) Alkane hydroxylases involved in microbial alkane degradation. *Appl Microbiol. Biotechnol.* 74, 13–21.
7. Smits, T.H.M., Röthlisberger, M., Witholt, B., and van Beilen, J.B. (1999) Molecular screening for alkane hydroxylase genes in Gram-negative and Gram-positive strains. *Environ. Microbiol.* 1, 307–317.
8. Vomberg, A., and Klinner, U. (2000) Distribution of *alkB* genes within n-alkane-degrading bacteria. *J. Appl. Microbiol.* 89, 339–348.
9. van Beilen, J.B., Funhoff, E.F., van Loon, A., Just, A., Kaysser, L., Bouza, M. et al. (2006) Cytochrome P450 Alkane hydroxylases of the CYP153 family are common in alkane-degrading eubacteria lacking integral membrane alkane hydroxylases. *Appl. Environ. Microbiol.* 72, 59–65.
10. Kloos, K., Munch, J.C., and Schloter, M. (1996) A new method for the detection of alkane-monooxygenase homologous genes (alkB) in soils based on PCR-hybridization. *J. Microbiol. Meth.* 66, 486–496.
11. Sharma, S., Radl, V., Hai, B., Kloos, K., Mrkonjic-Fuka, M., Engel, M. et al. (2007) Quantification of functional genes from prokaryotes in soil by PCR. *J. Microbiol. Meth.* 68, 445–452.
12. Mc Crady, M.H. (1915) The numerical interpretation of fermentation-tube results. *J. Infect. Dis.*17, 183–212.
13. Cochran, W.G. (1950) Estimation of bacterial densities by means of the Most Probable Number. *Biometrics* 4, 105–106.
14. Lee, L.G., Connell, C.R., and Bloch, W. (1993) Allelic discrimination by nick translation PCR with fluorogenic probes. *Nuc. Acid. Res.*21, 3761–3766.
15. Alef, K., and Nannipieri, P. (1995) *Methods in Applied Soil Microbiology and Biochemistry*, Academic Press Inc, San Diego, USA, 576 pp.

Chapter 5

Application of Fingerprinting Molecular Methods in Bioremediation Studies

Dimitrios G. Karpouzas and Brajesh K. Singh

Abstract

Bioremediation has been identified as a beneficial and effective strategy for the removal of recalcitrant environmental contaminants. Bioaugmentation of polluted environments with exogenous degrading microorganisms constitutes a major strategy of bioremediation. However, the ecological role of these strains and their impact on the endogenous microbial community of the micro-ecosystems where they are released should be known. Fingerprinting PCR-based methods, like denaturating gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (TRFLP), could be used in studies exploring the ecology of pollutant-degrading microorganisms and their effects on the structure of the soil microbial community. This chapter provides a brief outline of the technical details involved in the application of DGGE and TRFLP fingerprinting in soil microbial ecology, with particular reference to bioremediation studies.

Key words: Bioremediation, bacteria, DGGE, TRFLP, degradation, organic pollutants.

1. Introduction

Bioremediation has been recognized as an attractive decontamination strategy compared to physical and chemical treatments. This is due to its lower cost and little environmental disturbance taking place during its application (1). Biostimulation and bioaugmentation have been the main bioremediation strategies. Biostimulation relies on the optimization of the metabolic activity of the indigenous soil microflora for effecting pollutants removal, while bioaugmentation involves inoculation of contaminated environmental matrices with pollutant-degrading microorganisms to achieve accelerated decontamination.

S.P. Cummings (ed.), *Bioremediation*, Methods in Molecular Biology 599,
DOI 10.1007/978-1-60761-439-5_5,

The first step in bioaugmentation is the isolation, characterization and large-scale cultivation of pollutant-degrading microorganisms. Commonly, enrichment cultures in selective media where the pollutant serves as the sole source of C and/or N, P, S are employed for the isolation of such microorganisms. It is inevitable that enrichment techniques introduce a bias and the bacteria obtained may not have a significant role in the in situ degradation of the pollutant. Although several individual microorganisms or microbial consortia capable of rapidly degrading various pollutants have been isolated and characterized, their true ecological significance should be investigated before their release as bioaugmentation agents.

Molecular fingerprinting techniques were introduced in soil microbial ecology in the past 15 years and allowed the study of the ecology of microorganisms which could not be cultivated in synthetic media yet constitute the majority of soil microorganisms (2). Denaturating gradient gel electrophoresis (DGGE) and terminal restriction fragment length polymorphism (TRFLP) are two of the most popular fingerprinting methods used in microbial ecology (3). DGGE and TRFLP alone or in combination with cloning and sequencing have been used in different steps of bioaugmentation strategies. DGGE has been used in conjunction with culture-dependent techniques in order to elucidate the phylogeny but mainly the role and the ecological significance of the different members of complex pesticide-degrading microbial consortia (4–9). Other studies have used DGGE and TRFLP analysis in order to trace the fate of degrading microorganisms released in polluted soil ecosystems (10). In addition, DGGE (1, 11) and TRFLP (12) have been used to study possible perturbations in the soil microbial community, caused during bioaugmentation or biostimulation by the release of pollutant-degrading microorganisms. Here we present a methodological approach for the application of DGGE and TRFLP in bioremediation studies.

2. Materials

2.1. Denaturating Gradient Gel Electrophoresis

2.1.1. PCR Procedures (see Note 1)

1. 10× Mg^{++}-free DyNAzyme EXT buffer (Finnzymes).
2. 50 mM $MgCl_2$ solution (Finnzymes): 1.5 mM concentration in the reaction is used.
3. dNTPs (HT Biotechnology LTD): A working solution (10 mM concentration of each dNTP) is prepared while the concentration for each dNTP in the reaction is 0.2 mM.
4. Primers: A 10 pmol/μL working solution of each primer in sterile double distilled (dd)H_2O is prepared and their amount in the reaction ranges from 5 to 20 pmol.

5. Bovine serum albumin (BSA): Add in PCR reactions (200–400 ng/μL) with soil DNA in order to prevent inhibition of polymerase by co-extracted humic acids.

2.1.2. DGGE Procedures

1. DGGE system (INGENY phorU-2X2 or BioRad DCode).
2. 40% bisacrylamide 37.5:1 acrylamide:bisacrylamide solution
3. Deionized formamide solution
4. 50× TAE: 242 g Tris, 57 mL acetic acid and 100 mL of 0.5 M EDTA (PH 8.0) are dissolved in 1 L of dH_2O.
5. 10% ammonium persulphate solution (APS): 0.1 g APS is dissolved in 1 mL sterile dH_2O (stored at –20°C until use). Before gel casting one tube is thawed, each aliquot is for a single use.
6. TEMED (*N*,*N*,*N*′,*N*′-tetramethylenediamine)
7. GelBond PAG films (Camprex)
8. 0 % denaturant solution: 20 mL acrylamide and 2 mL 50× TAE in 100 mL of dH_2O (*see* **Note 2**).
9. 100% denaturant solution: 20 mL acrylamide, 40 mL formamide, 2 mL 50× TAE, 42 g urea made upto a final volume of 100 mL in dH_2O (*see* **Note 2**).
10. SYBR Gold Nucleic Acid Gel stain (Invitrogen): 1× working solution is prepared by dissolving 50 μL of the 10000× stock solution (stored at –20°C) in 500 mL 1× TAE.

*2.1.3. Silver Staining Reagents (*see Note 3*)*

1. Fixing solution I: 100 mL absolute ethanol, 5 mL glacial acetic acid, 895 mL dH_2O.
2. Staining solution: $AgNO_3$1 g/ L.
3. Developing solution: Dissolve 0.1 g $NaBH_4$ in 1 L of 1.5% NaOH (w/v), add 4 mL of formaldehyde.
4. Fixing solution II: $Na_2CO_3$7.5 g/ L.
5. Preservative solution: Mix 250 mL absolute ethanol and 100 mL glycerol (v/v), the volume is made to 1 L with dH_2O.

2.1.4. Cloning Procedures

1. PCR clean up kit (Macherey-Nagel GmbH & Co., or Qiagen).
2. pGEM-T Easy cloning vector (Promega).
3. Competent *E. coli* cells.
4. LB agar plates and broth: 10 g NaCl, 10 g cazein, 5 g yeast extract and 15 g agar (for preparation of agar plates) are dissolved in 1 L dH_2O, sterilized, and when cool antibiotics are added.

5. Ampicillin solution: stock solution of 100 mg/mL in ddH_2O is prepared, filter sterilized and 500 μL aliquots are added to 500 mL LB (already autoclaved) for a final concentration of 100 μg/mL.
6. IPTG (0.1 M): 0.238 g IPTG (stored at –20°C) dissolved in 10 mL ddH_2O and the solution is filter sterilized (stored at 4°C).
7. X-GAL (2% solution): 0.2 g X-GAL (stored at –20°C) is dissolved in 10 mL dimethylformamide and the solution is filter sterilized (stored at –20°C).
8. 96-well plates

2.2. Terminal Restriction Fragment Length Polymorphism

The reagents are the same as the ones described for DGGE, except primers are labelled with a fluorescent dye at the 5′ end.

2.2.1. PCR Procedures

2.2.2. PCR Product Purification by Ethanol Precipitation

Sodium acetate trihydrate (3 M): Add *ca.* 50 mL dH_2O to 40.824 g sodium acetate, mix and heat. Take to pH 4.6 with glacial acetic acid, and then make upto 100 mL. The solution should be autoclaved before use.

2.2.3. Restriction Digestion Reagents

1. Restriction enzymes
2. Appropriate buffer
3. Acetylated BSA

2.2.4. Sequencing Reagents and Instruments

1. Standard size marker
2. Deionized formamide
3. Running buffer and EDTA
4. DNA sequencer

3. Methods

3.1. Denaturating Gradient Gel Electrophoresis

3.1.1. PCR Procedures

PCR is a crucial step and allows the user to select which microbial groups to study. Thus, PCR aiming to amplify the 16S rRNA gene of the whole bacterial community will inevitably lead to a rather complex fingerprint, which can be difficult to analyse. In order to avoid this, group-specific primers that amplify variable regions of the small ribosomal subunit may be used in bioaugmentation studies, where the aim is to identify possible effects of inoculation on the structure of certain microbial guilds that are involved in important soil functions like nitrification or carbon assimilation. Group-specific primers that have been used in such studies are

Table 5.1
A list of group-specific primers that are used in DGGE fingerprinting studies

Primers	Microbial group
CTO189f ABC – CTO654r (13)	Ammonia-oxidizing bacteria
F243 – 513r (14)	Actinomycetes
Sphingo108f – Sphingo420r (15)	Sphingomonas
ITS1F – ITS4B (16)	Basidiomycetes
ITS1F – ITS4A (17)	Ascomycetes
NS31 – AM1 (18) and NS31 – Glo1 (19)	Arbuscular mycorrhiza fungi (AMF)

listed in **Table 5.1**. Universal bacterial or fungal primers can be alternatively used when the studied soils are expected to have low microbial diversity, such as heavily polluted soils or when a phylogenetic analysis of an enrichment culture is needed. In the former case, universal bacterial or fungal primers could be utilized in bioremediation studies in order to trace the fate of the inoculum and at the same time identify perturbations in the structure of the microbial community. In the latter case, DNA extracted from (i) the original soil, (ii) the enrichment culture and (iii) the individual isolated strains is subjected to PCR with universal bacterial primers (357f – GC and 534r) and the fragments derived are analysed in a comparative DGGE analysis in order to identify the ecological role and significance of the members of degrading consortia in the in situ degradation of a pollutant.

Several group-specific or universal primers provide relatively large products (400–700 bp), which either could not be analysed in DGGE (>600 bp) or provide poor resolution (400–600 bp). This has been the case with primer sets CTOs and NS31-AM1 (**Fig. 5.1**), which give products of 465 and 550 bp, respectively. Thus, we commonly follow a nested or semi-nested PCR approach to obtain PCR products for DGGE analysis. In this procedure, in the first PCR round soil DNA is amplified with group-specific primers and the product obtained is subjected to a second, nested-PCR with universal primers. It should be noted that in the second PCR round one of the two primers should carry a so-called GC-clamp', a 40-bp G- and C-rich sequence, at its 5′ end to prevent complete denaturation of the dsDNA fragments at high denaturant concentrations. Thus the second PCR round produces shorter products with a GC clamp attached, which are suited for DGGE analysis.

3.1.2. DGGE Procedures

In DGGE analysis DNA fragments of the same length but different nucleotide sequences are separated based on their different

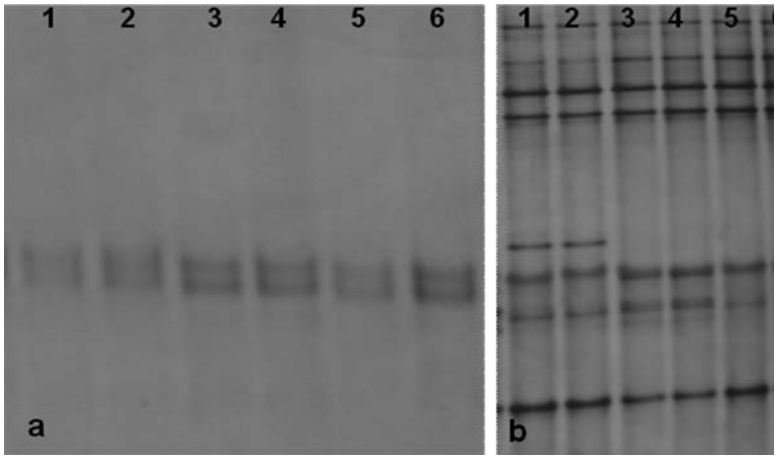


Fig. 5.1. DGGE analysis of the same community of AMF in roots of *Vicia faba* plants, which have been treated with different levels of olive mill wastewater 30 days before. Panel (**a**) fingerprints obtained with NS31-GC and AM1 primer set (600 bp product), Panel (**b**) fingerprints obtained after nesting of the PCR product obtained by primers NS31 – AM1 with nested primers NS31-GC and Glo1 (250 bp product).

mobility in polyacrylamide gels in a linear gradient of denaturating agents. The DGGE fingerprint of a soil microbial community comprises numerous bands, and each of them ideally represents a single taxa. However, it is possible, especially in more complex microbial communities, that two DNA fragments of different nucleotide sequence could migrate to the same position in the gel.

Before DGGE analysis, soil DNA extraction, PCR amplification and preparation of all reagents and solutions should be completed. We routinely use the following protocol for DGGE analysis with the INGENY system:

1. The pressure units are placed in the electrophoresis cassette, with the circular protuberances facing the cassette to come into good contact with the small glass plate.
2. A GelBond PAG film is cut carefully based on the shape and dimensions of the back glass plate (large notches) and it is subsequently firmly attached on it **(** *see* **Note 4)**.
3. The glass-plate assembly is set up by placing the white teflon spacer, which has been already greased, onto the back glass plate and on top of that the second glass plate is placed with the small notches facing down (*see* **Note 5**). It should be noted that the small enforcement at the bottom of the spacer must fit firmly against the bottom of the glass plate with the small notches.
4. The glass-plate assembly is placed in the electrophoresis cassette and the spacer is pulled up as far as it goes without lifting the glass-plate assembly. This is essential in order to insulate the bottom of the glass sandwich and minimize the danger of leaks during gel casting.

5. The screws are tightened and the combs are placed at the top of the glass-plate assembly.
6. The denaturants solutions (0% and 100%) should be mixed in appropriate volume ratios in order to achieve the desired gradient solutions. A guide for the preparation of gradient solutions is listed:

Denaturants Concentration (%)	0% solution (mL)	100% solution (mL)
20	20	5
30	17.5	7.5
40	15	10
50	12.5	12.5
60	10	15
70	7.5	17.5

7. The gradient solutions are transferred to the gradient-making device with the high- and low-concentration solutions placed in the left (connected to the tubing) and right leg of the device, respectively.
8. Aliquots of 100 μL APS and 10 μL of TEMED are added into the gradient solutions and are mixed briefly using a magnetic stirrer (*see* **Note 6**).
9. A pipette tip is adjusted on the free end of the tubing and placed centrally between the glass plates.
10. The connection valve in the mixing device and the peristaltic pump are switched on concurrently and the acrylamide is transferred to the glass-plate assembly, while gradient solutions in both legs of the gradient-making device are continuously mixed on a magnetic stirrer.
11. When the level of the acrylamide in the glass-plate assembly reaches just below the combs, gel casting is halted and the acrylamide is left to polymerize for an hour.
12. After polymerization is completed, approximately 4 mL of 0% denaturant solution amended with 20 μL APS and 2 μL TEMED (stacker solution) is added to the top of the gel for the wells to be formed (*see* **Note 7**).
13. After complete polymerization of the stacker (an hour), all screws are loosened gently (just to touch the pressure unit) and the cassette is placed inside the buffer tank where the temperature has been set to 60°C.
14. The buffer flow tube is connected with the blue connector of the cassette to ensure buffer circulation in the upper buffer reservoir and the combs are removed (*see* **Note 8**).

15. The spacers are pushed all the way down and the bubbles formed under the gel should be removed by briefly holding the cassette at an angle.
16. The upper two screws are tightened and the electrodes are connected to the electrophoresis cassette.
17. The samples are loaded on the wells using long pipette tips (*see* **Note 9**).
18. The buffer tank is connected to an external power supply and the gel is run at 75 V for 960 min (1200 volt-hours) (*see* **Note 10**).
19. On completion of electrophoresis, the cassette is removed from the tank and the gel is detached from the glassplate assembly, rinsed briefly with water and stained.
20. Silver staining provides the best resolution and the clearest banding patterns, but its use should be avoided when DGGE band excision is required since it leads to low DNA recoveries. The silver staining protocol we regularly use is as follows:
    a. Gel is placed in a plastic tray with 500 mL of fixing solution I and left to agitate gently for 2 h.
    b. Gel is transferred to 500 mL of staining solution and agitated for 20 min.
    c. Gel is transferred to 500 mL of developing solution and agitated for 10–20 min until the banding patterns are clearly visible and well stained.
    d. Gel is placed in 500 mL of fixing solution II and left to agitate for 20 min.
    e. Gel is removed, cut to appropriate size and placed in water-tight plastic bags with 10 mL of preservative solution until photographed.

    In all steps the gel should be fully immersed in the solutions used.
21. *SYBR Gold staining procedures*: This procedure is preferred when DGGE bands are going to be excised. Other nucleic acid stains such as ethidium bromide or SYBR Green can also be used although SYBR Gold provides comparably higher resolution. SYBR Gold staining involves gel immersion in 400–500 mL of 1× SYBR Gold solution, gentle agitation for 40 min and then visualization under UV light.
22. *DGGE analysis*: Image analysis software is used to analyse the banding patterns of DGGE gels. GelCompar is the most popular of those which digitizes the gel and converts it into a matrix of binary or relative abundance data for statistical analysis using multivariate techniques. Principal

coordinate analysis, cluster analysis, canonical variance analysis and other multivariate techniques are used for statistical interpretation of DGGE fingerprinting using statistical packages such as Genstat, Statistica or MultiVariate Statistical Package (MVSP). A recent comprehensive review on the multivariate analysis of fingerprinting data is available by Marzorati et al. (20).

3.1.3. Band Excision, Cloning and Sequencing

In studies looking at the impact of bioaugmentation on the structure of the microbial community, a common strategy to acquire phylogenetic information for bands whose presence or absence appears to be related to specific treatments (e.g. inoculated with pesticide-degrading bacteria) is the direct excision and sequencing of the band. In our experience this approach is time-consuming and troublesome, especially in complex communities where bands are not well separated. We have observed that upto three or four cycles of band excision, re-amplification and repeated DGGE screening against the environmental sample are required to derive the selected bands in a pure state.

We use a different approach for elucidating phylogenetic information from DGGE fingerprints. Direct cloning of the initial PCR round (600–1000 bp product) and subsequent screening of the clones in a second DGGE round against the environmental samples offer a more convenient approach for phylogenetic analysis of microbial communities fingerprinted by DGGE. In addition, this approach allows sequencing of large PCR products (600–1000 bp), thus providing more reliable phylogenetic analysis compared to band excision, which leads to sequencing of small DNA fragments (150–300 bp). The protocol we routinely use is described briefly:

1. The products from the first PCR round are thawed and equal volumes of the replicates of a single treatment are mixed to a final volume of 60 μl (*see* **Note 11**).
2. The PCR product is purified using a standard spin-column PCR-clean-up procedure, with elution made in 30 μL sterile ddH_2O (*see* **Notes 12 and 13**).
3. We routinely use pGEM-T-Easy cloning vector (*see* **Note 14**). Depending on the DNA concentration of the purified PCR product, 0.5–4 μl of the DNA insert is used in the ligation reaction with pGEM-T-Easy (*see* **Note 15**).
4. The ligation reaction is mixed and incubated overnight at 4°C (*see* **Note 16**).
5. Transformation into competent cells is performed according to manufacturers' instructions. The whole volume of the transformation culture (*ca.* 320 μL) including competent cells (60 μL), plasmid DNA (2 μL), IPTG (50 μL), X-GAL (10 μL) and LB (200 μL) are spread in LB plates

containing ampicillin (*see* **Note 17**). Three plates per transformation culture are usually spread to obtain adequate number of clones.

6. The plates are incubated overnight at 37°C, and next morning the efficiency of transformation is visually checked. Subsequently, plates are stored in the fridge before final selection of positive clones.
7. Next day, 30–50 white colonies from each treatment are selected with a toothpick. For each colony selected, the toothpick is first dipped into a 96-well plate containing the PCR mix and then in the corresponding well of a second 96-well plate containing 150 μL of LB + ampicillin. For the colony-PCR amplification, a 10× colony-PCR buffer that facilitates lysis of cells is used along with the set of primers which were utilized at the nested-PCR round aiming to acquire a product which will be appropriate for DGGE analysis (*see* **Note 18**).
8. Further DGGE screening of the selected cloned fragments against the environmental sample is done using the same electrophoresis and gradient conditions. In a gel with 32 wells, we tend to analyse 24 clones plus three marker lanes and three lanes with the environmental sample (*see* **Note 19**).
9. Clones showing the same migration with certain bands in the environmental sample are further processed for sequencing by extraction of the plasmid from the sub-culture of the clone in the 96-well plates (*see* Step 7 in **Section 3.1.3**). It is common that several clones show the same migration pattern as can be seen in **Fig. 5.2**. In that case two or more of the clones with the same migration pattern are sequenced. This way we avoid underestimating the diversity of the community since it is possible that different sequences with the same GC% ratio will show the same migration pattern.

3.2. Terminal Restriction Fragment Length Polymorphism

Terminal restriction fragment length polymorphism (TRFLP) is an automated and sensitive method that can be used to compare microbial communities and monitor changes in community structure. A fluorescently labeled primer is used for the PCR, and after restriction digestion, fragments of varied length are generated (*see* **Note 20**). The sequencer recognizes only the fluorescently labelled terminal fragments and, therefore, in principle, each fragment represents a unique genome in the sample (21). The TRFLP, just like DGGE, can be used to examine both the survival and the abundance of bioaugmentation agents in polluted environment as well their effects on indigenous populations. TRFLP has some advantages over DGGE in the sense that the data are semi-quantitative and, therefore, can be used to monitor relative abundance of bioremediation agents over a

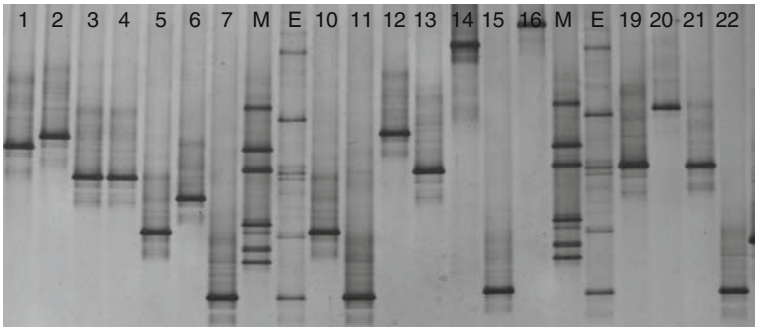


Fig. 5.2. DGGE screening of the migration pattern of 18 clones (1–20) of the ITS region of the community of basidiomycetes against the fingerprint of the soil sample from where they were originated (E). M, fungal marker lanes containing 20 ng/μL of the ITS-PCR products of the following fungi with the sequence appearing on the gel from top to bottom: *Pleurotus djamor, Fusarium oxysporum radici lycopersici, F. solani, P. eryngii, P. ostreatus, P. cystidiosus*.

period of time. Also, TRFLP is high-throughput method; therefore, a large number of samples can be analysed simultaneously. It can also be used to identify microbial strains using several public databases without a need of cloning and sequencing although at a lower resolution. TRFLP can also be used to examine the effect of biostimulation process on microflora of polluted environment.

The PCR procedure for TRFLP is the same as for DGGE except that here one or both primers used are fluorescently labelled at the 5′ end.

3.2.1. Purification of PCR Products

1. There are several commercial kits available, which can be used for cleaning PCR products and two of them were mentioned in previous sections. For economic reasons, several laboratories still use classical ethanol precipitation approach. **Table 5.2** describes in detail the ethanol precipitation procedure both for a single tube and in 96-well plate format.

3.2.2. Restriction Digestion of Purified PCR Products

1. The selection of restriction enzymes for digestion depends on the heterogeneity of the target sequence. There are several software packages and public databases available to help selecting appropriate restriction enzyme(s) for 16S rRNA genes. For example, MiCA (http://mica.ibest.uidaho.edu/digest.php) can be used to predict the TRFs for all known 16S rDNA available in public database using a single or multiple restriction enzymes. For other genes, the available sequences can be obtained from public databases such as NCBI or EMBL and then use REMA (www.macaulay.ac.uk/rema) software to predict the expected TRFs and select the best discriminatory restriction enzymes for digestion.

Table 5.2
A brief outline of the ethanol precipitation procedure of PCR products

Step	Specifics	
	Tube	Plate (make mix)
Add ethanol	100 μL	25,000 μL
Add sodium acetate	5 μL	1,250 μL
		Mix
		Dispense 100 μL per well
Incubate −20°C for at least 20 min (o/n is ok)		
Spin 30 min at max speed	14,100 × *g*	1109 × *g*
Remove s/n	Use pipette to remove s/n taking care not to take pellet	Place plate upside down gently over blue paper towel. Check pellet is still in plate and not on blue paper towel.
		Put plate over fresh blue paper towel and spin briefly upside down. Check pellet is still inside wells.
Add 70% ethanol 150 μL per well or per tube		
Incubate at −20°C for 10 min		
Spin 20 min at maximum speed	14,100 × *g*	1109 × *g*
Remove s/n	Use pipette to remove s/n taking care not to take pellet	Place plate upside down gently over blue paper towel. Check pellet is still in plate and not on blue paper towel.
	Air dry pellet in desiccator	Put plate over fresh blue paper towel and spin briefly upside down. Check pellet is still inside wells.
Dissolve pellet in 10–20 μL nuclease-free water		

2. The concentrations of the different reagents recommended for restriction digestion are listed in **Table 5.3**.
3. Samples should be incubated at the optimum temperature of the restriction enzymes for at least 15 min followed by a deactivation step at 95°C for 10 min.

3.2.3. Fragment Size Analysis on a DNA Sequencer

1. The size (in base pair) of the TRF is determined by capillary electrophoresis on a sequencer. Here we describe a methodology and chemistry based on Applied Biosystem Instruments (ABI) sequencer. However, the principle is applicable to sequencers from all other manufactures.

Table 5.3
A list of the reagents used in restriction digestion in TRFLP analysis

Reagents	Supplier	μL added per initial 50 μL reaction
Enzyme	Promega	2
BSA (100×)	Promega	0.2
10× buffer	Promega	2
Sample	–	Ideally ~500 ng sample – upto 15.8 μL
dH_2O (autoclaved)	MilliQ	Upto 20 μL

2. After digestion, 1 μL of the DNA is transferred to an optical 96-well reaction plate and is mixed with 0.3 μL of LIZ-labelled GeneScan^{TM}-500 internal size standard and 12 μL of Hi-Di^{TM} (highly deionized) formamide. Again, several different size markers (with different colour and base pair size) are available from various suppliers.
3. After mixing with the marker, samples are denatured at 95°C for 5 min and then chilled on ice for 5 min.
4. The sample DNA is then loaded by injection into the capillaries and the DNA fragments are separated according to their base pair size (*see* **Note 21**).
5. Fragment analysis

Description	POP 4	Unit
Oven temperature	60	°C
Poly fill volume	7300	Steps
Current stability	5	μAmps
Pre-run volt	15	kVolts
Pre-run time	180	s
Injection volt	1.6	kVolts
Injection time	15	s
Volt steps	20	nk
Volt step interval	15	s
Data delay time	750	s
Run volt	15	kVolts
Run time	2500	s

50 cm capillary. Module: FragmentAnalysis50_POP4_1. Dye Set G5.

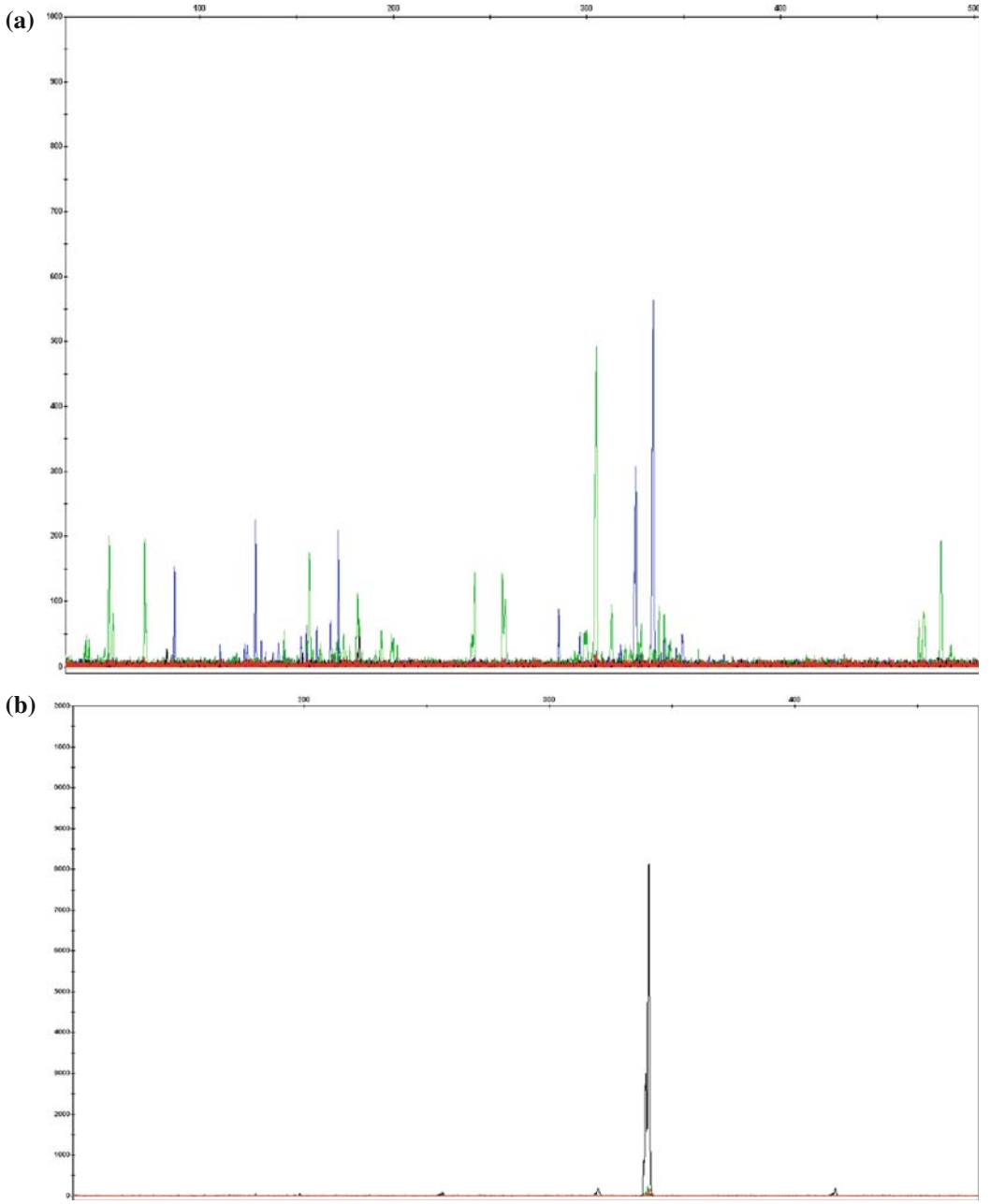


Fig. 5.3. (**a**) A typical TRFLP profile from an environmental sample obtained when both forward and reverse primers (with FAM and VIC respectively) are labelled. Value on X-axis represents base pair size for each fragment, which can be used for identification of taxa. Value on Y-axis represents the fluorescent unit, which is directly proportional to relative abundance of a particular TRF in the profile. (**b**) A typical TRFLP profile from a single bacterial species where only forward primer (with NED) is labelled.

3.2.4. Fragment Size Data Analysis

1. We add an example of data analysis using software GeneMapper (ABI, version 3.7). Please note that fragment data analysis software varies depending on the manufacturer of the sequencers (*see* **Note 22**). **Figure 5.3** shows the output data obtained for a typical soil community (**Fig. 5.3a**) and for a single bacterial species (**Fig. 5.3b**). GeneMapper provides a range of algorithms for data analysis. Please see manual for the detail of algorithm approaches and their suitability for different applications.
2. For TRFLP profiles obtained from complex communities (soil and water samples), it is advisable that only peaks corresponding to fragments between 50 and 500 bp taken into account as most TRFs fall in this range. This will exclude TRFs caused by primer-dimers (*see* **Note 23**). However, if necessary, peak size larger than 500 bp can also be obtained using internal markers, which have a larger range (ABI provides makers upto 1000 bp long).
3. TRFLP provides semi-quantitative (relative abundance) as well as binary data (presence and absence of TRFs). The table obtained from the GeneMapper includes information on number of TRFs, TRF size (in base pairs), height and area (in fluorescence units). The relative abundance of an individual TRF in a profile is obtained from the height or the area of the TRF, which is directly proportional to the TRF copy number present in the sample. The relative abundance of a particular TRF within a profile is calculated as the peak height (or area) of that TRF divided by the total peak height (or area) of all TRFs together in the profile (*22*).
4. For community profiles, it is advisable that any TRF that accounts for less than 0.5–1% of the total peak fluorescence is excluded from further analysis. This approach minimizes the effect of variations in the TRFLP profiles caused by the quantity of DNA analysed.

4. Notes

1. We prefer to use higher volumes in PCR reactions (50 μL) in order to have excess volumes of product if cloning is going to be performed
2. Denaturant solutions 0% and 100% should be made at low volumes (100 mL) in order to use them relatively fresh for the preparation of gradient solutions. Denaturants

solutions should be stored in the dark. In preparation of the 100% denaturant solution, urea is difficult to dissolve, and it is recommended to gently warm the solution and use a magnetic stirrer to facilitate dissolution.

3. The staining and the developing solutions should be prepared on the day of use. In the developing solution formaldehyde should be added first. $AgNO_3$, $NaBH_4$ and formaldehyde are toxic and they should be disposed of in appropriate waste containers.
4. GelBond PAG films facilitate safe handling of the delicate DGGE gels. However, due to their high cost, we tend to use them only for the production of publication quality DGGE gels with silver nitrate staining. When the film is attached on the glass plate, its hydrophobic side should be facing the glass plate. Firm attachment is achieved by addition of water in the interface between glass and film. Excess water is removed by rolling a pencil or a ruler on the surface of the film.
5. Before the preparation of the glass-plate assembly, a thin layer of silicon grease should be spread on both sides of the spacer to prevent any leaks during gel casting. Excess greasing might diminish the quality of the DGGE, especially at the side lanes.
6. After APS and TEMED have been added in the gradient solutions, polymerization of acrylamide:bisacrylamide starts immediately at room temperature. Therefore, gel casting should be initiated as soon as possible to avoid polymerization of acrylamide inside the tubing. After APS and TEMED addition and before gel casting, gradient solutions should be kept on ice to retard acrylamide polymerization.
7. The stacker should be preferably added with a pipette or a syringe in order to guarantee formation of nice and robust wells.
8. The combs should be removed only after the upper buffer reservoir of the cassette is full with buffer in order to ensure that the wells are filled up with buffer.
9. Preferably, two lanes on both sides of the gel are left empty since possible "smiling" effects might diminish the quality of the fingerprints in those lanes. In the first DGGE analysis of a particular set of samples, we tend to load 1 μL and 1.5–2 μL for strong and faint PCR products, respectively, with 5 μL of loading buffer. The quality of the fingerprinting obtained will be used as a guide for optimization of the amount of DNA loaded in subsequent gels. In each gel two or three marker lanes should be loaded. This not only provides a control of DGGE gels inherent heterogeneity

but also allows gel comparisons. Several people use size standards as markers, which is rather meaningless. We have prepared and routinely use our own standards for bacterial and fungal communities. These were made by mixing equal portions of PCR products from clones showing different migration.

10. The exact running time depends on the size of the product and the resolution required. Generally, it is expected that electrophoresis at low voltage for longer time provides improved resolution, but in most cases the decision is based on previous experience. The buffer flow at the upper buffer reservoir should be maintained closed for the first 5 min of electrophoresis in order to prevent any flushing out of the loaded samples from the wells. The running buffer (1× TAE) should be replaced after every three to four electrophoreses.
11. For PCR products that have been stored in the freezer for long, it would be preferable to follow an A-tailing procedure in order to replace any A-overhangs that tend to fall by prolonged storage. This short intervention would ensure adequate ligation efficiency into your cloning vector.
12. We use sterile ddH_2O instead of the elution buffer for the elution of DNA from the spin columns. We have observed that in this way no extra salts and buffers are introduced in the ligation reaction with the DNA and this leads to optimum cloning efficiencies.
13. The purity and the amount of the purified PCR product derived from PCR cleanup should be routinely checked by agarose gel electrophoresis. This provides a crude measure of the amount of PCR product and facilitates the optimization of the volume of PCR-insert, which will be used in the ligation.
14. In our experience, pGEM produces relatively higher and consistent cloning efficiency with relatively lower cost. On the other hand, TOPO cloning does not involve ligation and thus the time required for completion of cloning is significantly less. However, it should be noted that TOPO is slightly more expensive compared to pGEM.
15. The amount of the cloning vector used in the ligation reaction is estimated based on the size and the amount of the cloned insert and we usually aim for a 3:1 molar ratio between insert: vector. However, in our experience deviations from this ratio could provide high ligation efficiency after empirical assessment.

16. Overnight incubation maximizes ligation efficiency compared to the 1 h incubation, which is alternatively proposed by the pGEM-T-Easy manufacturers.
17. We have been using competent cells from different companies, including DH5A (Invitrogen), XLBlue (Stratagen) and JM109 (Promega), with good efficiency. Relatively higher transformation efficiency was evident when SOC instead of LB is used according to manufacturers' instruction.
18. This rapid colony-PCR procedure enables (i) the screening of the selected clones for positive acquisition of the insert and at the same time (ii) produces PCR products with the GC-clamp attached, which could be directly screened in a second DGGE round against the environmental samples from where they are originated
19. The number of clones selected for screening depends not only on the complexity of the microbial community but also on the instrument availability. For complex microbial communities we screen 40–50 clones, while in less complex communities where only few bands are targeted 25–30 bands are commonly screened. We usually load 0.5–1 μl of the colony-PCR product in each DGGE lane. The exact amount depends on the strength of band given by the PCR product in the agarose gel. The amount loaded corresponds to 10–20 ng of DNA per lane.
20. For TRFLP, several different types of fluorescent dyes are used for primer labelling (FAM, HEX, VIC, NED, PET are just a few examples). It is important to check the colour filter of the sequencer optimized for each dyes individually and in combination. Please see sequencer manual for detail.
21. Care should be taken in the amount of DNA loaded on the capillary. It is advisable for community analysis to use between 500 and 1000 ng of DNA for digestion in 20 μL reaction and only 1 μL should be loaded on the DNA sequencer. However, this concentration of DNA is also dependent on the number of different fragments expected in each profile. If DNA concentration is very high and the numbers of fragments in a profile are low, then the profile produces several artefact TRFs, a process called capillary bleeding. Therefore, it is important that optimized concentrations of DNA should be loaded on the sequencer.
22. There is always a minor shift in position for the same TRFL when run separately. However, most of the software packages available in the market have functions to take this into account. Depending on the quality of data between one

and three base pairs difference can occur when same sample is run on different sequencers. This should be taken into account for data analysis. However, if all profiles were generated in one run and on the same sequencer, the difference should be less than one base pair.

23. It is necessary that if two different set of samples to be compared, the analysis method should be the same. Any change in the analysis parameter may result in very different profiles.

References

1. Miyasaka, T., Asami, H., and Watanabe, K. (2006) Impacts of bioremediation schemes on bacterial population in naphthalene-contaminated marine sediments. *Biodegradation* **17**, 227–235.
2. Kirk, J.L., Beaudette, L.A., Hart, M., Moutoglis, P., Klironomos, J.N., Lee H., et al. (2004). Methods of studying soil microbial diversity. *J. Microbiol. Meth.* **58**, 169–188.
3. Lynch, J.M., Benedetti, A., Insam, H., Nutti, M.P., Smalla, K., Torsvik, V., et al (2004) Microbial diversity in soil: ecological theories, the contribution of molecular techniques and the impact of transgenic plants and transgenic microorganisms. *Biol. Fertil. Soils* **40**, 363–385.
4. Singh, B.K., Walker, A., Morgan, J.A.W., and Wright, D.J. (2003). Effects of soil PH on the biodegradation of chlorpyrifos and isolation of a chorpyrifos-degrading bacterium. *Appl. Environ. Microbiol.* **69**, 5198–5206.
5. Singh, B.K., Walker, A., Morgan, J.A.W., and Wright, D.J. (2003). Role of soil pH in the development of enhanced biodegradation of fenamiphos. *Appl. Environ. Microbiol.* **69**, 7035–7043.
6. Bending, G.D., Lincoln, S.D., Sorensen, S.R., Morgan, J.A.W., Aamand, J., and Walker, A. (2003). In-field spatial variability in the degradation of the phenyl-urea herbicide isoproturon is the result of interactions between degradative *Sphingomonas* spp. and soil PH. *Appl. Environ. Microbiol.* **69**, 827–834.
7. Dejonghe, W., Berteloot, E., Goris, J., Boon, N., Crul, K., Maertens, S., et al., (2003) Synergistic degradation of linuron by a bacterial consortium and isolation of a single linuron-degrading *Variovax* strain. *Appl. Environ. Microbiol.* **69**, 1532–1541.
8. Shi, S., and Bending, G.D. (2007) Changes to the structure of *Sphingomonas* spp. communities associated with biodegradation of the herbicide isoproturon in soil. *FEMS Microbiol. Lett.* **269**, 110–116.
9. Breugelmans, P., D'Huys, P-T., Mot, R.D., and Springael, D. (2007) Characterization of novel linuron-mineralizing bacteria consortia enriched from long-term linuron-treated agricultural soils. *FEMS Microbiol. Ecol.* **62**, 374–385.
10. Cunliffe, M., and Kertesz M.A. (2006) Effect of *Sphingobium yanoikuyae* B1 on bacterial community dynamics and polycyclic aromatic hydrocarbon degradation in aged and freshly PAH-contaminated soils. *Environ. Pollut.* **114**, 228–237.
11. MacNaughton, S.J., Stephen, J.R., Venosa, A.D., Davis, G.A., Chang Y.-J., and White D.C. (1999) Microbial population changes during bioremediation of an experimental oil spill. *Appl. Environ. Microbiol.* **65**, 3566–3574.
12. Raina, V., Suar, M., Singh, A., Prakash, O., Dadhwal, M., Gupta, S.K., et al. (2007) Enhanced biodegradation of hexachlorocyclohexane (HCH) in contaminated soils via inoculation with *Sphingobium indicum* B90A. *Biodegradation* **19**, 27–40.
13. Kowalchuk, G.A., Stephen, J.R., DeBoer, W., Prosser, J.L., Embley, T.M., and Woldendorp. J.W. (1997). Analysis of β-Proteobacteria ammonia-oxidizing bacteria in coastal sand dunes using denaturating gradient gel electrophoresis and sequencing of PCR amplified 16 rDNA fragments. *Appl. Environ. Microbiol.* **63**, 1489–1497
14. Heuer, H., Krsek, M., Baker, P., Smalla, K., and Wellington, E.M. (1997) Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturating gradients. *Appl. Environ. Microbiol.* **63**, 3233–3241.
15. Leys, N.M.E.J., Ryngaert, A., Bastiaens, L., Verstraete, W., Top, E.M., and Springael

D. (2004) Occurrence and phylogenetic diversity of *Sphingomonas* strains in soils contaminated with polycyclic aromatic hydrocarbons. *App. Environ. Microbiol.* **7**, 1944 –1955.
16. Gardes, M., and Bruns T.D. (1993). ITS primers with enhanced specificity for basidiomycetes – application to the identification of mycorrhizae and rusts. *Molec. Ecol.* **2**, 113–118.
17. Larena, I., Salazar, O., Gonzalez, V., Julian, M.C., and Rubio, V. (1999) Design of a primer for ribosomal DNA internal transcribed spacer with enhanced specificity for ascomycetes. *J. Biotech.* **75**, 187–194.
18. Helgason, T., Daniell, T.J., Husband, R., Fitter, A.H., and Young J.P.W. (1998) Ploughing up the wood-wide web? *Nature* **394**, 431.
19. Liang, Z., Drijber, R.A., Lee, D.J., Dwiekat, I.M., Harris, S.D., and Wedin, D.A. (2008) A DGGE-cloning method to characterize arbuscular mycorrhizal community structure in soil. *Soil Biol. Biochem.* **40**, 495–504.
20. Marzorati, M., Wittebolle, L., Boon, N., Daffonchio D., and Verstraete, W. (2008) How to get more out of molecular fingerprints: practical tools for microbial ecology. *Environ. Microbiol.* (**10**, 1571–1581 doi:10.1111/j.1462-2920.2008.01572.x).
21. Singh, B.K., Nazaries, L., Munro, S., Anderson, I.C., and Cambell, C.D. (2006) Use of multiplex terminal restriction fragment length polymorphism for rapid and simultaneous analysis of different components of the soil microbial community. *Appl. Environ. Microbiol.* **76**, 7278–7285
22. Singh, B.K., and Thomas, N. (2006) Multiplex terminal restriction fragment length polymorphism. *Nature Prot.* **1**, 2428–2433.

Chapter 6

Using Terminal Restriction Fragment Length Polymorphism (T-RFLP) Analysis to Assess Microbial Community Structure in Compost Systems

Sonia M. Tiquia

Abstract

Terminal restriction fragment length polymorphism (T-RFLP) analysis of PCR-amplified genes is a widely used fingerprinting technique in composting systems. This analysis is based on the restriction endonuclease digestion of fluorescently end-labeled PCR products. The digested product is mixed with a DNA size standard, itself labeled with a distinct fluorescent dye, and the fragments are then separated by capillary or gel electrophoresis using an automated sequencer. Upon analysis, only the terminal end-labeled restriction fragments are detected. An electropherogram is produced, which shows a profile of compost microbial community as a series of peaks of varying height. This technique has also been effectively used in the exploration of complex microbial environments and in the study of bacterial, archaeal, and eukaryal populations in natural habitats.

Key words: T-RFLP, fingerprinting technique, microbial composition, composting, PCR-based technique, culture-independent method.

1. Introduction

Composting is a biological conversion of solid organic wastes into stable materials such as fertilizers (1). The decomposition of organic matter during composting is mediated by a succession of microbial communities. The initial phase of composting process is characterized by the growth and activity of mesophilic microbes, which in turn leads to a rapid increase in temperature. At the next stage, the thermophilic microbes become responsible for the degradation process. The final stage, which includes

S.P. Cummings (ed.), *Bioremediation*, Methods in Molecular Biology 599,
DOI 10.1007/978-1-60761-439-5_6,

cooling-down and maturing phases, is characterized by the development of a new mesophilic community (2–3). The optimization of compost quality is directly linked to the composition and succession of microbial communities in the composting process. Therefore, it is important to monitor the succession of microbial communities for effective management of the composting process.

Culture-independent methods have been recently employed to monitor the succession of microbial communities during the composting process. One of the most important methods for the survey of compost microorganisms is the analysis of a clone library (4) or, more and more promisingly, the analysis of a metagenomic library (5). However, due to the complexity of the compost communities and the effort required for these analyses, clone libraries have been restricted to the analysis of a single sample or a few samples. To circumvent the limitation of the clone library approach, several PCR-based methods now exist to allow rapid fingerprinting and monitoring of the composting process. These techniques include terminal restriction fragment length polymorphism (T-RFLP) (6), denaturing gradient length polymorphism (DGGE) (2), single-strand conformation polymorphism (SSCP) (7), and phospholipid fatty acid analysis (PLFA) (8). In recent years, T-RFLP has been widely used for the analysis of microbial communities during composting process (6, 9–12) due to its high throughput and phylogenetic resolution.

Typically, T-RFLP analysis involves amplification of target genes from whole-community DNA extracts by using specific primer pairs, one of which is fluorescently labeled. Subsequently, amplicons are digested with restriction enzymes (usually tetranucleotide recognizing) and fragments are size-separated by electrophoresis on automated sequencers, whereby only the labeled terminal fragments (T-RFs) are detected and quantified. Individual T-RFs can be assigned presumptively to operational taxonomic units, which ideally correspond to phylogenetically related microorganisms, based on in silico search for matching restriction sites in sequences from clone libraries established in parallel from the same sample. In general, the T-RFLP technique has been proven to be reproducible as an accurate tool for community fingerprinting (13–16) . However, since T-RFLP is based on PCR amplification, all biases related to this technique apply (17).

2. Materials

2.1. DNA Extraction and PCR Amplification

1. Kit for extracting environmental DNA. We routinely use the Power Soil DNA extraction kit (MO BIO Laboratories, Inc., Carlsbad, CA) for compost samples (*see* **Note 1**).

Alternatively, good results are obtained from Soil FASTDNA kit or from the extraction protocols developed by Yang et al. (18).

2. Double distilled water (ddH_2O) sterilized by autoclaving or filtering. Prepare 100 μL aliquots before sterilization and keep at −20°C. Discard the aliquot after use.
3. 10× PCR buffer: 500 mM KCl, 10 mM Tris−HCl, 0.1% Triton X-100, pH 9.0
4. 50 mM $MgCl_2$ stock solution (Invitrogen, Carlsbad, CA)
5. Stock solution of a mixture of dexoyribonucleotide triphosphates (dNTPs): 2 mM of each dNTP in ddH_2O. Prepare aliquots of 20 μL and store at −20°C.
6. *Taq* DNA polymerase (Invitrogen, Carlsbad, CA) or other thermostable DNA polymerase
7. Primers for the amplification of the gene of interest: For instance on 16S rDNA, 8f primer (5′-AGAG TTTGATCCTTGGCTCAG-3) and 1492r primer (GCYTACCTTGTTACGACTT) give good results (*see* **Note 2**). 8f primer is labeled at the 5′ end with 6-FAM (6-carboxyfluo-rescein) fluorescent dye (Applied Biosystems, Foster City, CA). Prepare stock solutions of primers at 20 μM and store at −20°C.
8. Bovine serum albumin (BSA): This protein eliminates PCR inhibitors in compost DNA samples.
9. Dimethyl sulfoxide (DMSO): Aliquot 500 μL and keep at −20°C.

2.2. Gel Electrophoresis and PCR Purification

1. TAE buffer: 40 mM Tris-acetate, 1 mM EDTA, pH 8. Prepare a 50× stock solution.
2. Agarose (Sigma-Aldrich St. Louis, MO).
3. Ethidium bromide stock solution: 10 mg/mL. Store in a dark bottle. Caution: Ethidium bromide is a mutagen, suspected carcinogen, and at high concentrations is irritating to the eyes, skin, mucous membranes, and upper respiratory tract. Preparation of stock solutions and any operations capable of generating ethidium bromide dust or aerosols should be conducted in a fume hood to prevent inhalation. Nitrile gloves should be worn at all times.
4. 10× DNA-loading buffer: 70% (w/v) glycerol, 0.5% bromophenol blue. Store at 4°C.
5. DNA size marker: 100-bp or 1-kbp molecular marker (Invitrogen, Carlsbad, CA) for agarose gel electrophoresis.

6. Kit for purification of PCR products from unincorporated primers and salts. Good purification results are obtained with Qiagen PCR purification kit (Qiagen Inc., Valencia, CA).
7. Ethanol (100%)

2.3. Restriction Digestion and T-RFLP

1. Restriction enzymes with four-base recognition sequence (i.e., *Hha* I, *Msp* I, *Rsa* I) and their specific buffers (Roche Applied Science, Indianapolis, IN). Four base pair cutter restriction enzymes are most appropriate as the probability of having a restriction site within the amplicon is high. Various restriction enzymes can be used in single-enzyme reactions in order to determine which one yields the highest number and most even distribution of terminal restriction fragments (*see* **Note 3**).
2. Double-distilled water (Prepared in Section **2.2**.1).
3. TAMRA 500 (Applied Biosystems, Foster City, CA) molecular size marker for capillary electrophoresis.

3. Methods

3.1. DNA Extraction

Numerous methods are available for extracting community DNA from composts. It is important that the extraction procedure works both for Gram-positive bacteria and for Gram-negative bacteria. We employ a bead-beating technique using the Power Soil DNA extraction kit following the manufacturer's instructions, which briefly are as follows:

1. Transfer 0.25 g of compost sample to the PowerBead tubes and gently vortex to mix.
2. Add 60 μL of Solution C1 (contains SDS and other disruption agents required for complete cell lysis) and invert several times.
3. Secure PowerBead tubes horizontally using the MO BIO Vortex Adapter tube holder for the vortex or secure tubes horizontally on a flat-bed vortex pad with tape. Vortex at maximum speed for 10 min.
4. Centrifuge tubes at 10,000 × *g* for 30 s at room temperature.
5. Transfer the supernatant to a clean 2 mL collection tube.
6. Add 250 μL of Solution C2 (contains a reagent that precipitate non-DNA organic and inorganic materials) and vortex for 5 s. Incubate at 4°C for 5 min and then centrifuge the tubes at room temperature for 1 min at 10,000 × *g*.
7. Avoiding the pellet, transfer up to 600 μL of supernatant to a clean 2 mL collection tube 7. Add 200 μL of Solution

C3 (a second reagent to precipitate additional non-DNA organic and inorganic material) and vortex briefly. Incubate at 4°C for 5 min. Centrifuge the tubes at room temperature for 1 min at 10,000 × *g*.

8. Transfer up to 750 μL of supernatant to a clean 2 mL collection tube.
9. Add 1.2 mL of Solution C4 (a high concentration salt solution) to the supernatant and vortex for 5 s.
10. Load the supernatant spin filter and centrifuge at 10,000 × *g* for 1 min at room temperature. A total of three loads for each sample processed are required.
11. Add 500 μL of Solution C5 (an ethanol-based wash solution) and centrifuge at room temperature for 30 s at 10,000 × *g* . Discard the flow through from the 2 mL collection tube. Centrifuge again at room temperature for 1 min at 10,000 × *g* .
12. Carefully place spin filter in a clean 2 mL collection tube. Avoid splashing any Solution C5 onto the spin filter. Centrifuge at room temperature for 30 s at 10,000 × *g* . Discard the spin filter. The DNA in the tube is now ready for any downstream application.

3.2. PCR Amplification, Gel Electrophoresis, and Purification of PCR Products

1. Perform PCR amplification in a total volume of 50 μL. The PCR set up is laid out on **Table 6.1**. Consider preparing a master mix solution. For instance, for 10 samples, prepare a master mix solution for 11 reactions. Prepare a master mix containing 394.9 μL of dH_2O, 55 μL of 10× PCR

Table 6.1
PCR reaction

Components	Volume	Final concentration
10× PCR buffer	5 μL	1×
25 mM $MgCl_2$	2.5 μL	2.5 mM
10 mM PCR nucleotide mix	1 μL	0.2 mM each
10 pmol forward primer	1 μL	0.2 pmol
10 pmol reverse primer	1 μL	0.2 pmol
5U/μL *Taq* DNA polymerase	0.50 μL	2.5 U
BSA	0.60 μL	1.2%
DMSO	1.50 μL	3%
Environmental DNA (50 ng/μL)	1 μL	
Sterile dH_2O	To 50 μL	

buffer, 25.5 μL of 25 mM $MgCl_2$, 11 μL of 10 mM PCR nucleotide mix, 11 μL of each primer solution, 5.5 μL of *Taq* DNA polymerase (5 U/μL), 6.6 μL BSA, and 16.5 μL DMSO. Mix and aliquot 49 μL of master mix solution in 0.2 mL PCR tubes. Add 1 μL of template DNA (from extraction previously prepared at 50 ng/μL, *see* **Section 3.1**) to each PCR tubes containing the 49 μL of master mix.

2. The above PCR protocol has been optimized in an iCycler Thermal Cycler (Bio-Rad Laboratories, Inc., Hercules, CA). The reaction mixture, after incubation at 94°C for 2 min, is cycled through the following temperature profiles: 94°C for 30 s, 58°C for 1 min, and 72°C for 1 min for 25 cycles. Finally, the reaction mixtures are incubated at 72°C for 7 min.
3. Prior to the PCR product being analyzed, check 5 μL of each amplification mixture by agarose gel (1.0% w/v) electrophoresis in TAE buffer containing 1 μg/mL (w/v) of ethidium bromide.
4. If nonspecific products are observed, then the desired product should be purified by excising the respective part of the gel using a gel purification system. Otherwise, the PCR product is purified using commercial kit such as QIAquick PCR purification kit to remove excess primers.
5. It might be necessary to pool several PCR reactions to obtain enough products for further steps (200–300 ng of DNA recommended per restriction digest). The amplification efficiency of labeled primers tends to be lower than that of unlabeled primers, frequently leading to lower yields. The concentrate DNA and the volume of the pooled PCR reactions can be reduced to half to a fifth of the original volume using a Speedvac or ethanol precipitation.

3.3. Restriction Digestion of the PCR Products

1. Once purified, the PCR products are digested with a restriction enzyme. Restriction enzyme digestion of PCR product will generate products of varying length with respect to sequence diversity. For each digestion, 200–300 ng of purified PCR product (assuming a 50% loss during purification) and 10–20 U of restriction enzyme should be used. The incubation period can vary from 4 to 12 h at 37°C to assure complete digestion (*see* **Note 3**).
2. Restriction enzymes are inactivated by heating to 65°C for 20–25 min.
3. Separate the digested products by capillary electrophoresis on an automated sequencer. Inject 5 μL of digestion product with 0.5 μL of molecular weight standard TAMRA-500.

4. Run the electrophoresis as indicated by the manufacturer of the instrument.

3.4. Desalting of Digested PCR Products

In capillary electrophoresis the injection of DNA samples can be achieved by two methods. First, hydrodynamic injection requires pressure difference over the capillary. Alternatively, electrokinetic injection uses a combination of electrophoresis and electroendosmosis to inject the sample. PRISM 310 and 3100 Genetic Analyzers use the latter. The presence of ions can interfere with the uptake of DNA using electrokinetic injection because of preferential injection of higher charge-to-mass molecules (e.g., Cl$^-$ions). Therefore, it is essential to desalt the inactivated restriction digest with Microcon columns, Qiaquick Nucleotide Removal Kit, or conventional ethanol precipitation. In our case, the restriction products are diluted with water upto 500 μL, before concentration and desalinization on Microcon columns.

3.5. Analysis of T-RFLP Profiles

The profiles generated by T-RFLP can vary in two ways. First, there can be variation in the number and size (base pairs) of T-RFs present in a profile. For example, profiles from day 0 (DO) compost and day 28 (D28) compost are clearly different (**Fig. 6.1**). Secondly, differences can be found in the height (and consequently the area) of any particular peak. This variation can have a major influence in estimates of the biodiversity represented in the numerically rarer members of the community. Such a variation is clearly seen when comparing the heights of peaks in T-RFLP profiles from DO, D5, D12, D21, and D28 manure composts. The height of each peak can provide a measure of relative proportion of each component of a population, although biases caused by preferential annealing of the primer of templates (19) means that the absolute values should be treated with caution.

The second output generated from the analysis program is numerical and consists of a table, which includes the size in base pairs of each of the peaks (calculated by reference to the internal standard) and the height of each peak (relative to the amount of fluorescence detected) (**Table 6.2**). When analyzing any particular profile, a minimum threshold of fluorescence is first defined to exclude background noise. The minimum peak height is normally set at 50–100 fluorescent units (6, 11, 13, 16) (*see* **Note 4**). The calculation of T-RF size after comparison with internal standard can be estimated using the Local Southern size calling algorithm method on the GENESCAN software.

3.6. Computer Programs Used for Exploratory Analysis T-RFLP Microbial Community Fingerprints

The output generated from automatic sequencers is rarely suitable for statistical analysis and for assigning detected T-RFs and their relative abundances to appropriate ribotypes. The process of manually sorting and manipulating profiles into the desired format is tedious, time-consuming, and often influenced human error. The

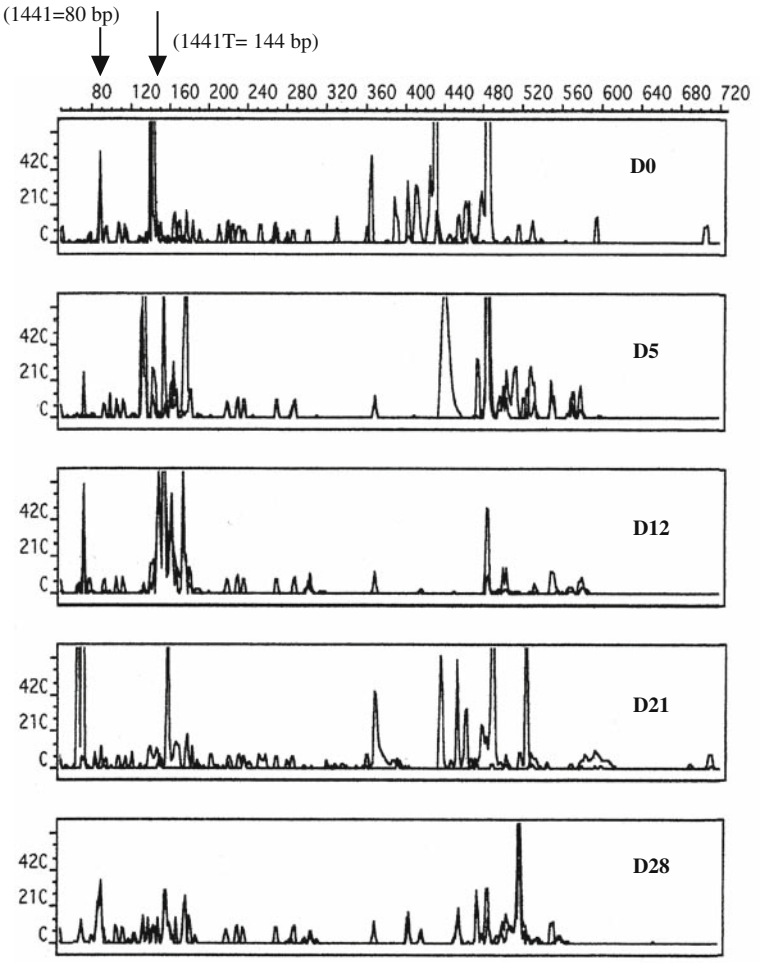


Fig. 6.1. T-RFLP profiles from *Hha* I analysis of 16S rRNA gene PCR products from DNA isolated from manure compost at different stages of composting (D0, day 0; D5, day 5; D12, day 12; D21, day 21; D28, day 28).

following are computer programs that are available for various analyses of T-RFLP data:

1. RIBOSORT is a computer package used for editing of automates T-RFLP data (20). It is designed to eliminate the laborious task of manually classifying community fingerprints in microbial ecology studies. The program automatically assigns detected fragments and their respective relative abundance to appropriate ribotypes.
2. T-RFLP FRAGSORT correlates multiple 16S rRNA gene T-RFLP profiles with corresponding in silico amplification and Digestions of Ribosomal Database Project II Alignments (http://www.oardc.ohio-state.edu/trflpfragsort).

Table 6.2
Example of numerical T-RFLP output of 3′ fragments generated by *Hha* I digestion of 16S rRNA from PCR products directly amplified from D0 (day 0) manure compost

Dye/sample peak[a]	Minutes[b]	Size[c]	Peak height[d]	Peak area[e]	Data point[f]
G, 17	91.68	199.68	167	1659	3438
G, 18	93.23	203.68	127	1658	3496
G, 19	93.60	204.65	431	4517	3510
G, 20	93.97	205.62	335	3747	3524
G, 21	94.29	206.45	198	2390	3536
G, 22	94.69	207.50	108	1259	3551
G, 23	95.17	208.75	162	3086	3569
G, 24	97.47	214.80	156	1584	3655
G, 25	97.81	215.72	553	6019	3668

[a]G indicates green, i.e., 3′ fragments. 17–25 represents the number of each fragment in the profile.
[b]Time in minute of migration of the fragment during electrophoresis before it reaches the detector.
[c]Size of fragments in base pairs.
[d]Peak height is given as fluorescent units.
[e]Peak area is a function of the peak height and the relative spread of each peak.
[f]Each peak is assigned a data point.

3. TAP-TRFLP uses virtual digests of sequence databases to determine predicted fragment lengths to ribosomal DNA (21).
4. TRAMPR matches a database of eukaryotic T-RFLP profiles from multiple restriction digests to environmental samples (22).
5. T-RFLP PROFILE calculates the similarity between profiles (https://rdp8.cme.msu.edu/cgis/trflp.cgi).
6. T-RFLP STATS has been developed for clustering T-RFLP profiles from multiple communities (23).
7. T-ALIGN is a program that compares multiple T-RFLP profiles to identify shared and unique components of microbial communities and also constructs consensus profiles from multiple T-RFLP profiles (24); http://inismor.ucd.ie/~talign/index.html).
8. FRAGMATCH matches a database of eukaryotic T-RFLP profiles from multiple restriction digests to environmental samples (25).

9. TRFCUT uses virtual digests of sequence databases to determine predicted fragment lengths to functional marker genes (26).
10. MiCA T-RFLP Analysis (APLAUS+) compares the data from one or more T-RFLP profiles to the outcomes of in silico analyses of sequences in the database done using the same primers and enzymes (http://mica.ibest.uidaho.edu/pat.php).
11. MiCA: T-RFLP Analysis (PAT+) a phylogenetic assignment tool that enables investigators to quickly find possible phylogenetic assignments based on data from series of restriction enzyme digests (http://mica.ibest.uidaho.edu/pat.php).
12. T-RFLP PROFILE MATRIX has been developed for clustering T-RFLP profiles from multiple communities (https://rdp8.cme.msu.edu/cgis/trflp.cgi).

3.7. Application of Diversity Statistics to Describe Diversity Patterns of T-RFLP Profiles

Diversity of T-RFLP patterns, such as the Shannon index ($\hat{H}$), equitability index (J), richness (d), and evenness (e), can be used as means of evaluating microbial diversity of compost samples using the formula suggested by Atlas and Bartha (27). The T-RFLP-based diversity statistics can be calculated as follows:

$$\text{Shannon index}\,(H) = \frac{C}{N} N \log_{10} N - \sum ni \log_{10} ni,$$

where $C = 2.3$; $N =$ sum of peak areas in a given T-RFLP; $n_i =$ area of T-RF i; and $i =$ number of T-RFs of each T-RFLP pattern. This calculation was derived on Shannon and Weaver's formula based on the

$$\text{Equilitability index}\,(J) = \frac{H}{H_{\max}},$$

where $\hat{H}$ = Shannon-diversity index and $H_{\max}$ theoretical maximal Shannon index for the T-RFLP examined, assuming that each peak represents only one member.

$$\text{Richness}\,(d) = \frac{S-1}{logN},$$

where $S =$ number of T-RFs, $N =$ sum of all peak areas in a given T-RFLP pattern.

$$\text{Evenness}\,(e) = \frac{H}{logS},$$

where $\hat{H} =$ Shannon index, $S =$ total number of T-RFs.

3.8. Application of Multivariate Statistical Analyses to Interpret T-RFLP Data

T-RFLP experiments generate large data sets, and a major challenge in T-RFLP experiments is to extract meaningful information out of the data. Too often, noise and bias from large data sets cloud data analysis and thus hinder our ability to learn about what basic principles control microbial community diversity and composition, microbial processes, and interspecies interactions (28). The management of microbial communities for practical applications such as bioremediation and waste treatment is also impeded by our inability to predict community dynamics and function under different environmental conditions. The most simplistic T-RFLP community analysis approach is to compare the presence or absence of different peaks. Such an approach is valid; however, it lacks the benefits of quantitative analysis. Many different statistical methods have been used for analyzing T-RFLP data, such as principal components analysis (6, 29–30), cluster analysis (6, 9–10, 13), self-organizing maps (28), and multidimensional scaling (31). The combination of these analyses offers a balance between noise elimination and information retention, yielding a powerful and yet easily interpreted method to examine community patterns based on T-RFLP data.

4. Notes

To be useful as a biomarker in composting systems, T-RFLP data need to be highly reproducible and must reflect compost microbial community composition. Like any other method, T-RFLP suffers from its own inherent pitfalls that need to be taken account with. Below are the crucial steps in T-RFLP that require attention.

1. Sample preparation and DNA extraction have the potential to influence T-RFLP fingerprint of microbial community, thus the representative sample size and DNA extraction protocol should be selected and verified carefully to minimize the possibility of later misinterpretation of results. In order to minimize the inherent random bias in compost sample composition, numerous replicate samples should be analyzed or even replicate extractions pooled. Subsequent steps in T-RFLP analysis are highly dependent on the starting DNA purity and extent of shearing. It would be worthwhile to consider a DNA extraction protocol with significantly remove contaminants that co-elute with DNA and reduce the degree of shearing. It is needless to say that these factors should be kept at minimum or at least constant across various samples and replicates.
2. Primer selection and PCR conditions can be improved to increase specificity and reduce bias during PCR. The numbers of ribosomal, housekeeping, and other functional

gene sequences in databases have increased exponentially, thus enabling constant improvement in primer quality through their evaluation using freely available tools such as Amplicon (http://sourceforge.net/projects/amplicon), BLAST http://www.ncbi.nlm.nih.gov/BLAST/ and FunGene (http://flyingcloud.cme.msu.edu/fungene//). Classical PCR optimization of PCR composition and thermal cycling parameters should also not be neglected.

3. The choice of restriction enzyme to be employed should be based on those that cut frequently (i.e., four-base cutters) and produce unique patterns between operational taxonomic units. PCR product digestion with endonucleases should be explored to determine which enzyme results in the highest number of peaks detected, therefore yielding best enzyme for each primer and PCR condition set. The use of multiple enzymes is also suggested in order to obtain better resolution between different communities. By choosing the appropriate number and types of restriction endonucleases, the probability that the resulting T-RF size distributions more accurately reflect the natural diversity of microbial populations within a sampled community is increased. In some instances, enzymatic digestions of PCR products can yield incomplete digests due to various reasons such as template purity, complexity, PCR salt interference, or traces of PCR enhancers and additives. Longer restriction times and higher enzyme concentration in restriction reactions of PCR products from environmental samples (i.e., composts, soils, sludge) are therefore suggested.
4. For the analysis of chromatogram, peaks below 50–100 units of fluorescence are excluded because of their low level of reproducibility. However, differences in DNA loading can also generate slightly different profiles. For this reason, it could be useful to standardize the DNA quantities loaded into the capillary. There is yet, no agreed-upon method for normalizing samples with different amounts of DNA, which would allow easy comparison of profiles with different total amounts of fluorescent label (32). Kaplan et al. (33) present a method for standardizing T-RFLP patterns based on T-RF peak area. The amount of DNA loaded onto a gel or a capillary is estimated as the sum of all T-RF peak areas in a pattern (total peak area). Dunbar et al. (34) propose a method for standardizing T-RF patterns based on peak height. The sum of peak height values is then standardized between samples by proportionally decreasing the height of each peak in the profiles until the sum of peak heights (total fluorescence) for each profile equals the lowest value represented among the samples.

References

1. Golueke, C.G. (1977) *Biological Reclamation of Organic Wastes.* Rodale Press, Emmaus, PA.
2. Ishii, K., Fukui, M., and Takii, S. (2000) Microbial succession during composting process as evaluated by denaturing gradient gel electrophoresis analysis. *J. Appl. Microbiol.* **89**, 768–777.
3. Ryckeboer, J., Mergaert, J., Coosemans, J., Deprins, K., and Swings, J. (2003) Microbiological aspects of biowaste during composting in a monitored compost bin. *J. Appl. Microbiol.* **94**, 127–137.
4. Freidrich, U., Prior, K., Altendorf, K. and Lipski, A (2002) High bacterial diversity of a waste gas-degrading community in an industrial biofilter as shown by 16S rDNA clone library. *Environ. Microbiol.* **4**, 721–734.
5. Tringe, S.G., and Rubin, E.M. (2005) Metagenomics: DNA sequencing of environmental samples. *Nat. Rev. Gen.* **6**, 805–814.
6. Tiquia, S.M. (2005) Microbial community dynamics in manure composts based on 16S and 18S rDNA T-RFLP profiles. *Environ. Technol.* **26**, 1104–1114.
7. Peters, S., Koschinsky, S., Schwieger, F., and Tebbe, C.C. (2000) Succession of microbial communities during hot composting as detected by PCR-single-strand-conformation polymorphism-based genetic profiles of small-subunit rRNA genes. *Appl. Environ. Microbiol.* **66**, 930–936.
8. Klamer, M., and Bååth, E. (1998) Microbial community dynamics during composting of straw material studied using phospholipid fatty acid analysis. *FEMS Microbiol. Ecol.* **27**, 9–20.
9. LaMontagne, M.L., Michel Jr., F.C., Holden, P.A., and Reddy, C.A. (2001) Evaluation of extraction and purification methods for obtaining PCR-amplifiable DNA from compost for community analysis. *J. Microbiol. Method.* **49**, 255–264.
10. Michel Jr., F.C., Marsh, T.L., and Reddy, C.A. (2002) Characterization of Microbial Community structure during composting using analysis of terminal restriction fragment length polymorphisms of 16S rRNA genes, in *Microbiology of Composting* (Insam, H., Riddech, N., and Klammer, S. eds.) Springer Verlag, Heidelberg.
11. Tiquia, S.M., and Michel, F.C., Jr. (2002) Bacterial diversity in livestock manure composts as characterized by terminal restriction fragment length polymorphisms (T-RFLP) of PCR-amplified 16S rRNA gene sequences, in *Microbiology of Composting.* (Insam, H., Riddech, N., and Klammer S., eds.) Springer Verlag, Heidelberg, Germany. pp. 64–82.
12. Tiquia, S.M., Ichida, J.M., Keener, H.M., Elwell, D., Burt, Jr., E., and Michel, Jr., F.C. (2005) Bacterial community structure on feathers during composting as determined by terminal restriction fragment length polymorphism analysis of 16S rDNA genes. *Appl. Microbiol. Biotechnol.* **67**, 412–419.
13. Liu, W.T., Marsh, T.L., Cheng, H., and Formey, L.J. (1997) Characterization of microbial diversity by determining terminal restriction fragment length polymorphism of genes encoding 16S rRNA. *Appl. Environ. Microbiol.* **63**, 4516–4522.
14. Clement, B.G., Kehl, L.E., Debord, K.L., and Kitts, C.L. (1998) Terminal restriction fragment patterns (TRFLPs), a rapid, PCR-based method for the comparison of complex bacterial communities. *J. Microbiol. Method.* **31**, 135–142.
15. Marsh, T.L. (1999) Terminal restriction fragment length polymorphism (T-RFLP): an emerging method for characterizing diversity among homologous populations of amplification products. *Curr. Opin. Microbiol.* **2**, 323–327.
16. Osborn, A.M., Moore, E.R.B., and Timmis, K.N. (2000) An evaluation of terminal restriction fragment length polymorphism (T-RFLP) analysis for the study of microbial community structure and dynamics. *Environ. Microbiol.* **2**, 39–50.
17. von Wintzingerode, F. Goebel, U.B., and Stackerbrandt, E. (1997) Determination of microbial diversity in environmental samples-pitfalls of PCR-based rRNA analysis. *FEMS Microbiol. Rev.* **21**, 213–229.
18. Yang, Z.H., Xiao, Y., Zeng, G.M., Xu, Z.Y., and Liu, Y.S. (2007) Comparison of methods for total community DNA extraction and purification from compost. *Appl. Microbiol. Biotechnol.* **74**, 918–925.
19. Suzuki, M.T., and Giovannoni, S.J. (1996) Bias caused by template annealing in the amplification mixtures of 16S rRNA genes by PCR. *Appl. Environ. Microbiol.* **62**, 625–630.
20. Scallan, U., Liliensiek, A., Clipson, N., and Connolly, J. (2008) RIBOSORT: A program for automated data preparation and exploratory analysis of microbial community fingerprints. *Mol. Ecol. Resources.* **8**, 95–98.
21. Marsh, T.L., Saxman, P., Cole, J., and Tiedje, J. (2000) Terminal restriction

fragment length polymorphism analysis program, a web-based research tool for microbial community analysis. *Appl. Environ. Microbiol.* **66**, 3616–3620.
22. Fitzjohn, R.G., and Dickie, I.A. (2007) Trampr : An R package for analysis and matching of terminal-restriction fragment length polymorphism (TRFLP) profiles. *Mol. Ecol. Notes.* **7**, 583–587.
23. Abdo, Z., Schuette, U.M.E., Bent, S.J., Williams, C.J., Forney, L.J., and Joyce, P. (2006) Statistical methods for characterizing diversity of microbial communities by analysis of terminal restriction fragment length polymorphisms of 16S rRNA genes. *Environ. Microbiol.* **8**, 929–938.
24. Smith, C.J., Danilowicz, B.S., Clear, A.K., Costello, F.J., Wilson, B., and Meiier, W.G. (2005) T-align : a web-based tool for comparison of multiple terminal restriction fragment length polymorphism profiles. *FEMS Microbiol. Ecol.* **54**, 375–380.
25. Saari, T.A., Saari, S.K., Campbell, C.D., Alexander, I.J., and Anderson, I.C. (2007) Fragmatch: a program for the analysis of DNA fragment data. *Mycorrhiza.* **17**, 133–136.
26. Ricke, P., Kolb, S., and Braker, G. (2005) Application of a newly developed arb software-integrated tool for in silico terminal restriction fragment length polymorphism analysis reveals the dominance of a novel *pmo* A cluster in a forest soil. *Appl. Environ. Microbiol.* **71**, 1671–1673.
27. Atlas, R.M., and Bartha, R. (1997) *Microbial ecology: Fundamentals and Applications. Fourth Edition.* Benjamin/Cummings Publishing Company, CA.
28. Dollhopf, S.L., Hashsham, S.A., and Tiedje, J.M. (2001) Interpreting 16S rDNA T-RFLP data: application of self-organizing maps and principal component analysis to describe community dynamics and convergence. *Microb. Ecol.* **42**, 495–505.
29. Wang, M., Ahrne, S., Antonsson, M. and Molin, G. (2004) T-RFLP combined with principal component analysis and 16S rRNA gene sequencing: an effective strategy for comparison of fecal microbiota in infants of different stages. *J. Microbiol. Method.* **59**, 53–69.
30. Pett-Ridge, J., and Firestone, M.K. (2005) Redox fluctuation structures microbial communities in a wet tropical soil. *Appl. Environ. Microbiol.* **71**, 6998–7007.
31. Wolsing, M., and Prieme, A. (2004) Observation of high seasonal variation in community structure of denitrifying bacteria in arable soil receiving artificial fertilizer and cattle manure by determining T-RFLP of *nir* gene fragments. *FEMS Microbiol. Ecol.* **48**, 261–271.
32. Blackwood, C.B., Marsh, T.L., Kim S.H., and Paul, E.A. (2003) Terminal restriction fragment length polymorphism data analysis for quantitative comparison of microbial communities. *Appl. Environ. Microbiol.* **69**, 926–932.
33. Kaplan, C.W., Astaire, J.C., Sanders, M.E., Reddy, B.S., and Kitts, C.L. (2001) 16S ribosomal DNA termination restriction fragment pattern analysis of bacterial communities in feces of rats fed Lactobacillus acidophilus NCFM. *Appl. Environ. Microbiol.* **67**, 1935–1939.
34. Dunbar, J., Ticknor, O., and Kuske, C.R. (2001) Phylogenetic specificity and reproducibility and new method for analysis of terminal restriction fragment profiles of 16S rRNA genes from bacterial communities. *Appl. Environ. Microbiol.* 67, 190–197.

Chapter 7

Combination of Fluorescence In Situ Hybridization with Staining Techniques for Cell Viability and Accumulation of PHA and polyP in Microorganisms in Complex Microbial Systems

Jeppe Lund Nielsen, Caroline Kragelund, and Per Halkjær Nielsen

Abstract

Fluorescence in situ hybridization (FISH) can be combined with a number of staining techniques to reveal the relationships between the microorganisms and their function in complex microbial systems with a single-cell resolution. In this chapter, we have focused on staining methods for intracellular storage compounds (polyhydroxyalkanoates, polyphosphate) and a measure for cell viability, reduction of the tetrazolium-based redox stain CTC. These protocols are optimized for the study of microorganisms in waste-water treatment (activated sludge and biofilms), but they may also be used with minor modifications in many other ecosystems.

Key words: FISH, ecophysiology, polyhydroxyalkanoate, polyphosphate, Neisser, CTC, tetrazolium, respiratory activity, intracellular storage.

1. Introduction

Microorganisms in mixed communities can be visualized, identified and quantified at single-cell level by fluorescence in situ hybridization (FISH) using fluorescently labelled oligonucleotide probes targeting the ribosomal RNA. Importantly, FISH can also be combined with a number of other staining techniques to understand the relationships between the microorganisms and their function while still maintaining a single-cell resolution. During the past 10 years, a range of such methods has been developed

S.P. Cummings (ed.), *Bioremediation*, Methods in Molecular Biology 599,
DOI 10.1007/978-1-60761-439-5_7,

Table 7.1
Overview of methods used for single-cell level ecophysiological characterization in mixed microbial ecosystems. All methods are combined with FISH

Information	Method	References
Uptake of substrates under defined conditions	Microautoradiography (MAR) Quantitative MAR (Q-MAR) Heterotrophic CO_2 assimilation ($HetCO_2$-MAR)	(1) (5) (6)
Uptake of substrates under defined conditions	NanoSIMS	(7, 9)
Uptake of substrates under defined conditions Surface structures	microRaman	(8, 10)
Storage compounds (PHA, lipids)	Sudan Black Nile Blue	(15, 16) (17, 18)
Storage compounds (S-compounds) (Poly-P)	 Thiosulphate Neisser, DAPI	(23) (14)
Cell respiratory activity/viability	Reduction of CTC	(11, 13, 12)
Exoenzyme expression to aid substrate uptake	ELF BODIPY	(30, 31) (32, 33)
Surface properties	Degree of hydrophobicity Presence of proteinaceous amyloidal fibrils	(34, 35, 36) (37, 38)

(**Table 7.1**) and applied in various ecosystems, although primarily related to biological wastewater treatment.

In particular, the combination of FISH with microautoradiography (MAR) has been widely used to reveal important physiological traits of uncultured microorganisms (1,2). This is a powerful technique to study the direct uptake of radiolabelled substrates under defined incubation conditions, enabling the link between physiological capability and identity (1, 3, 4). Variations of the MAR protocol such as quantitative MAR and $HetCO_2$-MAR allow for quantification of substrate uptake and more detailed physiological investigations (5, 6). New combinations along the same lines are NanoSIMS-FISH and microRaman-FISH, which basically offer similar type of information, but with increased resolution and information on the fate of the transformed compounds, and by using non-radioactive substrates (7 – 10).

However, several other FISH combinations do not require expensive and specialized equipment and can answer important questions related to microbial activity in mixed communities. FISH combined with the redox dye CTC (5-cyano-2,3-ditolyl

tetrazolium chloride) has been used to show respiratory activity in individual cells (11, 12) through reduction of CTC by the respiratory electron transport chain into insoluble, fluorescent formazan crystals (CTF), which precipitates inside the cells (11). The method is regarded as a good estimator for cell viability, rather than cell activity. However, CTC does not penetrate all bacteria, e.g. some filamentous *Alphaproteobacteria* (Kragelund, unpublished results), and will, in activated sludge and perhaps also in other ecosystems, underestimate the true number of viable cells (12, 13). However, a positive signal is always considered to indicate a viable cell, so the method can be used to estimate the minimum number of viable cells and to investigate whether probe-defined cells are viable.

The presence of storage compounds such as polyhydroxyalkanoates (PHA) or other lipid storage compounds (14) in cells can be visualized by combining staining (Sudan Black (15, 16) or Nile Blue (17, 18, 19)) with FISH. These storage compounds may be important under feast-famine conditions often encountered in many ecosystems, since the storage material can be utilized as energy and/or reserves of carbon during periods of unbalanced growth due to unfavourable conditions (20). Signal intensities are proportional to the amount of material and can thus provide the basis for a quantitative method at single-cell level through image analysis (17, 18, 19). PHA determined by Nile Blue also stains waxes and fats besides PHA present in cells (21).

Intracellular polyphosphate (polyP) inclusions can be detected by Neisser or DAPI (4′-6-diamidino-2-phenylindole) staining (15, 16, 22). Intracellular elemental sulphur granules can be visualized by phase contrast microscopy (23). DAPI can also be used for the detection of lipid granules. It is possible to distinguish between lipids and polyP, as the signal from lipids fades quickly as opposed to the signal from polyP, which appears strongly yellow. Both stains have successfully been combined with FISH (15, 16, 24), although combinations with DAPI can give high background intensities (16, 24). Lowering the DAPI concentration and staining for longer time can reduce this background signal.

In this chapter, we have concentrated on the methods and protocols that have been optimized and extensively applied for the study of ecophysiology of microorganisms in wastewater treatment (activated sludge and biofilms), but they may also be used with minor modifications in many other ecosystems. We have recently described in detail the MAR-FISH protocol, so these are not included here and can be found elsewhere (4). Very detailed protocols of FISH are available elsewhere (25, 26), but a brief description has been included here. The combinations of the various staining techniques with FISH described in the following methods are best performed by following a standard FISH

procedure, *see* **Section 3.1**. The more sensitive CARD-FISH procedure can generally also be applicable, but will not be described in this chapter. For detailed protocols on CARD-FISH see (27) and Pernthaler.The chapter focuses on the use of FISH combined with staining for respiratory activities, storage products such as PHA and polyphosphate. The combination of FISH with other stains for extracellular enzymes and surface properties can be found in Chapter 8.

1.1. Combining FISH with Other Stainings

When combining FISH with other staining techniques, it must be carefully considered which of the two techniques should be carried out first. The FISH procedure treatment (fixation, dehydration with ethanol, high salt concentrations, heating, rinsing, etc.) will often result in the subsequent staining technique acting on chemically altered microorganisms. On the other hand, performing the staining technique prior to the FISH will often cause bleaching or degeneration of the staining signal during the FISH procedure.

Limitations of traditional bandpass filters to distinguish two or more fluorophores with only slightly different (or even highly overlapping) emission spectra or crosstalk between emissions can be solved by a technique called emission fingerprinting. With this technique, the fluorescent signal is split into its spectral components, which can then be collected by a multichannel detector. This allows a comparison and separation of the spectral emission profiles of each fluorochrome present in the sample, and it is then possible to use dyes in combinations that are normally impossible to separate (28). Equipment for such separation of the fluorescence components is only available for certain microscopes (e.g. LSM 710/510 META, Carl Zeiss), and their use will not be further described in this chapter.

One very effective way to combine fluorescent staining and still be able to overcome the effects of spectral overlapping or signal bleaching through the use of chemicals necessary for FISH is by the application of relocation. An automatic stage controller on the microscope can usually relocate the stored position within an accuracy of 1 μm, and even after physical removal of the slide from the stage. It becomes possible to record the image and its position on the slide after the initial FISH or staining procedure, then remove the slide to perform the second technique followed by relocation and recording of the second image. If the proper control is exerted to avoid overlap of signals, this will provide a powerful approach to perform two or more different staining techniques on the same sample simultaneously. Manual relocation can also be performed, but requires a great deal of patience.

Typically, the combinations of fluorescent staining techniques with FISH consist of the following steps:

- Incubation of biological sample with (fluorescent) substrate (e.g. CTC), staining with the fluorescent dye (e.g. PHA)
- Microscopic evaluation of the obtained results (positions of interest are marked using stage controller)
- FISH procedure (fixation, hybridization and washing)
- Microscopic evaluation of the FISH (after relocation of the point of interest)
- Comparison of fluorescent signals by the staining/substrate and the FISH data by image analysis

2. Materials

2.1. Fluorescence In Situ Hybridization (FISH)

1. Containers for dehydration of slides in 50, 80 and 96% ethanol
2. Slides or cover glass (gelatine or poly-L-lysine coated)
3. 50 mL Falcon tubes
4. Antifading mountant (e.g. CitiFLUOR®, Vectrashield® or mixtures thereof)

2.2. Materials (PHA-FISH)

1. Nile Blue (1% aqueous solution)
2. Acetic acid (8% aqueous solution)
3. Coplin jar
4. Microscope slides (coated with gelatine or poly-L-lysine).

2.3. Materials (Neisser Staining and DAPI Combined with FISH)

1. Solution A: 0.1 g methylene blue, 5 mL 95% ethanol, 5 mL glacial acetic acid, 100 mL dH_2O.
2. Solution B: 3.3 mL crystal violet (10 % w/v in 95% ethanol), 6.7 mL 95% ethanol, 100 mL dH_2O.
3. Solution 1: Mix two parts by volume of solution A with one part of solution B.
4. Solution 2: 33.3 mL bismark brown (1% aqueous), 66.7 mL dH_2O.
5. Microscope slides (gelatine coated, see procedure in **Section 3.1**).
6. Coplin jars.
7. DAPI (4′,6-diamidino-2-phenylindole dihydrochloride) solution (1 μg/L).

2.4. Materials (CTC-FISH)

1. 50 mM CTC aqueous solution (Polyscience Inc.).
2. Rotary shaker.

3. Method

3.1. Procedure for Gelatine Coating of Microscopic Glass Slides

1. Place the slides in a beaker containing preheated (60°C) 1 M HCl solution. Let the slides stand for at least 8 h. After cooling, rinse the slides carefully in dH_2O. Rinse the slides in 95% ethanol and let air-dry.
2. Rinse the slides (either normal glass slides or Teflon-coated slides) in 70% ethanol, let air-dry, dip in 0.5% gelatine solution for 5 min at 70°C.
3. Remove the slides and let them air-dry in vertical position in a dust-free environment.

3.2. Fluorescence In Situ Hybridization (FISH)

1. Immobilized biofilm or activated sludge on gelatine-coated slides or cover glass is fixed by dipping in 4% paraformaldehyde at 4°C (Gram-negative cells) or 50% ethanol (Gram-positive bacteria) for 3 h.
2. Wash by dipping the slide in dH_2O.
3. Let the slide air-dry.
4. Dehydrate for 2 min each in 50, 80 and 96% ethanol.
5. Let the slide air-dry.
6. 2 mL of hybridization buffer with the proper stringency (salt, formamide) for the applied oligonucleotide probe are prepared according to **Table 7.2**. If probes have been optimized to work at different temperature, *see* **Note 1**.

Table 7.2
Hybridization buffer composition

Reagent	Amount	Final Concentration
5 M NaCl	360 μL	0.9 M
1 M Tris–HCl, pH 8.0	40 μL	20 mM
Formamide	0–1600 μL[1]	0–80%[1]
Distilled H_2O	Add to 2 mL	
10% SDS	2 μL	0.01%

[1] Depending on the T_d of the probes used, *see* **Note 3**.

7. 8 μL of hybridization buffer is transferred onto the slide within a small area (prepare one slide at a time to avoid evaporation of hybridization buffer and thus changed stringency).
8. Add 1 μL of each gene probe (probe concentration 50 ng/μL) and mix carefully (avoid contact with the sample) with the hybridization buffer. If more gene probes are added on the same slide, the order is not important

−1 μL of each of the probes is added to the well. Equimolar concentrations of each competitor probe are added if needed. If probes optimized for different formamide concentrations are required on the same slide, they must be applied in a double hybridization (*see* **Note 2**).

9. Place the slide horizontally in a moisture chamber (50 mL Falcon® tube with *ca.* 1.5 mL hybridization and a piece of paper tissue). The slide is hybridized in an oven (46°C) for at least 1.5 h; longer incubations will increase hybridization to less accessible regions of the ribosome (*see* **Note 3**).
10. During hybridization the washing buffer is prepared in a 50 mL polyethylene tube (formamide is replaced by NaCl, according to **Table 7.3**). The washing buffer is preheated in a 48°C water bath.

Table 7.3
Washing buffer composition

Formamide (%)	1 M Tris/HCl pH 8.0 (μL)	10% SDS (μL)	5 M NaCl (μL)	0.5 M EDTA (μL)
0	1000	50	9000	0
5	1000	50	6300	0
10	1000	50	4500	0
15	1000	50	3180	0
20	1000	50	2150	500
25	1000	50	1490	500
30	1000	50	1020	500
35	1000	50	700	500
40	1000	50	460	500
45	1000	50	300	500
50	1000	50	180	500
55	1000	50	100	500

The slides are gently rinsed by pouring a few milliliter of preheated washing buffer over the sample.

11. Each slide is transferred to a 50 mL polyethylene tube with the remaining preheated washing buffer, and incubated for 15 min at 48°C (water bath).
12. The slides are removed from the washing buffer and dipped in ice-cold dH_2O.
13. The slides are dried on the bench. The slides must be completely dry before continuing to the next step. Samples can be stored at −20°C at this point.

14. DAPI is added after FISH to a final concentration of 1 μg/mL where it stains for 15 min in the dark. Rinse with plenty of distilled water, and let the slide air-dry. If DAPI was added prior to FISH, *see* **Note 4**.

3.3. PHA-FISH

1. Dilute freshly collected activated sludge or biofilm (approximately 3 g dry weight/L) 1:4 with dH_2O, and transfer 20 μL to a gelatine-coated microscope slide. If sample homogenization is required, please *see* **Note 5**.
2. Let the slide air-dry.
3. Stain the sample by dipping the slide into a Coplin jar with 55°C warm 1% aqueous solution of Nile Blue for 10 min.
4. Remove the excess stain by carefully rinsing with dH_2O (room temperature).
5. Wash the stained cells for 1 min in 8% acetic acid (room temperature).
6. Remove the excess acetic acid by carefully rinsing with dH_2O (room temperature).
7. Let the slide air-dry.
8. Add a drop of dH_2O to the slide and evaluate it on an Epifluorescence microscope, using the appropriate filters (excitation wavelength 543 nm). A positive PHA staining will show clearly fluorescent PHA granules inside the cell. A positive signal should be clearly visible and easily distinguished from a negative signal using identical settings under the image acquisition.
9. Record any field of interest with a CCD camera or laser scanning microscope and store the coordinates for the field of interest on the microscope stage.
10. Remove the slide from the stage and let the slide air-dry.
11. Fix the sample directly on the slide. Different fixation procedures are followed depending on target of FISH probes applied (*see* **Note 6**).
12. For increased permeability of oligonucleotide probes, *see* **Note 7**.
13. Let the slide air-dry.
14. FISH is carried out as described in **Section 3.1**. For clear differentiation of the fluorescence signals from the PHA staining and FISH, only oligonucleotide probes labelled with a strong fluorochrome clearly distinguishable from the PHA emission spectra (such as the FLUOS, Cy5 or some of the Alexa dyes) should be used.
15. Relocate the co-ordinates where the PHA images were acquired on the microscope stage, and evaluate the FISH

signal. Make sure the amended FISH probes have been hybridized in this field of view. Record the image to be combined with the images of the PHA stain.

It is also possible to carry out the PHA staining on ethanol or PFA fixed samples. In that case the fixed samples are immobilized and stained as described above, except step 11 is excluded.

For quantification of the amount of PHA content inside bacteria, *see* **Note 8**.

3.4. FISH and polyP-Staining

3.4.1. FISH Combined with Neisser Staining (polyP-Staining)

1. Dilute freshly collected activated sludge or biofilm (approximately 3 g dry weight/L) 1:4 with dH_2O and transfer 20 μL to a gelatine-coated microscope slide. If sample homogenization is required, *see* **Note 5**.
2. Let the slide air-dry.
3. Perform FISH according to **Section 3.1**
4. Record any field of interest with a CCD camera or laser scanning microscope and store co-ordinates for the field of interest on the microscope stage.
5. Remove the slide from the stage and wash away the mountant by gently rinsing the slide with 70% ethanol. Let the slide air-dry.
6. Place the slide in solution 1 for 30 s in a Coplin jar. Rinse for 2 s with dH_2O.
7. Place the slide in solution 2 for 60 s in a Coplin jar. Rinse with water and dry carefully with tissue paper.
8. Examine the sample under microscope with 1000× magnification using oil immersions.
9. Relocate the co-ordinates where the FISH images were acquired on the microscope stage and evaluate the Neisser staining. Make sure the amended FISH probes have been hybridized in this field of view by including a specific as well as a general probe (e.g. group specific or the EUB mix probes). Record images to be combined with FISH images.

3.4.2. DAPI Combined with FISH (polyP-Staining)

1. Dilute freshly collected activated sludge or biofilm (approximately 3 g dry weight/L) 1:4 with dH_2O and transfer 20 μL to a gelatine-coated microscope slide. If sample homogenization is required, *see* **Note 5**.
2. Let the slide air-dry.
3. Fix the sample directly on the slide. Different fixation procedures are followed depending on the target of FISH probes applied (*see* **Note 6**). Samples fixed by 4% paraformaldehyde followed by thorough rinsing in dH_2O and air-dried can be stored at this point at −20°C for

several months without significant loss of signal. Avoid ethanol, as this will dissolve the precipitate.

4. For increased permeability of oligonucleotide probes, *see* **Note 7**.
5. Let the slide air-dry.
6. FISH is carried out as described in **Section 3.1**.
7. Add approximately 10 μL of DAPI solution for up to 1 h and incubate in the dark.
8. Wash the slide with dH_2O.
9. Let the slide air-dry.
10. Mount the slide with an antifading agent (e.g. Citi*fluor*) and examine under epifluorescence microscope. It is possible to record both FISH signal and DAPI signal simultaneously.

3.5. CTC-FISH

1. Dilute freshly collected activated sludge or biofilm (approximately 3 g dry weight/L) 1:4 with dH_2O. Transfer 2 mL to an incubation vial.
2. Add 10 μL CTC and incubate for 1 h on a rotary shaker.
3. Transfer 20 μL to a gelatine-coated microscope slide. If sample homogenization is required, Please *see* **Note 5**.
4. Examine the slide while it dries using an inverted fluorescence microscope.
5. Record any field of interest with a CCD camera or the laser scanning microscope and store co-ordinates for the field of interest on the microscope stage.
6. Remove the slide from the stage and let the slide air-dry completely.
7. Fix the sample directly on the slide. Different fixation procedures are followed depending on the target of FISH probes applied (*see* **Note 6**).
8. For increased permeability of oligonucleotide probes, *see* **Note 7**.
9. Let the slide air-dry.
10. FISH is carried out as described in **Section 3.1**.
11. Relocate the co-ordinates where the CTC images were acquired on the microscope stage and evaluate the FISH hybridization. Make sure the amended FISH probes have been hybridized in this field of view by including a specific as well as a general probe (e.g. group-specific or the EUB mix probes). Record images to be combined with CTC images.

4. Notes

1. Hybridizations are typically designed to occur at 46°C, but can be carried out at other temperatures by changing the matching formamide concentration according to the relation: 1% Formamide = 0.65°C.
2. Probes with different T_d (requiring different formamide concentrations) cannot be applied together, and must be applied in a double hybridization with two subsequent hybridizations starting with the highest formamide concentration.
3. Increased hybridization to less accessible regions of the ribosome has been shown to occur upon hybridization for up to 72 h (29).
4. DAPI added prior to the FISH procedure will fade, and combinations of FISH with DAPI staining should thus be performed after FISH.
5. The sample is smeared to cover an area of approximately 2 cm^2 using the side of a pipette tip. The sample can optionally be gently homogenized by a tissue grinder for 45 s prior to the immobilization for increased visibility of individual cells.
6. Fix the sample directly on the slide by adding 500 μL freshly prepared 4% paraformaldehyde (for Gram-negative cells) for 1–2 h or by dipping in a beaker with 50% ethanol (for Gram-positive cells) for at least 3 h.
7. For increased permeability of oligonucleotide probes, slides for Gram-negative cells should be further treated by washing in 50% ethanol for 2–4 h. For permeabilization of certain Gram-positive microorganisms, enzyme treatment must be applied at this point.
8. It is possible to quantify the amount of PHA present inside FISH-positive cells. Quantification of PHA formed inside bacteria (e.g. filamentous bacteria or single cells) after incubation under defined conditions can be measured using image analysis software such as ImageJ, an image-processing package that is available as freeware (http://rsb.info.nih.gov/ij/). After incubation with substrates under defined conditions, the sample should be fixed for FISH and appropriate FISH probes applied. The FISH-positive bacteria of interest are recorded, and the position marked by a stage controller. Afterwards, the sample is stained with Nile Blue and the previous FISH-positive bacteria are relocated and PHA content recorded. For quantification of Nile Blue, a number of images are recorded

(approximately 30 images, depending on standard deviation) and analysed. Segments of individual filaments or microcolonies are selected and analysed by recording the maximum intensity in each perpendicular line to the filament/microcolony image. An average intensity can then be calculated (based on minimum 100 cells) for each incubation. A number of images must be examined for each substrate incubation, and average fluorescence intensity and standard deviations can then be calculated. Statistical evaluations must be applied to find significant PHA formation from substrate addition, compared to the indigenous PHA content in the cells on a relative scale. We have not seen any studies which include a quantification of the absolute amount of PHA using this method, although it potentially is possible with proper standards.

References

1. Lee, N., Nielsen, P. H., Andreasen, K. H., Juretschko, S., Nielsen, J. L., and Schleifer, K. H., Wagner, M. (1999) Combination of fluorescent in situ hybridization and microautoradiography-a new tool for structure-function analyses in microbial ecology. *Appl. Environ. Microbiol.* **65**, 1289–1297.
2. Wagner, M., Nielsen, P. H., Loy, A., Nielsen, J. L., and Daims, H. (2006) Linking microbial community structure with function: fluorescence in situ hybridization-microautoradiography and isotope arrays. *Curr. Opin. Biotechnol.* **17**, 83–91.
3. Ouverney, C. C., and Fuhrman, J. A. (1999) Combined microautoradiography-16S rRNA probe technique for determination of radioisotope uptake by specific microbial cell types in situ. *Appl. Environ. Microbiol.* **65**, 1746–1752.
4. Nielsen, J. L., and Nielsen, P. H. (2005) Advances in microscopy: microautoradiography of single cells. *Methods Enzymol.* **397**, 237–256.
5. Nielsen, J. L., Christensen, D., Kloppenborg, M., and Nielsen, P. H. (2003) Quantification of cell-specific substrate uptake by probe-defined bacteria under in situ conditions by microautoradiography and fluorescence in situ hybridization. *Environ. Microbiol.* **5**, 202–211.
6. Hesselsoe, M., Nielsen, J. L., Roslev, P., and Nielsen, P. H. (2005) Isotope labeling and microautoradiography of active heterotrophic bacteria assimilation of $^{14}CO_2$ ($HetCO_2$-MAR). *Appl. Environ. Microbiol.* **71**, 646–655.
7. Behrens, S., Losekann, T., Pett–Ridge, J., Weber, P. K., Ng, W. O., Stevenson, B. S., et al. (2008) Linking microbial phylogeny to metabolic activity at the single-cell level by using enhanced element labeling-catalyzed reporter deposition fluorescence in situ hybridization (EL-FISH) and NanoSIMS. *Appl. Environ. Microbiol.* **74**, 3143–3150.
8. Huang, W. E., Stoecker, K., Griffiths, R., Newbold, L., Daims, H., Whiteley, A. S., and Wagner, M. (2007) Raman-FISH: combining stable-isotope Raman spectroscopy and fluorescence in situ hybridization for the single cell analysis of identity and function. *Environ. Microbiol.* **9**, 1878–1889.
9. Li, T., Wu, T. D., Mazeas, L., Toffin, L., Guerquin-Kern, J. L., Leblon, G., and Bouchez, T. (2008) Simultaneous analysis of microbial identity and function using NanoSIMS. *Environ. Microbiol.* **10**, 580–588.
10. Neufeld, J. D., and Murrell, J. C. (2007) Witnessing the last supper of uncultivated microbial cells with Raman-FISH. *ISME J.* **1**, 269–270.
11. Nielsen, J. L., Aquino, d. M., and Nielsen, P. H. (2003) Evaluation of the redox dye 5-cyano-2,3-tolyl-tetrazolium chloride for activity studies by simultaneous use of microautoradiography and fluorescence in situ hybridization. *Appl. Environ. Microbiol.* **69**, 641–643.
12. Creach, V., Baudoux, A. C., Bertru, G., and Rouzic, B. L. (2003) Direct estimate of active bacteria: CTC use and limitations. *J. Microbiol. Methods* **52**, 19–28.

13. Rodriguez, G. G., Phipps, D., Ishiguro, K., and Ridgway, H. F. (1992) Use of a fluorescent redox probe for direct visualization of actively respiring bacteria. *Appl. Environ. Microbiol.* **58**, 1801–1808.
14. Eikelboom, D. H. (2000) *Process control of activated sludge plants by microscopic investigation*. IWA Publishing, London.
15. Tsai, C. S., and Liu, W. T. (2002) Phylogenetic and physiological diversity of tetrad-forming organisms in deteriorated biological phosphorus removal systems. *Water Sci. Technol.* **46**, 179–184.
16. Liu, W. T., Nielsen, A. T., Wu, J. H., Tsai, C. S., Matsuo, Y., and Molin, S. (2001) In situ identification of polyphosphate- and polyhydroxyalkanoate-accumulating traits for microbial populations in a biological phosphorus removal process. *Environ. Microbiol.* **3**, 110–122.
17. Kragelund, C., Nielsen, J. L., Thomsen, T. R., and Nielsen, P. H. (2005) Ecophysiology of the filamentous *Alphaproteobacterium Meganema perideroedes* in activated sludge. *FEMS Microbiol. Ecol.* **54**, 111–122.
18. Kragelund, C., Kong, Y., van der Waarde, J., Thelen, K., Eikelboom, D., Tandoi, V., et al. (2006) Ecophysiology of different filamentous *Alphaproteobacteria* species from industrial waste water treatment plants. *Microbiology* **152**, 3003–3012.
19. Kong, Y. H., Xia, Y., Nielsen, J. L., and Nielsen, P. H. (2006) Ecophysiology of a group of uncultured Gammaproteobacterial glycogen-accumulating organisms in full scale EBPR wastewater treatment plants. *Environ. Microbiol.* **8**, 479–489.
20. Dawes, E. A. (1991) Storage polymers in prokaryotes in *Prokaryotic structure and function*. (Mohan, S., Dow, C. and Coles, J. A., ed.), Cambridge University Press, pp. 81–122.
21. Tandoi, V., Majone, M., May, J., and Ramadori, R. (1998) The behaviour of polyphosphate accumulating *Acinetobacter* isolates in an anaerobic-aerobic chemostat. *Water Res.* **32**, 2903–2912.
22. Crocetti, G. R., Hugenholtz, P., Bond, P. L., Schuler, A., Keller, J., Jenkins, D., and Blackall, L. L. (2000) Identification of polyphosphate-accumulating organisms and design of 16S rRNA-directed probes for their detection and quantitation. *Appl. Environ. Microbiol.* **66**, 1175–1182.
23. Nielsen, P. H., de Muro, M. A., and Nielsen, J. L. (2000) Studies on the in situ physiology of *Thiothrix* spp. present in activated sludge. *Environ. Microbiol.* **2**, 389–398.
24. Kawaharasaki, M., Tanaka, H., Kanagawa, T., and Nakamura, K. (1999) In situ identification of polyphosphate-accumulating bacteria in activated sludge by dual staining with rRNA-targeted oligonucleotide probes and 4 ′,6-diamidino-2-phenylindol (DAPI) at a polyphosphate-probing concentration. *Water Res.* **33**, 257–265.
25. Amann, R. I. (1995) In situ identification of micro-organisms by whole cell hybridization with rRNA- targeted nucleic acid probes in *Molecular microbial ecological manual.* (Akkermanns, A. D. L., van Elsas, J. D., de Bruijn, F. J., ed) Kluwer Academic Publications, London, pp. 1–15.
26. Daims, H., Ramsing, N. B., Schleifer, K. -H., and Wagner, M. (2001) Cultivation-independent, semiautomatic determination of absolute bacterial cell numbers in environmental samples by fluorescence in situ hybridization. *Appl. Environ. Microbiol.* **67**, 5810–5818.
27. Pernthaler, A., and Pernthaler, J. (2007) Fluorescence in situ hybridization for the identification of environmental microbes. *Methods Mol. Biol.* **353**, 153–164.
28. Chorvat, D., Jr., Kirchnerova, J., Cagalinec, M., Smolka, J., Mateasik, A., and Chorvatova, A. (2005) Spectral unmixing of flavin autofluorescence components in cardiac myocytes. *Biophys. J.* **89**, L55–L57.
29. Yilmaz, L. S., Okten, H. E., and Noguera, D. R. (2006) Making all parts of the 16S rRNA of *Escherichia coli* accessible in situ to single DNA oligonucleotides. *Appl. Environ. Microbiol.* **72**, 733–744.
30. Van Ommen Kloecke, F., and Geesey, G. G. (1999) Localization and identification of populations of phosphatase-active bacterial cells associated with activated sludge flocs. *Microb. Ecol.* **38**, 201–214.
31. Nielsen, P. H., Roslev, P., Dueholm, T. E., and Nielsen, J. L. (2002) *Microthrix parvicella*, a specialized lipid consumer in anaerobic-aerobic activated sludge plants. *Water Sci. Technol.* **46**, 73–80.
32. Xia, Y., Kong, Y., and Nielsen, P. H. (2007) In situ detection of protein-hydrolysing microorganisms in activated sludge. *FEMS Microbiol. Ecol.* **60**, 156–165.
33. Xia, Y., Kong, Y., Thomsen, T. R., and Nielsen, P. H. (2008) Identification and ecophysiological characterization of epiphytic protein-hydrolyzing *Saprospiraceae* ("*Candidatus* Epiflobacter" spp.) in activated sludge. *Appl. Environ. Microbiol.* **74**, 2229–2238.
34. Zita, A., and Hermansson, M. (1997) Deter-

mination of bacterial cell surface hydrophobicity of single cells in cultures and in wastewater in situ. *FEMS Microbiol. Lett.* **152**, 299–306.
35. Zita, A., and Hermansson, M. (1997) Effects of bacterial cell surface structures and hydrophobicity on attachment to activated sludge flocs. *Appl. Environ. Microbiol.* **63**, 1168–1170.
36. Nielsen, J. L., Mikkelsen, L. H., and Nielsen, P. H. (2001) In situ detection of cell surface hydrophobicity of probe-defined bacteria in activated sludge. *Water Sci. Tech.* **43**, 97–103.
37. Larsen, P., Nielsen, J. L., Otzen, D., and Nielsen, P. H. (2008) Amyloid-like adhesins produced by floc–forming and filamentous bacteria in activated sludge. *Appl. Environ. Microbiol.* **74**, 1517–1526.
38. Larsen, P., Nielsen, J. L., Dueholm, M. S., Wetzel, R., Otzen, D., and Nielsen, P. H. (2007) Amyloid adhesins are abundant in natural biofilms. *Environ. Microbiol.* **9**, 3077–3090.

Chapter 8

Ecophysiological Analysis of Microorganisms in Complex Microbial Systems by Combination of Fluorescence In Situ Hybridization with Extracellular Staining Techniques

Jeppe Lund Nielsen, Caroline Kragelund, and Per Halkjær Nielsen

Abstract

Ecophysiological analysis and functions of single cells in complex microbial systems can be examined by simple combinations of Fluorescence in situ hybridization (FISH) for identification with various staining techniques targeting functional phenotypes. In this chapter, we describe methods and protocols optimized for the study of extracellular enzymes, surface hydrophobicity and specific surface structures. Although primarily applied to the study of microbes in wastewater treatment (activated sludge and biofilms), the methods may also be used with minor modifications in several other ecosystems.

Key words: FISH, ecophysiology, extracellular enzymatic activity, enzyme-labelled fluorescence, BODIPY, microsphere adhesion to cells, antibodies, cell surface hydrophobicity.

1. Introduction

Microorganisms in mixed communities can be visualized, identified and quantified at single-cell level by fluorescence in situ hybridization (FISH) using fluorescently labelled oligonucleotide probes targeting the ribosomal RNA. Importantly, FISH can also be combined with a number of other staining techniques to understand the relationships between the microorganisms and their function while still maintaining a single-cell resolution.

Enzyme-labelled Fluorescence combined with FISH (ELF-FISH) is a powerful technique for identifying cells with certain types of exoenzymatic activity, such as lipase (1, 2) or phosphatase activity (3, 4). The mechanism behind the ELF approach lies within a substrate linked to a fluorescent and

S.P. Cummings (ed.), *Bioremediation*, Methods in Molecular Biology 599,
DOI 10.1007/978-1-60761-439-5_8,

a quenching molecule. Upon degradation of the substrate, the quenching will cease and the fluorescent substrate or degradation products, thereof, will then precipitate near the site of action. Six ELF-labelled substrates are commercially available (for enzymatic detection of phosphatase, chitinase, β-glucuronidase, β-galactosidase, esterase and lipase activities).

BODIPY-FISH is also a powerful technique for the detection of certain types of exoenzymatic activity, such as protease activity, exemplified by BODIPY labelled casein or bovine serum albumin (BSA) (5, 6). Other BODIPY substrates exist, for example, BODIPY starch and BODIPY cellulose. The mechanism of the BODIPY technique is based on a heavy labelling of the substrate (exemplified by casein in the described method below), but due to the folding of the protein the conjugate fluorescence is almost totally quenched. Upon protease activity, the protein is cleaved and the quenching decreases. A part, but not all of the cleaved peptides, will subsequently precipitate near the protease site.

Cell surface structures and properties can be visualized by various methods. Bacterial cell surface hydrophobicity is an important factor that influences bacterial adhesion to surfaces (e.g. (7 – 9)). The surface hydrophobicity can be qualitatively detected by microsphere adhesion to cells (MAC) analysis, which is based on microscopic enumeration fluorescent microspheres with a hydrophobic surface attaching to the bacterial surface (2, 7 – 9). Combinations of MAC with FISH will thus enable identification of the cells producing hydrophobic cell surfaces (4, 10).

Presence of specific components on the cell surface, such as proteinaceous amyloidal fibrils, can be detected with appropriate antibodies (11, 12). By applying a range of oligonucleotide probes targeting different phylogenetic groups, it is possible after antibody labelling to identify the antibody-positive cells. Unspecific binding of the antibody can be minimized by blocking of unspecific sites with, for example, gelatine. The protocol has been tested with conformational antibodies for detection of amyloidic fibrils, but should be applicable with most types of antibodies (11, 12).

In this chapter, we have concentrated on the methods and protocols that have been optimized and extensively applied for the study of ecophysiology of microorganisms in wastewater treatment (activated sludge and biofilms), but they may also be used with minor modifications in many other ecosystems. We have focused on the use of FISH combined with staining for exoenzymes (ELF, BODIPY), and surface properties and components (MAC and antibodies). The use of FISH combined with stains for respiratory activities and storage products such as PHA and polyphosphate can be found in **Chapter 7**.

1.1. Combining FISH with Other Stainings

When combining FISH with other staining techniques, it must be carefully considered which of the two techniques should be carried out first. The FISH procedure treatment (fixation, dehydration with ethanol, high salt concentrations, heating, rinsing, etc.) will chemically alter the microorganisms and thereby change the conditions for the subsequent staining technique. On the other hand, performing the staining technique prior to the FISH will often cause bleaching or degeneration of the staining signal during the FISH procedure.

Limitations of traditional bandpass filters to distinguish two or more fluorophores with only slightly different (or even highly overlapping) emission spectra or crosstalk between emissions can be solved by a technique called emission fingerprinting. With this technique, the fluorescent signal is split into its spectral components, which can then be collected by a multichannel detector. This allows a comparison and separation of the spectral emission profiles of each fluorochrome present in the sample, and it is then possible to use dyes in combinations that are normally impossible to separate (13). Equipment for such separation of the fluorescence components is only available for certain microscopes (e.g. LSM 710/510 META, Carl Zeiss), and their use will not be further described in this chapter.

One very effective way to combine fluorescent staining and still be able to overcome the effects of spectral overlapping or signal bleaching through the use of chemicals necessary for FISH is by the application of relocation. An automatic stage controller on the microscope can usually relocate the stored position with a precision of 1 μm, even after physical removal of the slide from the stage. It becomes possible to generate two images by recording the position after the initial FISH or staining and then removing the slide to perform the second technique followed by relocating and recording the second image. If the proper control is exerted to avoid overlap of signals, this will provide a powerful approach to perform two or more different staining techniques on the same sample simultaneously. Manual relocation can also be performed, but requires a great deal of patience.

Typically, the combinations of fluorescent staining techniques with FISH consist of the following steps:

- Incubation of biological sample with (fluorescent) substrate/marker
- Microscopic evaluation of the result obtained (positions of interest are marked using stage controller)
- FISH procedure (fixation, hybridization and washing)
- Microscopic evaluation of the FISH (after relocation of the point of interest)

- Comparison of fluorescent signals by the staining/substrate/marker and the FISH data by image analysis

2. Materials

2.1. Fluorescence In Situ Hybridization (FISH)

1. Containers for dehydration of slides in 50%, 80% and 96% ethanol
2. Slides or cover glass (gelatine or poly-L-lysine coated)
3. 50 mL Falcon tubes
4. Antifading mountant (e.g. CitiFLUOR®, Vectrashield® or mixtures hereof)

2.2. ELF-FISH

1. ELF®97-labelled substrate (esterase, lipase, chitinase, galactosidase, glucuronidase and phosphatase) or ELF®97 phosphatase substrate (5 mM 2-(5′-chloro-2-phosphoryloxyphenyl)-6-chloro-4(3*H*)-quinazolinone) containing 2 mM sodium azide (Molecular Probes, Eugene, OR, USA)
2. Detection buffer (composition not available from supplier) (Molecular Probes, Eugene, OR, USA)
3. Microscope slides (gelatine-coated slide, see below)
4. Epifluorescence microscope (or laser scanning microscope)

2.3. BODIPY-FISH

1. BODIPY FL casein (or similar BODIPY FL substrate) (EnzChek® Protease Assay Kit; Molecular Probes)
2. Distilled water (dH_2O)
3. Tris buffer (10 mM Tris/HCl, pH 7.8)
4. Epifluorescence or laser scanning microscope with filters matching the emission and excitation wavelengths of the BODIPY fluorochrome
5. Microscope slides (gelatine-coated, see procedure above)
6. Aluminium paper
7. Antifading mountant (e.g. CitiFLUOR, Citifluor Ltd., London, UK)
8. Paraformaldehyde (4%) (in phosphate-buffered saline, pH 7.0)
9. Inhibitors: 50 mM sodium fluoroacetate (Sigma-Aldrich Logistik, Gmbh, Germany), 100 mM sodium azide (Sigma-Aldrich Logistik, Gmbh, Germany), 100 mM sodium iodoacetate (Merck KGaA, Darmstadt, Germany).

2.4. MAC-FISH

1. Fluorescent polystyrene microspheres, FluoSpheres (diameter 0.1 or 0.01 μm, with a modified surface (sulphate-modified for hydrophobic, or carboxylate-modified for hydrophilic) (Molecular Probes, Eugene, OR, USA). We recommend yellow green (505/515) sulphate-modified beads with a diameter of 0.01 μm.
2. Sonicator
3. Inverted epifluorescence microscope

2.5. Antibodies (Ab)-FISH

1. Tissue grinder (Thomas Scientifics®, USA)
2. Phosphate-buffered saline (PBS)
3. 2% gelatine
4. 10% Tween 20
5. 10% Triton X-100

3. Method

3.1. Procedure for Gelatine Coating of Microscopic Glass Slides

1. Place the slides in a beaker containing preheated (60°C) 1 M HCl solution. Let the slides stand for at least 8 h. After cooling, rinse the slides carefully in dH_2O. Rinse the slides in 95% ethanol and let air-dry.
2. Rinse the slides (either normal glass slides or Teflon-coated slides) in 70% ethanol, let air-dry, dip in 0.5% gelatine solution for 5 min at 70°C.
3. Remove the slides and let them air-dry in vertical position in a dust-free environment.

3.2. Fluorescence In Situ Hybridization (FISH)

Please *see* **Chapter 7** for details.

3.3. ELF-FISH

1. 100 μL of fresh biomass (suspension of activated sludge/biofilm) is mixed with 0.5 μL 5 mM ELF®97 phosphatase substrate (or ELF®97acetate, ELF®97 β-D -glucuronide or ELF®97 NAG), for all other exoenzyme 2μl 10mM is used (*see* **Note 1** for the corresponding exoenzyme).
2. Prepare a negative control with pasteurized biomass (10 min at 80°C, performed just prior to the incubation, to avoid activity from sporulated organisms).
3. Incubate in the dark at room temperature for at least 60 min (incubation may be extended for several hours depending on the activity in sample).

4. The phosphatase reaction can be stopped by the reaction by addition of 100 μL PBS including 25 mM EDTA and 5 mM levamisol (pH 8.0) (optional).
5. Transfer 15 μL of the reaction mixture onto a gelatine-coated microscope slide, and smear the sample using the side of the pipette tip. Let the slide air-dry.
6. Add a drop of dH_2O and evaluate the slide on an epi-fluorescence microscope using the proper filters (Ex/Em of ELF product: 345/530 nm, and can thus be visualized by a normal DAPI long-pass filter). A positive ELF-signal viewed with a LP-DAPI filter should emit an intensely fluorescent yellow-green precipitate at the site of enzymatic activity, while soluble ELF®substrates fluoresce only weakly in the blue range.
7. Record any field of interest with a CCD camera or laser scanning microscope and store the co-ordinates for the field of interest on the microscope stage.
8. Remove the slide from the stage, and let the slide air-dry.
9. Fix the sample directly on the slide. Different fixation protocols are followed depending on the target of FISH probes applied (*see* **Note 2**). Samples are fixed by 4% paraformaldehyde followed by thorough rinsing in dH_2O and air-dried, and can be stored at this point at −20°C for several months without significant loss of signal. Avoid ethanol as this will dissolve the precipitate.
10. For increased permeability of oligonucleotide probes, *see* **Note 3**.
11. Let the slide air-dry.
12. FISH is carried out as described in **Chapter 7**, **Section 3.1.** For clear differentiation of the fluorescence signals from the ELF and FISH, oligonucleotide probes labelled with the cyanine dye Cy3 (or similar) should be used.
13. Relocate the same position where the ELF images were taken on the microscope stage, and evaluate the FISH signal. Make sure the amended FISH probes have been hybridized in this field of view. Record images to be combined with the images of the ELF stain.
14. Compare the fluorescence signal of the positive signal on the sample with the negative control.

The protocol for lipase and β-D-galactosidase vary slightly from the protocol described above due to the substrates' low solubility in water. Thus, before the incubation, the biomass sample (e.g. activated sludge) must be dissolved in DMF and methanol.

The alterations for these two substrates to the protocol above are:

1. 100 μL activated sludge is centrifuged (4500×*g* for 8 min, room temperature).

2. The pellet is resuspended in 100 μL DMF and 2 μL 10 mM ELF®97 palmitate is added (for the lipase assay), or it is resuspended in 100 μL 10 mM methanol with 2 μL 10 mM ELF®97 β-D-galactopyranoside (for the β-D-galactosidase assay).
3. Mix carefully.
4. Incubate in the dark for 60 min or more at room temperature.
5. Continue with step 5 in the protocol above for the ELF®97 phosphatase assay.

3.4. BODIPY-FISH

1. 400 μL freshly collected activated sludge (approximately 3 g/L dry weight) are centrifuged (4500 × *g* for 8 min, room temperature).
2. The volume of supernatant is replaced by Tris/HCl buffer (10 mM, pH 7.8) and the pellet resuspended.
3. Inhibitors can be added at this point if necessary. Add 2 mM iodoacetate, 2 mM azide and 1 mM fluoracetate to block certain types of metabolism to prevent soluble fluorescent substrate to be taken up by the cells.
4. BODIPY FL casein is added to the sludge (final amount 10–50 mg/g dry weight), depending on the characteristics of the sludge. Final volume ca. 600 μL. Mix carefully.
5. The incubation bottle must be wrapped in aluminium paper to exclude light.
6. Incubate the bottle on a rotary shaker (150–200 rpm at room temperature) for 1 h.
7. Transfer 15 μL of BODIPY incubated sludge to a microscope slide, and smear the sample using the side of the pipette tip, and let it air-dry at room temperature in the dark.
8. Add a drop of dH_2O and evaluate the slide on an epifluorescence microscope using the appropriate filters. A positive green BODIPY signal is distinguished from a negative signal by comparison of signals using identical settings under the image acquisitions.
9. Record any field of interest with a CCD camera or laser scanning microscope and store the co-ordinates for the field of interest on the microscopic stage.
10. Remove the slide from the stage, and let the slide air-dry.
11. Fix the sample directly on the slide, *see* **Note 2**.
12. For increased permeability of oligonucleotide probes, *see* **Note 3**.
13. Let the slide air-dry.

14. FISH is carried out as described in **Chapter 7**, **Section 3.1**. For clear differentiation of the fluorescence signals from BODIPY and FISH, only oligonucleotide probes labelled with the cyanine dye Cy3 should be used.
15. Relocate the same position where the BODIPY images were taken on the microscope stage, and evaluate the FISH signal. Make sure the amended FISH probes have been hybridized in this field of view. Record images to be combined with the images of the BODIPY stain.

3.5. MAC-FISH

1. 10 μL of fluorescent microspheres are diluted in 90 μL dH_2O in an Eppendorf tube.
2. The spheres are sonicated for 100 min in a sonicator (60 W). After sonication the spheres should be used within 2 h to avoid aggregation.
3. 10 μL of fresh sludge is diluted by the addition of 100 μL of sterile filtered effluent water from the same wastewater treatment plant, and then 10 μL of microspheres are added.
4. Vortex the mixture for 2 min (set at 1400 rpm).
5. Transfer 3 μL of the mixture to a gelatine-coated microscope slide. Examine the sample as it dries using an inverted microscope with the proper filters (depending on the chosen fluorophores). The more fluorescent microspheres attached to a cell surface, the more hydrophic the cell is compared to other cells in near vicinity. Fluorescent spheres emit a strong easily recognizable fluorescent signal. The sample should not dry out as this would cause free spheres to be adsorbed to all surfaces (*see* **Note 6**).
6. Record any field of interest with a CCD camera or laser scanning microscope and store the co-ordinates for the field of interest on the microscope stage.
7. Remove the slide from the stage, and let the slide air-dry.
8. Fix the sample directly on the slide, *see* **Note 2**.
9. For increased permeability of oligonucleotide probes, *see* **Note 3**.
10. Let the slide air-dry.
11. FISH is carried out as described **Chapter 7**, **Section 3.1.** For clear differentiation of the fluorescence signals from the microspheres and FISH, only oligonucleotide probes labelled with strong fluorophores like the cyanine dye Cy3 should be used when combined with green fluorescent microspheres.

3.6. Ab-FISH

1. 1 mL freshly collected activated sludge/biofilm (approximately 3 g/L dry weight) is diluted 1:4 with dH_2O.
2. The sample is gently homogenized with a tissue grinder for 45 s (optional).
3. Two 80 μL aliquots are transferred to two 1.5 mL Eppendorf tubes (the sample and a control).
4. The tubes are centrifuged (12,000 × *g* for 4 min).
5. Discard the supernatant.
6. Resuspend the pellet in a mixture of 40 μL of PBS and 40 μL of 2% gelatine.
7. Fix the cells by addition of 1 μL 4% Na-azide to each tube.
8. Incubate the mixtures at 37°C for 1 h.
9. Dilute the primary antibody stock in PBS (depending on the binding characteristics and concentration of the antibody in PBS; should be determined empirically for each sample and Ab).
10. Add 1.5 μL of this primary antibody working solution to the sample (but not to the control).
11. Add Tween 20 to both tubes to a final concentration of 0.6 mg/L.
12. Incubate the mixture at 37°C for 2 h.
13. Centrifuge the sample (13,000 × *g* for 4 min, room temperature) and discard the supernatant.
14. Wash the pellet once with 83 μL mixture of PBS/gelatin/Triton X 100 (41 μL of PBS, 41 μL 2% gelatine and 0.82 μL of 10% Triton X).
15. Centrifuge the sample (13,000 × *g* for 4 min, room temperature) and discard the supernatant.
16. Add 40 μL of 2% gelatine and 40 μL secondary antibody working solution (e.g. Goat-anti-mouse); proper dilution should be optimized for each sample.
17. Add 0.2 μL of 10% Tween 20.
18. Incubate the mixture at 37°C for 1 h.
19. Wash three times with PBS containing 0.1%Trition X 100 (83.5 μL of PBS + 0.84 μL of 10% Triton X).
20. Resuspend the pellet in 84 μL PBS.
21. Transfer 15 μL of antibody incubated sludge to a microscopic slide, and smear the sample using the side of the pipette tip, and let it air-dry at room temperature in the dark.
22. Let the slide dry and evaluate it on an epifluorescence microscope using the appropriate filters. A positive anti-

body signal is distinguished from a negative signal by comparison of signals using identical settings under the image acquisitions.

23. Record any field of interest with a CCD camera or laser scanning microscope and store the coordinates for the field of interest on the microscope stage.
24. Remove the slide from the stage, and let the slide air-dry.
25. Fix the sample directly on the slide, *see* **Note 2**.
26. For increased permeability of oligonucleotide probes, *see* **Note 3**.
27. Let the slide air-dry.
28. FISH is carried out as described in **Chapter 7**, **Section 3.1**. For clear differentiation of the fluorescence signals from the antibody and FISH, only oligonucleotide probes labelled with the cyanine dye Cy3 should be used when combined with green fluorescent labelled secondary antibodies.
29. Relocate the same position as the antibody images were taken on the microscope stage, and evaluate the FISH signal. Make sure the amended FISH probes have been hybridized in this field of view. Record images to be combined with the images of the Ab-stain.

4. Notes

1. Several ELF-labelled substrates are available for detection of different exoenzymatic activities. ELF®97-2-(5′-chloro-2-phosphoryloxyphenyl)-6-chloro-4(3*H*)-quinazolinone) is used for the detection of endogenous phosphatase activity. ELF®97 palmitate is used for the detection of lipase activity, ELF®97acetate as substrate for esterase activity, ELF®97 β-D-galactopyranoside as substrate for β-D-galactosidase, ELF®97 β-D-glucuronide as substrate for β-D-glucuronidase, ELF®97 *N*-acetylglucosaminide (NAG) for chitinase/ *N*-acetylglucosaminidase activity.
2. Fix the sample directly on the slide by adding 500 μL freshly prepared 4% paraformaldehyde (for Gram-negative cells) for 1–2 h, or by dipping in a beaker with 50% ethanol (for Gram-positive cells) for at least 3 h.
3. For increased permeability of oligonucleotide probes, slides for Gram-negative cells should be further treated by washing in 50% ethanol for 2–4 h. Enzyme treatment must be applied at this point for permeabilization of primarily Gram-positive microorganisms.

4. Several BODIPY substrates are available with various fluorescence markers, but in our experience the pH-insensitive green fluorescent BODIPY FL is superior for combinations with FISH. The following protocol describes in detail how BODIPY-FISH should be carried out to obtain optimal and reproducible results.
5. The degree of hydrophobicity is arbitrary, and can only be used for comparison between different cells within the same sample. Even comparison between samples or between different experiments should be taken with caution. The hydrophobicity can be measured as the number of spheres per cell or by intensity per area. An example of interpretation could look like this: very hydrophilic (no spheres), moderate hydrophilic (few spheres), intermediate (~6 spheres per cell), hydrophobic (~20 spheres per cell), very hydrophobic (cells covered by spheres) (9).
6. Spheres not bound to any surfaces will, during the drying phase, accumulate on any surfaces and thus cause false-positive MAC signals to occur. Thus great care should be taken during recording of the MAC signal. The best way to avoid this problem is by transferring the MAC-incubated sample to a slide directly on an inverted microscope, and then record the images *during* immobilization of the sample.

References

1. Nielsen, P. H., Roslev, P., Dueholm, T. E., and Nielsen, J. L. (2002) *Microthrix parvicella*, a specialized lipid consumer in anaerobic-aerobic activated sludge plants. *Water Sci. Technol.* **46**, 73–80.
2. Kragelund, C., Remesova, Z., Nielsen, J. L., Thomsen, T. R., Eales K.l., Seviour, R. J., et al. (2007) Ecophysiology of mycolic acid containing *Actinobacteria* (Mycolata) in activated sludge foams. *FEMS Microbiol. Ecol.* **61**, 174–184.
3. Van Ommen Kloecke, F., and Geesey, G. G. (1999) Localization and identification of populations of phosphatase-active bacterial cells associated with activated sludge flocs. *Microb. Ecol.* **38**, 201–214.
4. Kragelund, C., Levantesi, C., Borger, A., Thelen, K., Eikelboom, D., Tandoi, V., et al. (2008) Identity, abundance and ecophysiology of filamentous bacteria belonging to the *Bacteroidetes* present in activated sludge plants. *Microbiology* **154**, 886–894.
5. Xia, Y., Kong, Y., and Nielsen, P. H. (2007) In situ detection of protein-hydrolysing microorganisms in activated sludge. *FEMS Microbiol. Ecol.* **60**, 156–165.
6. Xia, Y., Kong, Y., Thomsen, T. R., and Nielsen, P. H. (2008) Identification and ecophysiological characterization of epiphytic protein-hydrolyzing saprospiraceae ("*Candidatus* Epiflobacter" spp.) in activated sludge. *Appl. Environ. Microbiol.* **74**, 2229–2238.
7. Zita, A., and Hermansson, M. (1997) Determination of bacterial cell surface hydrophobicity of single cells in cultures and in wastewater in situ. *FEMS Microbiol. Lett.152,299–306.*
8. Zita, A., and Hermansson, M. (1997) Effects of bacterial cell surface structures and hydrophobicity on attachment to activated sludge flocs. *Appl. Environ. Microbiol.* **63**, 1168–1170.
9. Nielsen, J. L., Mikkelsen, L. H., and Nielsen, P. H. (2001) In situ detection of cell surface hydrophobicity of probe-defined bacteria in activated sludge. *Water Sci. Tech.* **43**, 97–103.

10. Kragelund, C., Nielsen, J. L., Thomsen, T. R., and Nielsen, P. H. (2005) Ecophysiology of the filamentous *Alphaproteobacterium Meganema perideroedes* in activated sludge. *FEMS Microbiol. Ecol.* **54**, 111–122.
11. Larsen, P., Nielsen, J. L., Otzen, D., and Nielsen, P. H. (2008) Amyloid-like adhesins produced by floc-forming and filamentous bacteria in activated sludge. *Appl. Environ. Microbiol.* **74**, 1517–1526.
12. Larsen, P., Nielsen, J. L., Dueholm, M. S., Wetzel, R., Otzen, D., and Nielsen, P. H. (2007) Amyloid adhesins are abundant in natural biofilms. *Environ. Microbiol.* **9**, 3077–3090.
13. Chorvat, D., Jr., Kirchnerova, J., Cagalinec, M., Smolka, J., Mateasik, A., and Chorvatova, A. (2005) Spectral unmixing of flavin autofluorescence components in cardiac myocytes. *Biophys. J.* **89**, L55–L57.

Chapter 9

Applications of DNA-Stable Isotope Probing in Bioremediation Studies

Yin Chen, Jyotsna Vohra, and J. Colin Murrell

Abstract

DNA-stable isotope probing, a method to identify active microorganisms without the prerequisite of cultivation, has been widely applied in the study of microorganisms involved in the degradation of environmental pollutants. Recent advances and technique considerations in applying DNA-SIP in bioremediation are highlighted. A detailed protocol of a DNA-SIP experiment is provided.

Key words: DNA, stable isotope probing, bioremediation.

1. Introduction

It has been widely recognised that the environment has been polluted by a number of synthetic compounds in the past century, especially after industrialisation. The past few decades have witnessed an increasing interest in bioremediation where living organisms (mainly microorganisms) are used to break down these pollutants in the environment to environmental-friendly compounds. To evaluate the role of microorganisms in the metabolism of certain pollutants in the environment, many techniques need to be employed, including biological and analytical methods, of which stable isotope probing (SIP) is of particular interest (**Table 9.1**).

Stable isotope probing (SIP) relies on the incorporation of stable isotopes (^{13}C, ^{15}N) into DNA (1), RNA (2) or phospholipid fatty acid (PLFA) (3–4). Subsequent separation and identification of labelled DNA, RNA or PLFAs offer

S.P. Cummings (ed.), *Bioremediation*, Methods in Molecular Biology 599,
DOI 10.1007/978-1-60761-439-5_9,

Table 9.1
Recent studies using DNA/RNA-SIP for identifying active microorganisms for bioremediation

Substrate	Habitat	Phylogenetic groups identified	Marker genes	Reference
^{13}C-pyrene	PAH-contaminated soil	Uncultivated γ-*Proteobacteria*	16S rRNA	(27)
$^{13}C_6$-benzene	Coal gasification soil	*Deltaproteobacteria*; *Clostridia*; *Actinobacteria*	16S rRNA	(28)
^{13}C-acetate + perchloroethene	Pristine river sediment	*Dehalococcoides*	16S rRNA	(29)
^{13}-polychlorinated biphenyls	Pine tree soil	*Pseudonocardia*; *Kribbella*; *Nocardioides*; *Sphingomonas*	16S rRNA; ARHDs[1]	(20)
^{13}C-phenanthrene,^{13}C-pyrene	PAH-contaminated soil	*Acidovorax*	16S rRNA	(30)
^{13}C-labelled 2,4-dichlorophenoxyacetic acid	Agriculture soil	β-*Proteobacteria* related to *Ramlibacter* (*Comamonadaceae*)	16S rRNA	(31)
^{13}C-pyrene	Bioreactor-treated soil	*Sphingomonas*; uncultivated β- and γ-*Proteobacteria*	16S rRNA	(32)
$^{13}C_6$-benzene	Gasoline-contaminated groundwater	*Azoarcus*	16S rRNA	(33)
$^{12}C_6$ salicylate; ^{13}C naphthalene phenanthrene	Bioreactor treating PAH-contaminated soil	*Acidovorax.*; *Pseudomonas*; *Ralstonia*	16S rRNA	(34)
^{13}C-labelled naphthalene and glucose	Soil	*Acidovorax*; *Pseudomonas*; *Intrasporangium*	16S rRNA	(35)

(continued)

Table 9.1
(Continued)

Substrate	Habitat	Phylogenetic groups identified	Marker genes	Reference
$^{13}C_7$-benzoate	Marine sediment or contaminated sediment	–	*nosZ*	(36)
^{13}C-phenol	Activated sludge	*Acidovorax*	16S rRNA	(37)
^{13}C-pentachlorophenol	Pristine grassland soil	*Pseudomonas*; *Burkholderia*; *Sphingomonas*	16S rRNA	(38)
^{13}C-phenol	Agriculture soil	*Kocuria*; *Staphylococcus*; *Pseudomonas*	16S rRNA	(39)
$^{13}CH_3Cl$	Soil	*Hyphomicrobium*; *Aminobacter*	*cmuA*	(40)
$^{13}CH_3Br$	Soil	*Burkholderia*	16S rRNA; *cmuA*	(19)
$^{13}C_6$-benzene; $^{13}C_7$-toluene	Garden soil	*Arthrobacter*; *Hydrogenophaga*; *Pseudomonas*; *Rhodococcus*	16S rRNA; *bphA*; *todC*	Vohra and Murrell; unpublished data
^{13}C-benzoic acid	Agriculture soil	*Burkholderia*	16S rRNA	(41)

[1]Aromatic ring hydroxylating dioxygenase
– Not available

culture-independent insight into microorganisms involved in the uptake of particular labelled compounds, therefore linking the phylogeny of microorganisms to their functions. The application of SIP in bioremediation has been discussed in several reviews (5–9). Here we describe a detailed protocol for DNA-based stable isotope probing.

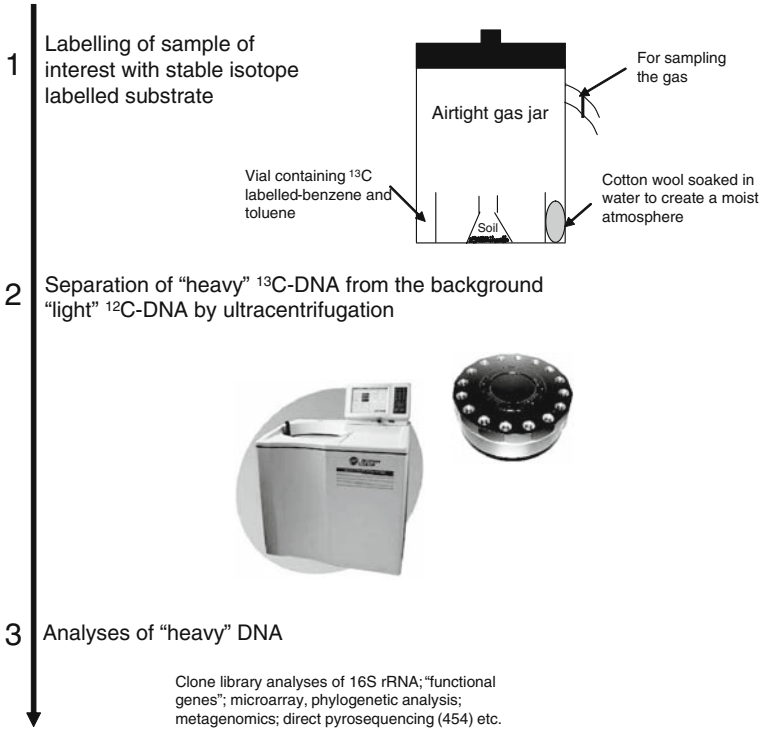


Fig. 9.1 Flow chart showing major steps in DNA-SIP analyses, involving: (1) labelling of sample of interest with stable isotope labelled substrate; (2) separation of "heavy" ^{13}C-DNA from the background "light" ^{12}C-DNA by ultracentrifuge; (3) analysis of "heavy" ^{13}C-DNA.

DNA-SIP involves three major steps (**Fig. 9.1**): (1) Labelling of the sample of interest with stable isotope-labelled substrate; (2) Separation of "heavy"^{13}C-DNA from the background "light" ^{12}C-DNA by ultracentrifugation; and (3) Analyses of "heavy" ^{13}C-DNA. The original DNA-SIP experiments only focused on the analyses of 16S rRNA genes and so-called functional genes involved in a particular bioprocess; initial studies in our laboratory (10–12) and more recently in the laboratory of Lidstrom and colleagues (13) have demonstrated the combined use of DNA-SIP and metagenomics.

2. Materials

2.1. Reagents

1. ^{13}C-labelled compounds, e.g. $^{13}C_6$-Benzene (> 99% ^{13}C, Sigma-Aldrich)
2. Agarose (Helena, UK)
3. 75% (v/v) ethanol (reagent grade, Sigma-Aldrich)
4. FAST PREP DNA® extraction kit (Qbiogen)
5. CsCl (Fisher Scientific) stock solution (density 1.890 g/mL in water)
6. Gradient buffer, containing 0.1 M Tris–HCl (pH 8.0), 0.1 M KCl and 1 mM EDTA, autoclaved
7. DNA precipitation buffer. 30% (w/v) polyethylene glycol 6000 and 1.6 M NaCl, autoclaved
8. Glycogen in water (20 mg/mL)
9. TE buffer. 10 mM Tris–HCl (pH 7.6) and 1 mM EDTA, autoclaved.

2.2. Equipment

1. Suitable devices for substrate exposure and labelling, e.g. BBL GasPak 150 anaerobic system, US916 (Rankin Biomedical Corporation) with flask inside to contain environmental samples (e.g. soil slurry).
2. Appropriate devices and methods for measuring substrate consumption or corresponding microbial activities, e.g. gas chromatograph equipped with flame ionisation detector (Agilent) to measure benzene concentrations during SIP incubations.
3. Bead beater (B. Braun, Melsungen AG)
4. Nanodrop 1000 spectrophotometer (NANODROP)
5. Centrifuge tubes (5.1 mL polyallomer, Beckman)
6. Ultracentrifuge (Beckman) and rotor VTi 65.2 (Beckman Coulter)
7. Tube sealer (Beckman Coulter)
8. Microcentrifuge (Eppendorf)
9. Microcentrifuge tubes (1.5 mL, Eppendorf)
10. High-performance liquid chromatography (HPLC) pump (Sykam) and corresponding tube (1.5 mm bore × 1.5 mm wall, Appleton Woods)
11. Pipettors (Gilson) and corresponding tips (Sarstedt, UK)
12. Needles, various sizes
13. Digital refractometer AR200 (Reichert).

3. Methods

3.1. SIP Incubations with ^{13}C

The first and crucial step in DNA-SIP is to label environmental samples with sufficient ^{13}C-substrate, yet minimise potential cross-feeding of ^{13}C-labelling, i.e. to ensure the incorporation of ^{13}C-labelled carbon into only the primary consumers of the target substrate. Unfortunately, there are no standard rules that one can follow and this needs repeated experiments to find out the appropriate amounts for a certain sample empirically. Typically, we found that ~50 μmol^{13}C per g (for soil/ sediment samples) and 5 μmol^{13}C per ml (for water samples) incorporation are sufficient to detect active microorganisms above background (*see* **Note 1–3**).

3.2. DNA Extraction

Standard protocols can be used for extracting DNA from SIP incubations. In our laboratory, the following protocols were routinely used for DNA extraction from soil/ sediment (bead-beating-based method using FAST PREP DNA extraction kit from Qbiogen), water samples (14–15) and high molecular mass DNA from soil/sediment (10, 16), respectively. Extracted DNA can be quantified using a spectrophotometer (e.g. NANODROP) or by agarose gel electrophoresis against known amounts of DNA standards. Additional purification of DNA may be required to remove contaminants, which can sometimes interfere with downstream analyses (*see* **Note 4**).

3.3. Ultracentrifugation

The original method for ultracentrifugation involves the use of ethidium bromide to visualise DNA after ultracentrifugation (1). This protocol requires >700 ng of labelled ^{13}C-DNA to form a visible band after ultracentrifugation (Vorha and Murrell, unpublished data) (15). This protocol is rarely used now since long incubation times are needed to achieve such a large amount of labelling. More recent studies deal with labelling requiring only a few nanograms of "heavy" DNA for detection. Thus, a fractionation-based method (17–18) was developed and we describe this method below.

1. Firstly, prepare gradient buffer and a CsCl stock solution with a density of 1.890 g/mL.
2. To set up the gradient including DNA and CsCl, mix DNA (500 ng–5 μg) and gradient buffer together in a total volume of 1.2 mL. In a 15 mL Falcon centrifuge tube, combine 4.8 mL of CsCl stock solution (density 1.890 g/mL) and the 1.2 mL solution that contains DNA and gradient buffer. The average density of the final solution should be 1.725 g/mL (*see* **Note 5**).

3. Mix gently by inversion and add to an ultracentrifugation tube (5.1 mL) using either a Pasteur pipette, a syringe and needle or a 1000 μL pipettor with tip. Balance tubes and seal tubes carefully and start ultracentrifugation at 44,100 rpm in a VTi 65.2 rotor for 36–40 h at 20 °C with vacuum on, maximum acceleration and no brake.

3.4. Fractionation and DNA Precipitation

1. After centrifugation, take out the tubes from the rotors very carefully to avoid mixing. Fix the centrifuge tube to a clamp and very carefully pierce the bottom of the tube with a needle (19-gauge) after cleaning the tube with 75% (v/v) ethanol.
2. Displace the CsCl solutions from the top of the centrifuge tube with sterile deionised water using a HPLC pump. The flow rate of the pump should be controlled so that approximately 400 μL per fraction is collected every minute into a 1.5 mL microcentrifuge tube, resulting in 12–13 fractions per centrifuge tube (5.1 mL in size) (*see* **Note 6**).
3. Precipitate DNA from CsCl solutions for each fraction by adding two volumes of precipitation buffer. Addition of glycogen (~20 μg) is helpful as a carrier to ensure complete recovery of DNA present at low concentrations. Precipitated DNA should be washed once with ethanol 75% (v/v), air-dried and dissolved in 50 μL TE buffer (*see* **Note 7**).

3.5. Identification and Analyses of "Heavy" DNA

1. Identifying "heavy" DNA from the fractionated DNA is not trivial. We observed that environmental samples exposed to low concentrations of ^{13}C-labelling yielded a smear of DNA, usually from fraction 6 to 12.
2. Fingerprint methods, such as denaturing gradient gel electrophoresis (DGGE), are highly recommended to confirm the presence of the "heavy" DNA, which is normally on the edge of the smear (*see* **Note 8**).

Further analyses of "heavy" DNA can be performed to identify active microorganisms involved in a particular bioprocess (**Table 9.1**). The 16S rRNA gene is the obvious phylogenetic marker, considering that extensive sequences are available in databases (such as GenBank) and standard primer sets targeting Bacteria, Archaea and Eukaryotes can be used. PCR can also be done using functional gene markers targeting genes involved in a particular pathway, such as chloromethane utilisation (*cmuA*) (19) and ARHD (aromatic ring hydroxylating dioxygenases) (20). These PCR products can be cloned and sequenced for subsequent phylogenetic analyses (*see* **Note 9**).

"Heavy" DNA can also be used for metagenomic analyses (10–13), either by large-scale shotgun or parallel sequencing to reconstruct metabolic pathways from the dominant members

(21–22) or by functional metagenomic screening to identify genes encoding industrial/ medical relevant enzymes / bioactive compounds (23–24) (*see* **Note 10**).

4. Notes

1. It is highly recommended that potential target substrate degradation activity of a given environmental sample is measured before setting up SIP incubations. Inclusion of ^{12}C-substrate controls and replications of ^{13}C-substrate SIP incubations are also recommended.
2. The concentration of substrate for SIP incubations needs to be chosen with great care. Environmental pollutants are usually toxic for microorganisms; thus a low concentration of substrate in the SIP incubation is preferable. However, DNA-SIP requires a certain amount of labelling so that ^{13}C- DNA can be detected above background. Therefore, the overall solution will be to consider the balance of the concentration of labelled substrate, activity of the samples and incubation time.
3. To maximise the separation between labelled- and unlabelled DNA, >99% ^{13}C/^{15}N-labelled substrate is recommended. However, due to the complexity of environmental pollutants, usually it is difficult to purchase fully labelled substrate. Using such partially labelled substrates may result in difficulty in identifying "heavy" DNA and additional CsCl ultracentrifugation with bisbenzimide may be required to enhance separation of isotopically labelled DNA from unlabelled DNA (25).
4. Contaminants such as humic substances can inhibit downstream PCR amplification and need to be removed from DNA. Standard agarose gel electrophoresis can be used to remove these contaminants. Alternatively, repeated CsCl centrifugation can be applied. To avoid DNA shearing during purification, a low melting point agarose can be used in combination with β-agarase (New England Biolabs) digestion following standard protocols from manufacturers.
5. Successful separation of labelled and unlabelled DNA relies heavily on the accuracy of the average density of CsCl/DNA mixture. Keep in mind that the density of CsCl stock solution may vary between each batch. Therefore, before ultracentrifugation, the average density of the mixture should be measured and may need to be adjusted using

either CsCl stock solution or gradient buffer to make up to 1.725 g/mL.

6. Before precipitating DNA from CsCl solutions, density of each fraction should be measured to ensure the correct formation of the density gradient within the centrifugation tube. Measuring the density of CsCl using a digital refractometer (Digital refractometer AR200, Reichert) is recommended. Alternatively, an analytic balance can be used to weigh 100 μL from each fraction of the gradient. Expected density should be around 1.69–1.76 g/mL from top to bottom, with a median density of ~1.725 g/mL for fraction 6 or 7.
7. It is recommended that an aliquot (~5 μL) of the DNA from each fraction is run on an agarose gel (1%, w/v) to examine "heavy" DNA in fractions (normally fraction 6–8). Alternatively, real-time PCR can be carried out to evaluate "heavy" DNA fraction as described by Lueders et al. (17).
8. It is highly recommended, when setting up a DNA-SIP experiment, to include a ^{12}C-substrate-exposed control. Fingerprints between ^{13}C-labelled and ^{12}C-labelled fractions can therefore be compared and this is extremely useful in identifying "heavy" DNA from SIP incubations.
9. "Functional genes", i.e. genes encoding key enzymes involved in substrate utilisation, are not always available for the degradation of certain environmental pollutants simply because they may be poorly characterised. Therefore, isolation and characterisation of representative microorganisms in the degradation of those pollutants are also important.
10. To produce sufficient DNA for making a metagenome library or for shotgun sequencing, multiple displacement amplification may be performed from the "heavy" DNA using Phi 29 DNA polymerase (26).

References

1. Radajewski, S., Ineson, P., Parekh, N.R., and Murrell, J.C. (2000) Stable-isotope probing as a tool in microbial ecology. *Nature* **403**, 646–649.
2. Manefield, M., Whiteley, A.S., Griffiths, R.I., and Bailey, M.J. (2002) RNA stable isotope probing, a novel means of linking microbial community function to phylogeny. *Appl. Environ. Microbiol.* **68**, 5367–5373.
3. Boschker, H.T.S., Nold, S.C., Wellsbury, P., Bos, D., de Graaf, W., Pel, R., et al. (1998) Direct linking of microbial populations to specific biogeochemical processes by ^{13}C labelling of biomarkers. *Nature* 392, 801–805.
4. Bull, I.D., Parekh, N.R., Hall, G.H., Ineson, P., and Evershed, R.P. (2000) Detection and classification of atmospheric methane oxidizing bacteria in soil. *Nature* **405**, 175–178.
5. Wackett, L.P. (2004) Stable isotope probing in biodegradation research. *Trends Biotechnol.* **22**, 153–154.
6. Dumont, M., and Murrell, J. (2005) Stable isotope probing – linking microbial identity to function. *Nat. Rev. Microbiol.* **3**, 499–504.
7. Madsen, E.L. (2006) The use of stable isotope probing techniques in bioreactor and field studies on bioremediation. *Curr. Opin. Biotechnol.* **17**, 92– 97.

8. Friedrich, M.W. (2006) Stable-isotope probing of DNA, insights into the function of uncultivated microorganisms from isotopically labeled metagenomes. *Curr. Opin. Biotechnol.* **17**, 59–66.
9. Andreoni, V., and Gianfreda, L. (2007) Bioremediation and monitoring of aromatic-polluted habitats. *Appl. Microbiol. Biotechnol.* **76**, 287–308.
10. Dumont, M.G., Radajewski, S.M., Miguez, C.B., McDonald, I.R., and Murrell, J.C. (2006) Identification of a complete methane monooxygenase operon from soil by combining stable isotope probing and metagenomics analysis. *Environ. Microbiol.* **8**, 1240–1250.
11. Neufeld, J.D., Chen, Y., Dumont, M.G., and Murrell, J.C. (2008) Marine methylotrophs revealed by stable-isotope probing, whole genome amplification and metagenomics. *Environ. Microbiol.* **10**, 1526–1535.
12. Chen, Y., Dumont, M.G., Neufeld, J.D., Bodrossy, L., Stralis-Pavese, N., McNamara, N.P., et al. (2008) Revealing the uncultivated majority, combining DNA stable isotope probing, multiple displacement amplification and metagenomic analyses of uncultivated *Methylocystis* in acidic peatlands. *Environ. Microbiol.* **10**, 1526–1535.
13. Kalyuzhnaya, M.G., Lapidus, A., Ivanova, N., Copeland, A.C., McHardy, A.C., Szeto, E., et al. (2008) High-resolution metagenomics targets specific functional types in complex microbial communities. *Nat Biotechnol.* **26**,1029–1034.
14. Murray, A.E., Preston, C.M., Massana, R., Taylor, L.T., Blakis, A., Wu, K. et al. (1998). Seasonal and spatial variability of bacterial and archael assemblages in the coastal waters near Anvers Island, Antarctica. *Appl Environ. Microbiol.* **64**, 2585–2595.
15. Neufeld, J.D., Dumont, M.G., Vorha, J., and Murrell, J.C. (2007) Methodological considerations for the use of stable isotope probing in microbial ecology. *Micro. Ecol.* **53**, 435–442.
16. Zhou, J., Bruns, M.A., and Tiedje, J.M. (1996) DNA recovery from soils of diverse composition. *Appl Environ Microbiol* **62**, 316–322.
17. Lueders, T., Manefield, M., and Friedrich, M.W. (2004) Enhanced sensitivity of DNA- and rRNA-based stable isotope probing by fractionation and quantitative analysis of isopycnic centrifugation gradients. *Environ. Microbiol.* **6**, 73–78.
18. Neufeld, J.D., Vohra, J., Dumont, M.G., Lueders, T., Manefield, M., Friedrich, M.W., et al. (2007). DNA stable-isotope probing. *Nat. Protocols* **2**, 860–866.
19. Miller, L.G., Warner, K.L., Baesman, S.M., Oremland, R.S., McDonald, I.R., Radajewski, S., and Murrell, J.C. (2004) Degradation of methyl bromide and methyl chloride in soil microcosms: use of stable C isotope fractionation and stable isotope probing to identify reactions and the responsible microorganisms. *Geochim. Cosmochim. Acta* **68**, 3271–3283.
20. Leigh, M.B., Pellizari, V.H., Uhlík, O., Sutka, R., Rodrigues, J., Ostrom, N.E., et al. (2007) Biphenyl-utilizing bacteria and their functional genes in a pine root zone contaminated with polychlorinated biphenyls (PCBs). *ISME J.* **1**,134–148.
21. Tyson, G.W., Chapman, J., Hugenholtz, P., Allen, E.E., Ram, R.J., Richardson, P.M., et al. (2004) Community structure and metabolism through reconstruction of microbial genomes from the environment. *Nature* **428**, 37–43.
22. Venter, J.C., Remington, K., Heidelberg, J.F., Halpern, A.L., Rusch, D., Eisen, J.A., et al. (2004) Environmental genome shotgun sequencing of the Sargasso Sea. *Science* **304**, 66–74.
23. Schwarz, S., Waschkowitz, T., and Daniel, R. (2006) Enhancement of gene detection frequencies by combining DNA-based stable-isotope probing with the construction of metagenomic DNA libraries. *World J. Microb. Biot.* **22**, 363–367.
24. Schmeisser, C., Steele, H., and Streit, W.R. (2007) Metagenomics, biotechnology with non-culturable microbes. *Appl. Microbiol. Biotechnol.* **75**, 955–962.
25. Buckley, D.H., Huangyutitham, V., Hsu, S.F., and Nelson, T.A. (2007). Stable isotope probing with ^{15}N achieved by disentangling the effects of genome G+C content and isotope enrichment on DNA density. *Appl. Environ. Microbiol.* **73**, 3189–3195.
26. Blanco, L., Bernad, A., Lazaro, J.M., Martin, G., Garmendia, C., and Salas, M. (1989) Highly efficient DNA synthesis by the phage phi 29 DNA polymerase. Symmetrical mode of DNA replication. *J. Biol. Chem.***264**, 8935–8940.
27. Jones, M.D., Singleton, D.R., Carstensen, D.P., Powell, S.N., Swanson, J.S., Pfaender, F.K., and Aitken, M.D. (2008) Effect of incubation conditions on the enrichment of pyrene-degrading bacteria identified by stable-isotope probing in an aged, PAH-contaminated Soil. *Microb. Ecol.* **56**, 341–349.
28. Kunapuli, U., Lueders, T., and Meckenstock, R.U. (2007) The use of stable iso-

tope probing to identify key iron-reducing microorganisms involved in anaerobic benzene degradation. *ISME J.* **1**, 643–653.
29. Kittelmann, S., and Friedrich, M.W. (2008) Identification of novel perchloroethene-respiring microorganisms in anoxic river sediment by RNA-based stable isotope probing. *Environ. Microbiol.* **10**, 31–46.
30. Singleton, D.R., Hunt, M., Powell, S.N., Frontera-Suau, R., and Aitken, M.D. (2007) Stable-isotope probing with multiple growth substrates to determine substrate specificity of uncultivated bacteria. *J. Microbiol. Methods* **69**, 180–187.
31. Cupples, A.M., and Sims, G.K. (2007). Identification of *in situ* 2,4-dichlorophenoxyacetic acid-degrading soil microorganisms using DNA-stable isotope probing. *Soil Biol. Biochem.* **39**, 232–238.
32. Singleton, D.R., Sangaiah, R., Gold, A., Ball, L.M., and Aitken, M.D. (2006). Identification and quantification of uncultivated Proteobacteria associated with pyrene degradation in a bioreactor treating PAH-contaminated soil. *Environ. Microbiol.* **8**, 1736–1745.
33. Kasai, Y., Takahata, Y., Manefield, M., and Watanabe, K. (2006). RNA-based stable isotope probing and isolation of anaerobic benzene-degrading bacteria from gasoline-contaminated groundwater. *Appl. Environ. Microbiol.* **72**, 3586–3592.
34. Singleton, D.R., Powell, S.N., Sangaiah, R., Gold, A., Ball, L.M., and Aitken, M.D. (2005). Stable-isotope probing of bacteria capable of degrading salicylate, naphthalene, or phenanthrene in a bioreactor treating contaminated soil. *Appl. Environ. Microbiol.* **71**, 1202–1209.
35. Yu, C.P., and Chu, K.H. (2005) A quantitative assay for linking microbial community function and structure of a naphthalene-degrading microbial consortium. *Environ. Sci. Technol.* **39**, 9611–9619.
36. Gallagher, E., McGuinness, L., Phelps, C., Young, L.Y., and Kerkhof, L.J. (2005). ^{13}C-Carrier DNA shortens the incubation time needed to detect benzoate-utilizing denitrifying bacteria by stable-isotope probing. *Appl. Environ. Microbiol.* **71**, 5192–5196.
37. Manefield, M., Griffiths, R.I., Leigh, M.B., Fisher, R., and Whiteley, A.S. (2005) Functional and compositional comparison of two activated sludge communities remediating coking effluent. *Environ. Microbiol.* **7**, 715 – 722.
38. Mahmood, S., Paton, G.I., and Prosser, J.I. (2005) Cultivation independent *in situ* molecular analysis of bacteria involved in degradation of pentachlorophenol in soil. *Environ. Microbiol.* **7**, 1349–1360.
39. DeRito, C.M., Pumphrey, G.M., and Madsen, E.L. (2005) Use of field-based stable isotope probing to identify adapted populations and track carbon flow through a phenol-degrading soil microbial community. *Appl. Environ. Microbiol.* **71**, 7858–7865.
40. Borodina, E., Cox, M.J., McDonald, I.R., and Murrell, J.C. (2005) Use of DNA-stable isotope probing and functional gene probes to investigate the diversity of methyl chloride-utilizing bacteria in soil. *Environ. Microbiol.* **7**, 1318–1328.
41. Pumphrey, G.M., and Madsen, E.L. (2008) Field-based stable isotope probing reveals the identities of benzoic acid-metabolizing microorganisms and their *in situ* growth in agricultural soil. *Appl. Environ. Microbiol.* **74**, 4111–4118.

Chapter 10

Two-Dimensional Gel Electrophoresis: Discovering Biomolecules for Environmental Bioremediation

Om V. Singh and Anuj K. Chandel

Abstract

Environmental contamination has been viewed as an ecological malaise for which bioremediation can be prescribed as a "perfect medicine." The solution to the problems with bioremediation lies in analyzing to what extent the microbes' physiological machinery contributes to the degradation process and which biomolecules and their mechanisms are responsible for regulatory factors within the degradation system, such as protein, metabolite, and enzymatic chemical transformation. In the post-genomic era, recent advances in proteomics have allowed us to elucidate many complex biological mechanisms. Two-dimensional gel electrophoresis (2DE) in conjunction with mass spectrometry (MS) can be utilized to identify the biomolecules and their molecular mechanisms in bioremediation. A set of highly abundant global proteins over a pI range 4–7 was separated and compared by size fractionation (25–100 kDa) on 2DE. We identified a set of catabolic proteins, enzymes, and heat shock molecular chaperones associated with the regulatory network that was found to be overexpressed under phenol-stressed conditions. This chapter also offers optimized ideal directions for 2DE, followed by easy-to-follow directions for a protein identification strategy using MALDI-TOF and targeting novel proteins/enzymes for a universal set of experiments.

Key words: Proteomics, two-dimensional gel electrophoresis, environmental pollutants, phenol, bioremediation, microorganism.

1. Introduction

Toxic pollutants in the environment are of major concern worldwide; many toxic, mutagenic, and carcinogenic aromatic pollutants are posing serious threats to human health and the environment (1–4). Many site-specific microorganisms are capable of carrying out bioremediation (5–7). However, certain pollutants have been unusually recalcitrant: either the microbes are unable to completely mineralize them or their sister metabolites accumulate

S.P. Cummings (ed.), *Bioremediation*, Methods in Molecular Biology 599,
DOI 10.1007/978-1-60761-439-5_10,

in the environment and become a major threat (8). Exploring new catabolic pathways and studying regulatory control of primary and secondary metabolites are two possible solutions to produce effective bioremediation. The current sophisticated techniques in proteomics make it possible to dissect the regulatory network by exploring proteins and enzymes of interest that are being differentially expressed in microbes during bioremediation (9–11). The mainstay of protein expression profiling for the past 30 years has been two-dimensional gel electrophoresis (2DE), where proteins are separated by the complementary techniques of isoelectric focusing (IEF) in the first dimension and SDS-PAGE size fractionation in the second dimension.

2DE can efficiently explore the physiological state of an organism, leading to a differential expression in genes and proteins that induces a chain of enzymatic reactions to catalyze the mineralization of contaminants in the cell (12–16). When the cellular lysate of *Mycobacterium* sp. was profiled on a 2DE gel, an 81-kDa protein similar to catalase-peroxidase was shown to be significantly induced under pyrene exposure (12). Later, two ring-hydroxylating dioxygenases, Pdo1 and Pdo2, were induced during pyrene catabolism by another *Mycobacterium* sp. (13). About 80 unique proteins were identified by 2DE/MS from *Pseudomonas putida* KT 2440 cultured in the presence of six different organic compounds (14). Based on 2DE analysis, an enzymatic pathway has been proposed for phthalate metabolism in *Rhodococcus* sp. strain TFB (15). 2DE also provided quantitative mechanistic insights into the global response to phenol during phenol biodegradation by *Pseudomonas* sp. M1 (16).

2DE identification marks these proteins and enzymes as possible catalysts that can direct the use of biomolecules extracted or removed from cell and/or cell-free systems for field bioremediation. Newly introduced technologies such as difference in-gel electrophoresis (DIGE) and the metabolic labeling strategy known as SILAC (stable isotope labeling by amino acids in cell culture) have limitations that include labeling strategies, sample complexity, fractionation, relative versus absolute changes, and the range of differences. Therefore, 2DE remains the most frequently used method to easily estimate protein abundance in microbial cells. The current study provides explicit directions for using 2DE to discover biomolecules during bioremediation.

2. Materials

2.1. Cell Growth and Lysis

1. Microorganism *Pseudomonas putida*-11 was cultured in basal mineral salt medium containing (per liter): 1.5 g

Na_2HPO_4, 1.0 g KH_2PO_4, 0.4 g $(NH_4)_2SO_4$, 0.3 g $MgSO_4{\cdot}7H_2O$, pH 7.4, 2.0 mL trace element solution. The trace element solution contained (per liter) 0.05 g $SnCl_2{\cdot}2H_2O$, 0.05 g LiCl, 0.1 g $Al(OH)_3$, 0.05 g KI, 0.06 g $MnSO_4{\cdot}4H_2O$, 0.05 g H_3BO_3, 0.1 g $NiSO_4{\cdot}6H_2O$, 0.05 g $(NH_4)6Mo_7O_{24}{\cdot}4H_2O$, 0.05 g $BaCl_2$, 0.1 g $CoCl_2{\cdot}6H_2O$, 0.1 g $ZnSO_4{\cdot}7H_2O$.

2. Microbial wash solution: phosphate buffered saline (pH 7.0), without $CaCl_2$ and $MgCl_2$ (Gibco).
3. Modified 2D lysis buffer: 9 M urea, 4% CHAPS, 20 mM Tris–HCl, pH 8.8.

2.2. Sample Preparation and Two-Dimensional Gel Electrophoresis

1. Protein analysis and sample cleanup kits: RC-DC protein analysis kit (Bio-Rad, Hercules, CA), and 2D cleanup kit (GE Healthcare).
2. Rehydration buffer: 8 M urea, 2 M thiourea, 4% CHAPS, 40 mM dithiothreitol (DTT), 0.5% carrier ampholyte (pH 4–7), 0.002% bromophenol blue (w/v). Buffer should be stored at −20°C and thawed at room temperature prior to use.
3. Protein rehydration: passive rehydration in disposable rehydration/equilibration trays (Bio-Rad), and active rehydration in PROTEAN® isoelectric focusing (IEF) trays (Bio-Rad).
4. Immobilized pH gradient (IPG) strips: (ReadyStrip™, 17 cm, pH 4–7, Bio-Rad). IPG strips have to be stored at –20°C or as recommended by manufacturer.
5. First-dimensional IEF: PROTEAN® IEF cell (Bio-Rad); Make paper wicks in 5–6 mm size using Whatman #1 filter paper (Millipore, MA); mineral oil (Sigma, MO).
6. Equilibration buffer: Buffer I: 6 M urea, 2% SDS, 0.375 M Tris−HCl pH 8.8, 20% glycerol and each time add 130 mM fresh DTT. Buffer II: 6 M urea, 2% SDS, 0.375 M Tris−HCl pH 8.8, 20% glycerol and each time add 135 mM fresh iodoacetamide (IAA). Buffer needs to be stored at −20°C and thawed at room temperature prior to use.
7. Overlay agarose: 0.5% agarose (low melting grade) in 1× Tris/glycine/SDS buffer (1× TGS solution: 25 mM Tris, 192 mM glycine, and 0.1% (w/v) SDS, pH 8.3). 1× TGS can be stored at room temperature.
8. Second-dimension SDS-PAGE: 30% acrylamide/bis solution (37.5:1 with 2.6% C) (this is a neurotoxin when unpolymerized − care should be taken to avoid exposure) and *N*,*N*,*N*′,*N*′-tetramethyl-ethylenediamine

(TEMED, Bio-Rad). TEMED can be stored at room temperature.

9. Ammonium persulfate: Prepare 10% solution in water (*see* **Note 1**) and immediately freeze in single-use aliquots of 1 mL at −20°C.
10. Water-saturated isobutanol: Shake equal volumes of water and isobutanol in a glass bottle and allow to separate; use top layer. Solution can be stored at room temperature.
11. Running buffer: 1× TGS (Bio-Rad) (25 mM Tris, 192 mM glycine, and 0.1% (w/v) SDS, pH 8.3).
12. Second-dimensional gel electrophoresis: Ettan™ DALT six large Vertical System (GE Healthcare).
13. 2D gel image development: SilverQuest™ silver staining kit (Invitrogen).
14. Image acquisition: Molecular Imager FX (Bio-Rad).
15. Spot pattern analysis: Progenesis Nonlinear Dynamics software version 2005.

2.3. Protein In-Gel Digestion and Peptide Extraction

1. Protein spot excision: Spot Picker, 1.5 mm (The Gel Company, San Francisco, CA).
2. Destaining solution (*see* **Note 2**): Solution I: 30 mM potassium ferrocyanide; Solution II: 100 mM sodium thiosulfate.
3. Protein digestion solution: Sequencing-grade modified trypsin (Promega, Madison, WI). Stock solution: 500 ng/μL by adding 40 μL 1% acetic acid solution in one trypsin vial of 20 μg. Aliquot into eight tubes with 5 μL in each tube and store at −20°C. Working solution: Dilute stock solution into 10 ng/μL immediately before use by adding 2.5 μL to 122.5 μL of 10 mM ammonium bicarbonate (NH_4HCO_3).
4. Peptide extraction: 0.1% trifluoroacetic acid (TFA) in 60% acetonitrile (ACN).
5. Peptide purification: ZipTip C18, tip size – P10 (Millipore, MA).
6. Wetting solution: 50% ACN in 0.1% TFA solution in water.
7. Equilibration and washing solution: 0.1% TFA in water.
8. Wash solution: 5% methanol in 0.1% TFA in water.
9. Matrix solution: 10 mg matrix α-cyano-4-hydroxycinnamic acid (CHCA) (Sigma) in 60% ACN.
10. Vacufuge: Eppendorf, Brinkman Intr. Inc., NY.

2.4. Mass Spectrometry and Peptide Mass Fingerprinting

1. Peptide standards (Sigma): Prepare by adding 1.5 μL bradykinin, 0.5 μL ACTH, 2.0 μL insulin from 10 pmol/μL stock solution to 6 μL matrix α-cyano-4-hydroxy-cinnamic acid (CHCA) (Sigma) solution that is prepared by dissolving 10 mg in 60% ACN. All peptide stock solutions should be stored at −20°C.
2. Peptide-loading platform: MALDI plate (Applied-Biosystem).
3. Matrix-assisted laser desorption ionization (MALDI) – time of flight (TOF) mass spectrometer (MS) (Voyager DE-STR, Applied-Biosystem, MA).
4. Peptide mass fingerprinting (PMF): Online peptide search engines – i. ProFound-peptide mapping, ii. MS-Fit, and iii. MASCOT Peptide Mass Fingerprint equipped with NCBInr and Swiss-Prot databases.

3. Methods

3.1. Microbial Growth and Protein Extraction

1. Microorganism *Pseudomonas putida*-11 is grown in succinate-supplemented mineral salt medium as the carbon source (25 mM). A reliable cell number of *P. putida* is obtained in 200 mL culture medium grown in an orbital shaker (200 rpm) at 30°C in 500 mL Erlenmeyer flasks. The growth is monitored by culture optical density (OD) at 600 nm after every 6 h. The inoculum for phenol biodegradation is prepared using the exponential growth phase. Cells are grown in the exponential phase up to an OD_{600} of 0.2, and then phenol is added to obtain a final concentration of 500 mg/L along with control (negative phenol) in parallel. Cells are exposed to phenol for an additional 5 h.
2. Total protein extraction of phenol-stressed *P. putida* is obtained from cell pellets resulting from the centrifugation of 200 mL culture samples at 5000 × *g* at 4°C. The cell pellets are washed twice with phosphate buffer saline (PBS) followed by centrifugation at 5000 × *g* at 4°C.
3. Cell pellets are lysed by resuspending in 1 mL 2D lysis buffer, then sonicated using Sonic Dismembrator (Fisher Scientific) by immersing in ice (eight treatments of 8 s with 30 s intervals between treatments). The sonicated cellular lysate was incubated for 2 h on ice. The protein supernatant is recovered by centrifugation at 12,000 × *g* for 15 min at 4°C to remove the cell debris. The supernatant is collected and kept at −80°C to await further analysis of the protein.

3.2. Sample Preparation for 2D Gel Electrophoresis

1. 2DE is performed as previously described after multiple modifications and optimizations for the current study. The total protein content in the cell extract is quantified using a Bio-Rad RC-DC kit as recommended in the manufacturer's protocol, by removing the higher concentration of urea that interferes in the Lowery method to analyze the total protein content (*see* **Note 3**).
2. The total protein (100 μg) samples from the phenol-stressed cells and the control are cleaned using a 2D cleanup kit (GE healthcare) as described in the manufacturer's recommended protocol.
3. The final protein pellets are dissolved in 300 μL IEF rehydration buffer (*see* **Note 4**). The dissolved protein solution is centrifuged at 12,000 × *g* for 10 min at room temperature to make the impurities settle.

3.3. First-Dimensional Gel Electrophoresis

1. Using forceps, the paper wicks are placed at both ends of the PROTEAN IEF focusing tray. 10 μL of deionized water is pipetted onto each wick to wet them. Then the protein sample in rehydration buffer (300 μL) is loaded onto the rehydration tray, avoiding trapping air bubbles in the solution.
2. With forceps, the precast ReadyStrip IPG strip (17 cm, pH 4–7) is placed in the PROTEAN IEF focusing tray for active rehydration, gel side down onto the sample, making sure that the acidic end (marked + on IPG strip) is at the anode (red/+). Each of the strips is covered with 2 mL of mineral oil to prevent evaporation during the rehydration process. The rehydration tray is then covered with a plastic lid (*see* **Note 5**).
3. Rehydration was performed under active conditions for 12 h at 35°C on 50 V. IEF is carried out using the following conditions on the PROTEAN IEF cell: (i) 250 V for 20 min on linear ramp, (ii) 10,000 V for 2 h on linear ramp, (iii) 10,000 V at 45,000 V/h on rapid ramp, (iv) holding at 500 V on rapid ramp until IPG strips are removed from the first dimension (*see* **Note 6)**.
4. After the first dimension, the IPG strips are carefully removed and held vertically for about 10–15 s to remove the excess mineral oil, then rinsed with running water. IPG strips can also be placed onto a piece of dry filter paper and blotted with a second piece of wet filter paper (*see* **Note 7**).
5. Prior to the second dimension of gel electrophoresis, the focused proteins on the IPG strips are subjected to a two-step equilibration using equilibration buffer I followed by equilibration buffer II.

6. The IPG strips are equilibrated in a passive equilibration tray by keeping the gel side facing up and adding 5 mL of equilibration buffer I with freshly added 130 mM DTT, then gently shaking for 10 min on an orbital shaker at room temperature.
7. The equilibration buffer I is decanted and buffer II was added, including a freshly added 135 mM IAA. The IPG strips are incubated for 10 min, gently shaking on the orbital shaker at room temperature.
8. During the second equilibration step, the overlay agarose is melted in a microwave oven.

3.4. Preparation of Molecular Weight Marker for Second-Dimensional Gel Electrophoresis

1. Regardless of specified pH, the IPG strips (7 cm) are rehydrated under passive conditions in disposable rehydration/equilibration trays with 75 μL Precision Plus Protein Standards marker for 12 h at room temperature. Rehydrated IPG strips are stored at 4°C in the same rehydration tray facing gel side up (*see* **Note 8**).
2. Prior to second-dimensional gel electrophoresis, the IPG strips are clipped to 5–8 mm size and placed on top of the second-dimension SDS-PAGE.

3.5. Second-Dimensional Gel Electrophoresis on SDS-PAGE

1. This protocol assumes the use of an Ettan™ DALT six-large-vertical-gel system, but these instructions can easily be adapted to other formats, including minigels. It is critical that the glass plates for the 2D gels be scrubbed clean with laboratory-grade detergent after use and rinsed extensively with distilled water.
2. A 1.0-mm thick, 10% gel is prepared by mixing 16.6 mL 30% acrylamide/bis solution (37.5:1, 2.6% C) with 20 mL sterile deionized water, 12.5 mL Tris 1.5 M (pH 8.8), 0.5 mL 10% SDS solution, 0.5 mL 10% ammonium persulfate solution, and 20 μL TEMED. The gel is poured, leaving about 1.5 cm of space for the IPG strips, and overlaid with 1 mL water-saturated isobutanol. The total time for 24 cm gel polymerization is about 2–3 h longer than for the minigel (7 cm) (*see* **Note 9**).
3. 1× running buffer was prepared by diluting 500 mL 10× TGS buffer with 4500 mL of water in a measuring cylinder and pouring into the assembly up to the designated mark. The circulatory pump is then turned on to mix the buffer.
4. A 100 mL graduated cylinder or tube of the same length or longer than the IPG strips is filled with 1× TGS buffer. Bubbles on the surface of the buffer are removed using a Pasteur

pipette. Then, the IPG strips are picked up with forceps and immersed in the 1× TGS buffer, rinsing off the extra equilibration buffer.

5. The empty space at the top of the SDS gel is filled with 1× TGS buffer. IPG strips are placed on one side of the glass plate on top of the gel and slightly pushed into the solution using forceps and a spatula (the 1× TGS makes it easier to slide the IPG strips down on top of the SDS gel). The forceps and spatula should push on the plastic backing of the strip and not the gel matrix. Once the IPG strips are set on the top of the gel, excess 1× TGS is poured off by inclining the gel on either side. A pre-rehydrated piece of molecular weight marker is loaded toward the acidic end (positive) (*see* **Note 10**). The IPG strips are then overlaid with melted agarose covering solution.
6. Assembly of the gel unit is completed by filling it with 1× TGS as recommended by manufacturer and connecting it to a power supply. The second-dimension electrophoresis is performed at 50 V for 30 min, followed by 100 W until the blue dye front arrived at the bottom of the gel (~5–6 h). While it is running, overheating in the assembly can be avoided by attaching a water circulatory pump at 10°C. Alternatively, second-dimension gel electrophoresis can be performed overnight at 100 V until the blue dye front arrives at the bottom of the gel.

3.6. Image Acquisition and 2D Gel Spot Pattern Analysis

1. The 2DE gels are stained with silver stain using the MS-compatible SilverQuest™ Silver staining kit as described in the manufacturer's manual. Highly reproducible silver-stained gel images are acquired using Molecular Imager FX. **Figure 10.1** shows consistently reproducible images of the total protein profile of *P. putida* under control and phenol-exposed (500 mg/L) conditions at 30°C for 5 h.
2. Any two gels undergoing direct comparison should always be run and silver-stained in parallel. Gels can be stored in 30% ethanol at room temperature until punched for protein spots.
3. Consistently replicated images are further processed for spot detection, gel alignment, and spot quantification by match ratio using Progenesis Nonlinear Dynamics software. Once software-based automatic warping and matching is complete, an average gel of all three replicate gels was created using the average gel command.
4. After normalization, using Progenesis background correction, the software is used to detect the spot volume of all three-replicate gels. This normalization is corrected for

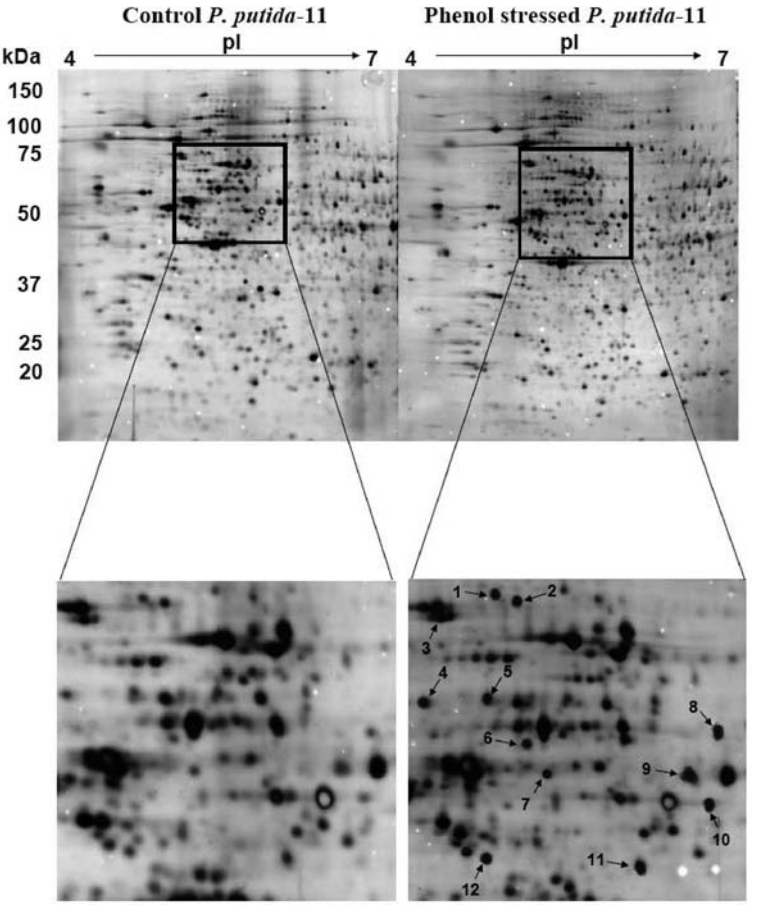


Fig. 10.1. A comparison in proteome map of *Pseudomonas sp.*-11. Cellular lysate was prepared by growing cells in succinate-supplemented carbon source (*left*, control) followed by an additional 5 h in phenol (500 mg/L) (*right*, sample stressed). Total protein (100 μg) underwent isoelectric focusing onto an IPG strip (pH 4–7 nonlinear) and separated on second dimension on 10% SDS-PAGE gel. Proteins were visualized by MS compatible silver staining. The images were scanned and analyzed for protein differential expression using Progenesis Nonlinear Dynamic software (version 2005). Zoomed central area from two gels show informative region that was identified having higher number of overexpressed proteins/ enzymes and molecular chaperones in phenol stressed (*right*) versus the control condition (*left*). *Arrows* indicating individual protein spots that were over expressed. The corresponding protein gel spots were excised, trypsinized, and analyzed by MALDI-TOF MS. The proteins were then subsequently identified by peptide mass fingerprints as show in **Table 10.1**.

loading differences, since the normalized intensities are given relative to the median of spot intensities, and it approximated the correction for nonlinear responses at the low and high ends of the intensity range. Progenesis software automatically calculates and reports the variation or error of all 2DE spots represented in a digitally constructed average gel.

5. To compensate for variations in gel staining and differences in protein spot numbers for each gel, normalized spot volumes are used to compare protein expression levels. The software calculated the normalized spot volume by dividing the single spot volume by the total spot volume and then multiplying by the total spot area. This normalization corrects loading differences, since the normalized intensities are given relative to the median of spot intensities, and it approximated the correction for nonlinear responses at the low and high ends of the intensity range. The applications of these corrections in silver-stained 2DE gels are shown in **Fig. 10.2**. Log normalization is performed by exporting data into an Excel sheet. Each data point is calculated as the mean ±SEM of spot volume relative to an average control gel. The analysis of variance is calculated and the *P* value <0.05 is considered significant.

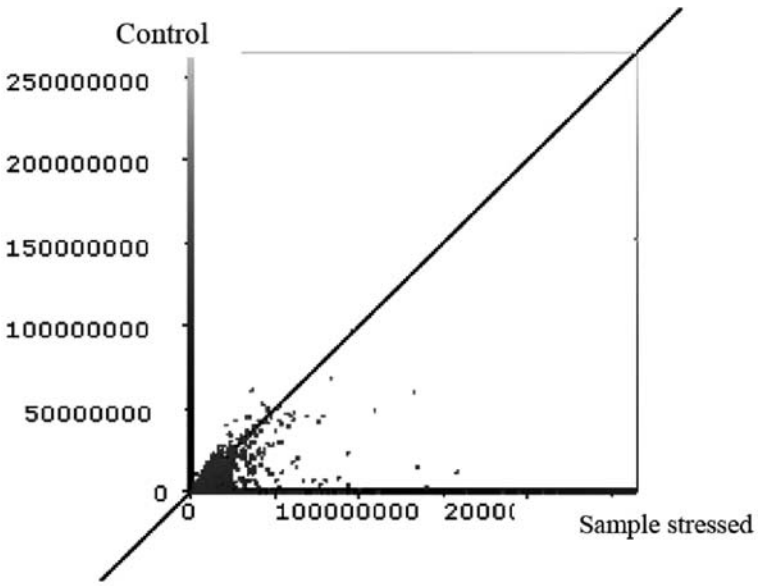


Fig. 10.2. Relative normal distribution of median protein spots volumes from three sets of experiment in control versus sample, i.e., phenol-stressed cellular lysate of *Pseudomonas* sp.-11 and its approximate corrections with silver-stained reference gel of control *Pseudomonas* sp.

3.7. Protein In-Gel Digestion

1. After gel image analysis, the protein spots of interest are excised manually using a plastic plunger (The Gel, San Francisco, CA) and digested in parallel with a blank gel as control in triplicate (*see* **Note 11**).
2. Protein gel pieces are placed in 1.5 μL Eppendorf tubes pre-rinsed with ACN. Gel pieces are washed twice with 50% methanol for 10 min, and can then be stored at −20°C.

3. The protein gel pieces are then destained by adding a 1:1 mix (100 μL + 100 μL) of destaining solution I and solution II until the gel pieces are no longer brown (~10–15 min) (*see* **Note 12**).
4. The destaining solutions are discarded and the gel pieces are washed three times with 200 μL water for 10 min each (additional washes can be done based on the clarity of the gel pieces).
5. Destained gel pieces were washed with 20 mM NH_4HCO_3(100 μL) and dehydrated with ACN (100 μL), twice for 10 min each. Finally, the dehydrated gel pieces are dried in a vacufuge until completely dry like rice grains (~15–20 min).
6. The dried gel pieces are swelled in 25–30 μL of protein digestion solution (10 ng/μL trypsin in 20 mM NH_4HCO_3) by incubating on ice for 45 min.
7. The excess trypsin solution around the gel pieces is removed and replaced by 25 μL of 10 mM NH_4HCO_3 (*see* **Note 13**).
8. Protein digestion is continued by incubating the gel pieces overnight at 37°C in a water bath with gentle shaking.
9. After a brief centrifugation at a higher speed, the supernatant is collected and stored in 1.5 mL Eppendorf tubes pre-rinsed with ACN.
10. Peptides are extracted from the gel pieces by adding 200 μL of 0.1% TFA in 60% ACN, followed by vigorous shaking for 60 min at 30°C.
11. The sample tubes are centrifuged briefly and the supernatants are mixed together. Then Step 10 is repeated.
12. The supernatants are collected and dried in a speed vac using a vacufuge (Eppendorf). The resulting peptides are solubilized in 10 μL 0.1% TFA and can then be stored at −20°C before purification by ZipTip.

3.8. Peptide Purification and Mass Spectrometry

1. Prior to peptide purification, the matrix-assisted laser desorption ionization (MALDI) plate is washed in four steps: 1−deionized water, 2–50% methanol; 3–100% methanol, and 4–100% ACN. These steps are repeated twice and then the MALDI plate is left to dry completely.
2. The frozen peptides are thawed on a bench and centrifuged briefly at high speed. The manufacturer's recommended protocol is followed for peptide purification, with slight modifications. The ZipTip C18 reverse phase column is wetted four times in wetting solution. The ZipTip is then equilibrated and washed four times in washing solution.

The dissolved peptides are bound to the column by filling the ZipTip with peptide solution about 10–15 times. The bound peptides are washed in wash solution four times, and finally released by rinsing the ZipTip with 2 μL CHCA matrix solution and spotting it directly onto the MALDI plate (*see* **Note 14**).

8. A standard solution of peptides is prepared in a 500 Eppendorf tube by adding 1.5 μL bradykinin, 0.5 μL ACTH, and 2.0 μL insulin from 10 pmol/μL stock solution in 6 μL matrix, and spotted on the same plate to calibrate the MALDI instrument.
9. Mass analyses are performed using a MALDI-TOF mass spectrometer (Voyager DE-STR, Applied-Biosystems. Framingham, MA) equipped with a 337 nm nitrogen laser.
10. Mass spectra are automatically calibrated upon acquisition using two-point residual porcine trypsin autolytic fragments (842.51 and 2210.10 [M+H+] ions) and matrix added standard bradykinin and ACTH peaks (757.39 and 2,465.19 [M+H+] ions). Raw spectra are baseline-corrected and noise-filtered (correlation factor = 7).
11. The spectra are deisotoped and the peaks automatically collected using the "copy peak list" feature of the software. Keratin and trypsin-derived extra peaks are manually removed from monoisotopic standard peak lists. All the submitted masses are accurate to the level of 25–50 ppm.

3.9. Identification of Proteins

1. Monoisotopic masses of each spectrum in triplicate are searched in the NCBInr databases (NCBInr 2005.01.03) using the MS-Fit search engine (The Rockefeller University Edition, version 2005.02.14). Identified proteins are further confirmed by searching in ProFound-Peptide Mapping and MASCOT Peptide Mass Fingerprint search engines using the NCBInr and Swiss-Prot 07.05.2006 databases.
2. The unmatched peptides and miscleavage sites are neglected. All mass searches are performed under all bacterial taxonomic categories in a pre-assumed experimental mass and pI range and cross-checked with other available search engines.
3. The search parameters are allowed complete modification with iodoacetamide (Cys). Peptides are matched with the theoretical peptide masses of all proteins against all bacterial entries in the NCBInr database with these parameters: i. peptide tolerance limit of 20–50 ppm or better mass accuracy; ii. number of peptides matched averaging more than 15 (minimum = 6); (iii) matched peptides covering at least 25% of the whole protein sequence with a significant Z score (>90 % probability) and higher Mowse score; (iv) each iden-

Table 10.1
Overexpressed identified protein/enzyme spots in 2DE by in-gel digestion and MALDI-TOF MS analysis from control versus phenol-stressed *Pseudomonas* sp.-11

Spot No	Accession No	Protein name	Theoretical Mr (kDa)/pI	Observed Mr (kDa)/pI	Matching peptides (*n*)	Unmatched peptides (*n*)	Sequence coverage (%)
I	Q88N13	Periplasmic beta-glucosidase	83.4/5.9	81.5/5.0	9	21	35
2	Q88DU2	Chaperone protein dnaK	68.9/4.8	70.2/5.2	28	11	48
3	A5W024	Isocitrate dehydrogenase	65.9/5.4	65.5/4.9	21	19	52
4	Q88MG1	CTP synthase	59.4/5.6	58.1/4.9	30	24	31
5	B0KI05	Phosphoenolpyruvate carboxykinase	55.5/5.1	59.1/5.1	31	29	46
6	B0KRB0	ATP synthase subunit alpha	55.3/5.3	55.2/5.0	16	20	39
7	Q8VMD8	AtpD beta synthase chain	Undefined	50.4/5.1	12	36	28
8	P48216	Chaperonin GroEL	56.1/5.0	56.5/6.0	28	12	51
9	Q99LE8	3-isopropylmatate dehydratase	51.2/5.5	54.1/5.8	24	32	32
10	Q31046	Dihydrolipoyl dehydrogenase	49.2/5.7	45.2/5.7	18	26	29
11	Q88L01	3-ketoacyl-CoA thiolase	41.5/6.5	40.5/5.4	12	18	26
12	Q88FC3	3-oxoacyl-(acyl-carrier-protein) synthase I	43.2/5.4	41.2/4.9	16	9	28

tified protein cross-referenced to the comparable pI and molecular weight (kDa) obtained from experimental image analysis on the 2D gel (*see* **Note 15**).

4. **Table 10.1** shows the identified proteins, the total number of hit peptides, and the coverage value of overexpressed proteins in *P. putida* under phenol-stressed conditions.

4. Notes

1. Unless stated otherwise, all solutions should be prepared in deionized water. This standard is referred to as "water" in the text.
2. Unless stated otherwise, all solutions should be stored at 4°C.
3. Always thaw protein samples on ice. Briefly centrifuge tubes at higher speed whenever protein needs to be thawed on ice from –80°C.
4. If pellets do not dissolve, vortex them for 5–10 min at room temperature. Do not overdry the protein pellets in step 2; overdried pellets may dissolve during the repeated freeze-and-thaw process.
5. Before they are loaded for active rehydration, the frozen ReadyStrip IPG strips are removed from −20°C and left to sit on the bench for 5–10 min. Then the cover sheets are carefully peeled from the ReadyStrip IPG strips using forceps and they are loaded into the solution facing gel side down onto the protein sample, making sure not to trap any air bubbles underneath the strips. If any air bubbles are trapped, hold one end of the IPG strip with forceps and move it back and forth slowly until the bubble moves to the edge of the strip. Slowly add mineral oil on the backing of the IPG strips.
6. Refer to the PROTEAN IEF cell manual from Bio-Rad to program the machine. During IEF, the user should watch to ensure that the current does not reach or exceed 50 μA/gel. If the current reaches this limit, it indicates that the protein sample contains salt impurities, which may cause horizontal lines in the second-dimensional gel image. To remove salt impurities from the protein sample, wash twice with the wash buffer in the 2D cleanup kit system under step 2.2 (1).
7. Try to remove excess mineral oil from the IPG strips, as it may create horizontal lines in the 2D gel.

8. The IPG strip gel should not dry out during storage. This can be avoided by sealing the rehydration tray with plastic wrap.
9. Gel polymerization efficiency can be examined by checking the leftover gel solution in the beaker. Do not leave polymerized gel inside the assembly overnight at room temperature; this may dry out the gel.
10. Always place a molecular weight marker at one end (either acidic, +/, or basic, −) to verify the polarity of the IPG strips and the orientation of the gel.
11. Extra care needs to be taken during spot punching to avoid possible contamination of keratin and overlapping of other spots. Excise a sufficient number of blank gel pieces.
12. Reagent A and Reagent B, available in the Invitrogen SilverQuest silver staining kit, can be used.
13. Add an extra 10 mM NH_4HCO_3 if necessary; gel pieces should not dry out during incubation.
14. Make a replica of the MALDI plate with the exact position of each spot and standards on a separate paper sheet to recognize the spotted samples.
15. Selection should be made based on the maximum coverage value. Prefer peptide error ±25 or less; otherwise the search may hit the wrong protein. The peptide sequence must have proline (P), which is an uncharged residue and flies faster in the MALDI-TOF tube; if it is not included in the search, then the bulky charged residue inside sequence should have alanine (A) or lysine (K).

Acknowledgments

Technical support rendered by Ms. Rashmi Singh for preparing this manuscript is gratefully acknowledged.

References

1. Perera, F. P. (1997) Environment and cancer: who are susceptible? *Science.* **278**, 1068–1073.
2. Chang, K. F., Fang, G. C., Chen, J. C., and Wu, Y. S. (2006) Atmospheric polycyclic aromatic hydrocarbons (PAHs) in Asia: a review from 1999 to 2004. *Environ Pollut.* **142**, 388–396.
3. Boström, C. E., Gerde, P., Hanberg, A., Jernström, B., Johansson, C., Kyrklund, T., et al. (2002) Cancer risk assessment, indicators, and guidelines for polycyclic aromatic hydrocarbons in the ambient air. *Environ. Health Perspect.* **3**, 451–488.
4. Belpomme, D., Irigaray, P., Hardell, L., Clapp, R., Montagnier, L., Epstein, S., et al. (2007) The multitude and diversity of environmental carcinogens. *Environ. Res.* **105**, 414–429.
5. Peng, R. H., Xiong, A. S., Xue, Y., Fu, X. Y., Gao, F., Zhao, W., et al., (2008) Microbial biodegradation of polyaromatic hydrocarbons. *FEMS Microbiol. Rev.*(Epub ahead of print) PMID: 18662317.
6. Díaz, E. (2004) Bacterial degradation of aromatic pollutants: a paradigm of metabolic versatility. *Int. Microbiol.***7**, 173–180.

7. Samanta, S. K., Singh, O. V., and Jain, R. K. (2002) Polycyclic aromatic hydrocarbons: environmental pollution and bioremediation. *Trends Biotechnol.* **20**, 243–248.
8. Esteve-Nunez, A., Caballero, A., and Ramos, J.L. (2001) Biological degradation of 2,4,6-trinitrotoluene. *Microbiol. Mol. Biol. Rev.***65**, 335–352.
9. Singh, O.V. (2007) Integrating "-omics" into biological processes and modeling for bioremediation. *OMICS.* **11**, 231–232.
10. Zhao, B., and Poh, C. L. (2008) Insights into environmental bioremediation by microorganisms through functional genomics and proteomics. *Proteomics.***8**, 874–881.
11. Singh, O. V. (2006) Proteomics and metabolomics: the molecular make-up of toxic aromatic pollutant bioremediation. *Proteomics.* **6**, 5481–5492.
12. Wang, R. F., Wennerstrom, D., Cao, W. W., Khan, A. A., and Cerniglia, C. E. (2000) Cloning, expression, and characterization of the *katG* gene, encoding catalase-peroxidase, from the polycyclic aromatic hydrocarbon-degrading bacterium *Mycobacterium* sp. strain PYR-1. *Appl. Environ. Microbiol.* **66**, 4300–4304.
13. Krivobok, S., Kuony, S., Meyer, C., Louwagie, M., Willison, J. C., and Jouanneau, Y. (2003) Identification of pyrene-induced proteins in *Mycobacterium* sp. strain 6PY1: evidence for two ring-hydroxylating dioxygenases. *J. Bacteriol.* **185**, 3828–3841.
14. Kim, Y.H., Cho, K., Yun, S.H., Kim, J.Y., Kwon, K.H., Yoo J.S., et al. (2006) Analysis of aromatic catabolic pathways in *Pseudomonas putida* KT 2440 using a combined proteomics approach: 2DE/MS and cleavable isotope-coded affinity tag analysis. *Proteomics.* **6**, 1301–1318.
15. Tomas-Gallardo, L., Canosa, I., Santero, E., Camafeita, E., Calvo, E., López, J. A., et al. (2006) Proteomic and transcriptional characterization of aromatic degradation pathways in *Rhodoccocus* sp. strain TFB. *Proteomics.* **6**, S119–S132.
16. Santos, P. M., Roma, V., Benndorf, D., von Bergen, M., Harms, H., and Sá-Correia, I. (2007) Mechanistic insights into the global response to phenol in the phenol-biodegrading strain *Pseudomonas* sp. M1 revealed by quantitative proteomics. *OMICS.* **11**, 233–251.

Chapter 11

The Application of Molecular Techniques to the Study of Wastewater Treatment Systems

Irena Wojnowska-Baryła, Agnieszka Cydzik-Kwiatkowska, and Magdalena Zielińska

Abstract

Wastewater treatment systems tend to be engineered to select for a few functional microbial groups that may be organized in various spatial structures such as activated sludge flocs, biofilm or granules and represented by single coherent phylogenic groups such as ammonia-oxidizing bacteria (AOB) and polyphosphate-accumulating organisms (PAO). In order to monitor and control engineered microbial structure in wastewater treatment systems, it is necessary to understand the relationships between the microbial community structure and the process performance. This review focuses on bacterial communities in wastewater treatment processes, the quantity of microorganisms and structure of microbial consortia in wastewater treatment bioreactors. The review shows that the application of molecular techniques in studies of engineered environmental systems has increased our insight into the vast diversity and interaction of microorganisms present in wastewater treatment systems.

Key words: Molecular technique, engineered microbial structure in wastewater treatment.

1. Bacterial Communities in Wastewater Treatment Processes

Selection of microorganisms through their ability to produce energy under specific conditions has been used as a basis for biological nutrient removal (BNR) plant designs. The periodic exposure of the microbial community to anaerobic, anoxic and aerobic conditions has enabled the selection of nitrifiers, denitrifiers and phosphate-accumulating microorganisms in one reactor system so that COD, N and P removal, which is rather problematical, can be accomplished in a single sludge. The wide range of operating

S.P. Cummings (ed.), *Bioremediation*, Methods in Molecular Biology 599,
DOI 10.1007/978-1-60761-439-5_11,

conditions and reactor configurations present in treatment plants results in a great complexity of microbial communities involved in pollutant removal (1).

In biomass responsible for nutrient removal, the dominant group is Betaproteobacteria, followed by Alphaproteobacteria, Gammaproteobacteria and in some cases Deltaproteobacteria (2). Other groups of bacteria, such as Planctomycetes, Firmicutes, Bacteriodetes and Cytophaga, are generally present, but often in relatively low numbers (3, 4). In wastewater treatment processes, the dissolved and particulate biodegradable constituents of wastewater are transformed into simpler end products and biomass. Microorganisms involved in pollutant removal can be organized in various spatial structures such as activated sludge flocs, biofilm or granules.

Activated sludge flocs are formed as a result of bioflocculation, a process where microbial cells (predominantly bacteria) and particles from the wastewater aggregate. Recalcitrant constituents and suspended and non-settable colloidal solids are then incorporated into biological flocs. Wilén et al. (5) indicated that effective bioflocculation is the key to efficient solid–liquid separation of activated sludge from treated waste. Decreased floc stability can be caused by environmental stresses such as sudden temperature changes, pH variations, toxic compounds or poor bioflocculation (6, 7). Deflocculation causes an increased number of small flocs and free bacteria, which cannot be separated from the water by means of settling, resulting in high effluent suspended solids (SS) concentrations. Both the physicochemical adhesion of cells and other particles and the formation of microbial colonies by cell division are important for bioflocculation to occur. However, only a minor fraction (5–20%) of the organic matter in the sludge floc is made up of bacteria, the rest consists mainly of extracellular polymeric substances (EPS) (8). The EPS holds the microorganisms together in a matrix onto which organic fibres, organic and inorganic particles as well as various colloids can adsorb; this close proximity between microorganisms and the organic material promotes its digestion by extracellular enzymes (9). It is believed that EPS is important for floc structure and stability as well as determining its physicochemical and biological properties (10). Different groups of bacteria have different abilities to flocculate due to differences in their physicochemical properties. These differences can be species or strain specific, or as a result of certain cellular activities. It has been shown that active aerobic metabolism of the bacteria is essential to maintain stable aggregates on a short-term basis (11).

Jorand et al. (12) proposed a model where the floc contains three structural levels, primary particles (single cells) are bound together with one type of EPS to secondary particles (microbial colonies), which are in turn bound together by another type

of EPS into a tertiary structure. The microstructure, defined as the position of specific bacterial populations, seems to be non-randomly distributed in the flocs (13). The macrostructure, i.e. the morphology of the overall floc, characterized by the parameters of floc size, abundance and type of filaments, density and overall shape, influences floc stability and settling characteristics. Bacteria are obviously an important component of activated sludge flocs and determining important aggregation properties; however, bacterial community composition and its possible correlation with floc settling and aggregation are poorly understood.

Immobilization of microorganisms into biofilms or granules, a process of cell-to-substratum or cell-to-cell attachment, is extensively employed in biotechnological applications (14). Traditionally, microbial biofilms were thought to be flat homogenous structures independent of the complex and dynamic environmental conditions under which they grew. Diffusion was considered as the predominant mechanism of mass transport, and the depth of penetration was thought to depend on biofilm thickness, regardless of the type of internal microstructure (15). However, it is now apparent that biofilms consist of microcolonies of cells and extracellular polymeric substances (EPS) separated by interstitial voids (16). The microcolonies are about 300 μm and the voids are about 100 μm wide. The voids are open channels connected with the bulk fluid. In the channels both diffusion and convection contribute to mass transfer, while in the microcolonies transport is determined by diffusion only. The architecture of a biofilm is affected by a multitude of external and internal factors, the surface loading rate, the shear rate (17) and the type of flow regime (laminar or turbulent conditions) (18).

The biofilm structure is important in genetic transfer by bacterial conjugation because it determines how deeply the donor cells can penetrate the biofilm. Open channels and pores allow more efficient donor transport and hence more frequent cell collisions, leading to rapid spread of the genes by horizontal gene transfer. Such insights into the physical environment of biofilms can be used for bio-enhancement of catabolic processes by the introduction of mobile genetic elements into an existing microbial community (19). In wastewater treatment systems, biofilm reactors are used when the reactor capacity obtained by using freely suspended organisms is limited by the biomass concentration and hydraulic residence time (20). In these situations, they are an effective solution to retain biomass in the reactors and to improve the volumetric biodegradation capacity, for example, in the case of slow-growing organisms such as nitrifiers or methanogens, or for diluted feed streams, in which only low biomass concentration can be achieved, without biomass retention. Traditional biofilm systems, such as trickling filters and rotating biological contactors, have small specific biofilm surface

areas available for substrate transport and reaction and a limited reactor capacity. However, a significant increase in biofilm surface area can be obtained by using small particles or highly porous materials as biomass carriers, as is achieved in upflow sludge blankets, fluidized bed reactors, expanded granular sludge blankets, biofilm airlift suspension reactors, internal circulation reactors and column reactors with biomass immobilized on porous ceramic carriers (20, 21).

Advances in granulation technologies have been achieved by focussing on general aspects of granule formation: effects of substrate type, loading rate, filling patterns, shear stress, dissolved oxygen concentration, COD/N ratio, settling velocity on granulation, and characteristics of granules such as granule size, morphology, sludge volume index (SVI), density, hydrophobicity, and conversion processes under different conditions (22). The formation of aerobic granules is crucial for their applicability in wastewater treatment and this process takes several weeks; therefore, the start-up time of an aerobic granular system is significantly longer than a conventional activated sludge process (23). It is well known that the aggregation of bacterial cells and the formation of flocs and even more dense aggregates can be enhanced by the addition of flocculants such as calcium, aluminium and iron. Similarly, the formation of granules may be enhanced by selected microbial cultures (24), for example, Limbergen et al. (25) proposed that the selection and application of floc-forming bacteria are important for good flocculation in an activated sludge system. This was demonstrated by the application of an enrichment culture, with an increased cell surface hydrophobicity, for a faster formation of mechanically stronger granules that formed within several days after inoculation and were suitable for reactors with mechanical stirring (26). Similarly, Tay et al. (27) applied acetate-fed granules as a starting seed for the development of phenol-degrading granules that developed within 1 week after initiating the reactor. Some studies have indicated that microbial granules cultivated under aerobic conditions are less stable than anaerobic granules. The poor stability of microbial granules can be a limiting factor in their application in real wastewater treatment practice. A possible reason for the poor stability of aerobic granules is the fast growth rate of heterotrophic bacteria, which dominate the granules. Studies on aerobic granulation indicated that the selection of slow-growing bacteria or the presence of slowly degrading compounds could lead to the formation of more stable granules (28). Therefore, it was hypothesized that the presence of a carbon source that is relatively recalcitrant might facilitate better granulation due to the selection of slow-growing bacteria (29).

Aerobically grown granules have a diverse microbial community, a complex spatial structure, coordinated physiological functions, and are characterized by specific temporal changes

(26). It was shown that microbial granules were composed of a variety of biological layers arranged as a sequence of obligate aerobic microorganisms, facultative anaerobic, and obligate anaerobic bacteria, and, finally, a core of dead and lysed cells (30). The microbial diversity of aerobic granules is closely related to their structure and the composition of the culture media in which they are grown. Heterotrophic, nitrifying and denitrifying populations can coexist in the microbial matrix of granules, with their respective activities being related to the nitrogen-loading rates and the availability of an external carbon sources. It has been shown that microbial granules subjected to alternating aerobic and anaerobic conditions are capable of removing organics and nitrogen from wastewater, and the coexistence of different species in the same microbial matrix provided a platform for bacteria to perform their respective functions synergistically (31). Aerobic granules contain channels and pores that penetrate to a depth of 900 μm below the surface (32), the porosity peaking at depths of 300–500 μm (22). These channels and pores could facilitate the transport of oxygen and nutrients into, and metabolites out of, the granules. Polysaccharide formation peaked at a depth of 400 μm below the granule surface while a layer of dead microbial cells was located at a depth of 800–1000 μm (33). In order to fully utilize the aerobic microorganisms in the granules, the optimal diameter should be less than 1600 μm, which is twice the distance from the granule surface to the anaerobic layer (34). Consequently, smaller granules will be more effective for aerobic wastewater treatment as they have more live cells within a given volume.

2. Biomass Sample Pre-processing for Molecular Analyses

DNA extraction procedures for environmental samples can involve cell extraction or direct lysis, depending on whether or not the microbial cells are isolated from their matrix. The protocol of DNA extraction requires three steps: cell lysis, removal of cell fragments and nucleic acid precipitation and purification. Cell lysis is a particularly critical step in DNA extraction from the environmental matrix. It is designed to release the DNA by breaking the cell wall and membranes of the microorganisms. Cell lysis can be achieved by incubation with detergent and/or with lytic enzymes, either by incubation with guanidium isothiocyanate (35) or by different mechanical treatments. Cell lysis generally combines detergent and lytic enzymes, the most widely applied detergent is sodium dodecyl sulfate (SDS), but sarkosyl is also used (36). The detergent can be used warm or cold to limit the extraction of humic substances. Lysozyme is the most

commonly used enzyme for the hydrolysis of the polysaccharide component of the bacterial cell wall; in addition, some proteases, like proteinase K, achromopeptidase or pronase can also help to liberate nucleic acids. Physical methods, including thermal shocks (repeated freezing and thawing of sample), bead-mill homogenization, bead-beating, microwave heating or the use of ultrasound have also been used for cell disruption. The simplest method, however, is grinding of sample in liquid nitrogen using a mortar and pestle or homogenizing with beads (silica, agate, glass) of various sizes and quantity (37, 38).

Nucleic acid-based methodologies for the monitoring of organisms or particular genes require nucleic acid extracts that are sufficiently free from inhibitory compounds, such as proteins, phenolic compounds, humic acids and heavy metals, to allow reliable PCR amplification, enzyme digestion, hybridization and/or reverse transcription (39 – 41). To this end, a number of nucleic acid purification strategies have been devised (42). However, the application of these strategies can reduce nucleic acid yield and, consequently, devalue the nucleic acids for the study of microbial diversity or environmental genomic analyses. At least four types of purification are commonly used: caesium chloride density gradient ultracentrifugation, chromatography, electrophoresis and dialysis followed by filtration. The efficiency of extraction/purification depends on the properties of the environmental sample, and each step of the extraction procedure must be adjusted for each sample (43). The choice of a protocol must be a compromise between the recovery of DNA that will be the most representative of the microbial community and the quality of the DNA (44). Most DNA extraction procedures focus on extraction and purification of DNA from soils and sediments, not from engineered biological systems. DNA extraction from activated sludge requires treatment to disrupt bacterial cells regardless of their biochemical composition or their localization in the floc. The efficiency of the extraction determines the quantity, quality and diversity of the extracted DNA or RNA. Griffiths et al. (45) described the first direct method for rapid coextraction of RNA and DNA from soil for the comparison of bacterial diversity by 16S rRNA reverse transcription-PCR (RT-PCR) and 16S ribosomal DNA (rDNA)-PCR. The efficacy and reproducibility of the method were confirmed by denaturing gradient gel electrophoresis (DGGE). In related studies, the method has been used to extract total nucleic acid from samples of activated sludge (45). According to Bourrain et al. (46), extraction of DNA from activated sludge samples required the following steps: sample dispersion, cell lysis treatment, DNA extraction purification and quantification. A preliminary dispersion step allowed cell lysis treatment to act more effectively on the bacteria inside the sludge floc structure. A cation-exchange resin (CER) treatment provided

higher DNA levels in sludge samples characterized by high values of organic matter, whereas sonication treatment proved more efficient in activated sludges characterized by poor compaction properties caused by a high density of filamentous bacteria.

There are many published methods for extracting DNA from environmental samples including aerobic granules, several of which have been tested using seeded samples (47 – 51). These studies seem to show that the inoculated bacteria were often more easily lysed than the autochthonous bacteria, so that they were more readily amplified.

The most commonly used technique for visualization and quantification of the microorganisms present in an engineered biological system is fluorescence in situ hybridization (FISH). Prior to FISH analysis, biofilm samples are fixed in 4% paraformaldehyde and frozen at −30°C. From the frozen biofilm samples, biofilm sections with 10 μm(52), 20 μm(53) or 25 μm thicknesses (54) are prepared by vertically cutting using a microslicer at −20°C. Thin sections of biofilm are placed in a hybridization well on a gelatin-coated microscopic slide and the standard hybridization protocol is used. A similar protocol was used by Tsuneda et al. (55) for the preparation of granulated activated sludge samples prior to hybridization.

3. Quantity of Microorganisms and Structure of Microbial Consortia in Wastewater Treatment Bioreactors

To design and control the activated sludge, granulated activated sludge or biofilm systems more efficiently, it is necessary to understand the relationships between the microbial community structure and the process performance (56, 57). Traditional microbiological techniques and conventional microscopy are insufficient to determine the composition, structure, stability, function and activity of bacteria involved in wastewater treatment processes. A wide range of molecular biology tools such as FISH with 16S rRNA-targeted oligonucleotide probes, DGGE or real-time PCR have to be used in order to gain an insight into the complex bacterial communities in engineered systems (53, 56, 58).

3.1. Quantification of Microorganisms

One of the most widely employed techniques for bacterial enumeration is FISH, a technique which allows the identification of microorganisms at any desired taxonomical level depending on the specificity of the probe used (59). Probes used in FISH analysis are short sequences of DNA labelled with a fluorescent dye, which recognize 16S rRNA sequences in the fixed cells and hybridize with them in situ. The majority of FISH applications in wastewater treatment have been directed towards the study of

those microorganisms taking part in the biological elimination of nitrogen and, to a lesser extent, phosphorous (59).

Nitrification, which converts ammonia to nitrite and nitrate, is the initial step of biological nitrogen removal carried out by two phylogenetically independent groups of autotrophic aerobic bacteria, namely ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). Nitrification is the rate-determining process in nitrogen removal. Nitrifying bacteria have slow growth rates and are sensitive to toxic shocks, pH and temperature changes. This explains why many sewage treatment plants fail to establish stable nitrification. Therefore, knowledge of the quantity of nitrifiers and their spatial organization and activities is important to improve treatment performance and process stability (60, 61).

The oldest method for the enumeration of AOB is culture-dependent MPN (most probable number). However, the culturing time is long and there is the possibility of underestimation due to difference of culturability among the species of AOB (2). In order to overcome these limitations, new methods have been developed, which include FISH (2, 62), dot blot hybridization using a 16S rRNA-targeted oligonucleotide probe (62), and an antibody method (63). Work on the detection limits of these methods (MPN: 7×10^4cells/mL, antibody: 1×10^4 cells/mL, FISH: 9×10^3 cells/mL and dot blot hybridization: 1×10^3 cells/mL) has shown that for relatively low ammonium-loaded suspended systems, such as the final effluent of municipal wastewater treatment plants, only dot blot hybridization can give reliable enumeration.

FISH has been successfully applied to quantify AOB in biofilm systems (64) and high ammonium-loading systems (2). Numerous probes used for the identification of Eubacteria, AOB and NOB have been published (65, 66); some examples are given in **Table 11.1**. Some data indicate that counting total numbers of nitrifying bacteria can be hampered because AOB forms dense clusters (67) and NOB cells are relatively small (68). The cluster sizes of ammonia-oxidizing bacteria in autotrophic nitrifying biofilm were found to be larger than those in the domestic wastewater biofilm, probably due to the higher ammonium-loading rate. Therefore, Okabe and Watanabe (68) used the surface fraction of total biomass area and probe-stained cell area (cluster of nitrifiers) to quantitatively characterize the population structure of nitrifying bacteria. A study to quantify, using FISH, the AOB and NOB in the biofilm of a sequence batch reactor (SBR) receiving reject water from sludge dewatering with a high concentration of ammonia (400–500 mg/L) and high salinity (conductivity 5000–6000 μS/cm) showed that ammonia oxidizers occupied a particularly high percentage of the total bacterial cell area (slightly more than 55%), while the combined cell area

Table 11.1
Selected probes used for the identification of Eubacteria and nitrifying bacteria

Probe	Sequence (5′–3′)	Target organisms	Reference
EUB338	GCTGCCTCCCGTAGGAGT	All Eubacteria	(65)
NEU	CCCCTCTGCTGCACTCTA	Halophilic and halotolerant members of the genus *Nitrosomonas*	(62)
Nso190	CGATCCCCTGCTTTTCTCC	Ammonia-oxidizing β-*Proteobacteria*	(62)
Nso1225	CGCGATTGTATTACGTGTGA	Ammonia-oxidizing β-*Proteobacteria*	(62)
Nsm156	TATTAGCACATCTTTCGAT	*Nitrosomonas* C-56, *Nitrosomonas europaea*, *Nitrosomonas eutropha*, and *Nitrosococcus mobilis*	(62)
Nsv443	CCGTGACCGTTTCGTTCCG	*Nitrosolobus multiformis*, *Nitrosospira briensis*, and *Nitrosovibrio tenuis*	(62)
NIT1	CACCTCTCCCGAACTCAA	*Nitrobacter* spp.	(58)
NIT2	CGGGTTAGCGCACCGCCT	*Nitrobacter* spp.	(58)
NIT3	CCTGTGCTCCATGCTCCG	*Nitrobacter* spp.	(58)
Nb1000	TGCGACCGGTCATGG	*Nitrobacter* spp.	(62)
Ntspa712	CGCCTTCGCCACCGGCCTTCC	Phylum *Nitrospira*	(66)
Ntspa662	GGAATTCCGCGCTCCTCT	Genus *Nitrospira*	(66)

of all nitrite-oxidizers was much smaller (about 8%). Among the nitrite-oxidizers, *Nitrospira*-like bacteria were clearly the dominant population, indicating that these bacteria should be regarded as the key organisms of nitrite oxidation in wastewater treatment plants (69).

Many studies deal with nitrifying trickling filters in municipal wastewater treatment plants. Biesterfeld et al. (57) showed that ammonia oxidizers composed as much as 17% of the total biofilm area, which was consistent with previous observations of 5–20% ammonia oxidizers in domestic wastewater systems (70). According to Persson et al. (71), the proportions of AOB varied from 16% to 45% in biofilm. Ballinger et al. (72) showed that the number of AOB depends on the C/N ratio in wastewater, while other work (73) revealed that the number of NOB is determined by free ammonia concentration.

Denitrification in wastewater treatment is the final step of nitrogen elimination following nitrification. Despite significant application of denitrification processes, knowledge about the bacteria involved in the process is relatively limited. An approach

involving 16S rRNA genes is not suitable for the investigation of communities of denitrifying bacteria in wastewater treatment processes because denitrification is widespread among phylogenetically unrelated groups (74). Biological denitrification is a respiratory process defined as the enzymatic, stepwise reduction of nitrogen oxides associated with electron transport phosphorylation and evolution of the gases: nitric oxide (NO), nitrous oxide (N_2O) and molecular nitrogen (N_2) (75). The key step in the process is catalysed by nitrite reductase (76). Two structurally different but functionally equivalent enzymes catalyse nitrite reduction, *nirK* and *nirS*. These genes are useful targets for PCR primers to detect communities of denitrifying bacteria in samples from activated sludge. A combination of DGGE analysis based on 16S rRNA genes and FISH technique (77) showed that the genus of *Thauera* was a major group of denitrifying bacteria in the denitrifying fluidized bed reactor (FBR). Excellent correlation between the *nirS* gene abundance and the denitrifying rates of the FBR was observed throughout the experimental period.

In an enhanced biological phosphate removal (EBPR) process, wastewater is treated in anaerobic–aerobic sequential conditions. During the anaerobic stage, polyphosphate-accumulating organisms (PAO) sequester short-chain organic acids in the wastewater forming intracellular polyhydroxyalkanoates, particularly polyhydroxybutyrate (PHB). Hydrolysis of intracellular polyphosphate provides the energy needed for this sequestration. During the subsequent aerobic stage, PHB is consumed to produce energy for bacterial growth and to restore the polyphosphate pool. As the quantity of phosphate removed from wastewater during the aerobic stage is greater than that released during the anaerobic stage, phosphate is accumulated in bacterial cells and removed from the system along with the waste sludge (78).

Successful operations of EBPR processes require the selection of PAOs over competitive organisms such as glycogen-accumulating organisms under optimal growth conditions, and thus it is important to identify which of these physiological groups are present in these communities (79). There are a number of probes used for the identification of PAOs (80 – 82) (**Table 11.2**).

Wong et al. (83) investigated activated sludge samples taken from different wastewater treatment plants with and without enhanced biological phosphorous removal, using FISH. The study showed that *Rhodocyclus*-related polyphosphate-accumulating organisms (PAOs) and glycogen-accumulating organisms from a gammaproteobacterial lineage GB were the predominant populations detected, representing 4–18% and 10–31% of total cells, respectively. However, there was poor correlation

Table 11.2
Selected probes used for the identification of bacteria responsible for phosphorus removal

Probe	Sequence (5′–3′)	Target organisms	Reference
ACA	ATCCTCTCCCATACTCTA	*Acinetobacter calcoaceticus*	(80)
MP2	GAGCAAGCTCTTCTGAACCG	*Microlunatus phosphorus*	(81)
PAO651	CCCTCTGCCAAACTCCAG	PAO-cluster	(82)
PAO846	GTTAGCTACGGCACTAAAAGG	PAO-cluster	(82)

between *Rhodocyclus*-related PAO population and sludge total phosphorous (TP) content.

Eschenhagen et al. (84) compared the bacterial composition of activated sludge from two laboratory plants with different modes of operation: anoxic/oxic (EBPR, no nitrification) and Phoredox-system (EBPR, nitrification and denitrification). In addition to FISH, they applied terminal restriction fragment length polymorphism analysis and comparative 16S rDNA analysis that indicated highly diverse microbial communities in both plants, suggesting substantial differences in the microbial structure. An enhanced biological phosphorus removal process combined with a biological nitrogen removal process is difficult to achieve because organic compounds expressed as COD are a limiting factor for phosphate release and denitrification at a low C/N ratio. The solution is the introduction of denitrifying phosphate-accumulating organisms (DNPAOs), which are capable of utilizing nitrate as an electron acceptor to simultaneously remove phosphorus and nitrogen from wastewater. A study to determine the presence of DNPAOs in activated sludge in SBRs operated under three different electron acceptor conditions (85) indicated that PAOs, responsible for phosphorus removal, consisted of at least three populations, including DNPAOs, in each reactor, and the microbial community structure was changed depending on the redox conditions.

Real-time PCR is a technique for collecting data, which follows the DNA production during each PCR cycle and quantifies it through a fluorescent reporter signal. This can be achieved using a variety of different fluorescent chemicals that correlate PCR product concentration and fluorescence intensity. Reactions are characterized by the point in time where the target amplification is first detected – this value is usually referred to as the cycle threshold (C_t). The amount of PCR product is positively correlated with the initial amount of target DNA. Real-time PCR assays are so accurate that it is even possible to detect a single copy of a specific transcript (86). Real-time quantification can be divided into two types: absolute and relative quantification (87). Absolute

quantification uses serially diluted standards of known concentrations to generate a standard curve that produces a linear relationship between the C_t value and initial amounts of sample, allowing the determination of unknowns based on their C_t values. In contrast, during relative quantification, changes in sample gene expression are measured based on either an external standard or a reference sample, also known as a calibrator.

The discharge of treated wastewater from wastewater treatment facilities to the surrounding environment can pose a threat to health, so molecular techniques that enable the monitoring of hazardous substances and microorganisms have significant applications. Quantitative real-time PCR can be applied to detect and enumerate bacterial pathogens during municipal wastewater treatment. Using this technique, the number of two enteric pathogens, *Salmonella* spp. and *Campylobacter jejuni*, and two bacteria commonly used as indicators, *Escherichia coli* and *Clostridium perfringens*, was monitored during municipal wastewater treatment and sludge composting. The bacterial taxa behaved differently during the process, and the main differences were observed during biological treatment in the activated sludge basins. *Salmonella* spp. and *C. jejuni* survived better during activated sludge treatment than *E. coli*, with *C. jejuni* being the most resistant to wastewater treatment among the four bacterial groups (88). The study concluded that the use of *E. coli* as an indicator of faecal contamination is debatable because of its different survival capacity in comparison with pathogenic bacteria. There has also been work (89) focused on the development of real-time assays for the quantifiable detection of antibiotic-resistance genes in different municipal wastewater samples (genes *vanA*, *ampC* and *mecA*). Despite the fact that the high bacterial diversity of wastewater represents a complex matrix for the application of molecular biology techniques, the resistance gene *vanA* was detected in 21% of the samples and *ampC* in 78%. The gene *mecA* was not found in municipal wastewater, but in two clinical wastewater samples. This approach has potential for the monitoring of antibiotic-resistant bacteria pathways and observing the fate of resistance genes within natural and engineered biocoenoses.

RT-PCR allows enumeration and investigation of the ecology of specific microorganisms responsible for key processes during wastewater treatment, e.g. ammonium oxidation during nitrification. Limpiyakorn et al. (90), examining activated sludge samples taken from five different sewage treatment systems, showed that the total number of ammonia-oxidizing bacteria (AOB) varied between $3.4\pm1.0\times10^9$–$1.1\pm0.3\times10^{10}$ cell/L, which can account for 0.13–0.33% of the total bacterial population. Moreover, in all systems the majority of the AOB population comprised *Nitrosomonas oligotropha* clusters commonly found in oligotrophic environments, including wastewater treatment

systems with low ammonium load. The study concluded that the low ammonium concentration and the low volumetric ammonia removal in the systems investigated were the main factors supporting the predominance of the *N. oligotropha* cluster.

Quantitative real-time PCR can also be a tool for monitoring the presence of microorganisms that pose a threat to the efficient operation of wastewater treatment plants, e.g. filamentous organisms such as *Microthrix parvicella* that lead to foaming and bulking of activated sludge when they proliferate uncontrollably. The obstructed retrieval of bulking and foaming activated sludge in secondary clarifiers may result in reduced wastewater treatment performance. Kaetzke et al. (91) developed a method for determining the relative frequency of the above-mentioned microorganisms in activated sludge samples by combining real-time PCR with both universal 16S rRNA gene primers and others specific for the *M. parvicella*. They analysed 32 activated sludge samples obtained from different wastewater treatment plants. And found the level of *M. parvicella* 16S rRNA gene copies in non-foaming samples was below 3% of the total number of 16S rRNA gene copies but between 0% and 18% in the 14 samples originating from wastewater treatment plants that displayed foaming and bulking problems. This research offers the opportunity to monitor the amount of *M. parvicella* in wastewater treatment plants and to change the operating strategy before bulking and foaming of activated sludge occurs.

Real-time multiplex PCR is a variant of PCR in which two or more loci are simultaneously amplified in the same reaction. The optimization of the protocol is crucial for the success of the experiment and critical factors, e.g. primer concentration or cycling profile have to be carefully analysed (92). Geets et al. (93) employed real-time PCR for the simultaneous quantification of nitrifying and denitrifying bacteria in activated sludge samples. Simultaneous quantification was possible along the 5-log dynamic range and with a high linear correlation ($R^2 > 0.98$). The abundance of ammonia oxidizers was from 10^6 down to 10^4/mL, whereas nitrite oxidizers were less abundant. As for the denitrification genes (*nirS*, *nirK*, *nosZ*), their abundance was generally in the order of 10^8–10^5/mL. The authors concluded that this approach can be applied as a fast and accurate tool for studying nitrifier and denitrifier community dynamics in engineered environmental samples.

It is worth stressing that the most comprehensive results can be obtained by combining particular techniques. For example, the results of PCR-DGGE and sequencing can be used for designing probes for FISH (56) or FISH and real-time PCR complement each other and, if combined, they give operators and researchers a broader assessment of the activated sludge community (94).

3.2. Spatial Organization of Bacteria

In order to study to understand how microbial aggregates form and how cells interact within them, visualizing and quantifying their spatial organization is required. Manser et al. (95) indicated a method to quantify nitrifying bacteria in activated sludge using FISH and epifluorescence microscopy. Combining FISH with confocal laser scanning microscopy (CLSM) allows the visualization of three-dimensional microbial structures of activated sludge flocs, biofilm and granules (59, 69, 71, 94, 96). Moreover, this technique allows the optical sectioning of samples nondestructively and thus the investigation of the spatial distribution of bacteria in activated sludge or biofilm samples (15, 58, 67).

The use of FISH and CLSM showed that the spatial organization of bacteria depended on the C/N ratio; in domestic wastewater treatment systems where the C/N ratio was high, heterotrophic bacteria occupied the outer part of the biofilm while AOB were distributed in the inner part. As the C/N ratio gradually reduced, nitrifying bacteria began to colonize the outer layer of the biofilm previously occupied by the heterotrophic bacteria (61).

Similarly, Jang et al. (54) showed that the bacterial distributions were significantly changed depending on the aeration. At a dissolved oxygen concentration of 2 mg O_2/L, heterotrophs dominated throughout the whole depth of the biofilm. At 10 mg O_2/L, the numbers of nitrifying bacteria were significantly increased suggesting that when oxygen is not limiting, those microorganisms with a slow growth rate in a mixed autotrophic/heterotrophic biofilm are typically more abundant in deeper sections of the biofilm. However, other work found aerobic ammonium- and nitrite-oxidizing microorganisms (*Nitrosomonas* and *Nitrobacter*) are dominant in the upper aerobic 100–200 μm of the biofilm (97, 98). Similar observations were made in the research of biomass in granules. Colonies of ammonia-oxidizing bacteria existed in the outer parts of the granules (55), with most of the nitrification occurring from the surface to a depth of 300 μm (99).

4. Activity of Particular Groups of Microorganisms in Wastewater Treatment Processes

The great challenge researchers are facing now is to relate the analysis of complex microbial consortia to the metabolic functions of specific groups of bacteria in the bioreactor. Research is increasingly focused on how environmental conditions influence microorganism's specific activity in wastewater treatment processes. The most credible indicator of living cells or a

specific activity in a complex microbial community is mRNA. This is due to the fact that the turnover of mRNA is rapid in living bacterial cells, with most mRNA having a half-life of only a few minutes (100). This information is extremely valuable when studying wastewater treatment processes because it allows differential detection of bacteria that are active in the treatment process, excluding those that are only present in a dormant form.

Relating biological activity to environmental conditions is economically important in the context of wastewater treatment. The application of molecular techniques based on RNA analysis poses an opportunity to identify which microorganisms in the biomass are active at given stages of wastewater treatment. Milner et al. (101) contrasted the presence and activity of the most abundant *Bacteria* in plug flow and completely mixed activated sludge plants. Their presence was detected by amplifying 16S rRNA gene fragments and their activity by amplifying native 16S rRNA using reverse-transcriptase PCR with *Bacteria*-specific primers, the amplified sequences were compared using DGGE. The plug flow plant exhibited a physicochemical gradient with an initial anoxic zone, whilst the two completely mixed reactors did not. Similarities were observed between the DGGE profiles for presence and activity; however, in the plug flow reactor one prominent band was detected in the active population but was absent from the corresponding profile of the 16S rRNA gene. The intensity of this band, identified as a *Nitrosomonas*-like sequence, varied along the physicochemical gradient of the plug flow reactor in a manner that coincided with the growth of AOB and the loss of ammonia.

It is also possible to monitor the activity of microorganisms introduced into activated sludge systems in order to enhance the removal of particular pollutants. Selvaratnam et al. (102) introduced a phenol-degrading bacterium *Pseudomonas putida* ATCC 11172 containing the *dmpN* gene, coding for phenol hydroxylase, an enzyme involved in the conversion of phenol to catechol, to a laboratory-scale SBR reactor. Applying reverse transcriptase PCR, the authors showed that the greatest *dmpN* gene expression was noted 15 min after the maximum concentration of phenol was observed in the reactor and 15 min after the start of aeration. Conversely, decreased phenol concentrations in the reactor corresponded to reduced levels of *dmpN* expression.

The expression of ammonia monooxygenase-encoding mRNA in aggregated sludge from a fluidized bed wastewater treatment system has been successfully used as an indicator of ammonia oxidation activity in order to monitor nitrifying bacteria in engineered systems. The work showed that in batch mode the sudden exposure to ammonia resulted in a quick response (1–2 h) of *amoA* mRNA transcription, while in continuous-feeding operation *amoA* mRNA level dynamically changed in response to the

change in the surrounding environmental conditions (103). Similarly, Cydzik-Kwiatkowska et al. (104) investigated transcription levels of *amoA* mRNA and 16S rRNA during the aeration phase in a sequencing batch reactor, using reverse transcription PCR. Expression of the *amoA* gene reached a detectable level 2 h after the beginning of the aeration phase and did not disappear until its end. Lack of detectable *amoA* expression at the beginning of the aeration phase was in agreement with nitrite concentration decrease. A gradual increase in *amoA* transcript level observed during the following hours indicated a rise in ammonia-oxidizing bacteria activity, and a detectable change in nitrite concentration was observed 2 h after the RT-PCR signal was provided for the first time. Changes in the 16S rRNA transcription level indicated that the metabolic activity of the activated sludge bacterial community increased gradually during the aeration phase.

A study of the expression of the NirS gene by mRNA analysis after shifting an aerobic culture of *Pseudomonas stutzeri* to nitrate respiration indicated that monitoring of mRNA levels allowed important questions to be answered regarding the maintenance and physiological status of a given gene in mixed culture and altering bioreactor operational strategies in order to obtain optimal pollutant removal (105).

Quantification of the signal given by rRNA-targeted oligonucleotides has been shown to allow the estimation of in situ growth rates of individual cells (106). When the majority of cells visualized in activated sludge flocs by staining with the DNA fluorochrome 4′,6-diamidino-2-phenylindole (DAPI) also hybridized with a probe specific for the domain *Bacteria*, this indicated not only in situ dominance of members of this domain but also high cellular ribosome contents and consequently high growth rates and high metabolic activity of the majority of cells. Witzig et al. (107) used the rate of oxygen consumption and cell detectability by FISH to determine the physiological state of the bacterial cells in an aerobic membrane bioreactor treating municipal wastewater. It was found that bacteria present in the highly concentrated biomass of the reactor used the energy supplied for their maintenance metabolism and were not in a physiological state characteristic for growth. This assumption could explain the zero net biomass production observed in the reactor.

5. Diversity and Composition Changes of Bacterial Consortia in Bioreactors

Many wastewater engineers contend that if reactors with activated sludge or other kind of biomass work properly resulting in a high pollutant removal rate, there is no need to apply complex molecular biology tools for detailed evaluation of biomass.

However, others argue that diversity within certain bacterial groups catalysing critical steps in nutrient removal is crucial to improving process stability and efficiency (69). Knowledge of how to increase the diversity of such bacterial groups by changes in the process regime makes it possible to design more robust and reliable wastewater treatment plants. If the microbial communities are confronted by unfavourable conditions, the stability of, for example, the ammonia-oxidation process should be higher in a reactor harbouring a more diverse ammonia-oxidizing community. The presence of many species able to perform the same ecological function increases the probability that a sudden change of environmental conditions will not decrease the effectiveness of wastewater treatment, because at least one of the species will manage to adapt and ensure maintenance of the specific metabolic pathway (108, 109). Taking such functional redundancy into consideration, it is likely that microbial population shifts and diversity measurements are a valuable tool in predicting wastewater treatment efficiency in bioreactors.

The diversity of microorganisms in activated sludge, biofilm or granulated activated sludge depends on the influent wastewater and the technological parameters of the process, e.g. the solids retention time. It is impossible to assess microbial diversity in a system by relying only on conventional microbiological techniques based on the isolation of pure cultures and morphological, metabolic and biochemical assays. Culture media tend to be highly selective for particular groups of bacteria. The application of molecular biology techniques, such as amplified ribosomal DNA restriction analysis (ARDRA), ribosomal intergenic spacer analysis (RISA) terminal restriction fragment length polymorphism (t-RFLP) or denaturing gradient gel electrophoresis (DGGE), to the study of microbial diversity in engineered systems overcomes this problem to some extent.

The studies of Fernandez et al. (110), based on amplified ribosomal DNA restriction analysis, showed that the process stability of methanogenic bioreactors depended on the diversity of functional groups within each trophic level. The authors investigated microbial populations from *Bacteria* and *Archea* in a methanogenic reactor operating over 2 years under constant conditions and with stable performance. The study showed that functional stability did not imply community stability and that an extremely dynamic community could be developed in a simple ecosystem fed with a single simple substrate (glucose). This implies that the dynamics of the microbial community was driven by the large number and diversity of minority populations present in the reactor.

Molecular techniques can be successfully employed for evaluating the impact of different physical or chemical factors on the microbial consortia, for example, the non-thermal effects of

microwave radiation on the biofilm in bioreactors fed with synthetic wastewater (111). In this study, eight trickling bioreactors were used in the experiment, four of which were located inside a microwave-exposed chamber, whereas the other four (control group) were placed in a chamber heated with warm air. The four research columns were irradiated with microwave radiation at 0.01 W/cm^3 of the reactor media. The results showed that microwave radiation positively influenced nitrification and denitrification efficiency (35.9% of nitrogen removal in microwave-radiated reactors versus 19.2% in control reactors), and the analysis of bacterial diversity based on DGGE patterns showed that significantly different communities developed in the reactors exposed to microwave radiation in comparison to the control reactors. Moreover, bacterial richness measured by the Shannon index was significantly higher in the microwave treated samples, suggesting that this biofilm was less prone to environmental disturbances.

It is also possible to observe changes within bacterial communities in time. Cydzik-Kwiatkowska (112) investigated the impact of ammonia and organic carbon load on ammonia-oxidizing bacteria (AOB) community in activated sludge. The experiment was carried out in two SBR reactors differing in ammonia load. Feeding conditions in the reactors were switched from those favouring autotrophic nitrification through to favouring heterotrophic processes and then back to autotrophic conditions. Observations of AOB community changes were based on PCR-RFLP analysis of amplified *amoA* gene fragments. AOB genetic diversity was evaluated on the basis of the number of different *amoA* gene forms. When only carbonates were introduced with wastewater, a stable ammonia-oxidizing bacteria community became established at about day 23 and 28 with an ammonia load of 250 and 130 mg $N\text{-}NH_4/d$, respectively. The diversity of ammonia-oxidizing bacteria was higher at lower ammonia load, and in both reactors a statistically higher diversity of AOB was observed when only carbonates were present in the wastewater in comparison to conditions in which sodium acetate was introduced to the reactors.

There are various plant designs in use for the treatment of wastewater. If two systems work equally efficiently in terms of pollutant removal, molecular biology techniques can be used for the evaluation of microbial community diversity in both systems in order to choose the one supporting the higher diversity. Rowan et al. (113) investigated the diversity of ammonia-oxidizing bacteria consortia in two full-scale treatment reactors, namely a biological aerated filter (BAF) and a trickling filter, each receiving identical wastewater. Diversity was evaluated on the base of DGGE profiles. The community structure of AOB was different in different sections of each of the reactors, and differences were also

noted between the reactors. The BAF reactor harboured a lower detectable diversity of AOB compared with the filter beds that, according to reports from practitioners, suffer less from nitrification failure (114). Cloning and sequencing of 16S rRNA gene fragments from AOB populations revealed that all samples analyzed appeared to be dominated by ammonia-oxidizing bacteria most closely related to *Nitrosococcus mobilis*.

The application of molecular biology techniques allows better understanding of the link between operational parameters and microbial community dynamics in wastewater bioreactors. LaPara et al. (115) studied the effect of temperature on bacterial communities in bioreactors treating pharmaceutical wastewater. The phylogenic diversity of the thermophilic (50–58°C) and mesophilic (28–32°C) bioreactors was studied by PCR-DGGE of the variable V3 region of the 16S rRNA gene and cloning and determination of the nucleotide sequence of nearly complete 16S rRNA genes amplified by PCR. PCR-DGGE results suggested that the bacterial community in mesophilic reactors supported a greater number of bacterial taxa than the thermophilic reactors. These results were confirmed by cloning of 16S rRNA genes. Collector's curves showed that the plateau level from plots of clones found in the mesophilic reactors (>31 unique phylotypes) was much greater than that of the two thermophilic reactors studied (9–10 unique phylotypes). The effects of C/N ratios (C/N 3:1, 5:1, 10:1) on the microbial community structure in wastewater in a suspended carrier biofilm reactor, with simultaneous nitrification and dentrification (SND), have also been studied using DGGE and FISH. The data showed that the total diversity of microbial community structure was positively correlated with C/N ratio, while that of the three communities of ammonia-oxidizing bacteria independent of it. The population of nitrifiers was inversely proportional to the C/N ratio, with the average fraction of AOB and NOB to all bacteria of 5.4, 4.8, 3.1% and 4.6, 3.5, 2.7%, respectively, as the C/N ratio changed from 3:1, 5:1 to 10:1 (116). Such studies demonstrate that molecular methods used to monitor shifts of microbial communities could offer guidance for process optimization and enhanced stability in wastewater engineering.

6. Bioaugmentation

Analysis of microorganisms within bioaugmented reactors is essential in understanding their fate and activity during engineered treatment processes. The availability of DNA probes for a number of degradative genes and the advent of the polymerase chain reaction (PCR) have provided new tools to achieve these aims. Many quantitative studies on bioaugmentation strategies

have been published (117, 118). One of the factors that determine the need for bioaugmentation is the presence or the absence of a bacterial population that is capable of degrading a target pollutant. To date, most bioaugmentation practices have been accomplished by enhancing the growth of indigenous microorganisms or by augmenting the existing microbial population with exogenous microorganisms capable of degrading the pollutant of interest. In bioreactors, conditions can be effectively optimized to achieve maximum microbial degradation of wastes and pollutants by bioaugmentation. However, one of the major constraints of this application is the ability to follow the added microorganism effectively and the ability to predict whether this organism will persist in the treatment system. Selvaratnam et al. (119) demonstrated not only the presence or absence but also the physiological status of added *Pseudomonas putida* by monitoring the *dmpN* gene during phenol degradation in a SBR. The evidence that enhanced phenol biodegradation was a result of microbial action was indicated by the persistence and expression of the *dmpN* gene for over 41 days. The data clearly showed that bacterial consortia monitoring with molecular techniques can successfully assess the state of a bacterium used in bioaugmentation and whether bioaugmentation had been effective in enhancing the degradation of phenol.

It is also possible to use genetically modified microorganisms containing heterologous genes that have been inserted into the chromosome to achieve pollutant removal. These microorganisms can be used for in situ degradation of different mixtures of chloro- and methylphenols in a laboratory-scale sewage plant. For example, Lee et al. (120) constructed a hybrid strain of *P. putida* that had the ability to utilize benzene, toluene, and *p*-xylene simultaneously. The hybrid strain contained genes encoding toluene dioxygenase from the *tod* pathway, and the entire TOL plasmid. Complete degradation of pollutants was achieved without metabolite accumulation.

Under practical conditions, target substances are in most cases mixed with a wide variety of other degradable materials. Many of these materials may be much more attractive to the bioaugmentation culture than the target substances, so they may be biodegraded preferably. In addition, target pollutants are often at a low concentration and occur occasionally in influent, so selection pressure from these pollutants may be too weak to maintain the specific degradation ability of the added bacteria. Comprehensive actions between the supplemented and the indigenous microbial community may also affect the results of bioaugmentation.

Several approaches, such as selection of an inoculum based on a comprehensive knowledge of the indigenous microbial community in the target habitat (121) and a slow release approach (122), have been explored to improve performance of

bioaugmentation. For reliable exploitation in bioaugmentation application, Thompson et al. (123) recently suggested the application of ecological theories for designing bioaugmentation strategies. In ecology, process reliability and stability are assumed to be related to functional redundancy, which is ensured by the presence of a reservoir of species able to perform the ecological function (124). Species are not functionally redundant for an ecosystem process through time (125), for example, one species within a group of *Pseudomonas* could increase in response to the reduction or loss of another taxa under randomly fluctuated environments and fill the functional niches occupied by the sensitive species (126).

The survival of the inoculated microbial culture is critical for successful bioaugmentation, but impossible to predict precisely. As an alternative strategy, bioaugmentation of a group of functionally similar strains may be an effective strategy for the stable performance of biological systems. However, the concern with the implementation of such a strategy is whether functionally similar strains can coexist in biological systems after bioaugmentation in the face of severe inter-strain competition and competitive exclusion. Competitive interaction, however, can be avoided and/or reduced by spatial isolation (127). Even competitively inferior strains could also become established in microbial communities in heterogeneous environments. Jiang et al. (50) evaluated simultaneous bioaugmentation of two functionally similar bacterial strains in aerobic granules. FISH and DGGE showed that the two strains could coexist in aerobic granules but not in activated sludge. These findings suggested that the compact structure of aerobic granules provided spatial isolation for coexistence of competitively superior and inferior strains with similar functions. Qu et al. (128) isolated *Sphingomonas xenophaga* QYY with the ability to degrade bromoamine acid (BAA) from sludge samples. In order to estimate the relationship between community dynamics and the function of augmented activated sludge, a combined method based on fingerprints (RISA) and 16S rRNA gene sequencing was used. The results indicated that the microbial community dynamics were substantially changed, and the introduced QYY strain was persistent in the augmented systems. Similarly, Fouratt et al. (129) demonstrated how community profiling of an undefined microbial population via temperature gradient gel electrophoresis (TGGE) can be used to examine the biological importance of organisms isolated from the same mixed population. They demonstrated that in biomass as well as in genomic DNA spiking experiments, a nitrifying bioaugmentation product (NBP) had to be present at a level of at least 5% of the total bacterial population in order to be detected whereas bioaugmentation at 1% of the total population was enough to yield significant improvements in nitrification efficiency.

References

1. Yuan, Z. and Blackall, L.L. (2002) Sludge population optimization: a new dimension for the control of biological wastewater treatment systems. *Water Res.* **36**, 482–490.
2. Wagner, M., Rath, G., Amann, R., Koops, H.P. and Schleifer, K.H. (1995) In situ identification of ammonia-oxidizing bacteria. *Syst. Appl. Microbiol.* **18**, 251–64.
3. Lee, N., La Cour Janssen, J., Aspegren, H., Henze, M.N.P.H. and Wagner, M. (2002) Population dynamics in wastewater treatment plants with enhanced biological phosphorus removal operated without nitrogen removal. *Water Sci. Technol.* **46**, 163–170.
4. Schmid, M., Thill, A., Purkhold, U., Walcher, M., Bottero, J.Y., Ginestet, P., Nielsen, P.H., Wuertz, S. and Wagner, M. (2003) Characterization of activated sludge flocs by confocal laser scanning microscopy and image analysis. *Water Res.* **37**, 2043–2052.
5. Wilén, B.M., Onuki, M., Hermanssonc, M., Lumleyd, D. and Minoa, T. (2008) Microbial community structure in activated sludge floc analysed by fluorescence in situ hybridization and its relation to floc stability. *Water Res.* **42**, 2300–2308.
6. Morgan-Sagastume, F. and Allen, D.G. (2003) Effects of temperature transient conditions on aerobic biological treatment of wastewater. *Water Res.* **37**, 3590–3601.
7. Henriques, I.D.S. and Love, N.G. (2007) The role of extracellular polymeric substances in the toxicity response of activated sludge bacteria to chemical toxins. *Water Res.* **41**, 4177–4185.
8. Frølund, B., Palmgren, R., Keiding, K. and Nielsen, P.H. (1996) Extraction of extracellular polymers from activated sludge using a cation exchange resin. *Water Res.* **30**, 1749–1758.
9. Laspidou, C.S. and Rittmann, B.E. (2002) A unified theory for extracellular polymeric substances, soluble microbial products, and active and inert biomass. *Water Res.* **36**, 2711–2720.
10. Raszka, A., Chorvatova, M. and Wanner, J. (2006) The role and significance of extracellular polymers in activated sludge. Part I: literature review. *Acta Hydrochim. Hydrobiol.* **34**, 411–424.
11. Wilén, B.M., Nielsen, J.L., Keiding, K. and Nielsen P.H. (2000) Influence of microbial activity on the stability of activated sludge flocs, *Colloid Surface B.* **18**, 145–156.
12. Jorand, F., Zartarian, F., Thomas, F., Block, J.C., Bottero, J.Y., Villemin, G., Urbain, V. and Manem, J. (1995) Chemical and structural (2D) linkage between bacteria within activated sludge flocs. *Water Res.* **29**, 1639–1647.
13. Wilén, B.M., Onuki, M., Hermansson, M., Lumley, D. and Mino, T. (2006) Influence of flocculation and settling properties of activated sludge in relation to secondary settler performance. *Water Sci. Technol.* **54**, 147–155.
14. Nancharaiah, Y.V., Joshi, H.M., Krishna Mohan, T.V., Venugopalan, V.P. and Narasimhan, S.V. (2008) Formation of aerobic granules in the presence of a synthetic chelating agent. *Environ. Pollut.* **153**, 37–43.
15. Wijeyekoon, S., Mino, T., Satoh, H. and Matsuo, T. (2000) Growth and novel structural features of tubular biofilms produced under different hydrodynamic conditions. *Water Sci. Technol.* **41**, 4–5, 129–138.
16. de Beer, D. and Stoodley, P. (1995) Relation between the structure of an aerobic biofilm and transport phenomena. *Water Sci. Technol.* **32**, 8, 11–18.
17. van Loosdrecht, M., Eikelboom, D., Gjaltema, A., Mulder, A., Tijhuis, L. and Heijnen, J.J. (1995) Biofilm structures. *Water Sci. Technol.* **32**, 8, 35–43.
18. Vieira, M., Melo, L. and Pinheiro, M. (1993) Biofilm formation: hydrodynamic effects on internal diffusion and structure. *Biofouling.* **7**, 67–80.
19. Wuertz, S., Okabe, S. and Hausner, M. (2004) Microbial communities and their interactions in biofilm systems: an overview. *Water Sci. Technol.* **49**, 11–12, 327–336.
20. Nicolella, C., van Loosdrecht, M.C.M. and Heijnen, S.J. (2000) Particle-based biofilm reactor technology. *Trends Biotechmol.* **18**, 312–320.
21. Zielińska, M. and Wojnowska-Barył a, I. (2007) Nitrification by biomass immobilized in porous carriers. *J. Environ. Eng. Sci.* **6**, 463–467.
22. Tay, J.H., Tay, S.T.L., Ivanov, V., Pan, S. and Liu, Q.S. (2003) Biomass and porosity profile in microbial granules sued for aerobic wastewater treatment. *Lett. Appl. Microbiol.* **36**, 297–301.
23. Moy, B.Y.P., Tay, J.H., Toh, S.K., Liu, Y. and Tay, S.T.L. (2002) High organic loading influences the physical characteristics of aerobic sludge granules. *Lett. Appl. Microbiol.* **34**, 407–412.

24. Beun, J.J., Hendriks, A., van Loosdrecht, M., Morgenroth, E., Wilderer, P.A. and Heijnen, J.J. (1999) Aerobic granulation in a sequencing batch reactor. *Water Res.* **33**, 2283–2290.
25. Limbergen, H.V., Top, E.M. and Verstraete, W. (1998) Bioaugmentation in activated sludge: current features and future perspectives. *Appl. Microbiol. Biot.* **50**, 16–23.
26. Ivanov, V., Tay, J.H., Tay, S.T.L. and Jiang, H.L. (2004) Removal of microparticles by microbial granules used for aerobic wastewater treatment. *Water Sci. Technol.* **50**, 147–154.
27. Tay, S.T.L., Moy, B.Y.P., Jiang, H.L. and Tay, J.H. (2005) Rapid cultivation of stable aerobic phenol-degrading granules using acetate-fed granules as microbial seed. *J. Biotechnol.* **115**, 387–395.
28. Liu, Y., Yang, S.F. and Tay, J.H. (2004) Improved stability of aerobic granules by selecting slow-growing nitrifying bacteria. *J. Biotechnol.* **108**, 161–169.
29. Nancharaiah, Y.V., Schwarzenbeck, N., Mohan, T.V.K., Narasimhan, S.V., Wilderer, P.A. and Venugopalan, V.P. (2006) Biodegradation of nitrilotriacetic acid (NTA) and ferrice NTA complex by aerobic microbial granules. *Water Res.* **40**, 1539–1546.
30. Ivanov, V., Wang, X.H., Tay, S.T.L. and Tay, J.H. (2006) Bioaugmentation and enhanced formation of microbial granules used in aerobic wastewater treatment. *Appl. Microbiol. Biot.* **70**, 374–381.
31. Qin, L. and Liu, Y. (2006) Aerobic granulation for organic carbon and nitrogen removal in alternating aerobic-anaerobic sequencing batch reactor. *Chemosphere.* **63**, 926–933.
32. Liu, Y. and Tay, J.H. (2004) State of the art of biogranulation technology for wastewater treatment. *Biotechnol. Adv.* **22**, 533–563.
33. Tay, S.T.L., Ivanov, V., Yi, S., Zhuang, W.Q. and Tay, J.H. (2002) Presence of anaerobic Bacteroides in aerobically grown microbial granules. *Microbial. Ecol.* **44**, 278–285.
34. Toh, S.K., Tay, J.H., Moy, B.Y.P., Ivanov, V. and Tay, S.T.L. (2003) Size-effect on the physical characteristics of the aerobic granule in a SBR. *Appl. Microbiol. Biot.* **60**, 687–695.
35. Porteous, L.A., Seidler, R.J. and Watrud, L.S. (1997) An improved method for purifying DNA from soil for polymerase chain reaction amplification and molecular ecology applications. *Mol. Ecol.* **6**, 787–791.
36. Carrigg, C., Rice, O., Kavanagh, S., Collins, G. and O'Flaherty, V. (2007) DNA extraction method affects microbial community profiles from soils and sediment. *Appl. Microbiol. Biot.* **77**, 955–964.
37. Kuske, C.R., Banton, K.L., Adorada, D.L., Stark, P.C., Hill, K.K. and Jackson, P.J. (1998) Small-scale DNA sample preparation method for field PCR detection of microbial cells and spores in soil. *Appl. Environ. Microbiol.* **64**, 2463–2472.
38. Miller, D.N., Bryant, J.E., Madsen, E.L. and Ghiorse, W.C. (1999) Evaluation and optimisation of DNA extraction and purification procedures for soil and sediment samples. *Appl. Environ. Microbiol.* **65**, 4715–4724.
39. Ogram, A. (2000) Soil molecular microbial ecology at age 20: methodological challenges for the future. *Soil Biol. Biochem.* **32**,1499–1504.
40. Niemi, R.M., Heiskanen, I., Wallenius, K. and Lindstrom, K. (2001) Extraction and purification of DNA in rhizosphere soil samples for PCR-DGGE analysis of bacterial consortia. *J. Microbiol. Meth.* **45**, 155–165.
41. Kemp, B.M., Monroe, C. and Smith, D.G. (2006) Repeat silica extraction: a simple technique for the removal of PCR inhibitors from DNA extracts. *J. Archaeol. Sci.* **33**, 1680–1689.
42. Roose-Amsaleg. C.L., Garnier-Sillam. E. and Harry, M. (2001) Extraction and purification of microbial DNA from soil and sediment samples. *Appl. Soil Ecol.* **18**, 47–60.
43. Thakuria, D., Schmidt, O., Mac Siurtain, M., Egan, D. and Doohan, F.M. (2008) Importance of DNA quality in comparative soil microbial community structure analyses. *Soil Biol. Biochem.* **40**, 1390–1403.
44. de Lipthay, J.R, Enzinger, C., Johnsen, K., Aamanda, J. and Sø rensen, S.J. (2004) Impact of DNA extraction method on bacterial community composition measured by denaturing gradient gel electrophoresis. *Soil Biol. Biochem.* **36**, 1607–1614.
45. Griffiths, R.I., Whiteley, A.S. and O'Donnell, A.G. (2000) Rapid method for coextraction of DNA and RNA from natural environments for analysis of ribosomal DNA- and rRNA-based microbial community composition. *Appl. Environ. Microbiol.* **66**, 5488–5491.
46. Bourrain, M., Achouak, W., Urbain, V. and Heulin, T. (1999) DNA extraction from activated sludges. *Curr. Microbiol.* **38**, 315–319.
47. Bej, A.K., Mahbubani, M.H., Dicesare, J.L. and Atlas, R.M. (1991) Polymerase chain reaction-gene probe detection of microorganisms by using filter concentrated samples. *Appl. Environ. Microbiol.* **57**, 3529–3534.
48. 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.

49. Quan, H., Shi, H., Liu, H., Lv, P. and Qian, Y. (2004) Enhancement of 2,4-dichlorophenol degradation in conventional activated sludge systems bioaugmented with mixed special culture. *Water Res.* **38**, 245–253.
50. Jiang, H.L., Maszenan, A.M., Tay, J.H. (2007) Bioaugmentation and coexistence of two functionally similar bacterial strains in aerobic granules. *Appl. Microbiol. Biotechnol.* **75**, 1191–1200.
51. Adav, S.S., Chen, M.Y., Lee, D.J. and Ren, N.Q. (2007) Degradation of phenol by aerobic granules and isolated yeast *Candida tropicalis*. *Biotechnol. Bioeng.* **96**, 5, 844–852.
52. Koch, G., Egli, K., van der Meer, J.R. and Siegrist, H. (2000) Mathematical modeling of autotrophic denitrification in a nitrifying biofilm of a rotating biological contactor. *Water Sci. Technol.* **41**, 4–5, 191–198.
53. Tsuneda, S., Miyoshi, T., Aoi, Y. and Hirata A. (2000) Tailoring of highly efficient nitrifying biofilms in fluidized bed for ammonia-rich industrial wastewater treatment. *Water Sci. Technol.* **42**, 3–4, 357–362.
54. Jang, A., Bishop, P.L., Okabe, S., Lee, S.G. and Kim I.S. (2002) Effect of dissolved oxygen concentration on the biofilm and in situ analysis by fluorescence in situ hybridization (FISH) and microelectrodes. *Water Sci. Technol.* **47**, 1, 49–57.
55. Tsuneda, S., Nagano, T., Hoshino, T., Ejiri, Y., Noda, N. and Hirata, A. (2003) Characterization of nitrifying granules produced in an aerobic upflow fluidized bed reactor. *Water Res.* **37**, 4965–4973.
56. Onuki, M., Satoh, H., Mino, T. and Matsuo, T. (2000) Application of molecular methods to microbial community analysis of activated sludge. *Water Sci. Technol.* **42**, 3–4, 17–22.
57. Biesterfeld, S., Figueroa, L., Hernandez, M. and Russell P. (2001) Quantification of nitrifying bacterial populations in a full-scale nitrifying trickling filter using fluorescent in situ hybridization. *Water Environ. Res.* **73**, 3, 329–338.
58. Wagner, M., Rath, G., Koops, H.P., Flood, J. and Amann, R. (1996) *In-situ* analysis of nitrifying bacteria in sewage treatment plants. *Water Sci. Technol.* **34**, 1–2, 237–244.
59. Sanz, J.L. and Köchling, T. (2007) Molecular biology techniques used in wastewater treatment: An overview. *Process Biochem.* **42**, 119–133.
60. Konuma, S., Satoh, H., Mino, T. and Matsuo, T. (2001) Comparison of enumeration methods for ammonia-oxidizing bacteria. *Water Sci. Technol.* **43**, 1, 107–114.
61. Aoi, Y. (2002) In situ identification of microorganisms in biofilm communities. *J. Biosci. Bioeng.* **94**, 6, 552–556.
62. Mobarry, B.K., Wagner, M., Urbain, V., Rittmann, B.E. and Stahl, D.A. (1996) Phylogenetic probes for analyzing abundance and spatial organization of nitrifying bacteria. *Appl. Environ. Microbiol.* **62**, 6, 2156–2162.
63. Takai, T., Niioka, H., Gao, Y., Matsumura, M., Inamori,Y. and Hirata, A. (1997) Development of rapid detection and quantification methods for nitrifying bacteria using monoclonal antibodies. *J. Jap. Soc. Wat. Environ.* **20**, 5, 318–323.
64. Schramm, A., Beer, D., Wagner, M. and Amann, R. (1998) Identification and activities in situ of *Nitrosospira* and *Nitrospira* spp. a dominant populations in a nitrifying fluidized bed reactor. *Appl. Environ. Microbiol.* **64**, 9, 3480–3485.
65. Wagner, M., Amann, R., Lemmer, H. and Schleifer, K.H. (1993) Probing activated sludge with oligonucleotides specific for Proteobacteria: inadequacy of culture-dependent methods for describing microbial community structure. *Appl. Environ. Microbiol.* **59**, 5, 1520–1525.
66. Daims, H., Nielsen, P.H., Nielsen, J.L., Juretschko, S. and Wagner, M. (2000) Novel Nitrospira-like bacteria as dominant nitrite-oxidizers in biofilms from wastewater treatment plants: diversity and in situ physiology. *Water Sci. Technol.* **41**, 4–5, 85–90.
67. Satoh, H., Okabe, S., Norimatsu, N. and Watanabe, Y. (2000) Significance of substrate C/N ratio on structure and activity of nitrifying biofilms determined by in situ hybridization and the use of microelectrodes. *Water Sci. Technol.* **41**, 4–5, 317–321.
68. Okabe, S. and Watanabe, Y. (2000) Structure and function of nitrifying biofilms as determined by in situ hybridization and the use of microelectrodes. *Water Sci. Technol.* **42**, 12, 21–32.
69. Daims, H., Purkhold, U., Bjerrum, L., Arnold, E., Wilderer, P.A. and Wagner, M. (2001) Nitrification in sequencing biofilm batch reactors: lessons from molecular approaches. *Water Sci. Technol.* **43**, 3, 9–18.
70. Okabe, S., Satoh, H. and Watanabe, Y. (1999) *In situ* analysis of nitrifying biofilms as determined by in situ hybridization and the use of microelectrodes. *Appl. Environ. Microbiol.* **65**, 3182–3191.
71. Persson, F., Wik, T., Sö rensson, F. and Hermansson, M. (2002) Distribution and activity of ammonia oxidizing bacteria in a large full-scale trickling filter. *Water Res.* **36**, 1439–1448.

72. Ballinger, S.J., Head, I.M., Curtis, T.P. and Godley, A.R. (2002) The effect of C/N ratio on ammonia oxidizing bacteria community structure in a laboratory nitrification-denitrification reactor. *Water Sci. Technol.* 46, 1–2, 543–550.
73. Simm, R.A., Ramey, W.D. and Mavinic, D.S. (2005) Nitrifier population dynamics in a bench-scale conventional activated sludge reactor following an induced perturbation. *J. Environ. Eng. Sci.* **4**, 385–397.
74. Michotey, V., Mejean, V. and Bonin, P. (2000), Comparison of methods for quantification of cytochrome cd1-denitrifying bacteria in environmental marine samples. *Appl. Environ. Microbiol.* **66**, 1564–1571.
75. Zumft, W.G. (1997) Cell biology and molecular basis of denitrification. *Microbiol. Mol. Biol. Rev.* **61**, 533–616.
76. Liu, X., Tiquia, S.M., Holguin, G., Wu, L., Nold, S.C., Devol, A.H., Luo, K., Palumbo, A.V., Tiedje, J.M. and Zhou, J. (2003) Molecular diversity of denitrifying genes in continental margin sediments within the oxygen-deficient zone off the Pacific Coast of Mexico. *Appl. Environ. Microbiol.* **69**, 3549–3560.
77. Araki, N., Tsukamoto, Y., Nagano, A., Yamaguchi, T. and Harada, H. (2006) Real-time PCR quantification of nitrite reductase (*nirS*) genes in a nitrogen removing fluidized bed reactor. *Water Sci. Technol.* **53**, 6, 59–65.
78. Liu, Y., Zhang, T., Herbert, H.P. and Fang, H.H.P. (2005) Microbial community analysis and performance of a phosphate-removing activated sludge. *Bioresource Technol.* **96**, 1205–1214.
79. Seviour, R.J., Mino, T. and Onuki, M. (2003) The microbiology of biological phosphorous removal in activated sludge systems. *FEMS Microbiol. Rev.* **27**, 99–127.
80. Wagner, M., Erhart, R., Manz, W., Amann, R., Lemmer, H., Wedi, D. and Schleifer, K.H. (1994) Development of an rRNA-targeted oligonucleotide probe specific for the genus *Acinetobacter* and its application for in situ monitoring in activated sludge. *Appl. Environ. Microbiol.* **60**, 3, 792–800.
81. Kawaharasaki, M., Takahiro, K., Tanaka, H. and Nakamura, K. (1998) Development and application of 16S rRNA-targeted oligonucleotide probe for detection of phosphate-accumulating bacterium *Microlunatus phosphorus* in the enhanced biological phosphorus removal process. *Water Sci. Technol.* **37**, 4–5, 481–484.
82. Crocetti, G.R., Hugenholtz, P., Bond, P.L., Schuler, A., Keller, J., Jenkins, D. and Blackall, L.L. (2000) Identification of polyphosphate-accumulating organisms and design of 16S rRNA-directed probes for their detection and quantitation. *Appl. Environ. Microbiol.* **66**, 3, 1175–1182.
83. Wong, M.T., Minob, T., Seviourc, R.J., Onukib, M. and Liu, W.T. (2005) In situ identification and characterization of the microbial community structure of full-scale enhanced biological phosphorous removal plants in Japan. *Water Res.* **39**, 2901–2914.
84. Eschenhagen, M., Schuppler, M. and Röske, I. (2003) Molecular characterization of the microbial community structure in two activated sludge systems for the advanced treatment of domestic effluents. *Water Res.* **37**, 3224–3232.
85. Ahn, J., Daidou, T., Tsuneda, S. and Hirata, A. (2002) Characterization of denitrifying phosphate-accumulating organisms cultivated under different electron acceptor conditions using polymerase chain reaction-denaturing gradient gel electrophoresis assay. *Water Res.* **36**, 403–412.
86. Palmer, S., Wiegand, A.P., Maldarelli, F., Bazmi, H., Mican, J.M., Polis, M., Dewar, R.L. and Planta, A. (2003) New real-time reverse transcriptase-initiated PCR assay with single-copy sensitivity for human immunodeficiency virus type 1 RNA in plasma. *J. Clin. Microbiol.* **41**, 4531–4536.
87. Wong, M.L. and Medrano, J.F. (2005) Real-time for mRNA quantitation. *BioTechniques.* **39**, 1, 1–10.
88. Wéry, N., Lhoutellier, C., Ducray, F., Delgenés, J.P. and Godon, J.J. (2008) Behaviour of pathogenic and indicator bacteria during urban wastewater treatment and sludge composting, as revealed by quantitative PCR. *Water Res.* **42**, 1–2, 53–62.
89. Volkmann, H., Schwartz, T., Bischoff, P., Kirchen, S. and Obst, U. (2004) Detection of clinically relevant antibiotic-resistance genes in municipal wastewater using real-time PCR (TaqMan). *J. Microbiol. Meth.* **56**, 277–286.
90. Limpiyakorn, T., Kurisu, F. and Yagi, O. (2006) Development and application of real-time PCR for quantification of specific ammonia-oxidizing bacteria in activated sludge of sewage treatment plants. *Appl. Genet. Mol. Biotechnol.* **7**, 1004–1013.
91. Kaetzke, A., Jentzisch, D. and Eschrich, K. (2005) Quantification of *Microthrix parvicella* in activated sludge bacterial communities by real-time PCR. *Lett. Appl. Microbiol.* **40**, 207–211.
92. Henegariu, O., Heerema, N.A., Dlouhy, S.R., Vance, G.H. and Vogt, P.H. (1997) Multiplex PCR: critical parameters and

step-by-step protocol. *BioTechniques.* **23**, 3, 504–511
93. Geets, J., de Cooman, M., Wittebolle, L., Heylen, K., Vanparys, B., de Vos, P., Verstraete, W. and Boon, N. (2007) Real-time PCR assay fort he simultaneous quantification of nitrifying and denitrifying bacteria in activated sludge. *Appl. Microbiol. Biotechnol.* **75**, 211–221.
94. Hill, S.J., Keller, J. and Blackall, L.L. (2003) Microbial quantification in activated sludge: the hits and misses. *Water Sci. Technol.* **48**, 3, 121–126.
95. Manser, R., Muche, K., Gujer, W. and Siegrist, H. (2005) A rapid method to quantify nitrifiers in activated sludge. *Water Res.* **39**, 1585–1593.
96. Gieseke, A., Arnz, P., Amann, R. and Schramm, A. (2002) Simultaneous P and N removal in a sequencing batch biofilm reactor: insights from reactor- and microscale investigations. *Water Res.* **36**, 501–509.
97. Jang, A., Okabe, S., Watanabe, Y., Kim, I.S. and Bishop, P.L. (2005) Measurement of growth rate of ammonia oxidizing bacteria in partially submerged rotating biological contactor by fluorescent in situ hybridization (FISH). *J. Environ. Eng. Sci.* **4**, 413–420.
98. Koch, G., Egli, K., van der Meer, J.R. and Siegrist, H. (2000) Mathematical modeling of autotrophic denitrification in a nitrifying biofilm of a rotating biological contactor. *Water Sci. Technol.* **41**, 4–5, 191–198.
99. Jang, A., Yoon, Y.H., Kim, I.S., Kim, K.S. and Bishop, P.L. (2003) Characterization and evaluation of aerobic granules in sequencing batch reactor. *J. Biotechnol.* **105**, 71–82.
100. Alifano, P., Bruni, C.B. and Carlomagno, M.S. (1994) Control of mRNA processing and decay in prokaryotes. *Genetica*. **94**, 157–172.
101. Milner, M.G., Curtis, T.P. and Davenport, R.J. (2008) Presence and activity of ammonia-oxidising bacteria detected amongst the overall bacterial diversity along a physio-chemical gradient of wastewater treatment plant. *Water Res.* **42**, 2863–2872.
102. Selvaratnam, S., Schoedel, B.A., McFarland, B.L. and Kulpa, C.F. (1995) Application of reverse transcriptase PCR for monitoring expression of catabolic *dmpN* gene in a phenol-degrading sequencing batch reactor. *Appl. Environ. Microbiol.* **61**, 11, 3981–3985.
103. Aoi, Y., Shiramasa, Y., Masaki, Y., Tsuneda, S., Hirata, A., Kitayama, A. and Nagamune, T. (2004) Expression of *amoA* mRNA in wastewater treatment processes examined by competitive RT-PCR. *J. Biotechnol.* **111**, 2, 111–120.
104. Cydzik-Kwiatkowska, A., Ciesielski, S. and Wojnowska-Baryła, I. (2007) Bacterial *amoA* and *16S rDNA* genes expression in activated sludge during SBR cycle. *Pol. J. Nat. Sci.* **22**, 246–255.
105. Hartig, E. and Zumft, W.G. (1999) Kinetics of *nirS* expression (cytochrome cd_1 nitrite reductase) in *Pseudomonas stutzeri* during the transition from aerobic respiration to denitrification: evidence for a denitrification-specific nitrate- and nitrite-responsive regulatory system. *J. Bacteriol.* **181**, 161–166.
106. Amann, R.I., Ludwig, W. and Schleifer, K.H. (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59**, 1, 143–169.
107. Witzig, R., Manz, W., Rosenberger, S., Krüger, U., Kraume, M. and Szewzyk, U. (2002) Microbiological aspects of a bioreactor with submerged membranes for aerobic treatment of municipal wastewater. *Water Res.* **36**, 394–402.
108. Briones, A. and Raskin, L. (2003) Diversity and dynamics of microbial communities in engineered environments and their implications for process stability. *Curr. Opin. Biotech.* **14**, 270–276.
109. LaPara, T.M., Nakatsu, C.H., Pantea, L.M. and Alleman, J.E. (2002) Stability of the bacterial communities supported by a seven-stage biological process treating pharmaceutical wastewater as revealed by PCR-DGGE. *Water Res.* **36**, 638–646.
110. Fernandez, A., Huang, S., Seston, S., Xing, J., Hickey, R.F., Criddle, C. and Tiedje, J. (1999) How stable is stable? Function versus community stability. *Appl. Environ. Microbiol.* **65**, 3697–3704.
111. Zieliński, M., Ciesielski, S., Cydzik-Kwiatkowska, A., Turek, J. and Dębowski, M. (2007) Influence of microbial radiation on bacterial community structure in biofilm. *Process Biochem.* **42**, 1250–1253.
112. Cydzik-Kwiatkowska, A. (2006) Examination of autotrophic nitrification in activated sludge with the use of PCR technique. Doctoral thesis (in Polish).
113. Rowan, A.K., Snape, J.R., Fearnside, D., Barer, M.R., Curtis, T.P. and Head, I.M. (2003) Composition and diversity of ammonia-oxidizing bacterial communities in wastewater treatment reactors of different design treating identical wastewater. *FEMS Microbiol. Ecol.* **43**, 195–206.
114. Dolan, J.F., O' Neill, M.J. and Horan, N.J. (1990) The options available for

ammonia removal at Davyhulme sewage-treatment works. *J. Inst Water Environ. Manage.* **4**, 457–468.
115. LaPara, T.M., Nakatsu, C.H., Pantea, L. and Alleman, J.E. (2000) Phylogenetic analysis of bacterial communities in mesophilic and thermophilic bioreactors treating pharmaceutical wastewater. *Appl. Environ. Microbiol.* **66**, 9, 3951–3959.
116. Xia, S., Li, J. and Wang, R. (2008) Nitrogen removal performance and microbial community structure dynamics response to carbon nitrogen ratio in a compact suspended carrier biofilm reactor. *Ecol. Eng.* **32**, 256–262.
117. Stackebrandt, E. and Goebel, B.M. (1994) Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. *Int. J. Syst. Bacteriol.* **44**, 846–849.
118. van Elsas, J.D., Duarte, G.F., Rosado, A.S. and Smalla, K. (1998) Microbiological and molecular biological methods for monitoring microbial inoculants and their effects in the soil environment. *J. Microbiol. Meth.* **32**, 133–154.
119. Selvaratnam, S., Schoedel, B.A., McFarland, B.L. and Kulpa, C.F. (1997) Application of the polymerase chain reaction (PCR) and reverse transcriptase / PCR for determining the fate of phenol-degrading *Pseudomonas putida* ATCC 11172 in a bioaugmented sequencing batch reactor. *Appl. Microbiol. Biotechnol.* **47**, 236–240.
120. Lee, J.Y., Jung, K.H., Choi, S.H. and Kim, H.S. (1995) Combination of the *tod* and the *tol* pathways in redesigning a metabolic route of *Pseudomonas putida* for the mineralisation of a benzene, toluene and p-xylene mixture. *Appl. Environ. Microbiol.* **61**, 2211–2217.
121. van der Gast, C.J., Whiteley, A.S. and Thompson, I.P. (2004) Temporal dynamics and degradation activity of an bacterial inoculum for treating waste metal-working fluid. *Environ. Microbiol.* **6**, 254–263.
122. Boon, N., Goris, J., de Vos, P., Verstraete, W. and Top, E.M. (2000) Bioaugmentation of activated sludge by an indigenous 3-chloroaniline-degrading *Comamonas testosterone* strain I2gfp. *Appl. Environ. Microbiol.* **66**, 2906–2913.
123. Thompson, I.P., van der Gast, C.J., Ciric, L. and Singer, A.C. (2005) Bioaugmentation for bioremediation: the challenge of strain selection. *Environ. Microbiol.* **7**, 909–915.
124. Naeem, S. and Li, S.B. (1997) Biodiversity enhances ecosystem reliability. *Nature.* **390**, 507–509.
125. Loreau, M., Naeem, S., Inchausti, P., Bengtsson, J., Grime, J.P., Hector, A., Hooper, D.U., Huston, M.A., Raffaelli, D., Schmid, B., Tilman, D. and Wardle, D.A. (2001) Biodiversity and ecosystem functioning. *Microbiol.* **68**, 326–334.
126. Ellis, R.J., Thompson, I.P. and Bailey, M.J. (1999) Temporal fluctuations in the *Pseudomonas* population associated with sugar beet leaves. *FEMS Microbiol. Ecol.* **28**, 345–356.
127. Zhou, J.Z., Xia, B.C., Treves, D.S., Wu, L.Y., Marsh, T.L., O' Neill, R.V., Palumbo, A.V. and Tiedje, J.M. (2002) Spatial and resource factors influencing high microbial diversity in soil. *Appl. Environ.* **68**, 326–334.
128. Qu, Y., Zhou, J., Wang, J., Fu, X. and Xing, L. (2005) Microbial community dynamics in bioaugmented sequencing batch reactors for bromoamine acid removal. *FEMS Microbiol. Lett.* **246**, 143–149.
129. Fouratt, M.A., Rhodes, J.S., Smithers, C.M., Love, N.G. and Stevens, A.M. (2003) Application of temperature gradient gel electrophoresis to the characterization of a nitrifying bioaugmentation product. *FEMS Microbiol. Ecol.* **43**, 277–286.

Chapter 12

Plasmid-Mediated Bioaugmentation of Wastewater Microbial Communities in a Laboratory-Scale Bioreactor

Stephan Bathe and Martina Hausner

Abstract

Xenobiotic degradation during biological wastewater treatment can be established or enhanced by bioaugmentation – the addition of biological agents carrying biodegradation genes required to perform the task. Whereas the addition of microbial cells carrying chromosomally encoded catabolic genes can be impaired by limited survival of the added microorganisms, the addition of donor organisms carrying a transmissible catabolic plasmid is a promising alternative. This plasmid can spread within the indigenous microbial community of the system, circumventing the need for extended survival of the introduced bacterial strain. Here we discuss how the catabolic plasmid pNB2 can be evaluated towards its potential to facilitate the degradation of a xenobiotic compound, 3-chloroaniline, and demonstrate the applicability of this plasmid to accomplish 3-chloroaniline degradation in a bioreactor setting after in situ transfer to suitable recipient strains.

Key words: Catabolic plasmid, *Pseudomonas putida*, transposon labelling, 3-chloroaniline, bioreactor, xenobiotic degradation, gfp, dsRed, *Comamonas testosteroni*, pNB2.

1. Introduction

Bioremediation, microbially mediated degradation or detoxification of contaminants, is a promising approach to remediating contaminated aqueous environments. Biofilms, flocs and other bioaggregates are self-organizing accumulations of microorganisms. Their products, extracellular polymeric substances (EPS), play an important role in both natural and man-made environments with respect to transformation and transport of contaminants. Many xenobiotics become adsorbed to biofilms (1) where they exert selective pressure on attached microbial communities,

S.P. Cummings (ed.), *Bioremediation*, Methods in Molecular Biology 599,
DOI 10.1007/978-1-60761-439-5_12,

resulting in adaptation of microorganisms to the contaminant. Microbial adaptation can be facilitated by (i) an increase in the numbers of specific degraders, (ii) microbial community adaptation through mutations or (iii) acquisition of relevant genetic information by the community through horizontal gene transfer (HGT), and thus an ultimate increase in the community's biodegradation potential (2). Biofilms represent environmental niches characterized by high cell densities and hence are ideal environments for HGT to occur (3, 4). Catabolic genes or those coding for antibiotic or metal resistance are often carried on plasmids. Plasmid transfer via conjugation (exchange of genetic information from one cell to another mediated by cell-to-cell contact) has been detected in many environments including soils, activated sludge, sediments, the rhizosphere and phyllosphere of plants, and model, engineered and natural biofilms. One common approach to initiating or accelerating bioremediation is bioaugmentation, the introduction of desired metabolic functions into an existing microbial community (5). This is often accomplished through the addition of a bacterial strain with the desired metabolic properties (i.e. the capability to degrade a relevant pollutant) to the indigenous microbial community (6). Alternatively, a mixed culture possessing the desired catabolic functions can be used (7). However, previous work has shown that strains added to existing microbial populations may not survive under the new environmental conditions and disappear to levels below detection limits (8, 9). On the other hand, bioaugmentation via in situ genetic manipulation (the introduction of catabolic genes into an existing indigenous community by means of HGT via conjugation or transformation) may lead to a successful introduction of degradative genes into an existing microbial community (10–12). In this way, genes encoding catabolic enzymes on mobile genetics elements harboured in a suitable bacterial host are introduced to indigenous populations. For example, studies have demonstrated that conjugative catabolic plasmids of the incompatibility group P1 are frequently transferred to a broad range of proteobacterial recipients (10). An example of such plasmids is plasmid pNB2 (11), which carries genes for the degradation of aniline and 3-chloroaniline.

Fluorescent proteins such as the green fluorescent protein (GFP) from *Aequorea victoria* (13, 14) or the red fluorescent protein DsRed (drFP583, commercially available as DsRed) isolated from a *Discosoma* sp. coral (15) have been used as markers for in situ monitoring of plasmid transfer (16). Fluorescent reporter genes have been used monitoring gene transfer in different environments, including agar surfaces, the phylloplane, microbial wastewater communities or biofilms (10, 17–19).

In this contribution, we describe the evaluation of the success of bioaugmentation of microbial wastewater communities in

laboratory-scale bioreactors with a donor strain carrying plasmid pNB2 (20, 11). We also outline techniques used to construct fluorescent protein-labelled donor strains and plasmids.

2. Materials

2.1. Media

1. LB-broth/agar 10.0 g/L tryptone, 5.0 g/L yeast extract, 5.0 g/L NaCl, 15 g/L agar.
2. Nutrient broth/agar: 5.0 g/L peptone, 3.0 g/L meat extract, 15 g/L agar.
3. R2A agar: 0.5 g/L yeast extract, 0.5 g/L peptone, 0.5 g/L casamino acids, 0.5 g/L dextrose, 0.5 g/L starch, 0.3 g/L sodium pyruvate, 0.3 g/L K_2HPO_4, 0.3 g/L $MgSO_4$, 15 g/L agar.
4. MMN-medium: Sterilize solutions a–c by autoclaving and the citrate solution by filter sterilization. For preparation of the medium, make up a 1× phosphate buffer solution (for plates, include 15 g/L of ultra-pure agar). Allow to cool to 50°C, then add the salt solution to 1× concentration and (if required) the ammonium solution to 1 mM. Citrate is added to a final concentration of 0.2%, and 3-chloroaniline (3CA) to 100–200 mg/L.
   a. 100× phosphate buffer solution: 141.96 g/L Na_2HPO_4, 136.09 g/L KH_2PO_4.
   b. 200× salt solution: 19.7 g/L $MgSO_4$, 1.18 g/L $CaCl{\cdot}2H_2O$, 0.23 g/L H_3BO_3, 0.56 g/L $FeSO_4{\cdot}7H_2O$, 0.34 g/L $MnSO_4{\cdot}H_2O$, 0.08 g/L $CuSO_4 \cdot 5H_2O$, 0.05 g/L $CoCl_2{\cdot}6H_2O$, 0.02 g/L $Na_2MoO_4{\cdot}2H_2O$, 0.64 g/L of Na_2EDTA.
   c. Ammonium solution: 1 M NH_4Cl.
   d. Carbon source solutions: 20% Sodium citrate, 3-chloroaniline, 10% sodium succinate.
5. Synthetic wastewater: The synthetic wastewater was prepared directly before use without sterilization using tap water. Stock solutions a and b do not require sterilization, but upon prolonged storage, precipitates may form in solution b, which should then be replaced. Add 2 mL of solution a and 12.5 mL of solution b to 985.5 mL of water to prepare 1 L of the final synthetic wastewater (SWW).
   a. 500× organic substrate solution: 64.0 g/L anhydrous sodium acetate, 66.8 g/L citric acid, 62.0 g/L sodium gluconate, 47.0 g/L anhydrous glucose, 33.0 g/L $NaH_2PO_4 \cdot H_2O$, 18.0 g/L KCl.

b. 80× salt solution: 22.1 g/L $NaHCO_3$, 11.4 g/L $MgSO_4 \cdot 7H_2O$, 8.6 g/L NH_4Cl.

2.2. Bacterial Strains and Plasmids

1. Wild-type plasmid pNB2 and host strain: Plasmid pNB2 (11) is a broad host range IncP1 plasmid (88 kb), which carries genes for mercury resistance and for the degradation of aniline and 3CA (20). The plasmid-carrying strain was *Comamonas testosteroni I2* obtained from a public culture collection (LMG 19558).
2. Experimental plasmid donor strain: *Pseudomonas putida* SM1443. Strain SM1443 is a modified *P. putida* KT2442 *lacI*^{q} strain with *npt* removed by site-specific recombination. This strain was constructed by Christensen and colleagues (18). For our work, we obtained *P. putida* SM1443 through a kind donation from Soren Molin (Department of Systems Biology, Technical University of Denmark, Lyngby).
3. gfp-transposon donor strain: *Escherichia coli* CC118 λpir – pUT*gfp*2x. We obtained this strain from Janet Jansson (Department of Microbiology, Swedish University of Agricultural Sciences). The pUT-derivative pUTgfp2x contains a kanamycin resistance cassette and two copies of the wild-type *gfp* gene under the control of a constitutive *psbA* promotor (14).
4. dsRed-transposon donor strain: *E. coli* CC118 λpir – pUT*dsRed*. This strain was obtained from Leo Eberl (Department of Microbiology, University of Zürich). The pUT-derivative used here contains a *dsRed* expression cassette under the control of an inducible *lac* promotor together with a gentamycin resistance gene (21).
5. Helper strain: *E. coli* HB101 – pRK2013. This strain can be obtained from the German Collection of Microorganisms and Cell Cultures (DSM No. 5599). Plasmid pRK2013 carries RK2 transfer genes as well as a kanamycin resistance cassette, and is a helper plasmid for mobilization of non-self-transmissible plasmids.

3. Methods

3.1. Labelling of Strains and Plasmids

This approach utilizes suicide vectors of the pUT-series based on transposon Tn5 (22). These vectors carry an antibiotic resistance gene cassette as well as a recognition site for the NotI restriction enzyme, surrounded by Tn5 recognition sequences. Additional genes (for example, *gfp* genes used for fluorescent "tagging" of the donor strain) can be cloned into the *Not*I recognition site.

The plasmid also carries the Tn5 transposase genes. The plasmid has a R6K origin of replication and can replicate only in bacterial strains producing the replication protein π. Examples of such strains are the *E. coli* λpir strains carrying the *pir* gene in a λ prophage. The plasmid can be mobilized for conjugation with a helper plasmid by means of a three-parental mating in which an *E. coli* λpir strain carrying a pUT plasmid (in our case *E. coli* CC118 λpir – pUT*gfp*2x or *E. coli* CC118 λpir – pUT*dsRed)* is mated with an *E. coli* strain harbouring the helper plasmid (*E. coli* HB101 – pRK2013) and a suitable recipient strain, which does not produce the replication protein π. The goal of the triparental mating is to introduce the pUT plasmid into the recipient strain, where it cannot replicate in the absence of the replication protein. In this way, only target strain colonies where transposon Tn5 with cloned information has been integrated into the chromosome are selected on minimal medium agar plates with appropriate antibiotics. The amino acid auxotrophic donor and helper *E. coli* strains are counter-selected by the use of an appropriate synthetic minimal medium or suitable antibiotics.

3.1.1. Chromosomal Labelling of Pseudomonas putida SM1443 with gfp

1. Grow individual 5 mL overnight cultures in LB medium with shaking at 150 rpm of the following strains:
   a. *E. coli* CC118 λpir – pUT*gfp*2x (with 50 μg/mL kanamycin at 37°C)
   b. *E. coli* HB101 – pRK2013 (with 50 μg/mL kanamycin at 37°C)
   c. *P. putida* SM1443 (at 30°C)
2. Centrifuge 1 mL of each culture for 5 min at 5,000*g*, discard supernatants and resuspend each cell pellet in 1 mL PBS.
3. Centrifuge again, discard supernatants, and resuspend each pellet in 50 μL PBS.
4. Mix the three cell suspensions (total volume 150 μL) in one reaction tube.
5. Transfer 100 μL of the cell suspension to an LB agar plate with a pipette (one big "drop" results on the LB plate).
6. Allow conjugation proceed overnight at 30°C.
7. Next day, remove the entire "patch" from the LB plate with a sterile inoculation loop and transfer the biomass to 1 mL PBS.
8. Vortex vigorously.
9. Centrifuge for 5 min at 5,000*g* and discard the supernatant. Resuspend again in PBS.
10. Spread-plate proportions of the cell suspension on MMN-agar plates containing 0.2% citrate and 100 μg/mL

kanamycin in order to obtain single colonies. This medium selects for *P. putida* transposon mutants (it does not support growth of the aminoauxotrophic *E. coli* strains).

11. Incubate at 30°C for 2 days.
12. Inspect plate with a transilluminator by exposing it to UV light (for a very short time) to select a green fluorescent colony (*P. putida* SM1443::*gfp*2x).

3.1.2. Labelling of Plasmid pNB2

The introduction of a fluorescent protein marker gene into pNB2 is performed in two steps, see also (10). First, a triparental mating between the original plasmid host, the *dsRed* donor strain, and the helper strain is conducted as described above to produce a *Comamonas* cell mixture with a transposon label either in the chromosome or in the plasmid. Second, a mating between this cell mixture and *P. putida* SM1443::*gfp*2x is performed, and *Pseudomonas* cells carrying a *dsRed*-labelled pNB2 plasmid are isolated by selecting for the antibiotic resistance cassettes associated with *dsRed*and *gfp*.

1. Grow individual 5 mL overnight cultures in LB medium with shaking at 150 rpm of
   a. *E.coli* CC118 λpir − pUT*dsRed* (with 25 μg/mL gentamycin at 37°C)
   b. *E.coli* HB101 − pRK2013 (with 50 μg/mL kanamycin at 37°C)
   c. *Comamonas testosteroni* I2 carrying plasmid pNB2 (at 30°C)
2. Centrifuge 1 mL of each culture for 5 min at 5,000*g*, discard supernatants and resuspend each cell pellet in 1 mL PBS.
3. Centrifuge again, discard supernatants, and resuspend each pellet in 50 μL PBS.
4. Mix the three cell suspensions (total volume 150 μL) in one reaction tube.
5. Transfer 100 μL of the cell suspension to an LB agar plate with a pipette (one big "drop" results on the LB plate).
6. Allow conjugation to proceed overnight at 30°C.
7. Next day, remove the entire "patch" from the LB plate with a sterile inoculation loop and transfer the biomass to 1 mL PBS.
8. Vortex vigorously.
9. Centrifuge for 5 min at 5,000*g* and discard the supernatant. Resuspend in 100 μL PBS.
10. Spread-plate the complete cell suspension on an MMN-agar plate containing 0.2% citrate and 25 μg/mL

gentamycin and incubate for 2 days at 30°C. This should select for a lawn of *Comamonas testosteroni* transposon mutants with the *dsRed* transposon integrated either in their chromosome or in plasmid pNB2.

11. Meanwhile, grow a 5 mL overnight culture of *P. putida* SM1443::*gfp*2x in LB medium containing 50 μg/mL kanamycin at 30°C. This should be ready at the end of the 2-day incubation of the MMN-agar plate from step 10.
12. In order to isolate cells with *dsRed*-labelled plasmids, the entire bacterial lawn is scraped off the MMN-plate with a sterile inoculation loop and transferred to 500 μL PBS.
13. Centrifuge 1 mL of the *Pseudomonas* culture and resuspend in 500 μL PBS.
14. Mix the two suspensions from steps 12 and 13, centrifuge, discard the supernatant, and resuspend the pellet in 100 μL of PBS.
15. Transfer the cell suspension to the surface of an LB agar plate with a pipette (one big "drop" results on the LB plate) and incubate overnight at 30°C.
16. Next day, remove the entire "patch" from the LB plate with a sterile inoculation loop and transfer the biomass to 1 mL PBS.
17. Centrifuge for 5 min at 5,000*g* and discard the supernatant. Resuspend again in PBS.
18. Spread-plate proportions of the cell suspension on MMN-agar plates containing 0.2% citrate, 25 μg/mL gentamycin and 100 μg/mL kanamycin to select for colonies of *Pseudomonas putida* SM1443::*gfp*2x cells possessing a *dsRed*-labelled plasmid pNB2.
19. Select a well-isolated colony and inspect it with a fluorescent microscope.
20. Upon microscopic observation and exposure to blue light, cells should show constitutive green fluorescence and no red fluorescence upon exposure to green light (red fluorescence should be suppressed as a result of the chromosomally encoded *lac*-Repressor in SM1443). However, growth of the cells on agar with 0.5 mM IPTG should result in the induction of red fluorescence in the SM1443 cells (*see* **Note 1**).

The constructed strain possesses chromosomal resistances against 100 μg/mL rifampicin and 50 μg/mL kanamycin as well as plasmid-conferred resistances against 25 μg/mL gentamycin and 5 μg/mL HgCl2 on LB medium. It should be noted that *Pseudomonas putida* SM1443::*gfp*2x – pNB2::*dsRed* did not

grow on MMN-medium containing 3-chloroaniline as the sole carbon source.

3.2. Microscopy

Three microlitres of a bacterial suspension are added to a well on a 6- or 10-well slide. GFP (excitation, 488 nm, emission, 509 nm) and dsRed (excitation, 563 nm, emission, 581 nm) fluorescence are investigated with a fluorescence microscope using appropriate filter sets and/or laser excitation wavelengths (488 nm and 543 nm, for GFP and dsRed, respectively) and emission filters (e.g. long-pass 505 nm or long-pass 560 nm for GFP and dsRed, respectively) for confocal laser scanning microscopy. For our work, we use a Zeiss LSM 510 (Carl Zeiss, Jena, Germany) confocal laser scanning microscope.

3.3. Evaluation of the 3-Chloroaniline Degradation Potential of Plasmid pNB2

Plasmid pNB2 was first isolated from a 3-chloroaniline-degrading strain of *Comamonas testosteroni*. After transfer of the plasmid to a *Ralstonia eutropha* strain, the newly formed transconjugants gained the ability to degrade aniline, but not 3-chloroaniline (23). However, this standard evaluation procedure of plasmid participation in a degradation pathway is restricted by the limited genetic background of a single recipient strain.

For a more thorough analysis, we, therefore, propose transfer of a potentially catabolic plasmid from a non-degrading host strain to a mixture of recipient strains, e.g. from activated sludge. Inoculation of the mating mixture into a medium containing the xenobiotic of interest as the sole carbon source can then reveal if this approach leads to degradation of the compound. This approach revealed that pNB2 carries part of the genes necessary for 3CA degradation (20), as outlined below.

3.3.1. Preparation of 3CA-Degrading Transconjugant Cultures

1. Prepare an overnight donor cell culture of *Pseudomonas putida* SM1443::*gfp*2x – pNB2::*dsRed* grown in LB medium with 25 μg/mL gentamycin and 50 μg/mL kanamycin.
2. Prepare a suspension of mixed recipient cells (e.g. fresh or pre-grown activated sludge or soil bacterial cell suspension). We used activated sludge that had been stored as a glycerol stock at −70°C and had been pre-grown overnight in synthetic wastewater.
3. Centrifuge 1 mL or donor cell culture and 2 mL of mixed recipient cell culture, centrifuge, discard the supernatants, and resuspend each culture in 500 μL of PBS.
4. Mix the two cell suspensions, centrifuge again, discard the supernatant, and resuspend the pellet in 100 μL of PBS.

5. Repeat steps 2 and 3 in order to obtain separate suspensions of donor and recipient cells to use as controls. Centrifuge these as well and resuspend in 100 μL of PBS each.
6. Pipette the three suspensions on separate places of an R2A agar plate (or on separate plates to avoid contamination) and incubate overnight at 30°C.
7. Scrape the mating spot from the agar surface and resuspend the cells in 1 mL of PBS (*see* **Note 2**). Repeat for donor and recipient cell control spots.
8. Centrifuge the suspensions, discard the supernatants, and resuspend the pellets in PBS.
9. Inoculate the complete suspensions into shake flasks containing 25 mL of synthetic wastewater with 100 μg/mL 3CA as the sole carbon source.
10. Shake the flasks at room temperature and 150 rpm. Every two days, remove a 500 μL sample and determine its 3CA concentration.

In our laboratory, the mating spot cultures showed a 3CA degradation after 6–10 days of incubation, whereas neither donor cell nor recipient control degraded the compound.

3.3.2. Spectroscopic Determination of the Concentration of 3CA in Liquid Samples

The aromatic ring of 3-chloroaniline shows an absorption maximum at 235 nm. We found this absorbance to be concentration-dependent according to Lambert-Beer's law with an extinction coefficient of 7.9/mM/cm. The concentration of 3CA in liquid samples can, therefore, be measured by simple UV spectroscopy, provided that no other aromatic compounds are present in the sample (11) (*see also* **Note 3**).

1. Take an appropriate amount of sample (dependent on the size of the spectrophotometer cuvette, mostly around 1 mL) and remove particles by filtration or centrifugation.
2. Measure the absorbance of the cleared sample in a UV spectrophotometer at 235 nm, blanked against the same medium without 3CA. If necessary, dilute the sample in order to remain within the linear range of the spectrophotometer.
3. Calculate the concentration from a standard curve made up from measuring solutions with known amounts of 3CA or by using Lambert-Beer's law and the extinction coefficient given above.

3.3.3. Isolation and Characterization of Transconjugants

Single strains carrying pNB2::*dsRed* can be isolated from enrichment cultures by selecting for the metabolic traits residing on the plasmid: degradation of aniline and 3-chloroaniline as well as resistances against gentamycin and mercury. We used the following procedure (*see* **Note 4**).

1. Prepare appropriate dilutions from the mating spot cultures in PBS and spread-plate them on R2A agar containing 25 μg/mL gentamycin and incubate for 3 days at 30°C.
2. Restreak around 50 single colonies grown on the agar surface (make short streaks of approximately 1 cm length) on the same medium. In this way, approximately 25 colonies per plate can be maintained for a limited period of time for PCR and microscopy analyses.
3. Prepare individual cell suspensions of the colonies by scraping off biomass with a pipette tip so that a small lump of cells is visible at the end of the tip. Insert the tip into a 0.5 mL PCR-cup containing 100 μL of ddH_2O and resuspend the cells by gentle up-and-down pipetting.
4. Immediately after preparation of the suspensions, check for red and green fluorescence.
5. Use 1 μL of the suspensions as template in a PCR reaction targeting the *tdnQ* gene residing on pNB2. Use a standard PCR mix (1× PCR-buffer, 2.0 mM of each dNTP, 2.5 mM of $MgCl_2$, 0.2 μM of each primer, 0.5 U Taq in a final volume of 25 μL). The primers are tdnQ-for (5′-GCGCAGCATCGAGGATGAATTG-3′), tdnQ-rev (5′-GATGCCAGCCCGAGACCAAG-3′), and 5 min of denaturation at 95°C followed by 30 cycles of 95°C for 30 s, 62°C for 30 s, and 72°C for 1 min.
6. Run a 2% agarose gel of the PCR products (5–10 μL each), stain and visualize the DNA. The *tdnQ* PCR product should have a size of 191 bp.
7. Colonies showing red fluorescence and a *tdnQ* PCR product are being regarded as transconjugants.

3.3.4. Differentiation of Transconjugant Strains by Rep-PCR Fingerprinting Using Primer BoxA1R

The transconjugant colonies isolated from the mating spot enrichment cultures above can represent different bacterial species and strains. In order to identify genetically different isolates, the isolated colonies can be screened by using a genetic fingerprinting approach. A PCR reaction is performed, which leads to a strain-specific product pattern that can be resolved by agarose gel electrophoresis. This procedure also helps to reduce redundancy in the isolate collection by elimination of clones of the same strain.

1. Use 2 μL of the transconjugant cell suspensions as template in a 25 μL PCR reaction containing the following ingredients: 1× PCR buffer, 6.25 mM $MgCl_2$, 1.25 mM of each dNTP, 10% DMSO, 1.5 units of Taq-polymerase, and 6 μM of primer BoxA1R (5′-CTACGGCAAGGCGACGCTGACG-3′). Perform the following thermal program in a PCR thermocycler: An initial denaturation of 10 min at 95°C followed by 25 cycles of

45 s at 94°C, 1.5 min at 50°C, and 8 min at 65°C. Finish the reaction sequence with an extension of 16 min at 65°C.

2. Prepare a 2% agarose gel of around 15 cm length, preferably with a thin comb (1 mm) to get sharp bands. Load half of the PCR reaction in a well and run the gel for the period of time enabling the bromophenol blue dye to migrate at least two thirds of the gel length (i.e. make sure you get a good separation of products by running the gel for a long time). Run an extended 100 bp DNA ladder (e.g. Fermentas 100 bp DNA ladder plus) as a size marker in every sixth lane to enable a good comparability between gel profiles.
3. Isolates with visually different fingerprints should be considered to be different strains. Select one representative of each group for phylogenetic characterization using comparative sequence analysis of the 16S rRNA gene and investigation of degradation capabilities (see below).

3.3.5. Screening of Transconjugant Degradation Capabilities

Confirmed and differentiated transconjugant strains should finally be screened for their ability to degrade 3-chloroaniline: the MNN-medium offers the possibilities to test for the utilization of 3-chloroaniline as a carbon source, as a nitrogen source, or both.

1. Grow 5 mL overnight cultures of transconjugant strains in liquid R2A medium containing 25 μg/mL of gentamycin.
2. Centrifuge an aliquot of each culture, remove the supernatant, and resuspend in an equal volume of PBS in order to remove nutrient traces.
3. Inoculate 100 μL of cell suspension into 5 mL of MNN-medium containing either:
   a. 200 mg/L of 3-chloroaniline (use as C- and N-source)
   b. 200 mg/L of 3-chloroaniline and 1 mM of NH_4Cl (use as C-source)
   c. 200 mg/L of 3-chloroaniline and 1 g/L of sodium succinate (use as N-source)
4. Incubate for up to 2 weeks with shaking at 150 rpm and 30°C.
5. Check for cell growth by analysing turbidity and for utilization of 3CA by spectrophotometry.

3.4. Bioaugmentation of Microbial Wastewater Communities

3.4.1. Reactor Setup

To test the feasibility of accelerated degradation of 3CA in laboratory-scale bioreactors using plasmid pNB2 and a *Pseudomonas putida* SM1443::*gfp*2x carrying a dsRed-tagged pNB2 plasmid derivative (**Section 3**), three Erlenmeyer flasks, each with a total volume of 1 L and a working volume of 500 mL, amended with wood chips were used as model laboratory-scale sequencing

batch moving bed reactors (SBMBRs; design adapted from the semi-continuous activated sludge reactors described by Boon and colleagues (6)). The reactors were set up and operated as described below:

1. Add 10 g of wood chips to each reactor as biofilm carrier material
2. Operate the reactors with a variable cycle duration and a volumetric exchange ratio of 100% per cycle
3. Provide mixing and aeration by continuous shaking on a rotary shaker at 120 rev/min, with a 3 h stationary period per day without shaking.
4. Use synthetic wastewater (SWW; **Section 2**) as feed.
5. Prepare an activated sludge culture inoculum as glycerol stock cultures (**Section 2**; Bathe, 2004).
6. Inoculate 10 mL of this activated sludge inoculum stock culture into 500 mL of SWW and incubate at 30°C and 150 rev/min overnight, pellet by centrifugation for 10 min at 10,000 *g* and resuspend in 1.5 L of fresh SWW.
7. Inoculate each reactor with 500 mL of this suspension.
8. On the following day, replace the bulk liquid in the reactors with fresh SWW.
9. Bioaugmentation of the reactors:
   a. Add the pNB2 donor strain to a final density of approximately 10^8 cells/mL to the test (bioaugmented) reactor.
   b. Add cells of a 3CA-degrading pNB2 transconjugant strain isolated above to a final density of approximately 10^8 cells/mL to a second reactor, designated as a positive degradation control.
   c. A third reactor (negative control) receives just the activated sludge inoculum and no additional culture.
10. Incubate all reactors overnight and on the following day (day "0" with respect to 3CA addition), withdraw the medium from the reactors and replace it with fresh SWW containing 3CA at a final concentration of 80 mg/L.
11. From this day on, exchange spent medium with fresh medium when 3CA in the test (bioaugmented) reactor was degraded.

The 3CA concentration in the positive control reactor should rapidly drop due to the direct addition of 3CA-degraders. The plasmid-bioaugmented reactor will likely display a slower onset of degradation, since 3CA-degrading cell populations formed by HGT need to grow to a relevant size. In our hands (5), 3CA was completely degraded in the positive control after 4 days, while the plasmid-bioaugmented reactor initially required 18 days to establish 3CA degradation.

3.4.2. Sampling and Processing of Samples for 3CA Concentration and COD Determinations

1. For 3CA and COD measurements, withdraw samples directly from the reactors and filter them through a 0.22 μm filter.
2. Determine 3CA concentration photometrically by measuring the absorption of its aromatic ring at 235 nm (**Section 3**).
3. Determine COD using commercially available cuvette tests (Dr Lange, Duesseldorf, Germany) according to standard American Public Health Association (APHA) procedures.

3.4.3. Sampling of Biofilms and DNA Extraction

1. Sample biomass by collection of a mixture of wood chips and reactor bulk liquid in a 50 mL test tube, whereby the volume of settled wood chips in the tube was approximately 25 mL and the liquid volume was approximately 15 mL, for a total volume of approximately 40 mL.
2. Suspend the biofilms in the bulk liquid by vigorous vortexing and shaking for 5 min.
3. Gently pour the suspensions off the wood chips and collect the biomass in the collected suspensions by centrifugation for 5 min at 10,000 *g*.
4. Wash the resulting pellet twice with 0.85% KCl and then store as a pellet at –20°C.
5. Extract DNA using a protocol for miniprep of bacterial chromosomal DNA (24):
   a. Suspend approximately 50 mg of the collected biomass pellet in 567 μL TE buffer.
   b. Add 30 μL of 10% SDS and 3 μL of Proteinase K (20 mg/mL) to the sample and incubate for 1 h at 37°C.
   c. Add 100 μL of 5 M NaCl and 80 μL of 10% CTAB/0.7 M NaCl, mix thoroughly, and incubate for 10 min at 65°C.
   d. Extract the solution with chloroform, phenol/chloroform, and again chloroform.
   e. Precipitate the DNA with 0.6 vol of isopropanol, pellet by centrifugation for 15 min at 15,000 *g* and wash with 1 mL of 70% ethanol.
   f. Resuspend in 100 μL of autoclaved molecular biology grade water.

3.4.4. Multiplex PCR for Analysis of Horizontal Gene Transfer of pNB2

This is a quick, semi-quantitative method to determine the presence/absence of donor strain and plasmid in relation to the total bacterial population (11). Quantitative data can be obtained by real-time PCR or microscopy (*see* **Note 5**).

1. Set up a PCR reaction containing 0.2 μmol/L of each primer (5′-TTTCAAGAGTGCCATGCCCGAAGG-3′) and GFPr (5′-CTGGTAAAAGGACAGGGCCATCGC-3′) targeting the wild-type *gfp* gene residing on the chromosome of the donor strain (10), 0.2 μmol/L of each primer tdnQ-for and tdnQ-rev (2), and 0.1 μmol/L of primers 517f (5′-GCCAGCAGCCGCGGTAATAC-3′) and 1072r (5′-CGAGCTGACGACAGCCATGC- 3′) targeting bacterial 16S rRNA genes, 2.0 mmol/L of $MgCl_2$, 0.2 mmol/L of each dNTP, 10 ng of template DNA and 0.01 U/μL Taq polymerase (Qiagen HotStarTaq, Hilden, Germany) in Qiagen PCR buffer in a final volume of 20 μL.
2. Set up the PCR program as follows: an initial denaturation step at 95°C for 15 min, followed by 28 cycles of 95°C for 30 s, 60°C for 1 min, 72°C for 2 min and a final extension at 72°C for 7 min.
3. Separate the DNA by electrophoresis on a 2%-TAE-agarose gel and visualize by staining with ethidium bromide.

4. Notes

1. The fluorescence intensities observed here can vary depending on the insertion site of the transposon in chromosome or plasmid and is also determined by maturation time and protein turnover in the host cell. In our experience, storage of plates at low temperature (e.g. in the fridge overnight) increases single-cell fluorescence. Additionally, rapidly maturing *dsRed* and *gfp* variants with high fluorescence intensities have been developed in the meantime and partly applied in microbial transposon labelling systems.
2. Microscopic observation of mating spot cells should show green fluorescent donor cells, red fluorescent transconjugant cells, as well as non-fluorescent recipient cells. The red fluorescence can vary considerably between individual cells depending on the microbial species and the time allowed for maturation. Again, prolonged storage under low temperatures can increase fluorescence intensities (but also have an influence on conjugation rates).
3. 3CA concentration can also be determined by HPLC. This is a more time-consuming strategy, but may be more accurate than spectrophotometry and also allows the use of complex media, which may absorb at 235 nm.
4. It should be taken into account here that, dependent on the media used, different subpopulations of transconjugants are being targeted that do not necessarily possess similar

traits. For example, strains isolated based on gentamycin resistance do not necessarily degrade 3-chloroaniline, even if the inoculum was a 3CA-enrichment culture. Similarly, non-transconjugants displaying similar properties can also be isolated. Separate screenings for plasmid presence by PCR and/or epifluorescence microscopy as well as testing of the degradative properties of the isolates are, therefore, required as well.

5. Besides assessment of the presences of plasmid and donor cells by multiplex-PCR, the detection of donor and transconjugant abundances is also possible by real-time PCR, analogous to the procedure described previously (25), using primers targeting (ii) plasmid-specific genes (*tdnQ* or *dsRed*), (ii) genes specific for the donor strain (e.g. *gfp*) and (iii) general bacterial primers (e.g. 341f/534r) in order to quantify donors only and plasmid-carrying cells (donors and transconjugants) in comparison to the total bacterial population. In principle, donors and transconjugants can also be visualized by epifluorescence microscopy of GFP and DsRed, but in our experiments, a quantitative analysis was not possible due to weak fluorescence signals and rapid bleaching. This can be overcome by the use of new-generation *gfp* and *dsRed* mutants available now.

Acknowledgements We would like to thank Professor Stefan Wuertz (UC Davis, CA) for initiating the plasmid transfer studies, which eventually led to the experiments described in this chapter, and to Professor Peter A. Wilderer for his support and interest in our studies. We would also like to acknowledge Dr Norbert Schwarzenbeck for his contribution and participation with regard to the bench-scale sequencing batch moving bed biofilm reactor experiments.

References

1. Wolfaardt, G.M., Lawrence, J.R., Headley, J.V., Robarts, R.D., and Caldwell, D.E. (1994) Microbial exopolymers provide a mechanism for bioaccumulation of contaminants. *Micro. Ecol.* **27**, 279–291.
2. Top, E.M, and Springael, D. (2003) The role of mobile genetic elements in bacterial adaptation to xenobiotic organic compounds. *Curr Opin. Biotechnol.* **14**, 262–9.
3. Hausner, M., and Wuertz, S. (1999) High rates of conjugation in bacterial biofilms as determined by quantitative in situ analysis. *Appl. Environ. Microbiol.* **65**, 3710–3.
4. Sorensen, S.J., Bailey, M., Hansen, L.H., Kroer, N., and Wuertz, S. (2005) Studying plasmid horizontal transfer in situ: a critical review. *Nat. Rev. Microbiol.* **3**, 700–10.
5. van Limbergen, H., Top, E.M., and Verstraete, W. (1998) Bioaugmentation in activated sludge: current features and future perspectives. *Appl. Microbiol. Biotechnol.* **50**, 16–23.
6. Kasai, Y., Kodama, Y., Takahata, Y., Hoaki, T., and Watanabe, K. (2007) Degradative capacities and bioaugmentation potential of an anaerobic benzene-degrading bacterium

strain DN11. *Environ. Sci. Technol.* **41**, 6222–7.
7. Dabert, P., Delgenès, J.P., and Godon, J.J. (2005) Monitoring the impact of bioaugmentation on the start up of biological phosphorus removal in a laboratory scale activated sludge ecosystem. *Appl. Microbiol. Biotechnol.* **66**, 575–88.
8. Bouchez, T., Patureau, D., Dabert, P., Juretschko, S., Doré, J., Delgenès, P., et al. (2000) Ecological study of a bioaugmentation failure. *Environ. Microbiol.* **2**, 179–90.
9. Tchelet, R., Meckenstock, R., Steinle, P., and van der Meer, J.R. (1999) Population dynamics of an introduced bacterium degrading chlorinated benzenes in a soil column and in sewage sludge. *Biodegradation* **10**, 113–25.
10. Bathe, S., Mohan, T.V., Wuertz, S., and Hausner, M. (2004) Bioaugmentation of a sequencing batch biofilm reactor by horizontal gene transfer. *Water Sci. Technol.* **49**, 337–44.
11. Bathe, S., Schwarzenbeck, N., and Hausner, M. (2005) Plasmid-mediated bioaugmentation of activated sludge bacteria in a sequencing batch moving bed reactor using pNB2. *Lett. Appl. Microbiol.* **41**, 242–7.
12. Newby, D.T., Gentry, T.J., and Pepper, I.L. (2000) Comparison of 2,4-dichlorophenoxyacetic acid degradation and plasmid transfer in soil resulting from bioaugmentation with two different pJP4 donors. *Appl. Environ. Microbiol.* **66**, 3399–407.
13. Chalfie, M., Tu, Y., Euskirchen, G., Ward, W.W., and Prasher, D.C. (1994) Green fluorescent protein as a marker for protein expression. *Science* **263**, 802–4.
14. Tsien, R.Y. (1998) The green fluorescent protein. *Ann. Rev. Biochem.* **67**, 509–44.
15. Matz, M.V., Fradkov, A.F., Savitsky, A.P., Zaraisky, A.G., Markelov, M.L., and Lukyanov, S.A. (1999) Fluorescent proteins from nonbioluminescent *Anthozoa* species. *Nat. Biotechnol.* **17**, 969–73.
16. Wuertz, S., Hendrickx, L., and Hausner, M. (2001) In situ quantification of gene transfer in biofilms. *Method. Enzymol.* **336**, 129–43.
17. Christensen, B.B., Sternberg, C., and Molin, S. (1996) Bacterial plasmid conjugation on semi-solid surfaces monitored with the green fluorescent protein (GFP) from *Aequorea victoria* as a marker. *Gene* **173**, 59–65.
18. Christensen, B.B., Sternberg, C., Andersen, J.B., Eberl, L., Moller, S., Givskov, M., and Molin, S. (1998) Establishment of new genetic traits in a microbial biofilm community. *Appl. Environ. Microbiol.* **64**, 2247–55.
19. Normander, B., Christensen, B.B., Molin, S., and Kroer, N. (1998) Effect of bacterial distribution and activity on conjugal gene transfer on the phylloplane of the bush bean (*Phaseolus vulgaris*). *Appl. Environ. Microbiol.* **64**, 1902–9.
20. Bathe, S. (2004) Conjugal transfer of plasmid pNB2 to activated sludge bacteria leads to 3-chloroaniline degradation in enrichment cultures. *Lett. Appl. Microbiol.* **38**, 527–31.
21. Tolker-Nielsen, T., Brinch, U.C., Ragas, P.C., Andersen, J.B., Jacobsen, C.S., and Molin, S., (2000) Development and dynamics of *Pseudomonas* sp. biofilms. *J. Bact.* **182**, 6482–9.
22. Herrero, M., de Lorenzo, V., and Timmis, K.N. (1990) Transposon vectors containing non-antibiotic resistance selection markers for cloning and stable chromosomal insertion of foreign genes in gram-negative bacteria. *J. Bact.* **172**, 6557–67.
23. Boon, N., Goris, J., De Vos, P., Verstraete, W., and Top, E.M. (2001) Genetic diversity among 3-chloroaniline- and aniline-degrading strains of the *Comamonadaceae*. *Appl. Environ. Microbiol.* **67**, 1107–15.
24. Ausubel, F.M., Brent, R., Kingson, R.E., Moore, D.D., Seidman, J.G., Smith, J.A., and Struhl, K. (eds) (1999) *Short Protocols in Molecular Biology* Wiley, New York, USA.
25. Bathe, S., and Hausner, M. (2006) Design and evaluation of 16S rRNA sequence based oligonucleotide probes for the detection and quantification of *Comamonas testosteroni* in mixed microbial communities. *BMC Microbiol.* **6**, 54.

Chapter 13

The Role of Decision Support for Bioremediation Strategies, Exemplified by Hydrocarbons for In Site and Ex Situ Procedures

Elizabeth E. Diplock, Dave P. Mardlin, Kenneth S. Killham, and Graeme I. Paton

Abstract

Despite the widespread availability of state-of-the-art biological techniques, remediation practitioners have been slow to adopt these technologies to assist in designing or indeed monitoring remediation strategies. In part, this is because practitioners are driven by cost and fail to see the benefit of emerging technologies, and in part because most companies have only a small portfolio of procedures available to them. Here, we review the component parts required to design a decision support tool, appraise one that the authors have developed and critically evaluate its application to case studies. If bioremediation is to become adopted, then it is likely to have to operate in parallel with other remediation methods. Furthermore, remediation strategies must couple effective technology with a transparency of information such that all parties (practitioners, developers and stakeholders) understand how decisions were reached.

Key words: Decision support tool, bioremediation, hydrocarbons, in site, ex situ.

1. Definitions

A clear outcome of the CLARINET group was that they highlighted that the term "decision support" has no clear universal meaning and that there is no real classification that constitutes what makes a decision support system or tool (1). The Oxford dictionary defines the words "decision", "support" and "tool" as:

S.P. Cummings (ed.), *Bioremediation*, Methods in Molecular Biology 599,
DOI 10.1007/978-1-60761-439-5_13, © Humana Press, a part of Springer Science+Business Media, LLC 2010

- *Decision*: "a conclusion or resolution reached after consideration; the action or process of deciding or the quality of being decisive".
- *Support*: "a person or thing that supports; the action of supporting or the state of being supported or assistance, encouragement or approval".
- *Tool*: "a device or implement used to carry out a particular function; a thing used to help perform a job or a person used by another".

So, in relation to remediation technologies, a decision support tool could be defined as*a device for providing assistance that results in a considered conclusion for the selection of the most appropriate remediation technology*. A great deal of attention has been focussed on risk assessment and hazard associated clean-up targets, with only tokenistic generalities being applied to the procedures and data requirements necessary to reach justifiable and transparent remedial decisions.

2. Background

The development of environmental decision support systems or tools (DSS/DST) is at the heart of environmental management. DSTs have the potential to integrate environmental data into a framework for undertaking site characterisation, monitoring and remediation decisions. Ideally, the DST should endeavour to integrate, analyse and present environmental information to remediation project managers, enabling a clear and cost-effective remediation strategy to be implemented. This tool should strike a balance between considering site complexity and simplicity of use. The key components that constitute decision-making include

- Site characterisation and site survey strategies:
  - data analysis including interpretation of site characterisation data and integration of information.
- Remediation strategies including tools for dealing with restrictions during the remediation planning process. Remedial action analysis which includes optimisation of design as well as a comparison between different technologies and risk management.
- Human health and ecological risk analysis (compliance with regulatory limits).
- Financial aspects (cost/benefit analysis) including financial optimisation and liability transfer.
- Socio-economic implications of the decision-making and the impacts of environmental improvement.

Decision-making with respect to the management of contaminated land has been transformed in recent decades, shifting

from cost-driven approaches, through technology feasibility studies, to risk-based criteria (2). Perhaps in the United Kingdom, the decision-making approach has evolved a step further because risk assessment criteria underpin the definition of contaminated land. Indeed, the ability to differentiate risk and hazard assessment from the decision support makes the process more streamlined and less ambiguous.

There are many technologies that have been designed and evolved over the past few decades, and these can target soils and/or controlled waters. Proven techniques for ex situ remediation are thermal treatment, extraction and biopiling, whereas for in situ remediation biorestoration, soil washing and soil venting are common. Successful containment techniques which prevent contamination from migrating consist of employing bentonite screens and soil vapour extraction.

Unbiased environmental decision-making is complex, multifaceted, and could involve many different stakeholders with different priorities or objectives: presenting exactly the type of problem that behavioural decision research shows humans are typically poor at solving, unaided. Most people, when confronted with such a problem will attempt to use intuitive approaches to simplify complexity until the problem appears manageable. In such a practice, vital information may be lost, opposing points of view discarded, elements of uncertainty may be ignored – in short, there are many reasons to expect that, on their own, individuals will often experience difficulty making informed decisions about subjects that involve uncertainties and tradeoffs. Furthermore, environmental decisions characteristically necessitate multidisciplinary knowledge that encompasses sciences of a physical, natural and social standpoint. Consequently, the tendency for environmental problems to require shared resources means that group decisions are often called for. These may have advantages over individual choices such as greater consideration as a higher chance of systematic thinking is likely to be involved.

The clean-up or "remediation" of a contaminated site aims to improve the quality of the site and minimise the risk potentially caused to humans, controlled waters or the environment. This process is a planned approach and the decision as to which remediation approach is most suitable is a crucial step. Indeed, the need to integrate justifiable risk and hazard assessment has led to great complexity in the evolution of decision support tools (3).

The selection of a remediation strategy is a complex task, but there is a lack of peer-reviewed papers that explain the steps adopted to reach such decisions. Poor selection of a remediation methodology may well end in failure to meet environmental standards and/or the defined budget. Accordingly, decision-makers often make inaccurate choices by opting for the most uncompromising technology, which could be the most expensive, to ensure remediation objectives are met (4).

In the United Kingdom, a risk-based approach has been adopted to the management of contaminated land in accordance with policy set out in *DEFRA's Circular 01/2006: Environmental Protection Act 1990: Part IIA Contaminated Land*. This necessitates that remediation is undertaken where:

- Contamination poses unacceptable actual or potential risks to health or the environment and that
- Suitable and cost-efficient means are accessible to do so, taking into account the actual or indented end-use of the site.

It has become commonplace to make use of multi-criteria decision analysis (MCDA) to rank remediation technologies and their suitability. A decision support tool should be designed to assist the decision-maker involved with contaminated land, in carrying out focussed analyses and making certain reproducible and transparent processes that consider all the variables involved (11, 5). This should be a balanced and systematic process founded on the principles of transparency. Decisions about which management options are most appropriate for a particular site need to be considered in a holistic manner (**Fig 13.1**).

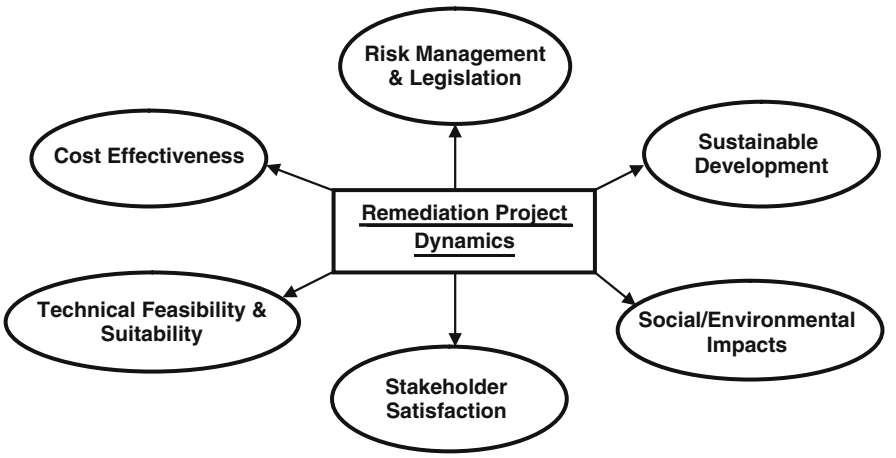


Fig. 13.1. Factors that require consideration when selecting an effective remediation solution to a contaminated land problem.

There has been a great deal of discussion about the nature and form that a DST ought to take, but there is little evidence that users have managed to actually develop or design one (1, 3, 6, 7, 8, 9, 10). In part, this is because there has been too much focus on trying to deal with the clean-up target as part of the tool. In reality, the clean-up is defined prior to the systematic selection of the strategy.

The decision-making process for any problem usually encompasses:

- A phase in which the problem is identified;
- A phase in which possible solutions are identified and developed;

- A selection phase in which the solution to be implemented is chosen;
- A monitoring phase to prove/disprove that the chosen option or set of options has been implemented.

Decision support integrates specialist expertise in a way that allows its reproducible use. It incorporates information concerning a site and general information such as legislation, guidelines and know-how, to produce decision-making knowledge in a way that is transparent, consistent and reproducible. Existing DS varies from simple diagrams derived from standards or regulations to software-based systems. Applications have been developed for contaminated site management, involving the characterisation of contamination, risk assessment phases, risk management, aftercare and monitoring.

Fundamentally, there are two different ways to the approach linking the most suitable remediation strategy to a given scenario. The first is to interpret the key systematic aspects that determine the most suitable technique. Conversely, the other is to relate all available technologies and modify these to suit site-specific scenarios.

3. Availability of Data and Value of Data in Decision-Making

First and foremost a detailed site investigation that characterises the chemicals of concern associated with the site, their extent and the nature of the harm that they pose is essential. So too is the detailed geochemical interpretation and an understanding of the physicochemical attributes underpinning the site. In this way, an estimate of the extent of the pollutant and the recalcitrance of the targets can be made. These data are essential in aiding the justification of a selected remediation strategy.

What makes bioremediation different from remediation is that there is a need to characterise and to understand the performance of a given biological process. Hence, if bioremediation is to be selected as a suitable approach then, in addition to the considerable volume of site data, there is a need to evolve a sampling regime capable of collating biological information.

4. Audit of Biological Methods to Aid Decision-Making in Bioremediation

Microbial bioassays have been widely reviewed for their applications in bioremediation sciences and application (11, 12). In summary, the assays operate at either the phenotypic, genotypic or functional level and have probably been of more value as academic examples rather than genuine examples.

Petroleum hydrocarbons are produced by natural geochemical processes. Hence they have been present in the environment long enough for an array of enzymes to have evolved, which can metabolise all of its components. Degrading microorganisms are ubiquitous in the soil and sub-soil environments; as a consequence, it is generally possible to instigate bioremediation of oil pollution without the use of inocula. Traditionally, it has been reported that the degrader community usually constitutes less than 1% of the microbial community, but in the presence of petroleum this rises to approximately 10% of the microbial community (13, 14). Petroleum pollution usually increases the total amount of microbial activity at a site (for example, as measured by respiration), but decreases the diversity of the microorganisms present (15). Studies carried out after the *Exxon Valdez* spill found average concentrations of oil-degraders of 10^6 cells/g, with enrichment factors as high as 10^4 (16). The increase in the numbers of oil-degraders was significant, but the degree of enrichment was variable.

The effects of oil pollution on microbial communities can be studied at a genetic level: the frequency and distribution of hydrocarbon-degrading genes can be assessed. A range of probes have been developed to monitor genes from pathways for aliphatic, monoaromatic, and polyaromatic hydrocarbon degradation (17). The probes can be applied to DNA, mRNA or rRNA; preliminary PCR is usually performed to enhance the sensitivity of detection (18). The probes can be applied to environmental isolates; however, a more valuable approach is to extract nucleic acids directly from soils, thus avoiding the bias associated with culturing (19, 20). Schmidt et al. (21) used six gene probes taken from aerobic hydrocarbon degradation pathways and two associated with methanogens to assess an aquifer contaminated with jet fuel. Almost all samples gave a positive response for all genes: radiolabelled hydrocarbons added to the samples were rapidly degraded.

Existing gene probes for hydrocarbon degraders have been shown to correlate with oil contamination and degradative potential. However, they are not able to completely characterise the ecological effects of oil pollution: several studies have reported that not all PAH degraders isolated from contaminated soils were able to hybridize with existing probes (22, 23, 24).

Despite the ability of indigenous microorganisms to degrade petroleum hydrocarbons, there are still situations where an inoculum might be used, such as the bioremediation of high molecular weight PAHs. Natural communities are often unable to degrade PAHs of four rings or greater, and are usually unable to degrade asphaltenes (14). It has been suggested that, because of the difficulties inherent in using microbial inocula, the use of cell-free immobilised enzyme systems might be preferable (25). A situation which would be likely to require seeding is when the

contaminant(s) being degraded is utilised as a co-substrate rather than as a sole carbon source; in this case, the indigenous degrading populations will not be able to multiply at the expense of the non-degraders (26). Inoculation may possibly be advantageous if rapid cleanup is desired, and there is a lag period before indigenous populations can adapt; however natural degrading populations often develop quickly (14).

Hydrocarbons are fully reduced compounds and thus do not undergo reductive degradation, but they can be metabolised under anaerobic conditions, provided that alternative terminal electron acceptors are present. After oxygen has been depleted, the commonest oxidation pathways for hydrocarbons in soil are (in order of use): nitrate reduction, iron reduction, sulphate reduction and methanogenesis (27). Manganese reduction can also take place. Anaerobic degradation has been established for representatives of all of the hydrocarbons found in petroleum products: monoaromatics including benzene (28, 29); PAHs up to four rings (30, 31, 32); *n* -alkanes, including long-chain alkanes (33); branched alkanes and cycloalkanes. There are many examples of intrinsic bioremediation projects where the demonstration of anaerobic degradation is a fundamental part of the assessment (e.g. 34). Anaerobic bioremediation is slower and less efficient than aerobic processes (27), and is therefore employed for in situ bioremediation of groundwater contamination most frequently in intrinsic bioremediation, when anaerobic processes are the norm for PHCs in groundwater, and rapid clean-up is not an aim. However, it is also possible to stimulate in situ anaerobic biodegradation by the addition of EAs such as nitrate directly to a polluted aquifer (35).

Petroleum and soil are both complex mixtures and, therefore, the environment in which bioremediation takes place is heterogeneous. Petroleum products can be present in soil in at least five discrete phases: in the vapour phase, in the aqueous phase, as NAPLs (non-aqueous phase liquids, i.e. oily liquids), adsorbed to particulate matter, and (as separated asphaltenes and resins) as solids. Therefore, the bulk of degradation will be carried out by microorganisms adhering to an interface between two phases – primarily at the water/NAPL boundary (36).

5. University of Aberdeen (UoA) – Remedial Decision Support Tool (DST) Appraisal

Decision support tools cannot solely deal with bioremediation approaches. Furthermore, it must be capable of being interpreted by non-experts, yet have such transparent data output that decision-making has clarity and logic. Indeed, an ideal system must be adaptable to the future and it must be directly integrated

with technology and able to evolve as new approaches become available. In summary the key features are:

- Users to identify and systematically compare information regarding each technology to meet remediation targets, highlighting their strengths and weaknesses.
- Establish a structure of technology evaluation and selection process, which simplifies decision-making and streamlines the factors involved in the remediation process.
- To define consistent, quantitative and qualitative indicators for key technical, environmental, economic and legal criteria that influences the selection and use of technologies.
- Provide a multi-criteria optimisation approach that determines whether a remedial option is feasible, effective/efficient and whether it satisfies all criteria and constraints identified by interested parties.
- Enable explanation and justification of the choice by providing evidence on the advantages and disadvantages of the potential options in a clear, concise and transparent manner.
- Enable a quicker turnaround of feasibility studies for remedial options.
- Allows site owners, consultants, stakeholders the opportunity to explore alternatives available to them.

5.1. Systematic Approach

After a comprehensive appraisal of the existing approaches, one of the most obvious features is that the available approaches are very bias and data demanding. Furthermore, they try to link risk assessment into the decision process. In this UoA DST, this has been circumvented by making a systematic tiered approach (http://www.abdn.ac.uk/remediation-dst/). This is a tool that addresses the concerns of uncertainty and enables a procedure to match specific issues of the site in question with the available technologies. Less hierarchy and rigidity enhances both the flexibility and the adaptability of the procedure. Tier 1 integrates generic and risk-derived assessment criteria laying out the general procedures that are required. Tier 2 matches the most suitable technology to the specific details of the given area of the site. Tier 3 describes specifically the steps required to optimise the remediation strategy and makes detailed recommendations as to what ought to be measured and how this can be effectively managed. Three case studies are detailed later in this chapter.

5.2. Application of UoA DST to On Site Hydrocarbon Remediation

5.2.1. Tier 1

The steps required at Tier 1 (**Fig 13.2**) are generic for each of the case studies. But the list of details serves to enable the practitioner

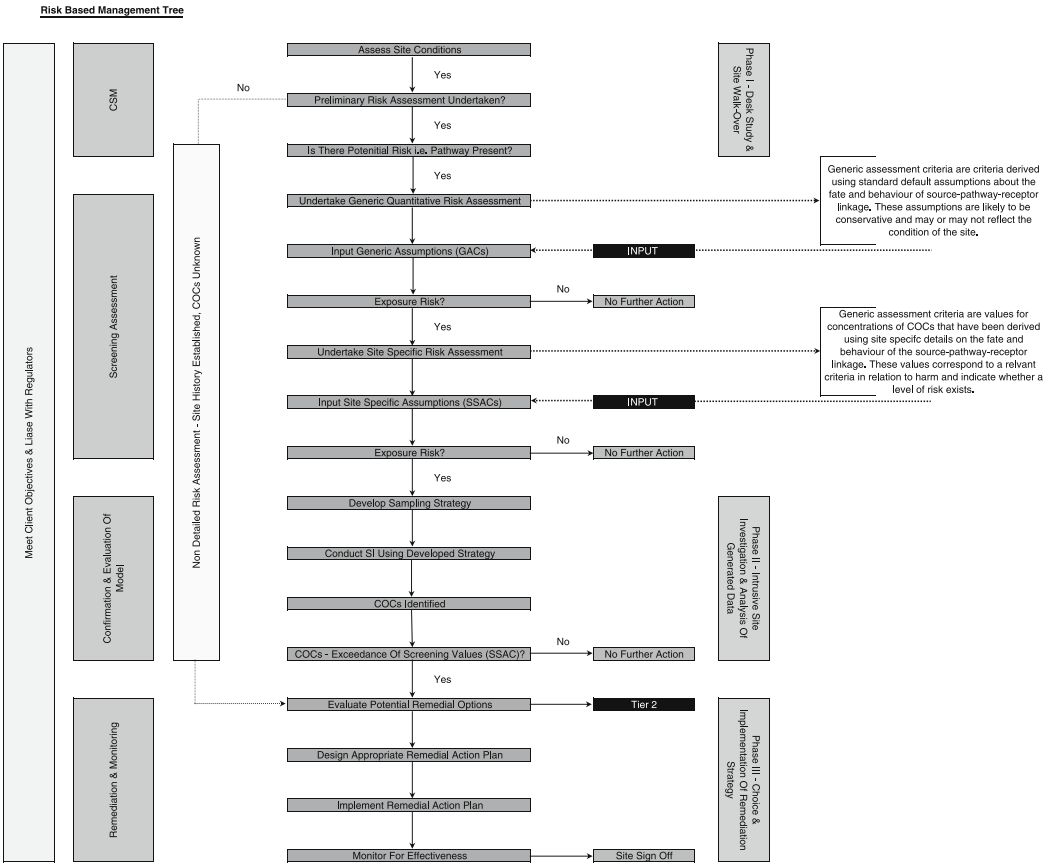


Fig. 13.2. Tier 1 of DST.

to follow the best practice under DEFRA Guidance and for the regulator to check the procedure against the proposed guideline. Most importantly, if there is no clean-up value at this stage, then it is not possible to determine the procedures for defining the suitability of a given strategy at a later stage.

5.2.2. Tier 2

This is the key matrix that enables the user to comparatively appraise the options available. Limited information can be used, but there is greater uncertainty with the outcome. The ranked appraisal is specific to the CoC or the previous land use (**Fig 13.3**). This tier enables transparency and clarity in the decision-making process. By selecting the nature and source of the industrial pollution and as a consequence measuring the CoC, it is possible to immediately dismiss as unsuitable certain approaches. The DST integrates the key components that enable decisions to be made, and as a consequence this acts as a transparent tool for both the practitioner (and client) and the regulator. The system also interfaces with impartial descriptors to aid parties. The approach is dynamic and simple to update, hence the DST can be extended and adapted to new technologies. Once

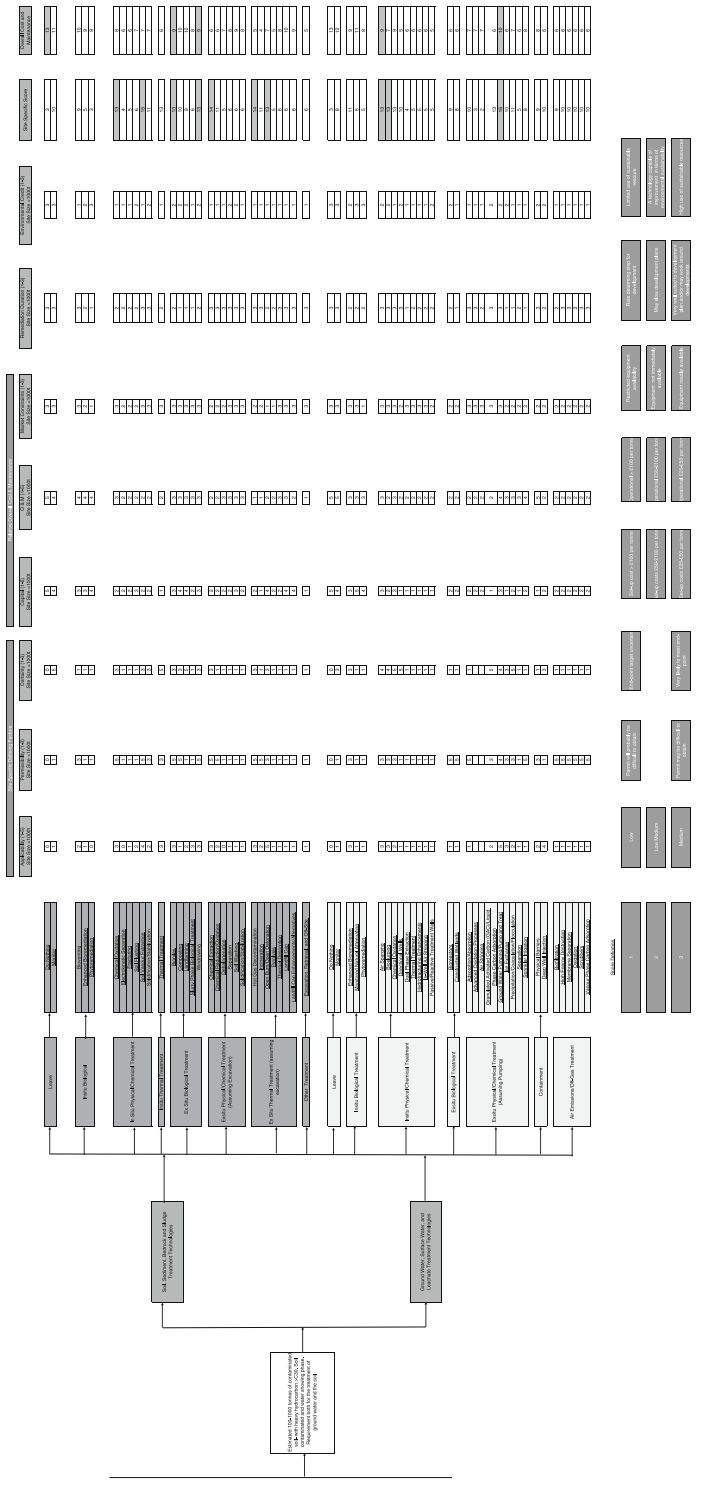


Fig. 13.3. Tier 2 of DST.

the appropriate methods have been highlighted, laboratory-scale testing is encouraged. Empirical testing of the data was conducted and interpreted in a risk-based framework.

In summary, the aspects of risk assessment and the corresponding clean-up targets have been appraised and hence the initial question relates to whether the target is defined by the soil concentration or the water concentration and which matrix will undergo the remediation. The key components that are then addressed are matching the site characteristics to the economics, licence and practicalities of the given remedial options through a rank systematic process that has been pre-rated. This rating can be adjusted as more information becomes available, and details of the procedure can be found at http://www.abdn.ac.uk/remediationdst/.

5.2.3. Tier 3

The DST is further enhanced by means of detailed analysis to optimise the procedures for best practice (**Fig 13.4**). This DST not only offers a valuable contribution to the design and implementation of remedial strategies but also enables the process of decision-making to be made on a more transparent basis, which is ultimately sought by the regulators. Such tools are invaluable and can be integrated with risk assessment approaches and updated in response to technology.

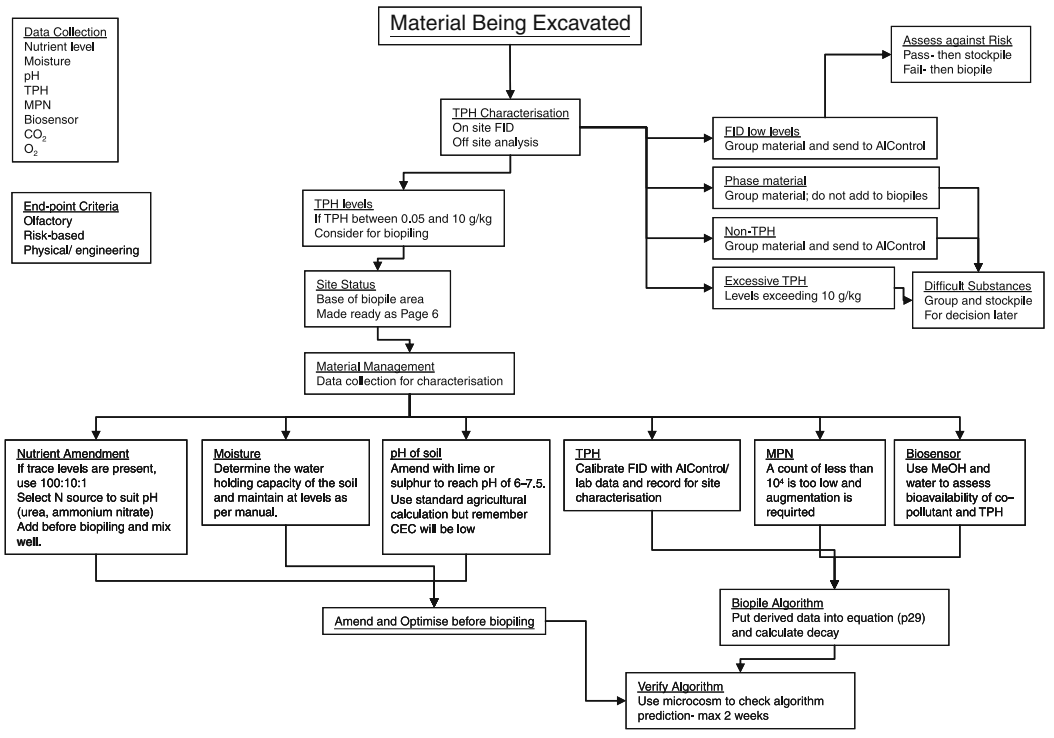
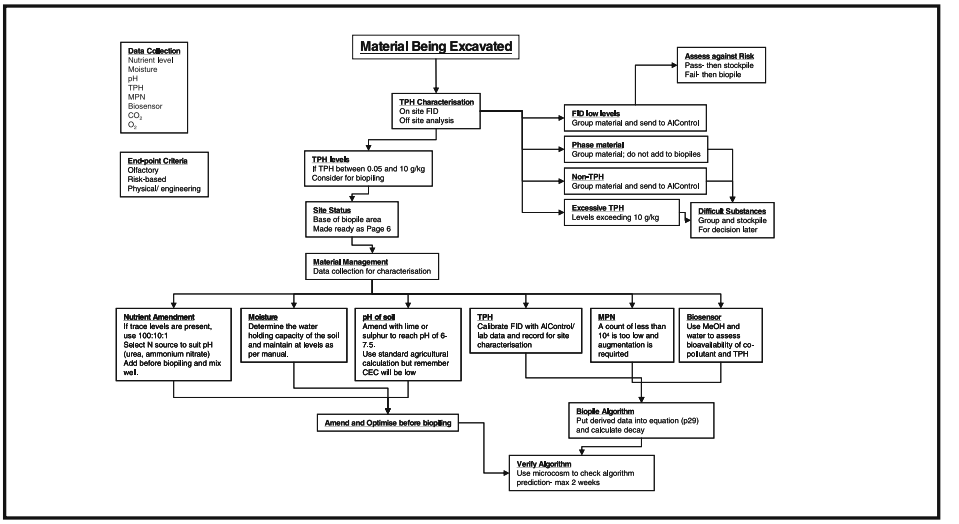


Fig. 13.4. Tier 3 of DST.

1. Former Railway Yard; UK

Problem:
This former railway yard offered a tremendous opportunity for development due to its prime location. The legacy of steam engines, diesel and electrical locomotives, however resulted in significant hydrocarbon contamination of the soil. Commonly coal and ash were dumped around sidings, solvents and lubrication oil handling was poor and fuel spillages were common.

DST Top 3 Technologies:
1. Biopiling
2. Windrowing
3. Landfilling



Remediation Solution:
To make best use of the limited space available the biopile treatment was carried out and then the soil which had met the remedial targets was stockpiled prior to backfilling. This was the unique aspect about this site in that the remediated soils were put directly to an end use. All of this was carried out in full consultation with Regulators. As one pile was competed the next one commenced.

Outcome:
Completion of the last biopile saw the remediation of 20,000 cubic metres equating to about 30,000 tonnes. The soil met the strict criteria that had been negotiated with the regulator within a suitable timescale. Had landfill been required, costs would have risen to many millions of pounds.

Fig. 13.5. Case study 1.

2. Industrial Site; Eastern Europe

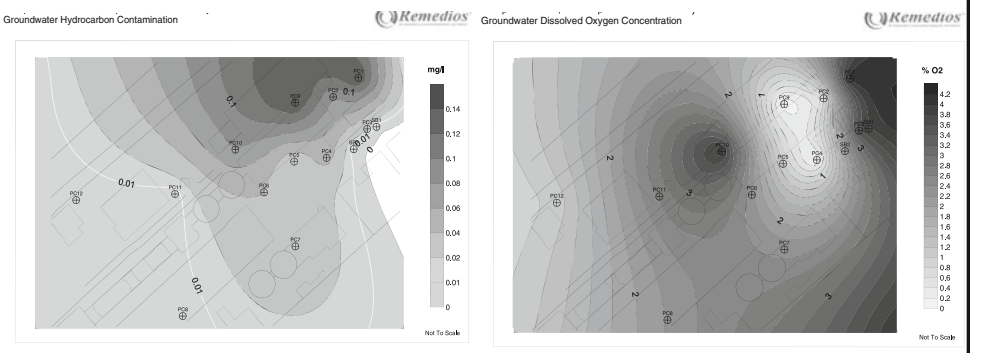
Problem:

A leading international cement and aggregate provider were concerned about the environmental liability associated with a cement plant located in the Eastern Europe, as there was a landfill site near the plant which had been used to dump various wastes over the years. A local environmental company had carried out a superficial site investigation which identified potential contamination at the site and as a result the company were very concerned about any future environmental implications.

DST Top 3 Technologies:
1. MNA
2. Windrow/ Biopile
3. Landfilling

Borehole ID	Hydrocarbon Concentration (mg/l)	Dissolved O_2 Concentration (%)
PC 1	0.14	4.2
PC 2	0.10	1.0
PC 3	0.02	3.2
PC 4	0.06	0.2
PC 5	0.02	1.0
PC 6	0.02	2.2
PC 7	0.02	2.2
PC 8	0.01	2.2
PC 9	0.14	0.2
PC 10	0.08	4.2
PC 11	0.01	2.4
PC 12	0.01	0.8
SB 1	0.01	4.0
SB 2	0.01	1.8

The DST enabled a systematic evaluation of the predicted rate of natural attenuation and the key gas measurements that were required to be made during the process. These were devised by using a range of MNA models that were integrated parallel to the DST. Microbial performance was key to meeting the defined risk derived criteria



Remediation Solution:

A detailed risk assessment of the soil and groundwater samples was undertaken. The results of the soil and groundwater risk assessments showed that there was minimal contamination in the site and groundwater samples. A report was produced that was compliant with UK legislation (in the absence of local legislation). It was recommended that routine monitoring of the existing boreholes should be carried out in order to be proactive in terms of any future liability and demonstrate best practice.

Outcome:

By assessing risks in a methodical manner, the design and implementation of an investigation strategy that directly assessed the conditions at the landfill site was achieved. A cost effective solution to the client was achieved and the client now feels they have this environmental issue under control.

Fig. 13.6. Case study 2.

6. Case Study Examples

The two case studies presented demonstrate the functionality of the UoA DST:

Case study 1: Former railway yard, UK (**Fig. 13.5**).

Case study 2: Industrial site, Eastern Europe (**Fig. 13.6**).

References

1. Bardos, R.P., Mariotti, C., Marot, F., and Sullivan, T. (2001) Framework for decision support used in contaminated land management in Europe and North America. *Land Contam. Reclam.* **9**, 149–163.
2. Plaza, G., Nalecz-Jawechi, G., Ulfig, K., and Brigmon, R.L. (2005) The application of bioassays as indicators of petroleum contaminated soil remediation. *Chemosphere*. **59**, 289–296.
3. Bezama, A., Szarka, N., Wolfbauer, J., and Lorber, K.E. (2007) Application of a balanced scorecard system for supporting decision-making in Contaminated Sites Remediation. *Wat. Air Soil Poll.* **181**, 3–16.
4. Bage, G.F., and Samson, R. (2002) A Technico-economic Approach for the selection of a site remediation strategy – Part A: Theory. *Environ. Manag.* **30**, 807–815.
5. Bardos P., Lewis A., Nortcliff S., Mariotti C., Marot F., and Sullivan T. (2003) Review of decision support tools for contaminated land management and their use in Europe. A report from the Contaminated Land Rehabilitation Network for Environmental Technologies (CLARINET) by the Decision Support Tools Working Group. Vienna (AT): Federal Environment Agency.
6. Nyer, E. (1996) Practical remediation technologies. *Ground Water Monit. Remed.* **16**, 54–57.
7. Critto, A., Cantarella, L., Carlon, C., Giove, S., Petruzzelli, G., and Marcomini, A. (2006) Decision support orientated selection of remediation technologies to rehabilitate contaminated sites. *Integ. Environ. Assess. Manag.* **2**, 273–285.
8. Boguski, T., Erickson, L.E., Fredkin, J., Green, R., Jamka, L., Norris, G., et al. (2007) Use the environmental knowledge and assessment tool to assist with environmental management. *Environ. Prog.* **26**, 251–262.
9. Geng, L., Chen, Z., Chan, C.W., and Huang, G.H. (2001) An intelligent decision support system for management of petroleum contaminated site. *Expert Syst. Appl.* **20**, 251–260.
10. Chen, Z., Huang, G.H., Chan, C.W., Geng, L.Q., and Xia, J. (2003) Development of an expert system for the remediation of petroleum contaminated sites. *Environ. Model. Assess.* **8**, 323–334.
11. Phelps, T.J., Pfiffner, S.M., Mackowski, R., Ringelberg, D., White, D.C., Herbes, S.E., and Palumbo, A.V. (1994) Application of microbial biomass and activity measures to assess in situ bioremediation of chlorinated solvents, in *Bioremediation of Chlorinated and Polycyclic Aromatic Hydrocarbon Compounds* (Hinchee, R.E., Leeson, A., Semprini, L. and Ong, S.K. ed.), Lewis Publishers, London, pp. 404–408.
12. Suflita, J.M. (1999) Intrinsic bioremediation of petroleum hydrocarbons in a gas condensate-contaminated aquifer. *Environ. Sci. Technol.* **33**, 2550–2560.
13. Atlas, R.M. (1991) Microbial hydrocarbon degradation – bioremediation of oil-spills. *J. Chem. Technol. Biotechnol.* **52**, 149–156.
14. Atlas, R.M., and Cerniglia, C.E. (1995) Bioremediation of petroleum pollutants. *BioSci.* **45**, 332–338.
15. Atlas, R.M., and Atlas, M.C. (1991) Biodegradation of oil and bioremediation of oil-spills. *Curr. Opin. Biotech.* **2**, 440–443.
16. Pollard, S.J.T., Brookes, A., Earl, N., Lowe, J., Kearney, T., and Nathanail, C.P. (2004) Integrated decision tools for the sustainable management of land contamination, *Sci. Total Environ.* **325**, 15–28.
17. Stapleton, R.D., and Sayler, G.S. (1998) Assessment of the microbiological potential for the natural attenuation of petroleum hydrocarbons in a shallow aquifer system. *Microb. Ecol.* **36**, 349–361.

18. Barkay, T., Nazaret, S., and Jeffrey, W. (1995) Degradative genes in the environment, in *Microbial Transformation and Degradation of Toxic Organic Chemicals* (Young, L.Y. and Cerniglia, C.E., ed.), Wiley-Liss, New York, pp. 545–577.
19. Rosenberg, E., Legman, R., Kushmaro, A., Taube, R., Adler, E., and Ron, E.Z. (1992) Petroleum bioremediation – a multiphase problem. *Biodegradation*. **3**, 337–350.
20. Atlas, R.M., and Bartha, R. (1992) Hydrocarbon biodegradation and oil-spill bioremediation. *Adv. Microb. Ecol.* **12**, 287–338.
21. Schmidt, L.M., Delfino, J.J., Preston, J.F., and St Laurent, G. (1999) Biodegradation of low aqueous concentration pentachlorophenol (PCP) contaminated water. *Chemosphere*. **38**, 2897–2912.
22. Lapham, L., Proctor, L., and Chanton, J. (1999) Using respiration rates and stable carbon isotopes to monitor the biodegradation of orimulsion by marine benthic bacteria. *Environ. Sci. Technol.* **33**, 2035–2039.
23. Berardesco, G., Dyhrman, S., Gallagher, E., and Shiaris, M.P. (1998) Spatial and temporal variation of phenanthrene-degrading bacteria in intertidal sediments. *Appl. Environ. Microbiol.* **64**, 2560–2565.
24. Ahn, Y., Sanseverino, J., and Sayler, G.S. (1999) Analyses of polycyclic aromatic hydrocarbon-degrading bacteria isolated from contaminated soils. *Biodegradation*. **10**, 149–157.
25. Fan, C-Y., and Krishnamurthy, S. (1995) Enzymes for enhancing bioremediation of petroleum contaminated soils: a brief review. *J. Air Waste Manag. Assoc.* **45**, 453–460.
26. Thurmann, U., Zanto, C., Schmitz, C., Vomberg, A., Puttmann, W., and Klinner, U. (1999) Correlation between microbial ex situ activities of two neighbouring uncontaminated and fuel oil contaminated subsurface sites. *Biotech. Techniques.* **13**, 271–275.
27. Mueller, J.G., Lantz, S.E., Devereux, R., Berg, J.D., and Pritchchard, P.H. (1994) Studies on the microbial ecology of polycyclic aromatic hydrocarbon biodegradation, in *Bioremediation of Chlorinated and Polycyclic Aromatic Hydrocarbon Compounds* (Hinchee, R.E., Leeson, A., Semprini, L. and Ong, S.K., ed.), Lewis Publishers, London, pp. 218–230.
28. Vidali, M. (2001) Bioremediation: an overview. *Pure Appl. Chem.* **73**, 1163–1172.
29. Anderson, R.T., Rooney-Varga, J.N., Gaw, C.V., and Lovley, D.R. (1998) Anaerobic benzene oxidation in the Fe(III) reduction zone of petroleum-contaminated aquifers. *Environ. Sci. Technol.* **32**, 1222–1229.
30. Pritchard, P.H., Mueller, J.G., Rogers, J.C., Kremer, F.V., and Glaser, J.A. (1992) Oil spill bioremediation: experiences, lessons, and results from the *Exxon Valdez* oil spill in Alaska. *Biodegradation*. **3**, 315–335.
31. Martin, S.R., Guwy, A.J., Ellis, B., Harold, P., Hawkes, F.R., and Hawkes, D.L. (2000) A novel method for measugin biomass activity applied to soils contaminated with oil refinery tar. *Environ. Technol.* **21**, 1019–1027.
32. McNally, D.L., Mihelcic, J.R., and Lueking, D.R. (1998) Biodegradation of three- and four-ring polycyclic aromatic hydrocarbons under aerobic and denitrifying conditions. *Environ. Sci. Technol.* **32**, 2633–2639.
33. Caldwell, M.E., Garrett, R.M., Prince, R.C., and Suflita, J.M. (1998) Anaerobic degradation of long-chain n-alkanes under sulfate-reducing conditions. *Environ. Sci. Technol.* **32**, 2191–2195.
34. Gieg, L.M., Kolhatkar, R.V., McInerney, M.J., Tanner, R.S., Harris, S.H., Sublette, K., and Suflita, J.M. (1999) Intrinsic bioremediation of petroleum hydrocarbons in a gas condensate-contaminated aquifer. *Environ. Sci. Technol.* **33**, 2550–2560.
35. Head, I.M., Singleton, I., and Milner, M.G. (ed) (2003) *Bioremediation – A Critical Review*. Horizon Scientific Press, Wiltshire, UK.
36. Rockne, K.J., and Strand, S.E. (1998) Biodegradation of bicyclic and polycyclic aromatic hydrocarbons in anaerobic enrichments. *Environ. Sci. Technol.* **32**, 3962–3967.

Chapter 14

Monitored Natural Attenuation

Kirsten S. Jørgensen, Jani M. Salminen, and Katarina Björklöf

Abstract

Monitored natural attenuation (MNA) is an in situ remediation technology that relies on naturally occurring and demonstrable processes in soil and groundwater which reduce the mass and concentration of the contaminants. Natural attenuation (NA) involves both aerobic and anaerobic degradation of the contaminants due to the fact that oxygen is used up near the core of the contaminant plume. The aerobic and anaerobic microbial processes can be assessed by microbial activity measurements and molecular biology methods in combination with chemical analyses. The sampling and knowledge on the site conditions are of major importance for the linkage of the results obtained to the conditions in situ. Rates obtained from activity measurements can, with certain limitations, be used in modeling of the fate of contaminants whereas most molecular methods mainly give qualitative information on the microbial community and gene abundances. However, molecular biology methods are fast and describe the in situ communities and avoid the biases inherent to activity assays requiring laboratory incubations.

Key words: Monitored natural attenuation, bioremediation, functional genes, anaerobic biodegradation.

1. Introduction

Monitored natural attenuation (MNA) is an in situ remediation method that relies on the monitoring of natural processes in soil and groundwater environments which act *without human intervention* to reduce the mass, toxicity, mobility, volume, or concentration of contaminants in those media (1). Complete mineralization of the contaminant is preferred upon the use of MNA as a remediation and risk management strategy. Monitored natural attenuation is thus best suited for biodegradable contaminants such as petroleum hydrocarbons. Like many active in situ

S.P. Cummings (ed.), *Bioremediation*, Methods in Molecular Biology 599,
DOI 10.1007/978-1-60761-439-5_14,

remediation methods, MNA may take time, but viewed on a long-term scale it may be more sustainable than many other remediation technologies, since no excavation and pumping of groundwater is required and neither transport nor final deposition places for the contaminated soil are needed.

Natural attenuation (NA) involves both aerobic and anaerobic degradation of the contaminants due to the fact that oxygen is depleted near the core of the contaminant plume. A sequence of anaerobic processes develops in the core of the plume indicating indirectly that the contaminants are being biodegraded. These anaerobic processes include denitrification, manganese reduction, iron reduction, sulfate reduction, and fermentation coupled with methanogenesis. Often one or more anaerobic processes become dominant. The US-EPA (1) was the first to propose that three lines of evidence for NA should be applied to demonstrate that natural attenuation is taking place: (i) documented decrease in contaminant concentrations at the site (historical data), (ii) documented geochemical conditions resulting from biological activities (indirect evidence), (iii) documented microbiological degradation activity (direct evidence). These principles have been adopted by many countries for the regulation of natural attenuation. Molecular biology methods provide direct demonstration of the third line of evidence (iii), and indirect evidence on criteria two (2). However, validation against chemical and other laboratory assays on microbial activity are of extreme importance for a more standardized use of molecular tools in the monitoring of bioremediation. We have investigated the progress of natural attenuation of petroleum hydrocarbons at a former landfill site, Trollberget in Southern Finland, and we have gained experience in the feasibility of different method in the assessment of natural attenuation (3, 4, 5, 6, 7). The petroleum hydrocarbon contamination at this site is mainly located in 1–4 m depth including both unsaturated and saturated zones. This chapter aims at evaluating these methods rather than giving complete method protocols for these. Most of the molecular techniques used in our study are described in detail in other chapters of this volume.

2. Materials

2.1. Sampling

1. Excavator or drilling rig
2. Groundwater and/or soil gas tubes to be installed
3. Spades and spoons
4. Containers (trays) for handling temporary soil batches
5. Eight-millimeter sieve

6. Sampling jars (preferably glass if the contaminants are petroleum hydrocarbons)
7. O_2 -free N_2 gas
8. Water sampling device
9. Portable gas analyzer
10. Means for cleaning sampling devices

2.2. Aerobic Microbial Activity Measurements (Non-molecular Biology)

1. 120 mL serum bottles with butyl rubber stoppers
2. 110 mL wide-mouth bottles and stoppers with attached NaOH traps
3. Syringes and needles
4. ^{14}C-labeled substrates: (e.g., UL-^{14}C-naphthalene (31 mCi/mmol, Sigma), 14,15-^{14}C-octacosane (20.5 mCi/mmol), or UL-^{14}C-toluene (2.8 mCi/mmol)
5. Scintillation vials and scintillation liquid
6. Liquid scintillation counter
7. CO_2 analyzer
8. Microtitre plates
9. Medium for MPN, Bushnell-Haas, (8)
10. Contaminant carbon source (pure)
11. Color indicator (iodonitrotetrazolium violet (INT), 0.3%, sterile filtered 0.2 μm)

2.3. Anaerobic Microbial Activity Measurement (Non-molecular Biology)

1. C_2H_2 gas
2. Syringes and needles
3. Gas chromatograph (GC-ECD) for N_2O quantification, Porapak Q column
4. Sterile 0.9% NaCl solution
5. SO_4^{2-} solution (0.5 mM in 0.9% NaCl)
6. Glove box with gas supply (85% N_2, CO_2 10%, and H_2 5%)
7. HEPES buffer (50 mM, pH 7)
8. Ferrozine (0.5 g/L in 50 mM HEPES, pH 7)
9. Spectrophotometer for Fe^{2+} quantification
10. 0.2-μm filters to be used before ion chromatography and Fe^{2+} determination
11. Ion chromatograph for SO_4^{2-} measurement
12. Gas chromatograph (GC-FID) for methane quantification

2.4. Molecular Biology Methods

1. DNA extraction kit (Fast DNA Spin kit for soil, BIO 191, Q-biogene, or PowerSoil DNA isolation kit (Mo Bio Laboratories)

2. Homogenizer for DNA extraction (e.g., FastPrep Instrument (Savant Instruments)
3. Specific primers for the organism of interest
4. PCR reagents
5. PCR machine
6. Real-time PCR machine
7. Gel electrophoresis and documentation equipment
8. Cloning kit
9. Enzymes for RFLP

3. Methods

3.1. Site Investigation and Sampling

Natural attenuation is always site-specific and, therefore, a careful site investigation must be performed when monitored natural attenuation is evaluated and used as a remediation technology. The purpose of the site investigation is to determine the spatial distribution of the contaminants, the risks for spreading and exposure to receptors, and to evaluate the extent and potential of biological degradation in situ to aid in the remediation of the site. The initial site investigation is extensive and, later on, one can focus on monitoring only selected parameters to follow the progress. Sampling is an integral part of natural attenuation because the result of any biological activity measurement only makes sense when it is seen in context of the time and sample origin. Therefore, sampling is included in this chapter in some detail. Chemical analyses will give data on contaminant concentrations and geochemistry at each sampling time, and with repeated sampling campaigns a time series can be obtained. On the contrary, biological assays give activity rates at each sampling time point because the samples are incubated in the laboratory after sampling. The steps in the site investigation are listed below:

1. Collection of historical data on the site use and, for example, possibly existing groundwater monitoring data and earlier soil sampling data.
2. Preparation of a sampling plan (*see* **Note 1**). Evaluate which environmental compartments and depths are to be investigated: e.g., soil, soil gas, sediment, and groundwater. Evaluate which chemical, geological, microbiological parameters are to be analyzed from the samples. Recommendations for minimum groundwater physical–chemical parameters: temperature, pH, contaminant concentration (e.g., petroleum hydrocarbons C_{10}–C_{40}), TOC, O_2, HCO_3^-, electrical conductivity, NO_3^-, Fe^{2+}, SO_4^{2-}, and CH_4. These parameters

include the contaminants and the indirect indicators of aerobic (O_2 and HCO_3^-) and anaerobic (NO_3^-, Fe^{2+}, SO_4^{2-}, CH_4, and HCO_3^-) microbial activity. Recommendation for minimum physical–chemical soil parameters: temperature, contaminant concentration (e.g., petroleum hydrocarbons C_{10}–C_{40}), soil organic matter, water content, soil gas composition (measured in situ).

3. Soil sampling. A joint sampling campaign for samples for different purposes is recommended if heavy drilling and excavation equipment is used. Samples for chemical, physical, and microbiological analyses are usually taken in separate bottles. The following procedure focuses on sampling for microbiological assays that need incubation.
   Soil sampling by drilling (*see* **Note 2**): the sample from a certain depth interval (e.g., 0.5-m intervals) should be collected separately and sieved. An 8-mm sieve is appropriate for this purpose, as it discards larger stones but retains soil aggregates. Triplicate samples from this homogeneous batch should be taken into glass jars or other appropriate containers (*see* **Note 3**). If the sample is from the anaerobic zone, the head space of an oxygen-tight sample bottle should be flushed with nitrogen in the field (**Fig14.1**). The samples should be transported below +10°C and stored at +4°C until

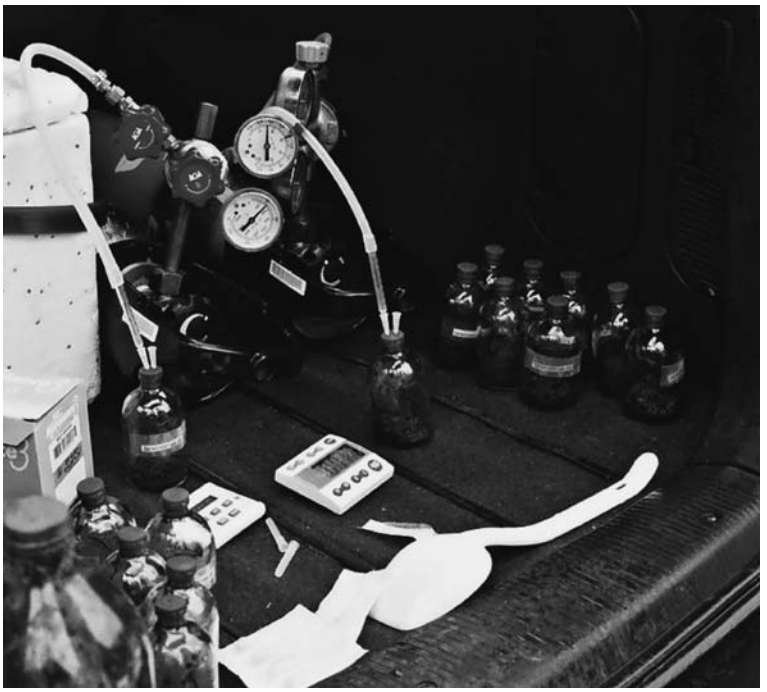


Fig. 14.1. Preparation of anaerobic microcosms in the field. The head space of 120 serum bottles containing soil are flushed with nitrogen gas in the trunk of a car.

the analyses. Samples for DNA extraction can be taken from the same sample bottles within a few days; but if the samples need to be stored for longer time, separate jars for storage at –20°C should be prepared (*see* **Note 4**).

4. Installation of soil gas monitoring wells. It is convenient to install soil gas monitoring tubes into the bore hole used for soil sampling (4). Insert a perforated tube, e.g., 25 mm in diameter and 0.5 m longer than the depth of the hole. Seal the top soil around tube with bentonite in order to prevent leakage of atmospheric air from above. Cap the tube with a tight stopper.
5. Soil gas sampling (*see* **Note 5**). The purpose of the soil gas sampling is to obtain information on the oxygen conditions in the subsurface and, based on this, to determine at what depth aerobic and anaerobic processes dominate (**Fig. 14.2**). The soil gas concentrations give a picture of the steady-state conditions in the soil, but do not as such measure any activity (3). The composition of soil gases at

Fig. 14.2. Sampling a gas monitoring well at the Trollberget site in Southern Finland using a portable gas analyzer. The analyzer has an air pump and sensors for measurement of O_2, CO_2, and CH_4. Two gas monitoring wells reaching different depths are installed next to each other.

the site can be measured from soil gas monitoring wells that have been installed at least 24 h earlier (4). Concentrations of O_2, CO_2, and CH_4 in the soil can be monitored at the site from the wells using a portable Dräger Multiwarn II multigas analyzer (Drägerwerk, Lübeck, Germany) (**Fig. 14.2**). Concentrations of methane can be confirmed in the laboratory (see below) from gas samples obtained by collecting a sample into a gas-tight glass vial in the field. If in situ gas production rates are required, the monitoring wells should be flushed with fresh atmospheric air and sealed for several hours before starting a gas measurement time series.

6. Groundwater sampling (*see* **Notes 6 and 7**). Groundwater samples can be taken using different types of samplers. We have used either an inertial pump consisting of a foot valve and piece of Teflon tubing or a bailer. Care should be taken to avoid oxygen contamination of the water sample. It is recommended to use a sampler that is designed for sampling of anaerobic groundwater when it is known that the well contains anoxic groundwater (*see* **Note 8**). Different sample bottles for organic and inorganic chemical and microbiological analyses are usually needed.
7. Preparation of microcosms assays. These microcosms can be used for contaminant degradation studies, aerobic respiration, methane production tests, or other tests (*see* **Note 9**). It is convenient to prepare several parallel bottles that can be used for destructive sampling over a time series. For the measurement of microbiologically produced gases, CO_2 and CH_4, use 120-mL serum bottles that can be closed by tight butyl rubber stoppers. Place 10–30 g of sieved soil into each preweighed bottle. The bottles can then be weighed upon return to the laboratory in order to obtain the exact sample weight. Three replicates should be made for each time point. After incubation and gas measurements, the bottles can be sacrificed for chemical analysis for the contaminant, DNA extraction, etc. A time series should have at least 5 time points inclusive of the 0-time (*see* **Note 10**).

3.2. Aerobic Microbial Activity Measurements (Non-molecular Biology)

1. Petroleum hydrocarbon degradation rates in microcosms. The degradation rate of petroleum hydrocarbons can be determined in controlled laboratory experiments by measuring the decrease in the original petroleum hydrocarbon concentration by time (3, 4). Degradation rates obtained with original contaminants reflect in situ degradation better than those obtained with spiked contaminants, because freshly added compounds may act very differently compared to aged contaminants. The 120-mL microcosm bottles prepared from the same batch of homogenous soil sample are

incubated at 7°C in the dark for 4 months (aerobic tests). Triplicate bottles are sacrificed for analysis of petroleum hydrocarbons at regular intervals. The disappearance rate of petroleum hydrocarbons is calculated. Since no amendments are made and the incubation is made at in situ temperature, the rate obtained is as close to in situ conditions as possible.

2. ^{14}C-mineralization tests. Mineralization of ^{14}C-labeled compounds gives evidence on the complete conversion of the contaminant to $^{14}CO_2$. However, petroleum hydrocarbon contamination is usually a mixture of hundreds of compounds and, therefore, it is necessary to select model compounds. ^{14}C-octacosane (e.g., hexadecane is also suitable), ^{14}C-toluene, and naphthalene can function as model compounds for alkanes, monoaromates, and polyaromates, respectively (6, 7) (**Fig. 14.3**). Place10-g subsamples of sieved soil into air-tight 110-mL bottles with stoppers that carry a cup containing 1 M NaOH to trap the produced ^{14}C-CO_2 in. One killed control and three replicates should be prepared for each soil sample. Add to every sample approximately 100,000 DPM of UL-^{14}C-naphthalene (31 mCi/mmol, Sigma), 14,15-^{14}C-octacosane (20.5 mCi/mmol), or UL-^{14}C-toluene (2.8 mCi/mmol). Incubate the samples at +7±1°C. Measure the radioactive $^{14}CO_2$ produced in the samples using a liquid scintillation counter over time.

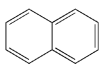
Model compound	Target gene	Target enzyme
$CH_3(CH_2)_{26}CH_3$	*alkB*	Alkane hydroxylase
14,15-^{14}C-octacosane		
	*xylE*	Catechol-2,3-dioxygenase
UL-^{14}C-toluene		
	*nahAc*	Naphthalene dioxygenase
UL-^{14}C-naphthalene		

Fig. 14.3. Example of available ^{14}C-labeled model compounds and the corresponding genes that encode key enzymes from aerobic degradation pathways. Alkanes, monoaromatic, and polyaromatic compounds are represented. ^{14}C-labeled substrates are used in mineralization assays and the corresponding functional genes can be quantified in soil samples by quantitative PCR.

3. Aerobic respiration. The total microbial CO_2 production is a measure of the overall microbial activity, but it does not confirm that the contaminant is degraded. If much other organic matter is present in the sample in addition to the

contaminant, this method does not tell very much about the specific degradation activity. Measure the CO_2 concentration in closed bottles (prepared as microcosms) over time. Gas samples of 0.5 mL are injected into a carbon analyzer.

4. Enumeration of aerobic PHC degraders by most-probable-number technique (MPN). The most-probable-number technique (MPN) is a method where bacteria, able to degrade specific compounds in the samples, are determined by culturing in the laboratory (8). This method is easy to perform and interpret. The method relies on the cultivation of microbes in liquid medium containing the contaminant as the sole C-source under laboratory conditions. Therefore, no direct conclusions about the number of microbes or their activity on the site can be made. Briefly, soil dilutions are distributed into microplates containing 1% diesel oil or lubrication oil as the only carbon substrate. The plates are incubated at 20°C for 4 weeks after which wells containing bacteria are detected by the addition of a color indicator. The most probable number of diesel and lubrication oil degraders is determined statistically.

3.3. Anaerobic Microbial Activity Measurements (Non-molecular Biology)

1. Petroleum hydrocarbon degradation rates in microcosms. The anaerobic degradation rate of petroleum hydrocarbons can be determined in controlled laboratory experiments by measuring the decrease in the original petroleum hydrocarbon concentration in the soil over time in anaerobic conditions (5). The 120-mL microcosm bottles containing 30 g of soil should be flushed with nitrogen for 4 min (**Fig. 14.1**). Incubate at 7°C in the dark for 10 months. Triplicate bottles should be sacrificed for analysis of petroleum hydrocarbons at regular intervals. The disappearance rate of petroleum hydrocarbons is calculated. Since no amendments are made and the incubation is at in situ temperature, the rate is as close to in situ conditions as possible.
2. Denitrification rates in microcosms. The denitrification rate is a measure of the ability of the microbial community to use nitrate as an alternative electron acceptor. NO_3^- is reduced to N_2 and the organic contaminant is oxidized to CO_2. We here measure the production of the intermediate N_2O by inhibiting the last step in the denitrificaiton process (5). The 120-mL microcosm bottles in triplicate containing 10 g of soil should be flushed with nitrogen for 4 min in the field. In the laboratory, add 10 mL of C_2H_2 gas to the bottles to inhibit the conversion of N_2O to N_2. If nitrate is not present in the samples, 0.5 mL of anoxic 10 mM KNO_3 should be added to the bottles. Incubate at 7°C in the dark for about 1 week. Measure the produced N_2O in the

headspace once a day by injecting 0.5 mL gas samples into a GC-ECD, and calculate the denitrification rate from the time points obtained. If the measurement is accompaingned by a simultaneous measurement of petroleum hydrocarbon disappearance from the same bottles, evidence for petroleum hydrocarbon degradation under denitrifying conditions can be obtained.

3. Iron reduction rate in soil slurries. The iron reduction rate is a measure of the ability of the microbial community to use Fe^{3+} as an alternative electron acceptor. $Fe3^{+}$ is reduced to Fe^{2+} and the organic contaminant is oxidized to CO_2. We here measure the production of soluble Fe^{2+} (5). The 120-mL microcosm bottles containing 30 g of soil should be flushed with nitrogen for 4 min in the field. In the laboratory, add 60 mL of anoxic 0.9% NaCl in a glove box and incubate under shaking for about 1 week and sample at regular intervals for Fe^{2+}. For the measurement of Fe^{2+} produced, withdraw anaerobically with a syringe and 0.2 μm filter a liquid sample of 20–600 μl, and add this directly into a preweighed volume of approximately 4 mL of ferrozine reagent (0.5 g/l in 50 mM HEPES, pH 7), and measure the developed color with a spectrophotometer at 562 nm. If the measurement is accompaingned by a simultaneous measurement of petroleum hydrocarbon disappearance from the same bottles, evidence for petroleum hydrocarbon degradation under iron-reducing conditions can be obtained.
4. Sulfate reduction rate in soil slurries. The sulfate reduction rate is a measure of the ability of the microbial community to use SO_4^{2-} as an alternative electron acceptor. SO_4^{2-} is reduced to H_2S and the organic contaminant is oxidized to CO_2. We here measure the consumption of soluble SO_4^{2-} in the water phase (5). The 120-mL microcosm bottles containing 30 g of soil should be flushed with nitrogen for 4 min in the field. In the laboratory, add 60 mL of anoxic 0.9% NaCl and 0.5 mM SO_4^{2-} in a glove box and incubate under shaking for about 1 month. Withdraw anaerobically 2 mL sample and filter it through a 0.22 μm filter and measure the SO_4^{2-} Concentration on an ion chromatograph. If the measurement is accompaingned by a simultaneous measurement of petroleum hydrocarbon disappearance from the same bottles, evidence for petroleum hydrocarbon degradation under sulfate-reducing conditions can be obtained.
5. Methane production rate in microcosms (4, 5). The methane production is a measure of the ability of the microbial community to convert acetate or H_2 and the CO_2 produced in fermentation processes to CH_4. Hydrocarbon fermentation is initiated by microbial fumarate addition. The

120-mL microcosm bottles containing 30 g of soil should be flushed with nitrogen for 4 min in the field. Incubate at 7°C in the dark for 10 months. Measure the CH_4 concentration regularly in gas samples from the headspace by injecting 0.5 mL samples into a GD-FID. If the measurement is accompanied by a simultaneous measurement of petroleum hydrocarbon disappearance from the same bottles, evidence for petroleum hydrocarbon degradation under fermenting and methanogenic conditions can be obtained (*see* **Note 11**).

3.4. Molecular Biology Methods

3.4.1. DNA Extraction

1. Extract two parallel 0.7 g soils samples from the same batch of soil using the FastDNA®SPIN Kit for Soil (Bio101) (or PowerSoil DNA isolation kit), which purifies genomic DNA from soil using the FastPrep Instrument (Savant Instruments).

3.4.2. Community Analysis (Cloning and Sequencing, RFLP)

Community analysis is used to study the microbial community composition and diversity based on the presence of 16S RNA genes in the soil sample. The analysis does not indicate whether those microbes in the sample are active or if they are involved in the degradation of the contaminants. However, based on known physiological properties of certain taxonomic groups, some conclusions can usually be drawn. Furthermore, the community composition may change over time or along gradients of environmental factors that may reveal the role of different members (*see* **Note 12**).

Steps in community analysis:

1. Amplification of the 16sRNA gene by PCR of the extracted community genomic DNA. A suggestion for general primers is given below.

Bacteria	PRBA338f	5′-ACTCCTACGGGAGGCAGCAG -3′ *(9)*
	Ds907r	5′-CCCCGTCAATTCCTTTGAGTTT-3′ *(10)*
Archaea	PREA46f	5′-(C/T)TAAGCCATGC(G/A)AGT -3′ *(9)*
	PRA1100r	5′-(T/C)GGGTCTCGCTCGTTRCC -3′ *(9)*,

and then primer pair

PARCH340f 5′-CCCTACGGGG(C/T)GCA(G/C)CAG-3′ *(9)*

ARC915r 5′-GTGCTCCCCGCCAATTCCT-3′ *(11)*

2. The following PCR conditions (*see* **Notes 13 and 14**) can be used with these primers: Use 0.2-mL thin-walled PCR tubes. The total volume of each PCR reaction mix is 50 μL and should contain 1 μM of each primer, 200 μM of

each dNTP, 2 U of Taq DNA polymerase (MBI Fermentas), 75 mM Tris–HCl, 20 mM $(NH_4)_2$ SO_4, 0.01% Tween 20, 2.5 mM $MgCl_2$, 1% of formamide, 0.5 mg/mL of BSA, and 1 μL of DNA sample. PCR should be run in a thermal cycler as follows for bacterial primers: 94°C for 60 s, after which 30 cycles of 94°C for 45 s, 46°C for 45 s, 72°C for 60 s, with a final extension of 6 min at 72°C. For archaeal primers the programme is 92°C for 120 s, after which 30 cycles of 92°C for 60 s, 65°C for 30 s, 72°C for 60 s, with a final extension of 6 min at 72°C.

3. Clean up the PCR products using Wizard®DNA cleanup.
4. Cloning. Clone purified PCR products using, e.g., the Promega pGEM®-T Easy Vector system according to the instructions of the manufacturer. It is recommended that at least 100 clones are sequenced per library (*see* **Note 15**).
5. Purification and analysis of diversity of clones, e.g., by RFLP before sequencing.
6. Sequencing of inserts in clones.
7. Matches to the Genbank by Blast.
8. Phylogenetic analysis.

3.4.3. Functional Genes (RLD-PCR, Real-Time PCR)

The abundance of functional (catabolic) genes is a measure of specific genes that encode specific enzymes in the degradation pathway of, e.g., petroleum hydrocarbons. Most frequently, key enzymes in the degradation pathway are used (**Fig. 14.3**). For aerobic degradation these are often oxidases. As both the enzymes and the degradation pathways are diverse and also vary between organisms, the detection of a limited set of genes may underestimate the true degradation potential (*see* **Note 16**). Despite these shortcomings, the functional gene abundances have proven to give robust estimates of degradation potential (*see* **Notes 17, 18**). Real-time PCR technology offers a very sensitive tool to quantify gene copies in environmental samples, but gene abundances can also be estimated by MPN-based dilution series like, e.g., the replicate limited dilution (RLD) technique (7) (*see* **Note 19**).

3.4.3.1. Gene Abundance by RLD-PCR Technique

1. From each soil sample, extract two replicate DNA samples and prepare from both of these two replicate 3× dilution series. Run PCR on all four-dilution series (12).
2. PCR amplification can be carried out in 0.2-mL thin-walled PCR tubes. Primers that we have tested are given below (6, 7) (*see* **Note 20**).

alkB	alkB703for	5′-TGGCCGGCTACTCCGATGA TCGGAATCTGG-3′ *(13)*
	alkB1572rev	5′-CGCGTGGTGATCCGAGTGC CGCTGAAGGTG-3′

xylE	xylEbf (482)	5′-AGGTATGGCGGCTGTGCGT TTC-3′ *(14)*
	xylEbr (950)	5′-TTCGTTGAGAATGCGGTCG TGG-3′
nahAc	Ac114F	5′-CTGGC(T/A)(T/A)TT(T/C) CTCAC(T/C)CAT-3′ *(15)*
	Ac596R	5′-C(G/A)GGTG(C/T)CTTCCA GTTG-3′

3. The final volume of 50 μL should contain 1 μM of each primer, 200 mM of each dNTP, 1 U of Taq DNA polymerase (MBI Fermentas), 75 mM Tris–HCl, 20 mM $(NH_4)_2$ SO_4, 0.01% Tween 20, 1.5 mM $MgCl_2$, and 1% nuclease-free bovine serum albumin (BioLabs). With primer pair Ac114F and Ac596R, run a touchdown PCR program according to (15). With primer pair alkB703for and alkB1572rev, run PCR with 45 cycles of 1 min at 95°C, 1 min at 60°C, 1 min at 72°C, and a final extension of 10 min at 72°C. With primer pair xylEbf and xylEbr, run PCR with 35 cycles of 1 min at 95°C, 1 min at 68°C, 1 min at 72°C, and a final extension of 5 min at 72°C. Verify the PCR products in a 1.5% (wt/vol) agarose gel in 0.5% TBE by running samples for 30 min at 150 V.
4. Calculate the number of gene copies (per mL of DNA extract or per gram of soil) from the highest dilution in which the PCR product was observed. The final gene copy abundance is an average of the four dilution series prepared from each soil sample (*see* **Note 21**).

3.4.3.2. Real-Time PCR for *nahAc* and *xylE*

1. For the preparation of a standard, use a purified PCR product of a pure culture of a bacterium that contains the gene in question, and make 10-fold dilution series.
2. Make standard curves for the quantification of the *nahAc* and *xylE* genes by the real-time quantitative PCR.
3. Run the standards in triplicate together with 3-, 9-, and 27-fold dilutions of the DNA extracts on a thermal cycler to quantify the number of the *nahAc* and *xylE* gene copies in soil samples. The total volume of the real-time PCR reaction is 20 μL and should contain 10 μL of the 2 × Quantitect SYBR Green mastermix (Qiagen GmbH, Hilden, Germany), 2.5 μM of each of the respective forward and reverse primers and 1 μL of the template DNA. The temperature program of the real-time PCR run for *nahAc* is 95°C, 15 min (first cycle only); 94°C, 15 s; 52°C, 30 s; 72°C, 30 s; altogether 40 cycles with a final extension at 72°C for 6 min. The temperature program of the real-time PCR run for *xylE* is 95°C,

15 min (first cycle only); 94°C, 15 s; 68°C, 30 s; 72°C, 30 s; altogether 40 cycles with a final extension at 72°C for 5 min.

4. Notes

1. Communicate with geologists, chemists, and engineers when planning and performing sampling.
2. Contaminated sites and aquifer sediments are frequently dominated by heterogeneity. Contaminant concentrations may vary in the scale of centimeters of tens of centimeters. Sampling by drilling is destructive. Repeated sampling from the same spot is not possible. However, replicate samples can be obtained from the same drilling batch of a certain depth.
3. Use true sample replicates rather than analysis replicates. For example, it is better to extract and analyze two parallel soil samples than to analyze the same extract sample twice.
4. Sampling for molecular biology assays. Some people prefer to sample soil directly into, e.g., 2-mL Eppendorf tubes and to transport the sample in a portable liquid nitrogen transporter to the laboratory and to store the soil samples at –80°C until DNA extraction. However, the storage of contaminated soil samples in plastic tubes in the –80° freezer together with clean samples or other live samples is not recommended. Samples containing organic contaminants should preferably be stored in jars made of glass or other inert gas-tight material. We have found that samples for DNA extraction can be stored at –20°C until DNA extraction if it is not possible to extract the DNA within a few days.
5. Soil gas measurement is a simple way of getting information about the in situ oxygen conditions.
6. Microbes are mainly attached to solid particles also in the saturated zone. In order to get a realistic measure of the microbial community and its activity in situ, the solid matrix should be included in the sampling of subsurface for community analyses and activity measurements. The microbial composition is different on the particles and in the free groundwater. However, repeated groundwater samples are easy to obtain and, e.g., useful for the monitoring of geochemical parameters.
7. Bacterial traps that are incubated in groundwater wells is a nice way of getting an enrichment of bacteria incubated in situ, but one has to keep in mind that the trap mainly

contains mobile free living bacteria from the groundwater, and the bacterial population living on the soil particles in the groundwater zone may have a different composition and activity.

8. Sampling from anaerobic depths should be performed with care. There are special samplers that are designed for sampling anoxic zones on the market. Groundwater sampling using a device that can take specifically in a certain depth is also on the market.
9. Apply data from the literature with care to your specific site. Microbial communities and activities are always site-specific.
10. Rates of degradation are of value for mathematical modeling of the distribution of the contaminants over time. Many other biological parameters, e.g., most molecular biology methods are not suitable for modeling, but have a qualitative value for the monitoring and evaluation of the progress of the biodegradation.
11. The presence of distinct physiological groups, e.g., nitrate reducers, iron-reducers, sulfate reducers, or methanogens, does not give any direct evidence for contaminant degradation if there is no measurement of the contaminant fate in the same samples. However, the dominance of a certain type of anaerobic metabolism at a certain depth may help to understand the progress of the contaminant degradation at the site.
12. Community composition does not give evidence for petroleum hydrocarbon degradation without corresponding degradation measurements. However, it seems that petroleum-contaminated anaerobic aquifers tend to develop a diverse but distinct community composition despite of their geographical origin (16, 17).
13. PCR contamination is a problem in many laboratories. In particular, the amplification of 16S RNA genes using general primers for the Bacteria domain is much more prone to contamination than amplification by using specific primers for a functional gene or for lower taxonomic groups. Several polymerase enzymes have been found to contain traces of bacterial 16S DNA. It may be a solution to change enzyme supplier and keep all reagents in fresh batches in case of contamination.
14. PCR contamination originating from tubes and pipettes may also be a problem. Do not autoclave disposable materials before use, since the autoclavation will not destroy the DNA, only living cells, and it may cause any present DNA to spread to other surfaces in the autoclave. Usually, factory-grade disposable materials are sufficiently clean. UV

irradiation is a more efficient way to destroy nucleic acids, but the distance to the UV source should be less than 1 m to be efficient. A UV lamp in the ceiling of the laboratory is not efficient enough.

15. Community analysis by cloning and sequencing gives evidence for the presence of different taxonomic group of bacteria, whereas DGGE does not reveal the species composition, but can be useful for comparison of samples or screening of many samples.
16. Comprehensive functional gene arrays have been developed, which are based on quantification by hybridization of probes of all known degradation genes (18) to community DNA from environmental samples. The problem is the sensitivity of the method, which is much lower than real-time PCR quantification.
17. Functional aerobic gene abundances are roughly correlated with aerobic mineralization rates of corresponding contaminants (6, 7).
18. The quantification of mRNA by reverse transcription real-time PCR can give an estimate of active populations.
19. Quantitative PCR of several functional genes are recommended for the same sample. Real-time PCR is much more sensitive than RLD-PCR (6). If the sample is from the anaerobic zone, the aerobic degradation genes are not so relevant to analyze. Our studies only assessed genes of aerobic pathways. Genes involved in the anaerobic degradation aromatic petroleum hydrocarbons can be assessed, e.g., with the *bss* gene, which encodes for benzyl succinate synthetase (19). There is a need for better knowledge on the anaerobic pathways. It seems in many cases the anaerobic pathways are more complicated and carried out be a consortium of organisms, e.g., syntrophs and methanogens.
20. The choice of primers – length of fragment: Amplification of fragments of different lengths is needed for different purposes. For example, real-time PCR using SYBR green only works for shorter fragments whereas traditional PCR works for larger fragments, e.g., of functional genes. Also DGGE works better for shorter fragments, whereas sequencing often is performed for the whole 16sRNA gene. This means that different PCR reactions using different primers have to be optimized.
21. Molecular biology methods are faster than longer laboratory incubations for microbial activity measurements, and they represent the in situ conditions. Molecular biology methods complement efficiently microbial activity and chemical measurements in the monitoring of natural attenuation.

References

1. EPA. (1999) Use of monitored natural attenuation at superfund, RCRA corrective action, and underground storage tank sites. Office of solid waste and emergency response. Washington DC, 9200.4–17p.
2. Jørgensen, K.S. (2007) In situ bioremediation. *Adv. Appl. Microbiol.* **61**, 285–305.
3. Björklöf, K., Salminen, J., Sainio, P., and Jørgensen, K. (2008) Degradation rates of aged petroleum hydrocarbons are likely to be mass transfer dependent in the field. *Environ. Geochem. Health* **30**, 101–107.
4. Salminen, J.M., Tuomi, P.M., Suortti, A.-M., and Jørgensen, K.S. (2004) Potential for aerobic and anaerobic biodegradation of petroleum hydrocarbons in boreal subsurface. *Biodegradation* **15**, 29–39.
5. Salminen, J.M., Hänninen, P.J., Leveinen, J., Lintinen, P.T.J., and Jørgensen, K.S. (2006) Occurrence and rates of terminal electron-accepting processes and recharge processes in petroleum hydrocarbon-contaminated subsurface. *J. Environ. Qual.* **35**, 1969–2440.
6. Salminen, J.M., Tuomi, P.M. and Jørgensen, K.S. (2008) Functional gene abundances (*nahAc, alkB, xylE*) in the assessment of the efficacy of bioremediation. Appl. Biochem. Biotechnol. **151**, 638–652.
7. Tuomi, P.M., Salminen, J.M., and Jørgensen, K.S. (2004) The abundance of *nahAc* genes correlates with the ^{14}C-naphthalene mineralization potential in petroleum hydrocarbon-contaminated oxic soil layers. *FEMS Microbiol. Ecol.* **51**, 99–107.
8. Haines J., Wrenn B., Holder E., Strohmeier K., Herrington R., and Venosa, A. (1996) Measurement of hydrocarbon-degrading microbial populations by a 96-well plate most-probable-number procedure. *J. Ind. Microbiol.* **16**, 36–41.
9. Teske, A., Wawer, C., Muyzer, G., and Ramsing, N.B. (1996) Distribution of sulfate-reducing bacteria in a stratified fjord (Mariager Fjord, Denmark) as evaluated by most-probable-number counts and denaturing gradient gel electrophoresis of PCR-amplified ribosomal DNA fragments. *Appl. Environ. Microbiol.* **62**,1405–1415.
10. Øvreås, L., Forney, L., Daae, F.L., and Torsvik. V. (1997) Distribution of bacterioplankton in meromictic Lake Saelenvannet, as determined by denaturing gradient gel electrophoresis of PCR-amplified gene fragments coding for 16S rRNA. *Appl. Environ. Microbiol.* **63**, 3367–3373.
11. Stahl, D.A., and Amann, R. (1991) Development and application of nucleic acid probes. In: Nucleic acid techniques in bacterial systematics (Stackebrandt, E. and e.Goodfellow, M., Eds.), pp. 205–248. Wiley, West Sussex, UK.
12. Chandler, D.P. (1998) Redefining relativity: quantitative PCR at low template concentrations for industrial and environmental microbiology. *J. Ind. Microbiol. Biotechnol.* **21**, 128–140.
13. Whyte, L.G., Greer, C.W., and Inniss, W.E. (1996) Assessment of the biodegradation potential of psychrotrophic microorganisms. *Can. J. Microbiol.* **42**, 99–106.
14. Jussila, M.M., Zhao, J., Suominen, L., and Lindström, K. (2007) TOL plasmid transfer during bacterial conjugation *in vitro* and rhizoremediation of oil compounds *in vivo*. *Environ. Pollut.* **146**, 510–524.
15. Wilson, M.S., Bakermans, C., and Madsen, E.L. (1999) In situ, real-time catabolic gene expression: Extraction and characterization of naphthalene dioxygenase mRNA transcripts from groundwater. *Appl. Environ. Microbiol.* **65**, 80–87.
16. Kasai, Y., Takahata, Y., Hoaki, T., and Watanabe, K. (2005) Physiological and molecular characterization of a microbial community established in unsaturated, petroleum-contaminated soil. *Environ. Microbiol.* **7**, 806–818.
17. 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. Environ. Microbiol.* **64**, 3869–3877.
18. He, Z., Gentry, T.J., Schadt, C.W., Wu, L., Liebich, J., Chong, S.C., et al. (2007) GeoChip: a comprehensive microarray for investigating biogeochemical, ecological and environmental processes. *ISME J.* **1**, 67–77.
19. Beller, H.R., Kane, S.R., Legler, T.C., and Alvarez, P.J.J. (2002) A real-time polymerase chain reaction method for monitoring anaerobic, hydrocarbon-degrading bacteria based on a catabolic gene. *Environ. Sci. Technol.* **36**, 3977–3984.

Chapter 15

Application of Bioassays for the Ecotoxicity Assessment of Contaminated Soils

María D. Fernández, Mar Babín, and José V. Tarazona

Abstract

The use of bioassays for soil characterization is receiving significant attention as a complementary tool to chemical analysis. Bioassays consist of direct toxicity assays of environmental samples that are transferred to the laboratory and analyzed for toxicity against selected organisms. Such soil samples contain the combination of the different pollutants present in situ and enable factors such as the bioavailability of contaminants or the interactions (synergic and antagonic) between them to be simultaneously studied.
In this chapter, methods for soil toxicity assessment based in the guidelines developed by OECD for single substances are described. These methods have been adapted for their application to the assessment of complex matrices such as soils. The field sample can be tested undiluted and/or diluted with "uncontaminated" soil to create a pollution gradient. In the diluted samples, concentration/response relationships may be obtained. Toxicity assays to soil organisms include earthworms, plants, and microorganisms tests. In addition, toxicity assays with soil extracts are recommended. Assays of extracts with algae, daphnia, and fish (in vitro test using fish cell lines) are also described.

Key words: Bioassays, contaminated soils, soil characterization, toxicity test, in vivo, in vitro.

1. Introduction

The term bioassay is used to describe ecotoxicity tests performed with environmental samples. They usually consist, in laboratory assays, of soil or water samples collected at the study site, which are tested to determine their toxicity to selected organisms. There is an increasing interest in incorporating bioassays in the characterization of contaminated soils as a complementary tool to chemical analysis (1, 2, 3). They allow the overall toxicity of the sample to be determined even if the contaminants in the soil are not

S.P. Cummings (ed.), *Bioremediation*, Methods in Molecular Biology 599,
DOI 10.1007/978-1-60761-439-5_15,

identified. Moreover, specific characteristics in the site, which influence soil toxicity, such as contaminant bioavailability and possible interactions between contaminants in the soil (synergic and antagonic effects) may be addressed. In addition to toxicity assays with soil samples, it is recommended that toxicity tests with extracts obtained from the soil are performed (4, 5, 6, 7). Toxicity studies on organisms from both terrestrial and aquatic environments allow a more complete soil toxicity assessment, involving a higher number of species and different methods of exposure. From the perspective of risk analysis, this approach will determine the transfer of contaminants from soil to water and take under consideration the risk of contamination of surface and ground waters due to runoff and leaching phenomena.

There are numerous methods to determine ecotoxicity to soil and aquatic organisms, which have been standardized by different associations (OECD, ISO, etc). However, these methods have been described for chemical compounds and require some adaptations prior to their application to the assessment of complex matrices such as soils. In this work, methods for soil toxicity assessment using bioassays are described. These methods are based in the guidelines developed by OECD for the assessment of single substances. The bioassays for soil toxicity are determined according to the following tests:

- Earthworms, acute toxicity tests. Modified test guideline N° 207 OECD (8)
- Terrestrial plants, seedling emergence and seedling growth test. Modified test guideline N° 208 OECD (9)
- Soil microorganisms, nitrogen transformation test. Modified test guideline N° 216 OECD (10)
- Soil microorganisms, carbon transformation test. Modified test guideline N° 217 OECD (11)

Toxicity to aquatic organisms: algae, daphnia, and fish are also described. The toxicity of soil extracts to algae and daphnia is determined according to:

- Algal growth inhibition test. Modified test guideline N° 201 OECD (12)
- *Daphnia* sp., acute immobilization test. Modified test guideline N° 202 OECD (13)

Assays to determine fish toxicity consist of an in vitro assay battery using RTG-2 fish cell lines (14). This assay is more appropriate for the analyses of soil extracts since it reduces the volume necessary to perform the assay. Only those modifications of standard methods necessary for their application to the assessment of contaminated soils will be described. A more detailed description of OECD methods may be found in http://oberon.sourceoecd.org (15).

2. Materials

2.1. General Material

1. Soil samples. The use of soils freshly collected from the field is recommended. However, soils may be stored in the dark at 4±2°C for a maximum of 3 months. During storage, aerobic conditions must be maintained.
   Control and dilution soil. Non-contaminated soil used as assay control and to prepare dilutions with test soil. Soils with high sand content (50–75%), low organic carbon levels (0.5–1.5%) and pH 5.5–7.5 are preferred (*see* **Note 1**).
   Reference soil. Non-contaminated soil with physicochemical characteristics similar to the test soil. Reference soils are sampled at non-polluted localities close to the contaminated site. When this is not possible, a natural or artificial soil with pH, soil organic matter content, soil texture, and nutrients comparable to the test soil should be used (*see* **Note 2**).
2. Control medium for toxicity tests to aquatic organisms. Two control media should be used in the assays with the soil extracts: (1) control medium recommended in the specific toxicity assay by OECD and (2) extracts obtained from reference soil under the same conditions used to obtain extracts from test soil (*see* **Note 3**).
3. Climatic cabinet or chamber at a controlled temperature between 20°C and 25°C (± 2°C) and continuous uniform illumination in the spectral range 400–700 nm. This can be obtained with universal white-type lamps. Additionally, a system to establish light-dark cycles is necessary for some tests.

2.2. Toxicity Assays to Soil Organisms

2.2.1. Soil Sample Preparation

1. Mechanical mixer: a large-scale laboratory mixer or small electric cement mixer (*see* **Note 4**).

2.2.2. Earthworms, Acute Toxicity Test

1. Assay organisms: The recommended species is *Eisenia foetida* although other terrestrial species may be used. Worms should be adults (at least 2 months old with clitellum) and weigh between 300 and 600 mg.
2. Climatic cabinet or chamber at a controlled temperature 20±2°C and continuous uniform illumination (400–800 lux) to ensure that earthworms stay in the test medium during the test period.

3. Glass container of about 1 L volume covered with a perforated plastic film.

2.2.3. Terrestrial Plants, Seedling Emergence and Seedling Growth Test

1. Assay organisms: Plant test species can be selected from OECD Guidelines (9), where a selection of plant species available for this test is shown. A minimum of three plant species should be used in the test. Assay should include one monocotyledonae and two dicotyledonae species belonging to three different families.
2. Climatic chamber or glasshouses that allow normal growth of testing plants. The growing conditions are selected according to the test species. Recommended conditions are temperature of 22±10°C, humidity of 70±25%, photoperiod of 16 h light as minimum and uniform illumination in the spectral range 400–700 nm with an intensity of 350±50 $\mu E/m^2/s$.
3. Plant containers of non-porous plastic or glazed material. The size of pots or containers must be adequate not to hamper the normal growth of selected species.

2.2.4. Soil Microorganisms, Nitrogen Transformation Test

1. Control and dilution soil. Soil with characteristics described in **Section 2.1**, step 1. Moreover, the carbon content of the microbial biomass should be at least 1% of the total soil organic carbon (16). It is preferable to use a recently sampled soil. If soil is stored, pre-incubation for 2–28 days is recommended at temperature and moisture content similar to that used in the test.
2. Organic nitrogen source, e.g., powdered lucerne meal. C/N ratio between 12/1 and 16/1 is recommended.
3. Test containers of inert material of approximately 100 mL with a top that allows gas exchange during the assay and minimizes water loss.
4. Climatic cabinet or chamber at a controlled temperature of 20±2°C.
5. Agitation device such as mechanical shaker or magnetic stirrer.
6. Potassium chloride solution 0.1 M stored at 4°C.
7. Filtration device, using nitrate-free filter paper.
8. Equipment for nitrate determination: spectrophotometer, nitrate electrode, etc.

2.2.5. Soil Microorganisms, Carbon Transformation Test

1. Control soil: soil with characteristics described in **Section 2.1**, step 1. Moreover, the carbon content of microbial biomass should be at least 1% of the total soil organic carbon. It is preferable to use a recent sampled soil. If soil is stored, pre-incubation for 2–28 days is recommended at temperature and moisture content similar to that used in the test.
2. Test containers of inert material of approximately 100 mL with a top that allows gas exchange during the assay and minimize water loss.
3. Climatic cabinet or chamber at a controlled temperature 20±2°C.
4. Glucose powder mixed with clean quartz sand (4 g glucose/10 g sand) (store at room temperature).
5. Equipment for the measurement of carbon dioxide production or oxygen consumption (17–20).

2.3. Toxicity Assays to Aquatic Organisms

2.3.1. Obtaining Soil Extracts

1. Agitation apparatus capable of rotating the extraction vessel in an end-over-end fashion at 30±2 rpm are preferred. Other shaking apparatus may be used if they are capable of providing a good contact between soil and extractant and do not produce a reduction of particle size.
2. Extraction vessels. Borosilicate glass bottles may be used for both organic and inorganic substances. For metals, it is recommended containers are made of polypropylene or linear polyethylene. For organics, plastic bottles shall not be used except polytetrafluoroethylene (PTFE) bottles. Volume must be sufficient to allow a good contact between the soil and the extraction solution (containers holding soil and extraction solution should not be more than half full).
3. Distillate water with conductivity less than 5 μS/cm.
4. Filters of pore size of 0.45 μm made of PTFE or of nylon rather than acetate or cellulose nitrate are recommended.
5. Filtration devices that allow vacuum filtration, made of inert material which will not leach or absorb waste components.
6. pH meter: The meter should be accurate to ±0.05 units at 25°C.
7. Conductivity meters with an accuracy of ±1 μS/cm.

2.3.2. Alga, Growth Inhibition Test

1. Assay organisms: fast-growing green algae species. The most widely used species are *Pseudokirchneriella subcapitata* (ATCC 22662), *Desmodesmus subspicatus* (86.81 SAG), and *Chlorella vulgaris* (CCAP 211/11b) (*see* **Note 5**).
2. Bunsen burner or sterile chamber.

3. Culturing apparatus: Cabinet or chamber capable of maintaining the incubation temperature in the range 21–25°C, controlled at ±2°C. Continuous uniform illumination with light intensity of 60–120 μE/m^2s (4440–8880 lux) in the range of 400–700 nm. These light conditions may be obtained with white type fluorescent lamps (light temperature of approximately 4200 K).
4. Light-intensity measurement instruments.
5. Growth, control, and dilution medium: 15 mg/L NH_4Cl, 12 mg/L $MgCl_2 \cdot 6H_2O$, 18 mg/L $CaCl_2 \cdot 2H_2O$, 15 mg/L $MgSO_4 \cdot 7H_2O$, 1.6 mg/L KH_2PO_4, 0.064 mg/L $FeCl_3 \cdot 6H_2O$, 0.1 mg/L $Na_2EDTA \cdot 2H_2O$, 0.185 mg/L H_3BO_3, 0.415 mg/L $MnCl_2 \cdot 4H_2O$, 3×10^{-3}mg/L $ZnCl_2$, 1.5×10^{-3}mg/L $CoCl_2 \cdot 6H_2O$, 1×10^{-5}mg/L $CuCl_2 \cdot 2H_2O$, 7×10^{-3}mg/L $Na_2MoO_4 \cdot 2H_2O$, 50 mg/L $NaHCO_3$. Substances must be of analytic grade. The pH of growth medium after equilibration with air is approximately 8. Stock solutions are sterilized by filtration (mean pore diameter 0.2 μm) or autoclaving and may be stored dark at 4°C for upto 2 months. A more detailed description of the preparation of this medium may be seen in OECD Guidelines (12).
6. Test containers made of chemically inert material. Material used in this test must be sterile in order to avoid contamination from bacteria or other algae.
   Sterile glass vessel of appropriate volume
   Sterile capped polypropylene test tube of 3 mL
   Sterile 96-well cell culture flat-bottom polystyrene microtitre plate
7. Apparatus to determine cell concentrations such as cell counts (electronic particle counter or microscope with counting chamber).
8. Fluorimeter and spectrophotometer microtiter plate reader (*see* **Note 6**).

2.3.3. Daphnia sp., Acute Immobilization Test

1. Assay organisms: *Daphnia magna* or any other suitable *Daphnia* species, less than 24 hours old. *Daphnia* should be apparently healthy and with a known history. All organisms used in the assay should come from the same culture stock. Conditions of culture and assay should be similar.
2. Normal laboratory material: pipettes, Petri plaques, etc., which should be all-glass or other chemically inert material.
3. Glass vessel of about 50 mL.
4. pH meters: The meter should be accurate to +0.05 units at 25°C.

5. Culture, control, and dilution water
   Natural water and reconstituted water (*see* **Note** 7) may be used as control and to prepare extract dilutions. pH water must be between 6 and 9. Water hardness between 140 and 250 mg/L (measured as $CaCO_3$) is recommended for *D. magna*. If natural water is used, the quality parameters should be measured at least twice a year. Absence of toxic substances should be checked and conductivity, total organic carbon (TOC), or chemical oxygen demand (COD) should be measured (*see* **Note** 8).

2.3.4. In Vitro Assay Battery to Detect Leachate Toxicity Using RTG-2 Cell Line

1. Assay organism: Rainbow trout gonadal RTG-2 cell line (ATCC CCL55) (*see* **Note** 9).
2. Cell culture flask (75 cm^2).
3. 96-well cell culture flat-bottom polystyrene sterile plate.
4. Micro pipettes and multichannel pipettes (*see* **Note** 10).
5. Cell incubator.
6. Vertical laminar flow cabinet.
7. Orbital shaker.
8. Spectrofluorimeter microtiter plate reader.

2.3.4.1. Cell Culture

1. Phosphate-buffered saline (PBS): prepare 10× stock with 1.37 M NaCl, 27 mM KCl, 100 mM Na_2HPO_4, 18 mM KH_2PO_4 (adjust to pH=7.4 with HCl if necessary) (storage at room temperature). Prepare working solution by dilution of one part with nine parts water and autoclave (store at 4°C).
2. PBS-EDTA: Prepare a 0.02% ethylenediamine tetraacetic acid disodium salt (EDTA) solution in PBS and autoclave (store at 4°C).
3. Trypsin–Versene (EDTA) mixture (1×) (Lonza, Switzerland) (store at –20°C).
4. Minimum essential medium with Earle's salts 10× (EMEM 10×) (Lonza, Switzerland) (store at room temperature).
5. Minimum essential medium with Earle's salts 1× (EMEM) (Lonza, Switzerland). Before use add 10% FCS, 1% L-glutamine, 1% Pen-Strep, 1% non-essential amino acids (store at 4°C).
6. Fetal calf serum (FCS) (heat inactivated) (Lonza, Switzerland) (store at −20°C).
7. L-glutamine 200 mM (Lonza, Switzerland) (store at −20°C).

8. Pen–Strep mixture: 5000 UI penicillin and 5000 μg streptomycin per mL in 0.85% saline (Lonza, Switzerland) (store at −20°C).
9. Non-essential amino acid solution 10 mM (Lonza, Switzerland) (stable at 4°C).
10. 0.89 M sodium bicarbonate ($NaHCO_3$). Weigh 7.5 g and make to 100 mL with distilled water (stable for 1 week at 4°C).
11. Gas cylinder (75% N_2; 20% O_2; 5% CO_2) (Air Liquide, France).

2.3.4.2. Tests

1. 0.825 μM 7-ethoxyresorufin. Stock solution: Weigh 1 mg and make to 3.5 mL with dimethyl sulfoxide (DMSO) (stable at room temperature). Dilute 4.1 μL/mL in culture medium. Prepare just before use.
2. 2.95 mM 4-methylumbelliferyl-β-D-galactopyranoside (MUG). Weigh 1 mg and make to 1 mL with DMSO. Prepare just before use.
3. Neutral red stain. 3-amino-7-dimethylamino-2-methylphenazine hydrochloride. Stock solution: Weigh 0.4 g and make to 100 mL with distilled water (dH_2O) (stable at 4°C). The day before use, make a 1 neutral red: 100 culture medium dilution and pre-incubate overnight at 37°C. Before use centrifuge (4000 rpm, 1 h) to remove precipitated dye crystals.
4. Formaldehyde–calcium chloride. Make two stock solutions A and B: Solution A (formaldehyde 10%): mix 10 mL formol (40%) with 40 mL dH_2O. Solution B (20 mM $CaCl_2$): weigh 5.4 g $CaCl_2$ and make to 50 mL with dH_2O. To prepare 100 mL, mix 10 mL solution A, 10 mL solution B, and 80 mL dH_2O (store at room temperature).
5. Acetic acid–ethanol: Mix 4 mL acetic acid, 200 mL ethanol, and 196 mL dH_2O (store at room temperature).
6. Coomassie Brilliant Blue G. Stock solution: Weigh 0.4 g and dilute in 250 mL ethanol, add 630 mL dH_2O and run through a filter paper (store at room temperature, protected against the light). Before use, dilute stock solution 7:1 with acetic acid.
7. Ethanol–glacial acetic acid–water. 10:5:85 (store at room temperature).
8. Desorb solution. Potassium acetate–ethanol: weigh 49.07 g potassium acetate and make to 500 mL with ethanol 70° (store at room temperature).

9. Ethanol 70°. 100 mL ethanol 96°:39 mL dH_2O (store at room temperature).

3. Methods

3.1. Toxicity to Soil Organisms

The ecotoxicity test with soil samples may be performed following two experimental designs:

1. Single concentration assessment
   Assessment is performed with no diluted samples of test soil. The effects on contaminated soil are compared to the reference soil. Data are expressed as a percentage of effects compared with the reference soil and significant differences are determined using statistical analyses.
2. Multiple concentration assessment
   Soils samples are diluted with the dilution soil and different soil concentrations are tested. The aim is to obtain a quantitative concentration–response relationship by regression analyses similar to that obtained assaying pure chemical substances. The results of the toxicity assessment are compared to control and dilution soil and expressed using conventional methods such as the equivalent to the LC50 (median lethal concentration) or EC50 (median effect concentration) expressed in terms of dilution (*see* **Note 11**).

Both approaches can be combined or selected on the basis of the expected level of pollution for soil toxicity tests (*see* **Note 12**). Assessment of leachates is performed in multiple concentration assessment.

3.1.1. Soil Sample Preparation

1. Sieve reference and test soils at a particle size ≤2 mm. Quantities of soil samples should be sufficient to support toxicity tests. A minimum of six replicates of control soil and test soil are recommended (*see* **Note 13**).

3.1.1.1. Single Concentration Assay

3.1.1.2. Multiple Concentration Assay

1. Sieve control and dilution soil, and test soil at a particle size ≤2 mm.
2. Weigh necessary quantities of dilution and test soils to obtain different soil concentrations. Ratios are calculated on a dry-weight basis. Tested concentrations are selected in geometric series. The recommended assay concentrations are 100, 50, 25, 12.5, and optionally 6.25% (w/w, test soil / total soil). The selected rates should include the concentration causing the 50% effect in order to obtain the L(E)C50. If results of the assay exceed the 50% effect level, lower concentrations in geometrical series should be used.

3. Mix thoroughly dilution and test soils, using a large-scale laboratory mixer or small electric cement mixer. Mixture may be also performed by handling in plastic bags, which are turned end-over-end enough times to ensure thorough mixing. Sufficient soil mixture must be obtained to support soil toxicity test.
4. Three replicates of test and control soil are used, except if otherwise indicated in the description of the corresponding test.

The following toxicity tests are described as multiple concentration tests. The methodology to perform single concentration tests is similar. Differences consist in (i) one concentration of contaminated soil is used rather than different concentrations, (ii) the number of replicates, which is higher in the single concentrations tests, as indicated above, and (iii) the statistical method used for data analysis (**Section 4**).

3.1.2. Earthworm, Acute Toxicity Test

This test determines the effects of contaminated soils in the survival of earthworms exposed to this soil. Earthworms are exposed to soil samples for 14 days and the survival is determined at 7 and 14 days of assay.

1. Place 750 g (d.w.) of soil samples into each glass container.
2. Adjust soil samples moisture at 60–80% of their water-holding capacity by adding distilled or deionized water. Lost water is replenished as necessary during the test time, by reweighing the containers periodically.
3. Wash earthworms and keep them for 24 h on moist filter paper to depurate the gut content. Earthworms are individually weighed and worms between 300 and 600 mg of wet weight are selected to be used in the assay.
4. Put ten weighed earthworms randomized into the soil surface of every container and cover the containers with a perforated plastic film. The test containers do not need to be aired during the assay.
5. Test containers are maintained for 14 days at temperature of $20\pm2^{\circ}$C and continuous illumination in order to ensure that earthworms stay in the soil.
6. At 7 and 14 days, pour the containers onto a tray and record the number of dead earthworms. Earthworms are considered as dead when they do not respond to a gentle mechanical stimulus to the front end.
7. Test is valid if mortality less than 10% is observed in the control.

8. The percentage of mortality at 14 days is calculated respect to the control and dilution soil, and the value of LC50 is determined (*see* **Note 14**).

3.1.3. Terrestrial Plants, Seedling Emergence and Seedling Growth Test

This test determines the effects of test soil in the emergence of seedling and in the growth of higher plants. Seeds of three different plant species are placed in the control and test soils. The number of emerged seedling and the effects in growth (shoot weight, shoot height, etc.) are measured at 14–21 days after 50% emergence in the control.

1. Transfer soil samples to pots or containers. Six replicates of controls and three replicates at each concentration are recommended (*see* **Note 15**).
2. Plant the seeds of selected species in each container at a depth of about 1 cm to facilitate seedling emergence. A minimum of seven seeds are recommended per replicate for optimal statistical analyses. Seeds should not be imbibed with water or coated with an insecticide or fungicide.
3. Temperature, moisture, and light conditions are selected to allow plants growing in optimum conditions.
4. Water the plants with dechlorinated or deionized water as needed. Bottom watering is preferable; however, top watering can be used, but avoid loss of the chemicals in the soil by water leaching.
5. Crops are maintained for 14–21 days after 50% of the control seedlings have emerged. During this period plants are observed weekly for visual phytotoxicity and mortality and the number of emerged plants is recorded.
6. At the end of the assay time, record the surviving number of emerged plants.
7. Harvest the shoots at the soil surface. The effects on growth are determined as wet or dry weight of shoots.
8. If wet weight is used to determine effects on growth, weigh individually plants immediately after harvest (*see* **Note 16**).
9. If dry weight is used to determine effects on growth, weigh total survival plants in each container after oven drying at 60°C to constant weight (24 h are usually sufficient). Total weight may be expressed as total biomass or on a per plant basis. Other variables which can be used to measure toxicity effects are root and shoot length.
10. The test is considered valid if a minimum of 65% of the seeds germinated and show a healthy growth in the control soil.

11. The percentage of inhibition of emergence and growth as compared with the control and dilution soil is calculated and the value of EC50 is determined for emergence and growth.

3.1.4. Soil Microorganisms, Nitrogen Transformation Test

The test determines the adverse effect of contaminated soils on the process of nitrogen mineralization in aerobic surface soils. The autochthonous soil microorganisms are used in the assay. Sieved test and control soils are amended with a source of organic nitrogen (powdered plant meal, lucerne) and samples are incubated for 28 days. After this time, samples are extracted with an appropriate solvent and the nitrate content of the soil is determined.

3.1.4.1. Incubation Procedure

1. Place 20 g of soil samples onto the appropriate containers and add 100 mg of powered lucerne meal (5 mg/g soil d.w.).
2. Add distilled or deionized water to adjust the moisture content of soil samples to between 40 and 60% of the maximum water-holding capacity of the soil. The weight of the containers is recorded and the soil moisture is maintained by reweighing the containers and adding distilled or deionized water every two or three days (*see* **Note 17**).
3. Incubate test and control soils for 28 days in the dark at a temperature of 20±2°C. Containers are loosely capped to minimize water loss but to allow the gas exchange.
4. Analyze soil samples for nitrate at the beginning and at the end of the incubation time (28 days).

3.1.4.2. Nitrate Analysis

1. Take an aliquot of 10 g (dry weight basis) of soil, add 50 mL of potassium chloride solution (0.1 M) to each sample and stir for an hour at 150 rpm.
2. Centrifuge the mixture (3000 rpm, 5 min) and filter it. Extracts may be stored prior to analysis at 4°C for a maximum of 3 days.
3. Measure nitrate content in the extracts by the selected method. (21). The quantities of nitrate formed (nitrate concentration at the end of assay minus initial concentration) are expressed in mg nitrate/kg soil d.w./day.
4. The test is considered valid if the variation between replicates control samples is less than 15%.
5. The nitrate formation rates in the control and test soils are compared. The percentage of inhibition of nitrate formation rate is calculated at each concentration and then used to determine the value of EC50.

3.1.5. Soil Microorganisms, Carbon Transformation Test

This test determines the effects of contaminated soils on the process of carbon transformation in aerobic surface soils. The autochthonous soil microorganisms are used in the assay. Test and control soils are incubated for 28 days. After incubation time, samples are mixed with glucose and the glucose-induced respiration rates are measured for 12 h. Respiration rates may be determined as oxygen consumed or carbon dioxide released.

3.1.5.1. Incubation Procedure

1. Place 20 g of soil samples into appropriate containers.
2. Add distilled or deionized water to each sample to adjust the moisture content of soils samples to between 40 and 60% of the maximum water holding capacity of the soil. The weight of each container is recorded and the soil moisture is maintained by adding distilled or deionized water every two or three days by reweighing the containers. (*see* **Note 17**).
3. Incubate test and control soils for 28 days in the dark at a temperature of 20±2°C. Containers are loosely capped to minimize water loss but to allow gas exchange.
4. Determine glucose-induced respiration rates of soil samples at the beginning and at the end of the incubation time (28 days).

3.1.5.2. Glucose-Induced Respiration Rates

1. Add glucose/sand mixture (2–4 g of glucose/kg soil d.w) to the soil and mix to homogeneity (*see* **Note 18**). Glucose-amended samples are incubated in an appropriate equipment at 20±2°C, during respiration measurement.
2. One hour after glucose addition, measure the oxygen consumed or dioxide carbon released continuously for 12 h. Mean respiration rates are determined and data expressed as carbon dioxide released (mg carbon dioxide/kg dry soil/hour) or as oxygen consumed (mg oxygen/kg soil d.w./hour) (*see* **Note 19**).
3. The test is considered valid if the variation between replicate control samples is less than 15%.
4. The mean respiration rates in the control and dilution soil, and test soil are compared and the percentage of inhibition is determined at each concentration and used to determine EC50 values.

3.2. Toxicity to Aquatic Organisms

3.2.1. Obtaining Extracts

Different standard methods may be used to obtain extracts from soils or waste samples. The main variables in these tests are the liquid-to-solid ratio, which ranges between 1:2 and 1:10, the time (12–24 h) and the potency of the shaking method. To obtain samples for ecotoxicity assays, the most used technique consists in shaking a sample of the soil using distilled or deionized water as extractant at 10:1 liquid to solid ratio (22, 23).

1. Sieve test soil and reference soil at <2 mm.
2. Place the soil sample (100±1 g d.w.) in the extraction vessels, add distilled or deionized water (1 L) (*see* **Note 20**).
3. Place in the rotary agitation apparatus and rotate at 30±2 rpm for 24 h at ambient temperature (15–25°C).
4. Following the agitation period, vacuum filter (0.45 μm) the liquid phase. A centrifugation step (3000 rpm, 15–30 min) can be performed first in order to facilitate the filtration process.
5. The volume, pH, and conductivity of the extracts are measured.
6. Extracts should be analyzed for toxicity as soon as possible following extraction. They may be stored at 4°C for a maximum of 72 h if cooling does not irreversibly affect the composition of the mixture.

3.2.2. Extract Sample Preparation

1. Prepare different concentrations of the extract as described in the specific toxicity assay.
2. Tested concentrations are selected in geometric series. At least five different concentrations should be tested. Initially, 100, 50, 25, 12.5, and 6.25% (v/v, extract/dissolution) may be selected. The dilutions tested should include the concentration causing the 50% effect used to obtain the L(E)C50. If the results of the assay exceed the 50% effect level, lower concentrations in the geometrical series should be used.
3. The pH of the test solutions should not be adjusted before the toxicity test.

3.2.3. Alga, Growth Inhibition Test ***(Use Sterile Equipment and Aseptic Techniques: Bunsen Burner)***

This test determines the inhibition of growth of exponentially growing culture of green algae exposed to extracts of test soil compared to control. Cultures of green algae in exponential growth are exposed to different extract concentrations under established conditions over several generations (72 h). Effects in growth are measured at 0, 24, 48, and 72 h as biomass yield or average specific growth rate.

3.2.3.1. Preparation of Alga Inoculum

1. Transfer stock algal cultures in agar tubes to fresh medium to be used in the test as following. Add growth medium (approximately 4 mL) in the agar tube in order to detach algae from agar and transfer the inoculum with a sterile pipette into a flask.
2. Stir algae suspension in a vortex or similar apparatus to ensure homogenous suspension. Take an aliquot, diluted with algal medium (1:10) and count the number of cells in a Neubauer chamber (N° cells/mL). This suspension is the algal inoculum (*see* **Note 21**).

3. The volume of algal inoculum necessary to obtain suitable initial cell concentration in the test samples is determined. The initial cell concentration in the test samples should be approximately 10^4cells/mL for *Pseudokirchneriella subcapitata* and *Desmodesmus subspicatus*, and an equivalent biomass if other species are tested.

3.2.3.2. Test Procedure

1. Test dilutions (2 mL) of extract at different concentrations are prepared in sterile capped polypropylene tubes. Mix the necessary volumes of extract sample and of algal inoculum with algal growth medium aliquots to obtain the dilutions of test solution. Tubes are shaken to keep the algae in suspension (*see* **Note 22**).
2. Transfer the test dilutions (200 μL) into individual wells of a sterile 96-well microtiter plate and place it in the culturing apparatus (*see* **Note 23**). It is recommended that three replicates at each concentration are used and at least three, but ideally six, replicates of controls are used.
3. Shake the culture microtiter plates in an orbital shaker at 100 rpm. During the test, algae should be kept in suspension and sufficient transfer of CO_2 from the atmosphere inside of well should be insured (e.g., by increasing the shaking rate). The cultures should be maintained at a temperature in the range of 21–25°C, controlled at ±2°C during 72 h and continuous uniform illumination (see illumination conditions in **Section 2.3.2**.).
4. After 0, 24, 48, and 72 h of exposure, measure the absorbance (λ; = 450 nm) or fluorescence (λ_{ex} = 430 nm; λ_{em} = 680 nm) in each well (*see* **Note 24**).
5. The test is considered valid if the cell concentration in the control cultures has increased by a factor of at least 16 within 3 days.

3.2.3.3. Data Analysis

The effects on the growth of algae may be determined through two response variables.

Biomass Yield. Biomass yield is calculated as the biomass at the end of the assay minus initial biomass. The concentration that produces 50% of biomass yield inhibition with respect to the controls (test control medium and the blank consisting in the extract obtained from an appropriate reference soil) is determined and expressed as $EC50_b$.

Average Specific Growth Rate. Average specific growth rate is the logarithmic increase in cell concentration per unit of time (μ;) at the end of the assay. This variable may be determined as:

$$\mu_{0-n} = \frac{\mathrm{Ln}N_n - \ln N_0}{t_n - t_0}$$

where

μ_{0-n}= average specific growth rate from t_0 to t_n

N_0 = number of cells /mL at the start of the assay

N_n = number of cells /mL at time t_n

t_0 = initial time of assay

t_n = time of measurement after beginning of test (24, 48, and 72 h).

The concentration that produces 50% of average specific growth rate inhibition respect to the control (test control medium and the blank consisting in the extract obtained from an appropriate control or reference soil) is determined and expressed as $EC50_r$.

3.2.4. ***Daphnia*** *sp. Acute Immobilization Test*

Daphnids less than 24 h are exposed to the extracts of soils. The effects of toxic substances in extracts on the swimming capability of *Daphnia magna* are determined after 48 h of exposure.

3.2.4.1. Preparation of Control and Dilution Solution

Natural or reconstituted water is bubbled with air for 6 h in order to ensure the dissolved oxygen concentration has reached saturation. The solution is kept for 12 h and the pH is measured. The pH must be between 6 and 9. Otherwise, the pH must be adjusted using HCl or NaOH solutions.

3.2.4.2. Test Procedure

1. Test dilutions of extract at different concentrations are prepared in the test vessels (at least 2 mL should be provided per organisms). Mix the appropriate volumes of extract sample and dilution solution to obtain test solutions of the chosen concentrations. Test samples are assayed without pH adjustment (*see* **Note 25**).
2. Transfer test organisms in the culture medium to a Petri plate using a 10 mL pipette. Take daphnids from the plate and place into test vessels with the aid of a Pasteur pipette. The daphnids should be gently placed in the vessel taking care that the pipette does not contact the solution. A minimum of 20 individuals must be used for each concentration, separated in 4 groups of 5 individuals or 2 groups of 10 individuals.
3. Cultures are maintained during 48 h at a constant temperature between 18°C and 22°C (±1°C). A light-dark cycle with a photoperiod of 16 h daylight and 8 h darkness is recommended. However, the assay may be also performed in

darkness. During the assay, test vessels are lightly covered to avoid water loss and dust deposition in the samples. Daphnia should not be fed and test vessels should not be aerated during the test.

4. The dissolved oxygen and pH in the control and test solutions are measured at the beginning and at the end of the assay. Dissolved oxygen must be ≥3 mg/L.
5. Count the number of immobilized daphnia in the test vessels at 24 and 48 h after starting the test. Daphnia are considered immobile if they are not able to swim within 15 s after gentle agitation of the test container.
6. The assay is valid if two conditions are fulfilled: (i) the number of daphnids immobilized (or showing other signs of disease or stress) in the control must be 10% or less of the total, and ii) the concentration of dissolved oxygen at the end of the test should be above or equal to 60% of the air saturation value at the temperature used (≥3 mg/L).
7. Daphnia immobilized in the test soils are compared to data from control medium and the blank consisting in the extract obtained from an appropriate control or reference soil. Percentages of daphnia immobilized for controls and each concentration at 48 h are calculated and the EC50 value is determined.

*3.2.5. In Vitro Assay Battery to Detect Leachate Toxicity Using RTG-2 Cell Line (*see *Note 26)*

This battery in vitro assay offers potential assessment of various aspects of cellular toxicity. The battery permits the determination of general cytoxicity by different end points such as cell viability using the neutral red assay, total protein content of the cells using the Kenacid blue protein (KBP) assay, cellular defense by a β-galactosidase assay, and selective cytotoxicity by the induction of cytochrome CYP1A, which is measured as 7-ethoxyresorufin-*O*-deethylase (EROD) activity.

3.2.5.1. Cellular Split on the Microtiter Plate (Using Sterile Equipment and Aseptic Techniques) (see Note 27)

1. Take the cells out of the incubator and inside the flow cabinet, aspirate out old media from the flasks containing the cells.
2. Wash the cells with 10 mL of PBS-EDTA. Aspirate out the PBS-EDTA.
3. Add 1 mL 0.05% Trypsin. Ensure that the entire surface of the flask is covered.
4. Wait, about 5 min, until the trypsinization is successful. Confirm the detachment under the microscope.
5. Add 5 mL of EMEM (10% FCS, 1% glutamine, 1% P+S, 1% non-essential amino acids). Pipette up and down a few

times to ensure that all the cells are resuspended in the media.

6. Transfer the cell solution to a sterile 10 mL capped polypropylene tube.
7. Centrifuge at 1500 rpm for 5 min.
8. Aspirate out the supernatant ensuring not to disturb the cell pellet.
9. Resuspend the cells in 10 mL of EMEM (10% FCS, 1% glutamine, 1% P+S, 1% non-essential amino acids) and proceed with the cell count.
10. Suspend the cells to be used at a density of 12.5×10^4/mL in EMEM (10% FCS, 1% glutamine, 1% P+S, 1% non-essential amino acids) medium.
11. 200 μL (2.5×10^4 cells) of these cell suspensions were transferred into individual wells of a 96-well microtitre tissue culture plate to achieve 60–70% confluence at the time of the addition of the samples.
12. Put the plate in a mini-chamber for regulating the gaseous environment. The mini-chamber can be constructed using a disposable plastic bag.
    a. Seal the mini-chamber with a heat sealer.
    b. Inject gas for several seconds through a tube connected to the gas cylinder with an insulin needle attached.
    c. After injection of gas is completed, the needle hole is closed with scotch tape.
13. The plate is incubated over night at 20°C to allow cell attachment.

3.2.5.2. Preparation of Samples

1. Prepare sample dilution in EMEM 10× culture medium (**Table 15.1**).
2. Prepare serial dilutions of the sample in EMEM 1× culture medium (**Table 15.2**).
3. Place serial dilutions in the 96-wells plate (**Table 15.3**).

3.2.5.3. Cells Poisoning

1. RTG-2 cells are maintained in culture and exposed to the sample over a broad range of concentrations (74%–0.14%) (8 wells, use one column of wells per concentration).
2. Open the mini-chamber gas and inside the flow cabinet, remove the culture medium. Media is eliminated by inverting the plate on a filter sheet.
3. Add 200 μL/well of the different sample concentrations to each column. Each concentration is transferred to all eight wells in a column from A to H.

Table 15.1
Sample dilution of the 10× medium culture

Component	Volume (μL)	Final
Sample or distilled water (10× negative control medium)	740.7	74%
EMEM 10×	100	1x
Non-essential amino acid solution 10 mM	10	0.1 mM
L-Glutamine 200 mM	10	2 mM
Penicillin–Streptomycin mixture	10	50 UI+50 μg
Fetal bovine serum	100	10%
Sodium bicarbonate 0.89 M	29.3	26 mM
TOTAL	1 mL	

Table 15.2
Serial sample dilution

Sample %	74	37	18.5	9.2	4.6	2.3	1.1	0.58	0.29	0.14
Dilution		1/2	1/4	1/8	1/16	1/32	1/64	1/128	1/256	1/512
Name	a	b	c	d	e	f	g	h	I	j

Table 15.3
Culture plate set-up

	Sample concentrations										Medium controls	
	1	2	3	4	5	6	7	8	9	10	11	12
A	a	b	c	d	e	f	g	h	i	j	1×	10×
B	a	b	c	d	e	f	g	h	i	j	1×	10×
C	a	b	c	d	e	f	g	h	i	j	1×	10×
D	a	b	c	d	e	f	g	h	i	j	1×	10×
E	a	b	c	d	e	f	g	h	i	j	1×	10×
F	a	b	c	d	e	f	g	h	i	j	1×	10×
G	a	b	c	d	e	f	g	h	i	j	1×	10×
H	a	b	c	d	e	f	g	h	i	j	1×	10×

4. Reserve two columns for the media controls (11 and 12). About 200 μL/well cell culture media (1× or 10×) is added and is defined as negative control values.
5. Put the plate (as previously described) in a mini-chamber for regulating the gaseous environment.
6. Suffuse gas for several seconds.
7. Incubate the cells with the sample dilutions and media controls at 20°C over night (*see* **Note 28**).

3.2.5.4. Test Procedure (*see* Note 29) (Sterile Equipment and Aseptic Techniques Are Not Necessary)

The versatility of this system enables the EROD, the βgal, the NR, and the KBP assays to be performed on the same microtiter plate.

Ethoxyresorufin-O-Deethylase Activity (EROD).

1. Open the mini-chamber gas and take out the microtiter plate.
2. Without removing the medium, add 50 μL of EROD substrate dilution (0.825 μM solution of 7-ethoxyresorufin in EMEM) to each well using a multi-channel pipettor.
3. Measure the fluorescence in a fluorimeter (λ_{ex} = 530 nm; λ_{em} = 590 nm) at 0, 30, and 60 min.

β-Galactosidase Activity (βgal).

1. Without removing the medium, add 20 μL of β-gal substrate dilution (MUG).
2. Measure the fluorescence in a fluorimeter (λ_{ex} = 360 nm; λ_{em} = 465 nm) at 0, 30, and 60 min.

*Neutral Red Uptake Assay (NR).*The assay assesses the incorporation of the neutral red dye by the lysosomes (Check) of living cells.

1. Remove the medium by inverting the plate on a filter sheet.
2. Wash twice with 200 μL PBS.
3. Add 100 μL of medium containing neutral red. Prior to use, the neutral red-containing medium has been preincubated overnight at 37°C and centrifuged (4000 rpm, 1 h) to remove precipitated dye crystals.
4. Incubating the plates for 3 h at 20°C.
5. Remove the colorant.
6. Fix the cells in 200 μL of formaldehyde:calcium chloride for 2 min at room temperature.
7. Remove the fix solution.
8. Extract the dye from the cells with 200 μL of acetic acid:ethanol.

9. Agitate the plate on a microtiter plate shaker for 20 min at room temperature.
10. Measure the absorption of neutral red at 540 nm in a spectrophotometer. The microtiter plate can remain at 4°C overnight.

Kenacid Blue Total Cell Protein Assay (KBP).

1. Remove the NR solution from microtiter plate.
2. Wash twice with 200 μL PBS.
3. Add 100 μL/well kenacid blue (Coomassie Brilliant Blue G) stain solution.
4. Agitate 30 min on a plate shaker at room temperature.
5. Remove the colorant.
6. Wash cells three times with 200 μL of ethanol:glacial acetic acid:water.
7. Add 100 μL of desorb solution.
8. Keep plate in an ultrasonic bath for 1–2 min.
9. Add a further 100 μL of the desorb solution.
10. Shake the plate for 30 min at room temperature.
11. Read in a spectrophotometer at 620 nm.

3.2.5.5. Data Analysis

Kinetic Parameters (EROD and βgal). EC50 inhibition value (EROD and βgal) is estimated from the area under the curves, using the OECD Guidelines (12). This method estimates growth inhibition in the cellular (algae) population and allows the use of all time point measurements, offering greater relevance and better statistical confidence than the single measurements at a fixed time point.

The area below the growth curve may be calculated according to the formula:

$$A = \frac{N_1 - N_0}{2} \times t_1 + \frac{N_1 + N_2 - 2N_0}{2} \times (t_2 - t_1)$$

$$+ \frac{N_{n-1} + N_n - 2N_0}{2} \times (t_n - t_{n-1})$$

where

A = area

$N_0, N_1, \ldots, N_{n-1}, N_n$ = sample concentrations

t_1 = time of first measurement after beginning of test (1 min)

t_n = time of nth measurement after beginning of test (30 and 60 min).

Non-kinetic Parameters (NR and KBP). The absorbance value at 540 nm is the amount of NR taken up by the cells. As cells lose viability, they lose the ability to take up NR.

Plot your values of NR uptake (Y axis), average of eight wells, versus concentration of sample (X axis), and determine the EC50 value. Data can also be presented as a percentage of the control.

4. Data Statistical Analyses

4.1. Single Concentration Assessment

1. Toxicity effects in the test sample are calculated as the percentage inhibition of the measured effect with respect to the reference soil (*see* **Note 30**).
2. Toxicity responses obtained in test soils are compared with those in the reference soil by one-way analysis of variance (ANOVA), with Fisher's least significant difference procedure (LSD, $P<0.05$). Significant differences between samples are established (*see* **Note 31**).

4.2. Multiple Concentration Assessment

1. Toxicity effects in the test sample are calculated as the percentage inhibition of the measured effect with respect to the control and dilution soil.
2. Plot the percentage of inhibition of the measured parameter (e.g., absorbance, plant weight, etc.) versus the logarithm of the concentration of sample. L(E)C50 value may be calculated by determining the X-axis value corresponding to one-half of the difference between the maximum (plateau) and the minimum (control medium) effect values. The quality of the data may be improved using a computerized statistical method.
3. The data (percentage of inhibition) are fitted to a linear regression by performing a transformation of response data, for instance, using a logit-probit (24–25) or Weibull units (26) (*see* **Note 32**). The regression analysis is performed using individual data rather than treatment group median. The measured effects should range from 20 to 80% and values of R^2 of 0.7 or higher should be obtained.
4. The concentration corresponding to at specified percentage effect (50%) relative to the control is estimated, through a process known as inverse estimation. Value of L(E)C50 and the 95% confidence intervals are calculated. Data are referred as the dilution of contaminated soil (g test soil/kg soil) or extract (mL extract/L dissolution) (*see* **Note 33**).

5. Notes

1. The characteristics of the dilution soil are selected to produce the minimum adsorption of chemicals in the soil and to provide maximum bioavailability of the substances.

2. The reference soil is necessary as its toxicity to organisms can vary with soil characteristics. Obtaining a reference soil is one key point in the soil assessment. The lack of reference soil is particularly critical for the toxicity test to plants, microorganisms, and algae.
3. Data from extract tests are compared to toxicity data from control medium in the test. However, effects due to nutrients in the soil (e.g., growth-stimulating effect on algae) can hide the toxic effects of soil contaminants. To minimize this phenomenon, it is recommended to use an additional control. This control consists of the extract obtained from an appropriate reference soil.
4. A mechanical mixer is necessary only if soil is tested at different concentrations. Soils may be also mixed manually in bags that are turned end-over-end enough times to ensure thorough mixing. However, mechanical mixing is recommended.
5. The strains recommended are available from the following collections:
   ATCC = American Type Culture Collection (USA)
   SAG = Collection of Algal Cultures (Germany)
   CCAP = Culture Center of Algae and Protozoa (UK)
6. Growth is quantified from measurements of the algal biomass. Direct measurement of weight biomass is difficult and, therefore, surrogate parameters are used. In the proposed method, absorbance or fluorescence measurements are recommended as the measured parameter. The conversion factor and linearity between cell biomass and surrogate parameters should be established.
7. Example of reconstituted test water (27).
   1. Prepare the following stock solutions:
      - Calcium chloride solution. Dissolve 11.76 g of $CaCl_2 \cdot 2H_2O$ in distilled or deionized water and adjust volume to 1 L with distilled or deionized water (stored at 4°C).
      - Magnesium sulfate solution. Dissolve 4.93 g of $MgSO_4 \cdot 7H_2O$ in distilled or deionized water and adjust volume to 1 L with distilled or deionized water (stored at 4°C).
      - Sodium bicarbonate solution. Dissolve 2.59 g of $NaHCO_3$ in distilled or deionized water and adjust volume to 1 L with distilled or deionized water (stored dark at room temperature for upto 2 months) (stored at 4°C).

- Potassium chloride solution. Dissolve 0.23 g of KCl in distilled or deionized water and adjust volume to 1 L with distilled or deionized water (stored at 4°C).
- Substances must be of analytical grade and the conductivity of distilled or deionized water should not be higher than 10 μS/m.

2. Mix 25 ml of each stock solution and adjust the volume to 1 L with distilled or deionized water. The solution is bubbled with air for 6 h in order to reach oxygen saturation in the solution. The solution is kept for 12 h and the pH is measured. This pH must range between 7.6 and 8.2. (stored at 4°C).

8. It is recommended that the water used in the test should be similar to the culture water to avoid the necessity of adaptation of the organisms prior to the test. Dechlorinated tap water may be also used as culture, control, and dilution water; however, maximum precautions should be taken since chlorine is highly toxic to *Daphnia*. If this water is used, a daily chlorine analysis must be performed.
9. This protocol can be made with any cell line.
10. We recommend using repeating pipettes, electronic digital pipettes, and multichannel pipettes for addition of the liquids to the wells of the 96-well plate.
11. LC50 (median lethal concentration): concentration that causes mortality in 50% of the test organisms.
    EC50 (median effect concentration): concentration that causes the measured effect in 50% of the test organisms.
    IC50 (median inhibition concentration): concentration that inhibits/reduces the response observed in the control by a 50%. This term is sometimes employed for non-quantal data (e.g., growth).
12. In the first approach, the selection of the reference soil is a crucial point. The use of an unsuitable reference soil may result in erroneous data. In the second approach, effects observed for different concentrations help to interpret results and to differentiate effects due to different physicochemical soil properties and those due to soil contaminants. However, the second method required a higher number of samples to be tested, and consequently is more expensive and time-consuming.
13. A high number of replicates are recommended in the assay with non-diluted samples. This is because data obtained in this approach are highly dependent on the number of

replicates. These methods take account of the multiple statistical tests by adjusting the statistical threshold for declaring significance. If experimental variability is relatively high, the sensitivity of the analyses to detect differences from the control will be relatively low.

14. Optionally, effects on growth may be determined. With this aim, earthworms are purged for 24 h on moist filter paper. Then, all individuals in each container are weighed and the weight is expressed on a per earthworm basis. The percentage of inhibition of growth as compared with the control and dilution soil is calculated and the value of EC50 is determined.
15. Variability between replicates in the plant test is higher than other toxicity tests, consequently a higher number of replicates of control soil are recommended in order to obtain the statistical power desirable.
16. Plant weight must be recorded immediately after harvest. Otherwise, loss of water in the plants affects the weight measures, and consequently the toxicity test.
17. Soil moisture is a key factor in the tests based on microbial processes. The moisture content of the soil sample should be between 40% and 60% of the maximum water-holding capacity during the assay time. Distilled or deionized water is added as necessary.
18. Glucose added should be sufficient to obtain the maximum respiratory response. This amount may be determined in a previous test using a series of glucose concentrations. The proposed amount (2–4 g of glucose/kg soil d.w.) weight soil is sufficient for soil with characteristics of control soil proposed in this work.
19. Of particular importance in these experiments is the selection of the mineralization rate. Usually, carbon mineralization curves show an initial period having a zero slope, followed by an increase of the mineralization rate. In this test, the effects on carbon mineralization should be estimated from the maximum respiration rate.
20. The quantity of sample recommended in most of the standard methods to obtain extracts from wastes is 100 g. Soil samples are more homogenous that other solid wastes, consequently, the size of sample may be less than 100 g. However, enough soil should be used for extraction, so that the volume of extract will be sufficient to support all of the analyses required.
21. Inoculum culture in test medium may be prepared 2–4 days before start of the test, in order to adapt the culture to the

test conditions. The increase of biomass may be measured to ensure the growth is exponential.

22. In extracts, nutrient added from the soil may have a growth-stimulating effect on algal biomass. The extracts should fulfill the following criteria:

P	≤ 0.7	mg/L
N	≤ 10	mg/L
Chelators	$\leq 10^{-3}$	mmol/L
Hardness (Ca + Mg)	≤ 0.6	mmol/L

Otherwise, stimulating effects of nutrients may hide toxic effects due to soil contaminants. The use of extracts from reference soil as a control is necessary for determining the inhibition of average specific growth rate for algae.

23. The algal assay may also be performed in a conical flask. However, a higher volume of sample is necessary.
24. The algal biomass may be determined by cell counts. Sample in the wells are taken, diluted with algal medium (1:10), and transferred to a Neubauer chamber. This method is more time-consuming than direct measurement using fluorimetric or spectrophotometric methods.
25. If pH in the test medium does not fulfill the test conditions (pH=6–9), an additional assay with pH adjustment may be performed. The pH adjustment must be made ensuring that contaminant concentration in the extract is not changed and no chemical reaction or precipitation is caused.
26. Cell viability can be measured in a variety of ways, most of which are based on indicators that measure fundamental cellular functions. Processes like membrane permeability, lysosomal integrity, and enzymatic metabolism are examples of such functions that are essential for proper cell integrity and growth. However, the toxic threshold concentration at which a sample augments cell membrane permeability may not be the same as that affecting lysosomal integrity (uptake of neutral red dye) or modify enzymatic activities.
27. Culture medium, PBS, and any component must be room temperature warmed, prior to use.
28. Sample incubation times can be increased (48 or 72 h).
29. It may be convenient to dilute the solutions into a sufficient volume of culture medium to allow for the extra non-recoverable volume required when using reagent reservoirs and multichannel pipettes.

30. For single concentration assessment, effects of soil tests are compared to reference soil rather than control soil. In this way differences due to different physicochemical soil properties are minimized.
31. Dunnett's method is perhaps the most widely used method in ecotoxicology for comparing samples.
32. These methods are appropriate to use on quantal data (e.g., mortality). For continuous data (e.g., growth) other methods are more appropriate. (28).
33. Regression models provide estimations of the toxic effects with higher sensitivity and precision than analyses at a single concentration. Moreover, the use of different concentrations allows detecting cross-effects such as those due to different physicochemical properties of the test and control soil.

References

1. Stephenson, G. L., Kuperman, R. G., Linder, G. L., and Visser, S.w (2002) Toxicity tests for assessing contaminated soils and ground water, in *Environmental Analysis of Contaminated Sites* (Sunahara, G. I., Renoux, A. Y., Thellen, C., Gaudet C. L., and Pilon, A., eds.), John Wiley and Sons. Ltd, New York, NY, pp. 25–43.
2. Loibner, A. P., Szolar, O. H. J., Braun, R., and Hirmann, D. (2003) Ecological assessment and toxicity screening in contaminated land analysis, in *Chemical Analysis of Contaminated Land* (Thompson, K. C., and Nathanail, C. P., eds.), Blackwell Publishing Ltd., Oxford, UK, pp. 229–267.
3. Fernández, M. D., Vega, M. M., and Tarazona, J. V. (2006) Risk-based ecological soil quality criteria for the characterization of contaminated soils. Combination of chemical and biological tools. *Sci. Total. Environ.***366**, 466–484.
4. Van WGestel, C. A. M., van der Waarde, J. J., Derksen, J. G. M., van der Hoek, E. E., Veul, M. E. X., Bouwens, S., Rusch, B., et al. (2001) The use of acute and chronic bioassays to determine the ecological risk and bioremediation efficiency of oil-polluted soils. *Environ. Toxicol. Chem.***20**, 1438–1449.
5. Robidoux, P. Y., Gong, P., Sarrazin, M., Bardai, G., Paquet, L., Hawari, J., et al. (2004) Toxicity assessment of contaminated soils from an antitank firing range. *Ecotoxicol. Environ. Saf.* **58**, 300–313.
6. Fernández, M. D., Cagigal, E., Vega, M. M., Urzelai, A., Babín, M., Pro, J., and Tarazona, J. V. (2005) Ecological risk assessment of contaminated soils through direct toxicity assessment. *Ecotoxicol. Environ. Saf.* **62**, 174–184.
7. Eom, I. C., Rast, C., Veber, A. M., and Vasseur, P. (2007) Ecotoxicity of a polycyclic aromatic hydrocarbon (PAH)-contaminated soil. *Ecotoxicol. Environ. Saf.* **67**,190–205.
8. Organization for Economic Cooperation and Development (OECD) (1984) Guidelines for Testing of Chemicals. Earthworm, acute toxicity tests. Test guideline N° 207. Paris, France.
9. Organization for Economic Cooperation and Development (OECD) (2006a) Guidelines for Testing of Chemicals. Terrestrial Plant Test: Seedling Emergence and Seedling Growth Test. Test guideline N° 208. Paris, France.
10. Organization for Economic Cooperation and Development (OECD) (2000a) Guidelines for Testing of Chemicals. Soil microorganisms, nitrogen transformation test. Test guideline N° 216. Paris, France.
11. Organization for Economic Cooperation and Development (OECD) (2000b) Guidelines for Testing of Chemicals. Soil microorganisms, carbon transformation test. Test guideline N° 217. Paris, France.
12. Organization for Economic Cooperation and Development (OECD) (2006b) Guidelines for Testing of Chemicals. Alga, growth inhibition test. Test guideline N° 201. Paris, France.
13. Organization for Economic Cooperation and Development (OECD) (2004) Guidelines

for Testing of Chemicals. Daphnia s.p., acute immobilisation test. Test guideline N° 202. Paris, France.
14. Babin, M. M., and Tarazona, J.V. (2005) In vitro toxicity of selected pesticides on RTG-2 and RTL-W1 fish cell lines. *Environ. Pollut.* **135**, 267–274.
15. Organization for Economic Cooperation and Development (OECD) Guidelines for testing of chemicals. Last updated October 2008. OECD. http://oberon.sourceoecd.org.
16. Anderson, J. P. E., and Domsch, K. H. (1977) A physiological method for the quantitative measurement of microbial biomass in soils. *Soil. Biol. Biochem.* **10**, 215–221.
17. Anderson, J. P. E. (1982). Soil Respiration. in *Methods of Soil Analysis.* Part 2: *Chemical and Microbiological Properties* (Page, A. L., Miller. R. H., and Keeney, D.R. eds.), Agronomy Monograph N° 9. American Society of Agronomy, Madison, WI, **41**: pp. 831–871.
18. Heinemeyer, O., Insam, H., Kaiser, E. A., and Walenzik, G. (1989) Soil microbial biomass and respiration measurements, an automated technique base on infrared gas analyses. *Plant Soil* **116**: 77–81.
19. International Organization for Standardization (ISO) (1993). Soil quality. Methods for measuring the mineralization of organic chemicals in soil under aerobic conditions using laboratory incubation systems. ISO 11266-1: 1993, Geneva, Switzerland.
20. International Organization for Standardization (ISO) (1997). Soil Quality. Laboratory incubation systems for measuring the mineralization of organic chemicals in soil under aerobic conditions. ISO 14239: 1997, Geneva, Switzerland.
21. Eaton, A. D., Clesceri, L. S., and Greenberg, A. E. (ed.) (1995) *Standard Methods for the Examination of Water and Wastewater*. 19th. American Public Health Association, Baltimore, Maryland, pp. 4-85–4-90.
22. Deutsches Institut für Normung (DIN) (1984) Determination of leachability by water. German standard methods for the examination of water, waste water and sludge, sludge and sediments (group S). DIN 38414-4:1984. Part 4. Berlin, German.
23. European Committee for Standardization (2005) Characterization of waste. Preparation of waste samples for ecotoxicity tests. EN 14735: 2005. Brussels, Belgium.
24. Litchfield, J. T., and Wilcoxon, F. (1949) A simplified method of evaluating dose effect experiments. *J. Pharmacol.* **96**, 99–113.
25. Trevors, J. T. (1986) A basic program for estimating LD50 values using the IBM-PC. *Bull. Environ. Contam. Toxicol.* **37**, 18–26.
26. Christensen, E. R., and Nyholm, N. (1984) Exotoxicological assays with algae: Weibull dose–response curves. *Environ. Sci. Technol.***18**, 713–718.
27. International Organization for Standardization (1996). Water quality. Determination of the inhibition of the mobility of *Daphnia magna* Straus (Cladocera, Crustacea). Acute toxicity test. ISO 6341:1996, Geneva, Switzerland.
28. Organization for Economic Cooperation and Development (OECD) (2005) Current approaches in the statistical analyses of ecotoxicity data: a guidance to application. Paris, France.

Chapter 16

Community-Level Physiological Profiling

Kela P. Weber and Raymond L. Legge

Abstract

Community-level physiological profiling (CLPP) is a technique which offers an easily applied protocol yielding information regarding mixed microbial community function and functional adaptations over space and time. Different communities can be compared and classified based on sole carbon source utilization patterns (CSUPs) gathered using BIOLOG™ microplates. One of the most challenging aspects associated with the CLPP method is in the data analysis. This chapter describes the relatively simple CLPP laboratory protocol and provides a detailed description of different data analysis techniques.

Key words: Community-level physiological profiling (CLPP), BIOLOG™, carbon source utilization pattern (CSUP), microbial community, microbial ecology, multivariate analysis, principle component analysis (PCA).

1. Introduction

The term community-level physiological profiling (CLPP) was first coined by Lehman et al. (1) to describe the characterization and classification of heterotrophic microbial communities based on sole carbon source utilization patterns (CSUPs). Although CLPP is considered a broad term which could cover many different types of studies undertaken using a number of different assays, currently the term CLPP is used almost exclusively in reference to data collected using BIOLOG™ microplates. BIOLOG™ microplates are 96-well plates where each well contains a different carbon source and a redox dye indicator, most often tetrazolium violet. When a mixed microbial community sample is inoculated into each of the wells, the production of NADH via cell respiration reduces the tetrazolium dye to formazan, resulting in a

S.P. Cummings (ed.), *Bioremediation*, Methods in Molecular Biology 599,
DOI 10.1007/978-1-60761-439-5_16,

colour change within each individual well, which can be detected photometrically.

There are a number of different microplates manufactured by BIOLOG™ for CLPP use, with the three most popular being the GN2, GP2 and EcoPlates. The GN2 plate is the most recent version of the GN plate and is suitable for characterizing or identifying Gram-negative bacteria. The GP2 plate is the most recent version of the GP plate, and is suited to characterizing or identifying Gram-positive bacteria. The GN2 and GP2 plates both contain 95 different carbon sources with one of the 96 wells serving as a blank. Both the GN2 and the GP2 plates were originally developed for species identification (2), but are now commonly used for CLPP. The BIOLOG™ EcoPlate contains 31 different carbon sources and a blank in triplicate. Use of triplicates allows for increased confidence in statistical analysis of the resulting plate data. The EcoPlate was developed for environmental applications, which dictated the selection of carbon sources, with at least nine substrates considered constituents of plant root exudates (3,4).

BIOLOG™ offers a number of other plates suitable for CLPP studies. SF-N and SF-P microplates are alternatives to the GN2 and GP2 plates, as they provide the same corresponding substrates, but without the tetrazolium dye. Turbidity or a different metabolic indicator can be added to assess activity. MT microplates contain the same redox chemicals as the GN2 and GP2 plates, but do not contain any substrates. These plates allow for the creation of customized plates by adding suitable substrates for specific ecological studies. FF plates, which have been recently introduced by BIOLOG™ for the study of fungi and yeasts, contain a unique set of carbon sources and use both turbidity and/or reduction of tetrazolium as activity indicators. The GN plate and its corresponding successor, GN2, have been favoured in CLPP studies although other plates may offer greater relevance and analytical options (4).

Garland and Mills (5) were the first to use BIOLOG™ plates for characterizing heterotrophic soil bacterial communities, and a number of studies have since followed (*see* (4,6) for examples). The advantage of CLPP over both classic cell culturing and molecular level RNA/DNA amplification-based techniques is its relatively simple protocol and ease of use. Both classic cell culturing and molecular level RNA/DNA amplification-based techniques can be time consuming and require specialized expertise (7).

Limitations pertaining to the CLPP approach using BIOLOG™ microplates have been discussed in the literature (4,6,7). Limitations and pitfalls pertaining to data analysis have also been recently described (8). Some of the most pertinent limitations include the bias in the technique towards rapidly growing bacteria, the need to ensure similar inoculum sample sizes in the

wells, the need to reduce time between sampling and inoculation of the microplates and the difficulties with meaningful data analysis and interpretation.

Some of the limitations surrounding the CLPP method pertain to the long incubation times, the indirect measurement of microbial activity, and the use of high substrate concentrations. The use of lower substrate concentrations would allow for less selective enrichment within the wells as growth and incubation conditions would be more akin to those from which the sample originates. To allow for the use of decreased substrate concentrations, more sensitive and direct activity measurements would then be required. Newly developed CLPP methods include those of Degens et al. (9) and Garland et al. (10). Degens et al. (9) developed a method for the detection of CO_2 generation from mixed microbial communities utilizing a range of carbon substrates. Although Degens et al. (9) refer to their measurements as microbial catabolic diversity, the basis behind the study is similar to that of CLPP. Garland et al. (10) developed a fluorescent-based method of measuring O_2 consumption for mixed microbial communities utilizing a range of carbon substrates. Using this method, Garland et al. (10) found that incubation times could be reduced to less than 24 h, and the substrate concentrations could be reduced by a factor of 10–100 when compared to BIOLOG™ plates. Currently, the term CLPP almost exclusively refers to the use of BIOLOG™ microplates. Studies such as those conducted by Garland et al. (10) and Degens et al. (9) point to the expanding usage of the term CLPP. Improvements to the CLPP method both with and without the use of BIOLOG™ plates will no doubt lead to the evolution and changing and/or broadening of the term CLPP and its associated methods in the future.

CLPP is a technique which offers an easily applied protocol yielding large amounts of information regarding mixed microbial community function and functional adaptations over space and time. Carrying out the CLPP laboratory protocol is a relatively simple process, which has lead to its recent increase in popularity. However, the data analysis aspect associated with CLPP can be challenging, often requiring a background in multivariate analysis methods. Following is a description of the CLPP laboratory protocol and an in-depth description of the data analysis procedure.

2. Materials

1. Suspended mixed microbial community sample
2. BIOLOG™ microplate(s)

3. Microplate reader equipped with a 590 nm filter

Optional:

1. Buffer solution – May be needed if (i) performing a detachment protocol from a sediment sample to generate a suspended microbial community sample or (ii) performing serial dilutions before plate inoculation (*see* **Section 3.2**).
2. Incubator – May be needed if incubating samples at a temperature other than room temperature (*see* **Section 3.3**).

3. Methods

3.1. CLPP Protocol – General Description

Each well of the BIOLOG™ plate is inoculated with 150 μL of the sample of interest and incubated at temperatures generally ranging from 20 to 30°C. Absorbance readings (590– 600 nm) are performed as necessary using a microplate reader over an incubation period ranging from 10 to 200 h. The sample should be a uniform suspension, so if sampling sediment or biofilm, an appropriate detachment and/or homogenization protocol is necessary.

3.2. Inoculation

About 150 μL of a suspended mixed microbial community sample is inoculated into each of the 96 wells of the BIOLOG™ microplate. An undiluted sample is recommended as dilution of samples containing a mixed population has been shown to affect the resulting CLPPs (11,12). Analytical methods for dealing with small differences in inoculation densities are discussed later; however, it is important to ensure similar cell densities of the samples to reduce any error in the CLPP analysis (*see* **Note 1**). Although not recommended in all cases, if cell densities in the original samples are exceedingly high, serial dilutions may be needed before microplate inoculation. If the plates are inoculated at high cell densities, colour development may proceed at a rate where capturing meaningful data is difficult. It has been suggested that formazan production does not occur until cell densities between 10^5 and 10^8 cells/mL are reached (6,12). Lastly, the time between sampling and inoculation should be kept to a minimum in order to reduce cell death or community structural changes of the sample prior to plate inoculation.

3.3. Incubation

Plates can be incubated over a range of temperatures with room temperature being the most common. Incubation periods tend to range between 10 and 200 h; and standard incubation temperatures between 20 and 30°C. Incubation temperatures similar to those from which the sample was collected are ideal. There is some debate surrounding the effect that incubation tempera-

ture has on the resulting CLPPs. Christian and Lind (13) showed that temperature had an effect on the calculated CSUPs, whereas Classen et al. (14) showed CLPPs to be relatively insensitive to incubation temperature. Room temperature incubation has not been criticized in the literature, as the resulting CLPPs have proven useful and reasonable for most published studies.

The plates do not need to be agitated during incubation due to the relatively long incubation times. Stationary incubation at room temperature is the most common method, although shaking is required prior to plate reading to ensure uniform distribution of the formazan. Incubation periods will vary for different studies, but generally range from 10 to 200 h depending on the study and the inoculation density. Choice of a specific incubation time is not obvious, and is largely dependent on the subsequent data analysis (*see* **Section 4**). **Figure16.1** is a picture of a BIOLOG^{TM} EcoPlate after a 20 h incubation period following inoculation with interstitial water from a wetland mesocosm (15).

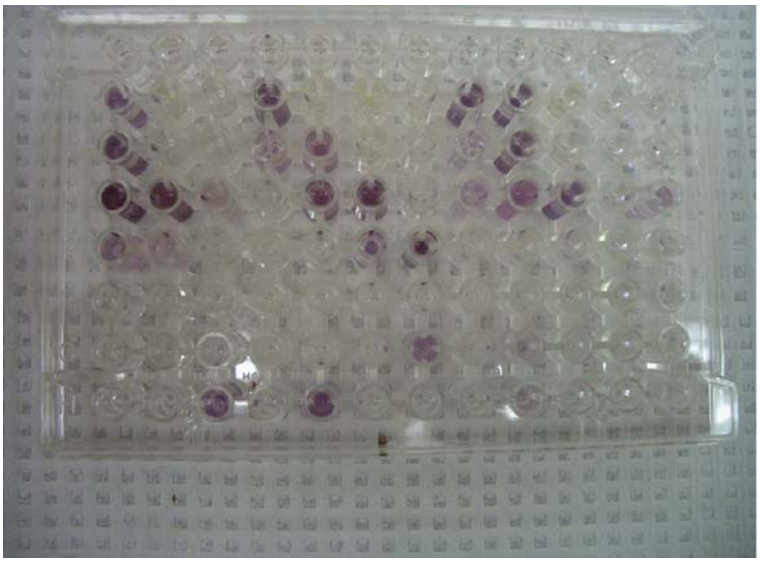


Fig. 16.1. BIOLOG^{TM} EcoPlate 20 h after inoculation with interstitial water from a wetland mesocosm system. Plate used in the study of Weber et al. (15).

3.4. Data Collection

Absorbance readings (590 nm) for all 96 wells are collected throughout the incubation period to reveal the kinetic profiles for each of the carbon sources. Reading frequency will vary based on the nature of the inoculum and the type of metric chosen for analysis (*see* **Note 2**); if data analysis does not require the fitting of kinetic profiles and a single time point is used for analysis (*see* **Section 4**), less frequent readings are reasonable. Plates should

be agitated before each reading to ensure sufficient colour distribution in each well.

An example of the type of data that can be collected when following the described protocol is provided in **Fig.16.2**. Colour development for individual wells for a single set of EcoPlate replicates is presented (31 different carbon sources and 1 blank); colour development curves show a general sigmoidal shape.

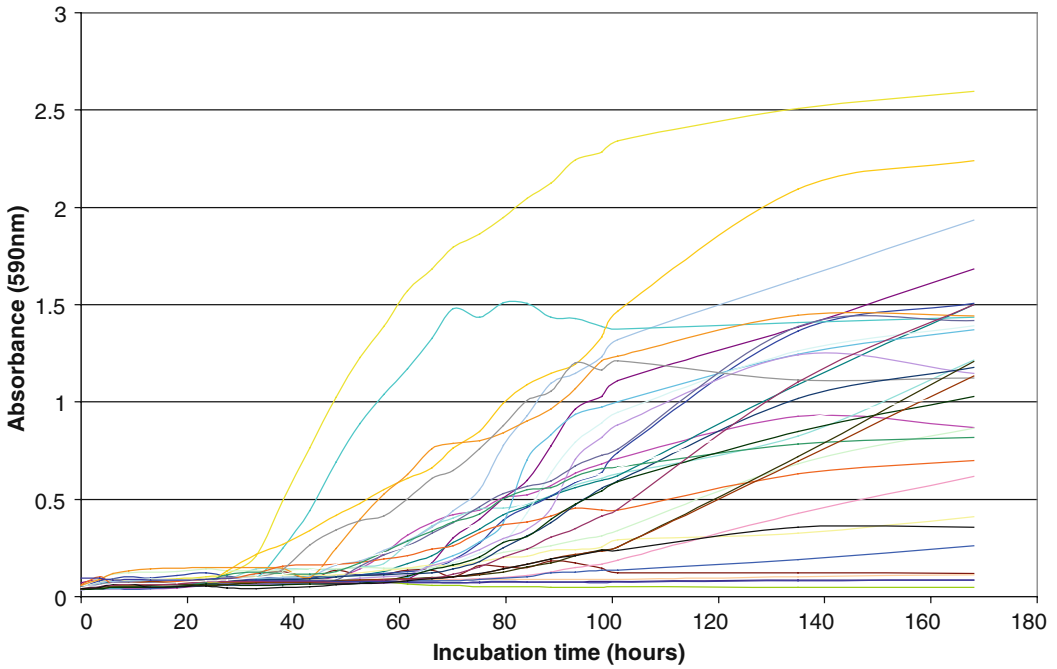


Fig. 16.2. Individual well colour development curves for a single set of EcoPlate replicates (31 carbon sources and 1 blank). Data from Weber et al. (15).

3.5. Anaerobic Protocol

A small number of earlier studies indicated that formazan is not produced in regular aerobic-usage BIOLOG™ plates incubated under anaerobic conditions (4,16). However, a number of recent studies have shown formazan production does occur under anaerobic conditions (13,17,18). BIOLOG™ manufactures an AN microplate for the identification of anaerobic bacteria although a number of anaerobic CLPP studies have favoured the use of GN or ECO microplates as these plates have been previously shown to be suitable for ecological and exploratory CLPP studies for mixed microbial systems. The anaerobic CLPP protocol is identical to the aerobic protocol described here, with a few changes focusing on minimizing the exposure to oxygen throughout the procedure (*see* **Note 3**). This can be accomplished through any number of standard anaerobic culturing and testing techniques.

3.6. Data Analysis

A large amount of data can be collected with a single BIOLOG™ plate; when numerous plates are used, the amount of data can then become overwhelming. To deal with the large amount of data and large number of variables to be processed, some form of multivariate analysis is required. Data analysis will be largely discussed with direction towards the use of principle component analysis (PCA) as this is the most commonly and easily used method for analysing and visualizing the CLPP data. A number of other techniques will also be discussed later.

3.7. Standard Analysis Method When Using Principle Component Analysis

Before reaching the point of performing a multivariate analysis technique (such as PCA), a number of steps should be followed:

(1) Decide what metric will be used for the data analysis

(2) Standardization of the data

(3) Assess heterogeneity, normality and the underlying factor structure of the data

(4) Perform a data transformation if required

3.8. Selecting a Metric

First a metric needs to be selected and extracted from the BIOLOG plate data to represent activity in each well. The three most commonly utilized metrics are:

(i) An absorbance value for each well for a specific incubation time point

(ii) An absorbance value for each well taken from a time point representing a specific average well colour development (AWCD) for that plate

(iii) Some type of logistic curve fitting value such as lag, maximum utilization rate (slope), area under the curve or an asymptote value

3.8.1. Selecting a Specific Incubation Time Point

To evaluate all plate data within a study, a specific incubation time point can be chosen as a metric, but choosing this time point may not be obvious. An increase in the differences (or variation) between well absorbance values indicates an increase in the amount of information contained within the data set. Using absorbance values taken early in the incubation time would yield little information, for at early stages of growth (for example, 10 h in **Fig.16.2**) the difference between well absorbance values is too small to yield useful information. Using absorbance values taken later during the incubation can provide more information regarding the CLPP of the microbial inoculum as long as the values are not above a value of 2. As seen in **Fig.16.2**, there is an increase in the dispersion of (or differences between) well absorbance values as the incubation proceeds. This dispersion of well absorbance values can be represented as the standard deviation calculated at each time point (**Table16.1**).

Table 16.1
Calculated AWCD, number of values above an absorbance of 2, and standard deviations for absorbance values over the 168 h incubation time for the plate shown in Fig. 16.2. Data from Weber et al. (15)

Incubation time (hours)	AWCD	# values above absorbance of 2	Standard Deviation
0	0.00	0	0.01
4	0.01	0	0.02
6	0.01	0	0.02
9	0.01	0	0.02
13	0.01	0	0.02
18	0.00	0	0.02
24	0.00	0	0.02
28	0.01	0	0.02
34	0.02	0	0.04
38	0.04	0	0.09
43	0.06	0	0.14
48	0.09	0	0.20
53	0.12	0	0.25
57	0.16	0	0.29
62	0.19	0	0.33
66	0.24	0	0.37
70	0.27	0	0.40
75	0.31	0	0.40
80	0.37	0	0.43
84	0.42	0	0.45
89	0.47	1	0.46
93	0.53	1	0.49
98	0.57	1	0.50
101	0.60	1	0.52
135	0.85	2	0.59
168	0.99	2	0.63

Absorbance readings above 2 contribute to measurement error as they are outside the linear absorbance range. An appropriate time point will be the time point that preserves the greatest variance between well responses while retaining the maximum number of wells within the linear absorbance range. For example for the data in **Table16.1**, Weber et al. (15) chose to use absorbance data from the 84 h time point for subsequent mul-

tivariate analysis (*see* **Note 4**). This study included a number of BIOLOG™ plates comparing a number of different microbial samples. The data from all plates were considered before a time point was chosen. When using this simplified method of choosing a specific time point for all analyses, similar inoculation densities for all plates is essential (12).

3.8.2. Selecting a Time Point Related to a Specific AWCD Reference Value

As recommended by Garland et al. (12), a specific AWCD value can also be chosen as a reference point for all plates analysed. Absorbance values used for subsequent multivariate analysis are extracted from each set of plate data at the specific time point where the reference AWCD occurs. Garland (7) showed that using AWCD reference values between 0.25 and 1.0 yields relatively similar CLPPs for use in community classification (*see* **Note 5**).

3.8.3. Kinetic Analysis

Kinetic analysis can also be performed on the well colour development data (for examples, *see 4, 12, 19, 20*). A number of different metrics can be chosen for use in multivariate analysis. Factors such as lag time, maximum utilization rate (slope), area under the curve or asymptote values have been used. A large amount of data needs to be acquired for a logistic curve to fit the data, and not all data are suited to a logistic fit. Deviation in absorbance readings and non-characteristic responses in some wells can have a large effect on curve fitting, making some data unfit for kinetic analysis.

Kinetic approaches have great potential, as a more detailed understanding of the nature of the color responses can be theoretically attained (4,12). However, a general lack of understanding regarding physiological or ecological bases for differences in the derived kinetic parameters limits the amount of information that can be extracted when using a kinetic approach (12). If the objective is to classify different microbial populations, using data from a single absorbance point reading may be more useful. Garland et al. (12) found that using a single absorbance point reading corrected by the AWCD was more successful than using kinetic parameters for classifying different soil bacteria populations, and remarked that the use of kinetic parameters for CLPP may provide some additional information, but only if the influence of inoculum density is carefully considered (*see* **Note 6**).

The overall incubation time for any one study is dependent upon what type of metric will be used and cannot be easily determined. It is preferable to run a number of test plates inoculated with microbial community samples similar to those to be characterized in the overall study before starting an experimental regime. Using these test plates, careful determination of which type of metric will be used and the resulting incubation time can be assessed.

3.9. Standardization of the Data

When performing a CLPP analysis, numerous plates are often used to study different mixed microbial communities in space or over time. As recommended by Garland (7), if the choice of metric is to use a single time point absorbance, and not perform a kinetic analysis of the data, an initial standardization of the data helps to reduce any bias due to inoculum density differences between samples. Standardization of the data involves correcting each absorbance value by its corresponding blank and then dividing by the AWCD for that time point. The standardized absorbance for well k can be calculated as:

$$\bar{A}_k = \frac{A_k - A_o}{\frac{1}{31}\sum_{i=1}^{31}(A_i - A_o)} \quad [1]$$

where A_i represents the absorbance reading of well i and A_0 is the absorbance reading of the blank well (inoculated, but without a carbon source). Where there is very little response in a well, negative values of standardized absorbance may occur and, since this is physically meaningless, they are coded as zeros for further analysis. Standardization of the data may not be needed when performing certain types of kinetic analyses.

3.10. Assess the Suitability of the Data Set for Multivariate Analysis

Efficient and meaningful statistical methods for dealing with a large number of interdependently correlated variables are needed when evaluating CSUPs from BIOLOG™ plates; most researchers have turned to some form of multivariate technique and, in particular, principal component analysis (PCA) (21). In performing PCA, each plate (p number of plates) is considered an object with n variables (31 for EcoPlates, 95 for GN2 or GP2 plates) giving a matrix with p rows and n columns. The transformation of BIOLOG™ plate data is an important aspect of multivariate analysis techniques such as PCA. Weber et al. (8) provide an in-depth study on data preparation techniques briefly described in the following section.

Many multivariate analysis techniques assume two fundamental properties of a data set: normality and homoscedasticity (that is, homogeneity of variance: all variables are assumed to have the same variance). In PCA, the dimensionality of the data set is reduced by extracting an orthogonal set of principal components (PCs) made up of linear subsets of the original ordinates; the extraction is designed so that the maximum amount of variance is concentrated in the first PC, with the second largest amount of variance contained in the second PC, and so on. This analysis technique is most powerful if the data have an underlying factor structure; that is, it is dependent on linear correlations between the different variables (22). Weber et al. (8) concluded that if

homoscedasticity, normality and the number of linear correlations within a data set are not evaluated and the possibility of transforming the data is not considered, erroneous analysis and misleading conclusions may arise when performing multivariate analysis on microplate data (*see* **Note 7**). Following is a short summary of useful data transformation techniques and data assessment methods used when working with BIOLOG™ microplate data.

3.10.1. Normality

Normality of BIOLOG™ microplate data can be evaluated through formal statistical tests. The kurtosis and skewness of each variable can be calculated and the standard errors found according to Equations (**15.2**) and (**15.3**) for kurtosis and skewness, respectively:

$$SE_{kurtosis} = \sqrt{\frac{24}{n}} \quad [2]$$

$$SE_{skewness} = \sqrt{\frac{6}{n}} \quad [3]$$

where $SE_{kurtosis}$ and $SE_{skewness}$ are the standard errors for kurtosis and skewness, respectively, and n is the number of observations. The corresponding z values can also be calculated as:

$$z_{kurtosis} = \frac{\text{kurtosis}}{SE_{kurtosis}} \quad [4]$$

$$z_{skewness} = \frac{\text{skewness}}{SE_{skewness}} \quad [5]$$

Either z value may be used in a formal statistical test of a null hypothesis that the data is normally distributed versus an alternative that it is not. A two-tailed test is used and the null hypothesis rejected with 95% confidence if $|z| > 1.96$.

Normality according to Weber et al. (8) can be assessed using both kurtosis and skewness, by calculating the mean value of the statistics across all variables as well as testing the individual variables. Note that the 95% significance level applies to the individual tests and not to the global set of tests of all variables; therefore, the number of significant results is considered to be indicative of the number of significantly non-normal variables tested. For a more detailed example see Weber et al. (8).

3.10.2. Homoscedasticity

Homoscedasticity is perhaps best assessed by a scatter plot of pairs of variables (a characteristic oval appearance will result for homoscedastic pairs); however, this is not feasible for so many variables, and instead a variance ratio can be calculated:

$$\text{variance ratio} = \frac{\text{highest variance}}{\text{lowest variance}} \quad [6]$$

This is adapted from the concept that a lesser degree of variation in separate variances contributed by many variables will constitute a lower ratio between the highest variance of any one variable and the lowest variance of any one variable in that data set (23). This cannot be tested formally and should only be considered indicative of the relative homogeneity of variance between data sets (lower values being relatively more homogeneous).

3.10.3. Underlying Factor Structure – Linear Correlations

The number of linearly correlated variables within a data set can be calculated by obtaining the correlation matrix and counting the number of correlation coefficients greater than Pearson's critical r value for the specified number of observations. This corresponds to a pairwise formal test of the null hypothesis of no correlation between variables versus an alternative of (positive or negative) correlation at a 95% confidence level. Again, in making multiple comparisons the global confidence level of the test (over all pairs) is lower than the nominal pairwise level, but the number of significant results can, for the sake of this assessment, be interpreted as an indicator of the suitability of the data for PCA. If a transformation significantly reduces the number of linear correlations between variables, then it can be suspected that this may cause a problem in subsequent analysis.

3.10.4. Perform a Data Transformation if Required

As presented in Weber et al. (8) two transformations commonly employed in ecological data analysis can also be used for BIOLOG™ microplate data: the Taylor power law transformation and the logarithmic transformation. The Taylor transformation (24) is commonly used to stabilize variances and make data conform to the assumptions of parametric analysis such as normality (22). It is based upon the assumption that:

$$S^2 = a\bar{y}^2 \qquad [7]$$

where S is the standard deviation of a sample variable, $\bar{y}$ is the mean of a sample variable and a is the sampling factor. This leads to

$$\log S^2 = \log a + b \log \bar{y}^2 \qquad [8]$$

where the slope, b, may be obtained by linear regression of the data for all variables. This leads to the conditional transformation:

$$y_i' = y_i^{(1-b/2)} \quad \text{for } b \neq 2 \qquad [9]$$

or,

$$y_i' = \ln(y_i) \quad \text{for } b = 2 \qquad [10]$$

where y_i' is the value of the transformed variable.

A logarithmic transformation can also often serve to normalize skewed data (22). A common logarithmic transformation used in ecological data analysis is of the form:

$$A' = \ln(\bar{A}_k + 1) \quad [11]$$

where A′ is the value of the transformed variable.

These two simple data transformation examples are given to provide a starting point for utilizing transformations when performing multivariate data analysis of microplate data. Many other data transformations exist and may be more suitable for specific data sets. See (22) and (25) for detailed discussions regarding different data transformation techniques.

3.11. Perform PCA on the Data Set

Principle component analysis (PCA) is the most commonly employed multivariate analysis technique when working with BIOLOG™ microplate data. PCA is based on an eigenanalysis of an R-mode (between variables) variance− covariance matrix (22). In short, PCA is able to take a high dimensional space (32 dimensions in this case) and ordinate samples (objects) on a two-dimensional plane while preserving the maximum allowable amount of variance within the data set. PCA is most commonly used to visualize data plotted on the first two principle component (eigenvector) axes for interpretation. Common uses include the study of ecological shifts over time and space (22). PCA analysis can preserve varying degrees of the original variance within the first two axes; values from 40 to 80% are commonly achieved. PCA ordinations allow the CSUPs from the bacterial community samples to be grouped and differentiated. PCs are most commonly extracted from the covariance matrix of the data. Use of the covariance matrix preserves scale.

PCA has been widely adopted for analysing CLPPs based on CSUPs generated using BIOLOG™ microplates. As outlined in previous sections, attention needs to be paid to the distribution of the underlying variables and the possibility of applying a transformation to the data to improve the analysis. One of the significant advantages of PCA is that it is robust and analyses remain valid even if the assumptions of normality and homoscedasticity are not met; however, the analysis can be improved if the data can be transformed to meet these assumptions. Recent CLPP example studies utilizing PCA analysis of BIOLOG™ microplate data include (15,26,27).

Included as **Table 16.2** is a "Quick Reference Guide", which attempts to briefly summarize the steps in the CLPP protocol within a single page for laboratory use.

Table 16.2
CLPP protocol – quick reference guide

I – Inoculation
→150 μL of a suspended mixed microbial sample into each of the 96 wells
NOTES: Time between sampling and inoculation should be minimized. Keep inoculation densities similar between plates.
II – Incubation
→ Incubate at room temperature
NOTES: Incubation period selected based on the type of metric to be used for data analysis (Step IV-A-1). Common incubation times between 10 and 200 h. Common incubation temperatures between 20 and 30°C.
III – Data Collection
→ Periodic absorbance (590 nm) readings taken for all wells during incubation period
NOTES: Plates should be shaken before each reading.
IV – Data Analysis
(A) PCA Analysis
(1) Choose a metric
(i) An absorbance value for each well from a specific incubation time point
(ii) An absorbance value for each well taken from a time point representing a specific AWCD for that plate
(iii) Some type of logistic curve fitting value such as lag, slope, area under the curve, or an asymptote value.
(2) Standardization of data if not performing a kinetic analysis
(3) Check data set for
(i) normality
(ii) homoscedasticity
(iii) underlying factor structure– liner correlations
(4) Perform a data transformation if required
(5) PCA analysis
(B) Other Analysis Methods
(1) Clustering analysis
(2) Diversity indices – substrate diversity, substrate richness, substrate evenness
(3) Alternative methods – factor analysis, PCoA, DCA, NMDS, DA, CCorA, RDA

3.12. Other Analysis Methods

In addition to PCA, a number of other analysis methods have been successfully utilized in garnering information from BIOLOG™ plates. Some of the more common methods include:

(1) Clustering analysis

(2) Diversity indices – substrate diversity, substrate richness, substrate evenness

(3) Alternative methods – factor analysis, PCoA, DCA, NMDS, DA, CCorA, RDA

3.12.1. Clustering Analysis

As recommended by Legendre and Legendre (22), clustering analysis is often performed to verify and validate results obtained using PCA. Clustering analysis allows for the CSUP similarities to be visualized in a dendogram for any given number of plates. An unweighted pair-group method using arithmetic averages (UPGMA) clustering analysis is often recommended. See (15,26,28) for recent examples of clustering analysis using CSUPs from BIOLOG™ microplates.

3.12.2. Substrate-Related Diversity Indices

BIOLOG™ plates have also been used, in a more traditional ecological sense, to calculate diversity indices based on CSUPs (29). The Shannon index or what is often called "diversity" is a common ecological metric used to track and understand shifts in communities over space and time. Using the CSUP gathered from a single BIOLOG™ plate, substrate diversity (H) can be calculated as:

$$H = -\sum p_i \ln(p_i), \qquad [12]$$

where

H – substrate diversity

p_i – ratio of the activity of a particular substrate to the sums of activities of all substrates

activity– chosen metric for analysis (absorbance value, kinetic parameter, etc.)

Two other parameters associated with substrate diversity which can be calculated using CSUPs are substrate richness (S) and substrate evenness (E). Substrate richness is a measure of the number of different substrates utilized by a microbial population. Substrate evenness is defined as the equitability of activities across all utilized substrates; substrate richness is calculated as the number of wells with a corrected absorbance greater than 0.25. Substrate evenness is calculated as:

$$E = H/H_{\max} \qquad [13]$$

Recent examples of studies utilizing the Shannon index include (15,26,27).

3.12.3. Multivariate Analysis Methods

Although PCA is the most popular approach, with proper data treatment essentially any multivariate analysis technique can be used to analyse the data matrix collected when applying the CLPP protocol to any number of mixed microbial community samples. A short list of reference studies utilising some of the less popular and/or more recently introduced multivariate methods either

examining or relating BIOLOG™ data to other data sets include: factor analysis (30), principle coordinates analysis (PCoA) (31), detrended correspondence analysis (DCA) (32,33), non-metric dimensional scaling (NMDS) (14), discriminant analysis (DA) (20), canonical correlations analysis (CCorA) (34), and redundancy analysis (RDA) (27). For an in-depth description of the mentioned multivariate methods, see Legendre and Legendre (22).

4. Notes

1. Using a minimum inoculation density of 10^5 cells/mL is the best way to reduce lag times although smaller inoculation densities can be used. True cell densities can be difficult to determine; therefore, an alternative inoculation approach is to dilute the sample to an optical density if ~0.2 at 420 nm for the suspended mixture. This inoculation approach may lead to inoculation density differences, but from a practical perspective is more easily controlled and implemented.
2. Reading frequencies can vary quite widely. For the data seen in **Fig.15.2**, a reading frequency of 4 h was used. This reading frequency provided enough data to decipher the sigmoidal shape of the colour development curves. However, if a larger inoculum density was used, a faster response would be observed, and therefore more frequent readings would be required in order to properly decipher and/or model the sigmoidal shape of the colour development curves. Preliminary trial runs using inoculum densities and bacterial communities similar to one's study samples is always a good idea. They can help one determine inoculum dilutions, reading frequencies and metric choices, which are essential in gathering meaningful data.
3. Following anaerobic inoculation, plates can be covered with non-slit silicon plate seal, or simply sealed around the edges with a generous amount of parafilm and masking tape. Both procedures have been proven effective. Microplate absorbance readings are then periodically taken without removing the plate lids. In the author's experience, overall profiles and readings have been shown to not be significantly affected by leaving the lid on during plate readings. Some plate readers require that the lids to be removed before absorbance readings can be taken. These plate readers unless equipped with or situated in a nitrogen purging area would not be suitable for anaerobic samples.
4. Each metric contains associated positives and negatives. Use of a single time point reading often guides the user towards using data points in an area where almost all carbon source

utilization curves are in a stationary (steady-state) phase. This can be useful when comparing plates over extended time periods, as the basis for comparison is relatively stable. However, in interpreting this type of data, one should be aware that the activity levels of the community on specific carbon sources is not emphasized due to the carbon utilization curves being in the stationary phase.

5. When choosing to use a reference AWCD, one should be aware that the carbon utilization curves can be in the lag, exponential growth or the stationary phase. Therefore, this method, although based on a fixed reference point, may not give stable comparison results over an extended time period for community monitoring studies. However, in comparison to a fixed time point, this method does emphasize activity in each well, which may be of interest to the user. It should also be mentioned that using an AWCD reference point may not be appropriate in studies where some plates contain a large number of unresponsive wells.
6. Kinetic analysis allows one to compare many different aspects of the carbon utilization curves, allowing the user to tune the analysis to emphasize a specific aspect of the community. However, in modelling the data sometimes poor-fit can occur, and in many instances kinetic analysis is not feasible due to time or instrument constraints.
7. The authors have found that with larger data sets (100+ objects), normality, homoscedasticity and the underlying factor structure of the data do not have as large an effect on PCA results and subsequent data interpretation when compared to smaller data sets. However, it should be emphasized that assessing the data set for normality, homoscedasticity and the underlying factor structure, and considering an initial data treatment are necessary steps in the data analysis procedure. If a large difference between PCA results from transformed and untransformed data is not observed, the data set was likely already suited for PCA or was of a size where a data transformation did not have a large effect on the PCA results.

References

1. Lehman, R. M., Colwell, F. S., Ringelberg, D. B., and White, D. C. (1995) Combined microbial community-level analyses for quality assurance of terrestrial subsurface cores. *J. Microbiol. Meth.***22**, 263–281.
2. Insam, H. (1997) A new set of substrates proposed for community characterization in environmental samples, in *Microbial Communities: Functional Versus Structural Approaches* (Insam, H., and Rangger, A., eds.), Springer-Verlag, Berlin.
3. Campbell, C. D., Grayston, S. J., and Hirst, D. J. (1997) Use of rhizosphere carbon sources in sole carbon source tests to discriminate soil microbial communities. *J. Microbiol. Meth.* **30**, 33–41.
4. Preston-Mafham, J., Boddy, L., and Randerson, P. F. (2002) Analysis of microbial community functional diversity using sole-

carbon-source utilisation profiles – a critique. *FEMS Microbiol. Ecol.* **42**, 1–14.
5. Garland, J. L., and Mills, A.L. (1991) Classification and characterization of heterotrophic microbial communities on the basis of patterns of community-level sole-carbon-source utilization. *Appl. Environ. Microbiol.* **57**, 2351–2359.
6. Konopka, A., Oliver, L., and Turco, R. F. (1998) The use of carbon substrate utilization patterns in environmental and ecological microbiology. *Microb. Ecol.* **35**, 103–115.
7. Garland, J. L. (1997) Analysis and interpretation of community-level physiological profiles in microbial ecology. *FEMS Microbiol. Ecol.* **24**, 289–300.
8. Weber, K. P., Grove, J. A., Gehder, M., Anderson, W. A., and Legge, R. L. (2007) Data transformations in the analysis of community-level substrate utilisation data from microplates. *J. Microbiol. Meth.* **69**, 461–469.
9. Degens, B. P., Schipper, L. A., Sparling, G. P., and Duncan, L. C. (2001) Is the microbial community in a soil with reduced catabolic diversity less resistant to stress or disturbance? *Soil Biol. Biochem.* **33**, 1143–1153.
10. Garland, J. L., Roberts, M. S., Levine, L. H., and Mills, A. L. (2003) Community-level physiological profiling performed with an oxygen-sensitive fluorophore in a microtiter plate. *Appl. Environ. Microbiol.* **69**, 2994–2998.
11. Franklin, R. B., Garland, J. L., Bolster, C. H., and Mills, A. L. (2001) Impact of dilution on microbial community structure and functional potential: Comparison of numerical simulations. *Appl. Environ. Microbiol.* **67**, 702–712.
12. Garland, J. L., Mills, A. L., and Young, J. S. (2001) Relative effectiveness of kinetic analysis vs single point readings for classifying environmental samples based on community-level physiological profiles (CLPP). *Soil Biol. Biochem.* **33**, 1059–1066.
13. Christian, B. W., and Lind, O. T. (2006) Key issues concerning Biolog use for aerobic and anaerobic freshwater bacterial community-level physiological profiling. *Internat. Rev. Hydrobiol.* **91**, 257–268.
14. Classen, A. T., Boyle, S. I., Haskins, K. E., Overby, S. T., and Hart, S. C. (2003) Community-level physiological profiles of bacteria and fungi: plate type and incubation temperature influences on contrasting soils. *FEMS Microbiol. Ecol.* **44**, 319–328.
15. Weber, K. P., Gehder, M., and Legge, R. L. (2008) Assessment of the changes in the microbial community in response to acid mine drainage exposure. *Water Res.* **42**, 180–188.
16. Winding, A., and Hendriksen, N. B. (1997) Biolog substrate utilization assay for metabolic fingerprints of soil bacteria: incubation effects, in *Microbial Communities: Functional Versus Structural Approaches* (Insam, H., and Rangger, A., eds.), Springer, Berlin.
17. Mills, A. L., and Garland, J. L. (2002) Application of physiological profiles to assessment of community properties, in *Manual of Environmental Microbiology 2nd Edition* (Hurst, C. J., Crawford, R. L., Knudsen, G. R., Mcinerney, M. J., and Stetzenbach, L. D., eds.), ASM Press, Washington, DC.
18. Beaumont, V. L. (2007) Investigation of Microbial Fuel Cell Performance and Microbial Community Dynamics during Acclimation and Carbon Source Pulse Tests. *MASc Thesis*, Department of Chemical Engineering, University of Waterloo. 200 pp.
19. Haack, S. K., Garchow, H., Klug, M. J., and Forney, L.J. (1995) Analysis of factors affecting the accuracy, reproducibility, and interpretation of microbial community carbon source utilization patterns. *Appl. Environ. Microbiol.* **61**, 1458–1468.
20. Mondini, C., and Insam, H. (2003) Community level physiological profiling as a tool to evaluate compost maturity: a kinetic approach. *Eur. J. Soil Biol.* **39**, 141–148.
21. Glimm, E., Heuer, H., Engelen, B., Smalla, K., and Backhaus, H. (1997) Statistical comparisons of community catabolic profiles. *J. Microbiol. Meth.* **30**, 71–80.
22. Legendre, P., and Legendre L. (1998) *Numerical Ecology* (2nd edition). Elsevier B.V. Amsterdam, Netherlands.
23. Lo, A. W., and MacKinlay, C. A., (1988) Stock market prices do not follow random walks: evidence from a simple specification test. *Rev. Financial Stud.* **1**, 41–66.
24. Taylor, L. R. (1961) Aggregation, variance, and the mean. *Nature.* **189**, 732–735.
25. Montgomery, D. C. (2001) *Design and Analysis of Experiments* (5th edition). John Wiley and Sons, Inc., New York.
26. He, X.Y., Wang, K., Zhang, W., Chen, Z., Zhu, Y., and Chen, H. (2008) Positive correlation between soil bacterial metabolic and plant species diversity and bacterial and fungal diversity in a vegetation succession on Karst. *Plant Soil.* **307**, 123–134.
27. Farnet, A.M., Prudent, P., Cigna, M., and Gros, R. (2008) Soil microbial activities in

a constructed soil reed-bed under cheese-dairy farm effluents. *Biores. Technol.* **99**, 6198–6206.
28. De Paolis, M. R., and Lippi, D. (2008) Use of metabolic and molecular methods for the identification of a Bacillus strain isolated from paper affected by foxing. *Microbiol. Res.* **163**, 121–131.
29. Zak, J. C., Willig, M. R., Moorhead, D. L., and Wildman, H. G. (1994) Functional diversity of microbial communities: a quantitative approach. *Soil Biol. Biochem.* **26**, 1101–1108.
30. Niklinska, M., Chodak, M., and Laskowski, R. (2005) Characterization of the forest humus microbial community in a heavy metal polluted area. *Soil Biol. Biochem.* **37**, 2185–2194.
31. Hackett, C. A., and Griffiths, B. S. (1997) Statistical analysis of the time-course of Biolog substrate utilization. *J. Microbiol. Meth.* **30**, 63–69.
32. Houlden, A., Timms-Wilson, T. M., Day, M. J., and Bailey, M. J. (2008) Influence of plant developmental stage on microbial community structure and activity in the rhizosphere of three field crops. *FEMS Microbiol. Ecol.* **65**, 193–201.
33. Garland, J. L. (1996) Analytical approaches to the characterization of samples of microbial communities using patterns of potential C source utilization. *Soil Biol. Biochem.* **28**, 213–221.
34. Leriche, F., Bordessoules, A., Fayolle, K., Karoui, R., Laval, K., and Dufour, E. (2004) Alteration of raw-milk cheese by *Pseudomonas spp.*: monitoring the sources of contamination using fluorescence spectroscopy and metabolic profiling.*J. Microbiol. Meth.* **59**, 33–41.

Subject Index

S.P. Cummings (ed.), *Bioremediation*, Methods in Molecular Biology 599,
DOI 10.1007/978-1-60761-439-5,

E

F

G

H

I

L

M

N