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Hydrocarbon and Lipid Microbiology Protocols

Pollution Mitigation and Waste Treatment Applications

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Preface to Hydrocarbon and Lipid Microbiology Protocols[1]

All active cellular systems require water as the principal medium and solvent for their metabolic and ecophysiological activities. Hydrophobic compounds and structures, which tend to exclude water, although providing *inter alia* excellent sources of energy and a means of biological compartmentalization, present problems of cellular handling, poor bioavailability and, in some cases, toxicity. Microbes both synthesize and exploit a vast range of hydrophobic organics, which includes biogenic lipids, oils and volatile compounds, geochemically transformed organics of biological origin (i.e. petroleum and other fossil hydrocarbons) and manufactured industrial organics. The underlying interactions between microbes and hydrophobic compounds have major consequences not only for the lifestyles of the microbes involved but also for biogeochemistry, climate change, environmental pollution, human health and a range of biotechnological applications. The significance of this "greasy microbiology" is reflected in both the scale and breadth of research on the various aspects of the topic. Despite this, there was, as far as we know, no treatise available that covers the subject. In an attempt to capture the essence of greasy microbiology, the *Handbook of Hydrocarbon and Lipid Microbiology* (http://www.springer.com/life+sciences/microbiology/book/978-3-540-77584-3) was published by Springer in 2010 (Timmis 2010). This five-volume handbook is, we believe, unique and of considerable service to the community and its research endeavours, as evidenced by the large number of chapter downloads. Volume 5 of the handbook, unlike volumes 1–4 which summarize current knowledge on hydrocarbon microbiology, consists of a collection of experimental protocols and appendices pertinent to research on the topic.

A second edition of the handbook is now in preparation and a decision was taken to split off the methods section and publish it separately as part of the Springer Protocols program (http://www.springerprotocols.com/). The multi-volume work *Hydrocarbon and Lipid Microbiology Protocols*, while rooted in Volume 5 of the Handbook, has evolved significantly, in terms of range of topics, conceptual structure and protocol format. Research methods, as well as instrumentation and strategic approaches to problems and analyses, are evolving at an unprecedented pace, which can be bewildering for newcomers to the field and to experienced researchers desiring to take new approaches to problems. In attempting to be comprehensive – a one-stop source of protocols for research in greasy microbiology – the protocol volumes inevitably contain both subject-specific and more generic protocols, including sampling in the field, chemical analyses, detection of specific functional groups of microorganisms and community composition, isolation and cultivation of such organisms, biochemical analyses and activity measurements, ultrastructure and imaging methods, genetic and genomic analyses,

[1] Adapted in part from the Preface to *Handbook of Hydrocarbon and Lipid Microbiology*.

systems and synthetic biology tool usage, diverse applications, and the exploitation of bioinformatic, statistical and modelling tools. Thus, while the work is aimed at researchers working on the microbiology of hydrocarbons, lipids and other hydrophobic organics, much of it will be equally applicable to research in environmental microbiology and, indeed, microbiology in general. This, we believe, is a significant strength of these volumes.

We are extremely grateful to the members of our Scientific Advisory Board, who have made invaluable suggestions of topics and authors, as well as contributing protocols themselves, and to generous *ad hoc* advisors like Wei Huang, Manfred Auer and Lars Blank. We also express our appreciation of Jutta Lindenborn of Springer who steered this work with professionalism, patience and good humour.

Colchester, Essex, UK — Terry J. McGenity
Braunschweig, Germany — Kenneth N. Timmis
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About the Editors

Terry J. McGenity is a Reader at the University of Essex, UK. His Ph.D., investigating the microbial ecology of ancient salt deposits (University of Leicester), was followed by postdoctoral positions at the Japan Marine Science and Technology Centre (JAMSTEC, Yokosuka) and the Postgraduate Research Institute for Sedimentology (University of Reading). His overarching research interest is to understand how microbial communities function and interact to influence major biogeochemical processes. He worked as a postdoc with Ken Timmis at the University of Essex, where he was inspired to investigate microbial interactions with hydrocarbons at multiple scales, from communities to cells, and as both a source of food and stress. He has broad interests in microbial ecology and diversity, particularly with respect to carbon cycling (especially the second most abundantly produced hydrocarbon in the atmosphere, isoprene), and is driven to better understand how microbes cope with, or flourish in hypersaline, desiccated and poly-extreme environments.

Kenneth N. Timmis read microbiology and obtained his Ph.D. at Bristol University, where he became fascinated with the topics of environmental microbiology and microbial pathogenesis, and their interface pathogen ecology. He undertook postdoctoral training at the Ruhr-University Bochum with Uli Winkler, Yale with Don Marvin, and Stanford with Stan Cohen, at the latter two institutions as a Fellow of the Helen Hay Whitney Foundation, where he acquired the tools and strategies of genetic approaches to investigate mechanisms and causal relationships underlying microbial activities. He was subsequently appointed Head of an Independent Research Group at the Max Planck Institute for Molecular Genetics in Berlin, then Professor of Biochemistry in the University of Geneva Faculty of Medicine. Thereafter, he became Director of the Division of Microbiology at the National Research Centre for Biotechnology (GBF)/now the Helmholtz Centre for Infection Research (HZI) and Professor of Microbiology at the Technical University Braunschweig. His group has worked for many years, *inter alia*, on the biodegradation of oil hydrocarbons, especially the genetics and regulation of toluene degradation, pioneered the genetic design and experimental evolution of novel catabolic activities, discovered the new group of marine hydrocarbonoclastic bacteria, and conducted early genome sequencing of bacteria that

became paradigms of microbes that degrade organic compounds (*Pseudomonas putida* and *Alcanivorax borkumensis*). He has had the privilege and pleasure of working with and learning from some of the most talented young scientists in environmental microbiology, a considerable number of which are contributing authors to this series, and in particular Balbina and Terry. He is Fellow of the Royal Society, Member of the EMBO, Recipient of the Erwin Schrödinger Prize, and Fellow of the American Academy of Microbiology and the European Academy of Microbiology. He founded the journals *Environmental Microbiology, Environmental Microbiology Reports* and *Microbial Biotechnology*. Kenneth Timmis is currently Emeritus Professor in the Institute of Microbiology at the Technical University of Braunschweig.

Balbina Nogales is a Lecturer at the University of the Balearic Islands, Spain. Her Ph.D. at the Autonomous University of Barcelona (Spain) investigated antagonistic relationships in anoxygenic sulphur photosynthetic bacteria. This was followed by postdoctoral positions in the research groups of Ken Timmis at the German National Biotechnology Institute (GBF, Braunschweig, Germany) and the University of Essex, where she joined Terry McGenity as postdoctoral scientist. During that time, she worked in different research projects on community diversity analysis of polluted environments. After moving to her current position, her research is focused on understanding microbial communities in chronically hydrocarbon-polluted marine environments, and elucidating the role in the degradation of hydrocarbons of certain groups of marine bacteria not recognized as typical degraders.

Applications I: Degradation – Pollution Mitigation and Waste Treatment Introduction

Michael J. Firth, Roger C. Prince, and Michel Boufadel

Abstract

Bioremediation of petroleum hydrocarbons can be a cost-effective part of site remediation programs for soil, ground water, shorelines, and, in some cases, sediments. Research documenting the effectiveness of bioremediation goes back to at least its use to remediate soil following a 1972 pipeline rupture in Ambler, PA [1]. Bioremediation has been successfully demonstrated in laboratory and field tests for refineries (e.g., [2–4]), for the treatment of oily sludges [5–7] and for remediating accidental petroleum releases such as oil spills [8, 9]. Research has documented the presence of native microbes capable of degrading hydrocarbons in most soils, the rate of biodegradation in various climates from temperate to polar, the potential benefits of using specific inocula to enhance degradation rates, and the optimal conditions (e.g., nutrients, pH, etc.) for biodegradation to occur. In 1993 the National Research Council [10] published a seminal guide supporting the use of bioremediation and documented the biology and state-of-practice at the time. Similarly, the Interstate Technology and Regulatory Council [11] published a series of case studies that demonstrated the effectiveness of bioremediation for petroleum hydrocarbons in ground water. With almost 40 years of documented support, bioremediation of petroleum hydrocarbons is now a proven technology. For other hydrocarbons, most notably chlorinated hydrocarbons, laboratory studies can often demonstrate biodegradation, but success in the field varies [12].

Keywords: Bioremediation, Biostimulation, Modeling

1 Introduction

Bioremediation of petroleum hydrocarbons can be a cost-effective part of site remediation programs for soil, ground water, shorelines, and, in some cases, sediments. Research documenting the effectiveness of bioremediation goes back to at least its use to remediate soil following a 1972 pipeline rupture in Ambler, PA [1]. Bioremediation has been successfully demonstrated in laboratory and field tests for refineries (e.g., [2–4]), for the treatment of oily sludges [5–7] and for remediating accidental petroleum releases such as oil spills [8, 9]. Research has documented the presence of native microbes capable of degrading hydrocarbons in most soils, the rate of biodegradation in various climates from temperate to polar, the

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potential benefits of using specific inocula to enhance degradation rates, and the optimal conditions (e.g., nutrients, pH, etc.) for biodegradation to occur. In 1993 the National Research Council [10] published a seminal guide supporting the use of bioremediation and documented the biology and state-of-practice at the time. Similarly, the Interstate Technology and Regulatory Council [11] published a series of case studies that demonstrated the effectiveness of bioremediation for petroleum hydrocarbons in ground water. With almost 40 years of documented support, bioremediation of petroleum hydrocarbons is now a proven technology. For other hydrocarbons, most notably chlorinated hydrocarbons, laboratory studies can often demonstrate biodegradation, but success in the field varies [12].

There are two ways that bioremediation can be applied on a site. It can be implemented in situ (for soil, groundwater, and sediment) with or without amendments to stimulate the degradation of the constituents of concern (COCs). Otherwise the contaminated media can be excavated and managed on the ground surface using amendments and active mixing to manage nutrient and oxygen levels. Ex situ bioremediation tends to be more effective than in situ approaches because of the ability to more efficiently ensure distribution of nutrients and oxygen, reducing much of the heterogeneity that can occur in natural systems, but it is likely to be rather more expensive.

In a risk management context, where soil requires remedial action because of estimated risks to human health or the environment, the remedial objective of a bioremediation project is to reduce constituent concentrations below those that pose a risk. As noted below, depending on the composition of the contaminants, this may or may not readily occur. In the case of petroleum hydrocarbon mixtures and many chlorinated hydrocarbons, there can be some recalcitrant components that do not degrade within what a regulator would consider an acceptable time frame. For complex petroleum hydrocarbon mixtures, a few of these recalcitrant constituents [typically pyrogenic polynuclear aromatic hydrocarbons (PAHs) such as benzo(a)pyrene] tend to be the primary drivers of the predicted chronic risk. In these cases, risk management alternatives that consider options for placing contaminated soil beneath caps or paved areas that will reduce the potential for exposure can be evaluated and implemented. For example, soil with residual constituent concentrations that are above soil cleanup goals developed for a residential exposure scenario (that is, unlimited potential contact with the soil by a child or resident living on the site) can be reused in areas that will be paved for parking lots or roads, assuming that this risk management approach is acceptable to the regulator and is managed appropriately for future property uses.

The utility of bioremediation depends on several factors. First, the COCs must be amenable to biodegradation by organisms either present naturally or potentially introduced as inocula. Naturally occurring microbes that can breakdown petroleum hydrocarbons occur in most environments, and low molecular weight petroleum hydrocarbons tend to be readily biodegraded. In contrast, microbes that effectively degrade chlorinated hydrocarbons are not always present, and chlorinated hydrocarbons range in degradability; some can be extremely recalcitrant to the point that degradation may not occur within a time frame determined to be reasonable in a regulatory context. Second, the physical and chemical conditions need to be conducive to degradation. The conditions that favor bioremediation differ for intrinsic and engineered bioremediation, although both require relatively low (less than 10,000 mg contaminant/kg solids) residual concentrations of nonaqueous-phase contaminants. In ground water, key site characteristics for intrinsic bioremediation include: consistent ground water flow throughout the seasons; the presence of minerals that can minimize pH changes; and high concentrations of oxygen, nitrate, sulfate, or ferric iron. For engineered bioremediation, the key site characteristics are permeability of the subsurface to fluids, and uniformity of the subsurface.

The biodegradability of organic constituents depends on their chemical structures and physical/chemical properties [e.g., water solubility, water/octanol partition coefficient (K_{ow})]. Highly soluble organic compounds with low molecular weights tend to be more rapidly degraded than slightly soluble compounds with high molecular weights. The low water solubilities of the more complex compounds render them less bioavailable to petroleum-degrading organisms, and likely their availability is a function of surface area available for dissolution and/or microbial attachment. If the contaminants are not bioavailable because of adsorption to organic matter in the soil, microbes cannot degrade the compound. Consequently, the larger, more complex chemical compounds may be slow to degrade or may even be recalcitrant to biological degradation (e.g., asphaltenes in No. 6 fuel oil, although reports are appearing that at least some asphaltenes are biodegradable [13, 14]). Of course heavy metals are not degraded, but under some circumstances physical and biological processes can reduce their bioavailability in the soil and reduce their potential risk. In addition, metals can be accumulated into plants in the process known as phytoremediation [15, 16].

Where conditions support biodegradation either intrinsically or through site engineering, regulatory agencies typically accept bioremediation if it can likely meet specified media-specific cleanup goals. In its favor, bioremediation destroys contaminants as it meets the primary objective of regulators as opposed to excavation and

moving the contamination to another site such as a landfill. On a site-specific basis, however, key regulatory concerns may include

- Potential current and future risks to human and ecological receptors
- Short-term control of sources so that contamination does not spread
- Time to meet site cleanup objectives
- Toxicity of degradation by-products (primarily a concern for chlorinated hydrocarbons)

As such, although regulatory agencies may accept bioremediation in theory, there are challenges to implementing it at a given site. Modeling to demonstrate the potential effectiveness (or to optimize treatment regimens of engineered systems) of bioremediation in groundwater and soil (see below) may or may not be required to gain regulatory acceptance. In addition, pilot studies or treatability studies may need to be conducted to support the selection of bioremediation as a preferred remedial alternative. As noted above, with appropriate risk management and regulatory flexibility, where needed, bioremediation can proceed as part of a successful site remediation.

2 Soil

Soil can either be bioremediated in place (in situ) or it can be excavated and bioremediated aboveground (ex situ). Guidance on designing biopiles, biocells, and related engineered systems is readily available on the internet (e.g., [17]) and Leeson et al. [18] provide a substantial list of guidance documents that have been published to guide such work. Several factors should be considered when assessing the appropriateness of soil bioremediation. As stated above, the most important criterion is whether the petroleum hydrocarbons can be degraded to meet the appropriate cleanup criteria for the site. In many cases, petroleum hydrocarbon contaminated soil that contains PAHs, which oftentimes have cleanup goals established at (or below) background, will not meet cleanup goals for unrestricted (or residential) use [19]. Depending on site-specific considerations, however, it may be possible to use bioremediation to address the majority of contamination and to reuse the remediated soil in areas where limited or no human exposure will occur. Another factor that should be considered is the need for sufficient land to contain the contaminated soils while the bioremediation is proceeding. This is not typically an issue for large sites, but many smaller sites may face time and space constraints. A final consideration is the cost relative to other potential remedial alternatives. Predicted costs used to compare the

bioremediation project to other remediation options should include design costs through final compliance sampling and analysis to demonstrate that cleanup goals have been met.

Advantages of bioremediation include

- Can be conducted on site,
- Transportation and landfill costs are not incurred,
- Backfill costs are not incurred, and
- Contamination is permanently eliminated.

Disadvantages of soil bioremediation include

- Design criteria for highly efficient remediation can require site-specific consideration, and
- Extensive monitoring during and following the remediation may be required.

3 Groundwater

Groundwater contamination with petroleum hydrocarbons has often been addressed through bioremediation (e.g., [20]). Indeed site remediation is a substantial industry; for example, McHugh et al. [21] point out that over 12,000 sites with groundwater contamination have been treated between 2001 and 2011 in California alone. Most of these sites received more than one technology, usually some active physical removal followed by a second technology such as air sparging that stimulates biodegradation. When looking across the sites evaluated [21], different treatments were clearly differentially effective at different sites, but on the whole treatments were effective. For example, the median decrease in benzene concentration from the date of detection to 2010 was 85%.

In situ groundwater bioremediation encourages growth and reproduction of indigenous microorganisms to biodegrade organic constituents in the saturated zone. Bioremediation, along with other natural processes, can effectively remediate most petroleum hydrocarbons. Some constituents like MTBE [22] and many chlorinated hydrocarbons [23] can also be degraded by microbes under some conditions, however, the rate of degradation may be very slow. Guidance is readily available on the use of in situ bioremediation to address ground water contamination (e.g., [24, 25]). In situ bioremediation of groundwater is often combined with other remedial technologies such as air sparging, soil vapor extraction, and bioventing to increase the rate of degradation and/or reduce the mass of contaminants that require biodegradation.

According to USEPA [24], the key parameters that determine the effectiveness of in situ groundwater bioremediation are

- Hydraulic conductivity of the aquifer, which controls the distribution of electron acceptors and nutrients in the subsurface;
- Biodegradability of the petroleum constituents, which determines both the rate and degree to which constituents will be degraded by microorganisms; and
- Location of petroleum contamination in the subsurface. Contaminants must be dissolved in groundwater or adsorbed onto more permeable sediments within the aquifer.

As with soil, this technology has been proven successful many times. Its success can be limited in conditions where the conditions for biodegradation are not favorable for the COCs.

Advantages of bioremediation include [24]:

- Remediates hydrocarbons that are dissolved and potentially those adsorbed onto or trapped within the aquifer matrix,
- Uses equipment that is widely available and easy to install.
- Creates minimal disruption and/or disturbance to on-going site activities.
- The time required for subsurface remediation may be shorter than other approaches (e.g., pump-and-treat).
- Generally recognized as being less costly than other remedial options.
- In many cases this technique does not produce waste products that must be disposed.

Disadvantages of bioremediation include [24]:

- Injection wells and/or infiltration galleries may become plugged by microbial growth or mineral precipitation.
- High concentrations (total petroleum hydrocarbons greater than 50,000 ppm) of low solubility constituents may be toxic and/or not bioavailable.
- Difficult to implement in low-permeability aquifers.
- Re-injection wells or infiltration galleries may require permits or may be prohibited. Some states require permits for air injection.
- May require continuous monitoring and maintenance.
- Remediation may only occur in more permeable layers or channels within the aquifer.

This section of "Protocols" deals with harnessing microbes to mitigate anthropogenic spills. There are protocols for assessing the bioremediation of highly contaminated materials (e.g., [26, 27]), assessing biofilms (e.g., [28]), assessing the biodegradation of compounds present at very low concentrations (e.g., [29]), and protocols for ecological risk assessment [30]. One might ask if there is a need for additional academic research into bioremediation,

given that numerous commercial entities are carrying out research on their own for competitive advantage. We are long past the need for simple demonstration projects, even though those were clearly essential for establishing the industry. On the other hand, there remains a need for experimentally verified predictive models that can be used to compare various potential treatments for a contaminated site. Such models will need input from multidisciplinary teams, integrating physical insights about a contaminated site with microbiological knowledge of biodegradation, acknowledging that heterogeneity in both areas will need to be considered.

4 Modeling to Predict Biodegradation Success

Because remediation is now a commercial enterprise (e.g., [21]), there is a real need for useful models that can predict the length of time likely to be required for successful bioremediation. At terrestrial sites this will allow an estimate of how the expenses associated with bioremediation compete with more rapid, but potentially much more expensive physical removal and disposal (with the potential for long-term liability for the disposed material). At sea, models can yield insights as to where oil may beach, and how long it can be expected to have an environmental impact. Obviously there are many levels of complexity that might to be considered for different scales, ranging from fundamental approximations of the microbial biodegradation process to those that consider ocean currents. Perhaps the simplest deal with highly soluble components (such as BTEX – benzene, toluene, ethylbenzene, and the xylenes), and rely on dissolution prior to biodegradation. Usually these models built on knowledge and experience from the field of wastewater treatment, and rely on Monod-type expressions [31] to describe the effect of substrate concentration on the rate of biodegradation – one example for bioremediation of groundwater is SEAM3D (Sequential Electron Acceptor Model for three-dimensional transport; [32]).

Predicting the rates of hydrocarbon biodegradation in more complicated matrices, such as soils or shoreline sediments, from basic principles is unlikely to be achieved within the next decade, and thus the best recourse may be to use calibrated models based on reasonable biological models (e.g., BIOB, [33–35]). Even so, predicted values are associated with a large degree of uncertainty and at present the goal is to determine if a compound biodegrades within 3 months or 3 years, but not to discriminate between 3 or 4 months. For this reason, biodegradation models should provide uncertainty ranges in their predictions. Nevertheless models should attempt to account for the environmental conditions (nutrients, salinity, temperature, and oxygen), interfacial area between low solubility hydrocarbons and water, and the density (or

concentration) of the microbial community. The impact of nutrient concentrations and the different electron acceptors likely to be involved in terrestrial sites has been investigated by several groups (e.g., [36–38]). The question of what level of nutrients to be aimed for in shoreline sediments has been one area of focus, and present experience suggests 1–3 mg-N/L [8, 9, 39] (Of course the environmental impact of any addition must be considered before beginning additions in the field.). The impact of oxygen concentrations on the aerobic biodegradation of oil has also been studied. In carefully controlled laboratory studies, the dissolved oxygen concentration needed for the biodegradation of hydrocarbons in open water can be as low as 0.1 mg/L [40–42]. However, results from field studies suggest that a concentration greater than 1.5 mg/L in the pore water is needed for the efficient biodegradation of oil [43–45]. Accounting for the interfacial area between low solubility hydrocarbons and water has not been pursued in the context of models for biodegradation, although experimental data are beginning to be acquired for droplets at sea [46, 47], and in groundwater [48]. However, another challenge emerges in relation to the microbial community, which may not be uniformly distributed in the porous medium or on the water–oil interface.

Modeling the biodegradation of dispersed oil may be a simpler problem since the dispersed oil can be assumed to be in a reasonably homogeneous milieu, but current models were initially designed to model physical processes such as spreading on the sea surface and evaporation. Examples include SIMAP [49] and OSCAR [50], and there are on-going efforts to improve the prediction of the biodegradation parts [51].

In any case, we can expect that as more experimental data is accumulated it will be possible to generate ever more predictive models to give regulators confidence that bioremediation will indeed be able to deliver on its promise as an environmentally friendly permanent solution to a range of environmental contaminants. The protocols in this volume will be an important part of that solution.

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Bioremediation of Hydrocarbons and Chlorinated Solvents in Groundwater: Characterisation, Design and Performance Assessment

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Abstract

Bioremediation is an accepted and widely implemented technology for the management of groundwater contaminated by hydrocarbon and chlorinated solvent compounds. This chapter reviews the general application of bioremediation processes within a cost–benefit and risk assessment framework, which considers different contaminant types and their properties, release contexts, and the strengths and limitations of available approaches. The pathways, reaction mechanisms and microorganisms responsible for biodegradation of hydrocarbons and chlorinated solvents under aerobic and different anaerobic conditions in groundwater are illustrated. The technical framework and methodology which underpins the characterisation of biodegradation processes for these organic compounds in groundwater is described, including relevant data reduction and interpretation techniques used for the performance assessment of intrinsic and engineered in situ bioremediation. This emphasises the integration of hydrochemical, stable isotope and molecular microbiological analysis with other data in site assessments for in situ bioremediation. Engineering scale-up of bioremediation in groundwater requires knowledge of scale-dependent processes which affect the implementation and performance assessment of this technology. Various methods are described to evaluate these. Comprehensive site investigation is necessary to design in situ bioremediation schemes, with focus on clear definition of the contaminant source and detailed subsurface characterisation of the aquifer geological, hydrogeological and geochemical properties which control groundwater flow and in situ biodegradation potential. This information is needed to develop conceptual site models supporting bioremediation implementation. Enhancement of bioremediation performance using methods based on bioaugmentation and biostimulation, and limitations related to contaminant bioavailability, are critically reviewed. Different design concepts can be devised to enhance and optimise treatment efficiency of engineered in situ bioremediation, by controlling the groundwater flow regime and amendment delivery. The monitoring requirements for process operation and verification are also discussed.

Keywords: Bioremediation, Chlorinated solvents, Contamination, Groundwater, Hydrocarbons, Microbiology

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1 Introduction

Contaminated soil and groundwater can cause detrimental effects on human health, the wider environment and the quality of water resources, as well as having major socio-economic implications. Recent estimates suggest that 2.5 M potentially contaminated sites could exist within European Union countries, with around 340,000 requiring remediation; the cost associated with managing this land is estimated to be €6.5B per year [1]. However, it is important to note that both former brownfield land and diffuse contaminant sources (e.g. from agriculture) can have significant detrimental effects on regional groundwater and surface water quality [2].

Many remedial actions can be used to mitigate the impacts of soil and groundwater contamination. These range from site management measures through to physical, chemical and biological remediation technologies [3–5], which may be applied in situ (i.e. the contaminated soil or groundwater is treated in place with minimal disturbance) or ex situ (i.e. the contaminated material is removed for treatment). Bioremediation technologies use the metabolic processes of bacteria, fungi and plants to reduce the mobility and toxicity of environmental contaminants of concern or to convert them to less hazardous end-products through metabolic activity [3, 5–9].

This chapter provides an overview of practical techniques that can be applied for the evaluation, implementation and verification of bioremediation for soil and groundwater contaminated with petroleum hydrocarbons and chlorinated aliphatic hydrocarbons (CAHs), which are important and common groups of environmental contaminants. Current best practice in the management of contaminated sites is first described to provide the context in which bioremediation processes are applied. The principles of bioremediation and its application for petroleum hydrocarbons and CAHs are then introduced (Sects. 3 and 4), followed by relevant characterisation and performance assessment techniques (Sect. 5), and the application of these in the engineering design of field-scale bioremediation processes (Sect. 6).

2 Management of Contaminated Sites

2.1 Site Assessment

Risk assessment is the preferred approach worldwide for the evaluation of potential impacts posed by land contamination and the definition of remediation objectives [10–14]. At a technical level, risk assessment considers 'contaminant linkages', which consist of:

- A **source**: the site or area from which the contamination originates, e.g. a landfill, former industrial site or zone of agricultural activity;

- A **pathway**: the means by which the contamination may move and be transported away from the source, e.g. dissolved in groundwater, as a vapour within ground gas, with soil-derived dust or by ingestion;
- A **receptor**: something that may be harmed by the contamination, e.g. current or future site occupants, groundwater resource, a water supply borehole, surface water, structures or crops.

All three components must exist for there to be a viable contaminant linkage and, consequently, a potential risk. Once contaminant linkages have been identified, appropriate qualitative and/or quantitative risk assessments must be undertaken to define those linkages which pose an unacceptable level of risk. The outcome of risk assessment will, in turn, inform the evaluation and specification of appropriate risk management options, including (but not limited to) remediation.

At any stage, broader factors such as sustainability objectives and government policy may form an important part of the overall risk evaluation process. Site-specific remediation sustainability assessment, considering environmental, economic and societal impacts of any proposed remediation measures, is becoming increasingly important to determine the benefits of undertaking remediation, selecting the methods to be applied and deriving performance objectives [15–18]. If remediation is necessary at a site, then the risks identified by the contamination can be mitigated to an acceptable level in one of three ways:

- By reducing the source concentration or extent; and/or,
- By reducing the flux of contaminants or preventing migration along the pathway(s); and/or, on rare occasions,
- By removing or isolating the receptor

2.2 Remediation Design, Operation and Performance Verification

Assessment of bioremediation performance is generally based on a 'lines of evidence' approach, whereby multiple sets of site data are collected to provide a high level of confidence that the technology is functioning correctly and has achieved the performance required [3, 15, 19–23].

The same evidence is also important in evaluating viable remediation options, process design and controlling operation. Lines of evidence can be categorised into three types:

– Field data showing a reduction in the contaminant concentration or flux over time at one or more points along the source–pathway–receptor chain;
– Field data demonstrating that the desired treatment processes are actively occurring. In the case of bioremediation this could include, for example, measurements of respiratory substrates/products or the use of stable isotope analysis to demonstrate the

mechanism of contaminant removal and the end-products generated;

– Supporting evidence relating to the process. Examples for bioremediation technologies include laboratory microcosm studies or the use of molecular biological techniques to demonstrate that the appropriate microbial population is present and active.

Not all will be required in every case, rather an appropriate level of data and interpretation will apply.

3 Bioremediation Technologies

3.1 Overview

The principal process types available for the implementation of bioremediation are summarised in Table 1 and further described in, for example, Bardos et al. [24], Environment Agency [23], National Academy of Sciences [21], CL:AIRE [25] and Clu-In [26]. The microorganisms involved in bioremediation may be indigenous to the contaminated site or isolated from elsewhere and introduced to the site to fulfil a specific function. It is useful to distinguish at an operational level between *intrinsic bioremediation* from *engineered or enhanced bioremediation* [5]. In the former, treatment occurs under existing conditions in an open system without intervention in the host environment – the existing biodegradation capacity of the system is used at rates determined by natural processes and metabolic capability of the indigenous microorganisms [3]. Intrinsic bioremediation is not synonymous with monitored natural attenuation (MNA), however – the key distinction is that only biological processes are considered in the former, whereas a wide range of abiotic processes (dilution, dispersion,

Table 1
Principal bioremediation process types in common use

Process	Matrix	
	Soil	Groundwater
In situ technologies		
Monitored natural attenuation (MNA)	N	Y
In situ bioremediation	Y	Y
Biobarriers	N	Y
Phytoremediation	Y	(Y)
Ex situ technologies		
Windrows, biopiles and related	Y	N
Slurry bioreactors	Y	(Y)
Water treatment bioreactors	N	Y

volatilisation, sorption and abiotic chemical reactions) are also evaluated in natural attenuation with regard to their contribution to risk reduction along the transport pathway [3, 5, 27]. This difference is reflected in the technical guidance developed for performance assessment [6, 19–23].

In engineered bioremediation direct intervention is used to create a closed system in the host environment which optimises conditions for treatment – the objective is to promote microbial activity and increase rates of processes that enhance the existing biodegradation capacity for contaminant removal. This process optimisation is most often achieved by adding one or more of electron donors, electron acceptors, nutrients and, in some cases, microorganisms through the contaminated subsurface to encourage microbial growth and enhance the treatment [8].

3.2 Applications

Bioremediation can be used to treat a wide range of contaminants and is applied in the clean-up of lagoons, sludges and process-waste streams, as well as soil and groundwater [3, 5, 6, 28]. It must frequently address multi-phase heterogeneous environments, such as soils and aquifers, in which organic contaminants may be present in as a separate phase (as a light (LNAPL) or dense (DNAPL) non-aqueous phase liquid), associated with soil particles, dissolved in soil water, present in the soil gas or co-existing in mixtures with different classes of organic compounds (e.g. hydrocarbons and chlorinated solvents) and inorganic contaminants (e.g. heavy metals) that also require treatment [29, 30]. Hence, bioremediation is often integrated with other remediation technologies, either sequentially or simultaneously, to treat different phases and/or contaminant groups [31].

The range of potential contaminants of concern is diverse but in general the oxidation state of target contaminants is the most important factor that determines whether the bioremediation process should be aerobic or anaerobic. Aerobic bioremediation is thermodynamically most favourable for the treatment of reduced compounds (e.g. hydrocarbons and some chlorinated compounds), whereas oxidised compounds (e.g. highly substituted chlorinated organic compounds) are more easily treated by anaerobic bioremediation [3]. Many sites will contain a complex mixture of contaminants, the remediation of which may pose conflicting demands and therefore require careful evaluation with possible application of multiple technologies and/or a combination of aerobic and anaerobic bioremediation processes.

In some cases the treatment timescales involved in bioremediation will be relatively long and the residual contaminant levels achievable may not always be appropriate. In these cases, options exist to use supplementary technologies to decrease remediation timescales, by either reducing the contaminant flux from sources or increasing the bioremediation capacity of the aquifer downgradient

Table 2
Example common contaminant types treatable using bioremediation

Contaminant	Typical bioprocess objectives
Inorganics	
Ammonia	Nitrification (oxidation to nitrate)
Nitrate, nitrite	Denitrification (reduction to nitrogen gas)
Perchlorate	Reduction to chloride
Cyanides	Mineralisation to carbon dioxide and nitrogen
Metals and metalloids	Immobilisation by oxidation/reduction reactions or precipitation; immobilisation by bioaccumulation
Organics	
Petroleum hydrocarbons	Mineralisation to carbon dioxide or methane
Chlorinated organics	Mineralisation to chloride and carbon dioxide, methane, ethane or ethene
Phenols	Mineralisation to carbon dioxide or methane
Alcohols, ketones, fatty acids, ethers	Mineralisation to carbon dioxide or methane
Explosives and other organo-nitrogen compounds	Mineralisation to inorganic nitrogen and carbon dioxide or methane; biodegradation to less hazardous products; immobilisation by sorption or bioaccumulation

of the source [15]. However, a great many contaminants can, in principle, be treated by appropriate bioremediation technologies (Table 2). While the methodologies are not technically complex, considerable experience and expertise is required to design and implement a successful bioremediation program, due to the need to thoroughly assess a site for suitability and to optimise conditions for satisfactory results [8].

Bioremediation uses relatively low-cost, low maintenance, low-technology techniques, which can often be implemented on site and coupled with other treatment methods [3, 6, 28]. In a survey of bioremediation performance at over 400 clean-up sites in the USA, costs of bioremediation were found to be 80–90% less than other remediation technologies based on physical and chemical methods [7]. Direct costs for ex situ bioremediation are usually higher than for in situ methods, due to the need for excavation of contaminated media for treatment, but ex situ remediation is generally faster and offers easier performance monitoring. In addition, biodegradation rates and the consistency of the process outcome differ between in situ and ex situ bioremediation methods, mainly due to the greater physical access that ex situ methods allow for enhancement and optimisation of the treatment [7]. The main difference between these two approaches is that engineered

bioremediation seeks to control microbiological processes to achieve the remediation objectives, by reducing or overcoming limitations on microbial activity, for example, by maximising delivery of growth factors in the treatment zone and active management of the groundwater flow field to ensure this.

It is therefore unsurprising that bioremediation technologies are widely applied with a global market of US $1.5B per annum, estimated by Singh et al. [32]. However, while bioremediation has many advantages compared with alternative remediation technologies (Table 3), it also has limitations [3, 5, 6, 28, 33]. Further, some

Table 3
Relative advantages and disadvantages of bioremediation for soil and groundwater treatment

Advantages	Disadvantages
As a natural process bioremediation is perceived by the public as an acceptable treatment option for contaminated soil and groundwater, since it is performed by naturally occurring microorganisms, which increase in number when the contaminant is present and decline when the contaminant is degraded, producing usually harmless treatment residues products such as carbon dioxide, water and cell biomass	Bioremediation is limited to those compounds which are biodegradable, although not all can be biodegraded at acceptable rates or completely
Theoretically, bioremediation can completely destroy or transform a wide variety of hazardous compounds to less harmful products, eliminating possible future liability associated with treatment and disposal of contaminated material	Some products of biodegradation may be more persistent or toxic than the parent compound
Complete destruction of target contaminants is possible within the host media, instead of transferring contaminants from one environmental medium to another, for example, from land to water or air, as may occur using other remediation methods	Biological processes are often highly specific and require site-specific assessment of factors which affect remediation performance (presence of metabolically capable microbial populations, suitable environmental growth conditions and appropriate levels of nutrients and contaminants)
Bioremediation can often be carried out on site without causing major disruption of normal operational activities, also eliminating the need to transport waste off-site and the potential threats to human health and the environment from such transportation	Results of bench-scale and pilot-scale studies are not easily extrapolated to full-scale field operations as predictors of remediation performance, due to different limitations (e.g. mass transfer) on processes at different scales
Bioremediation can be less expensive than other remediation technologies, particularly those which involve complex treatment processes or are energy intensive	Contaminants may be present as solids, liquids, and gases, which affect overall treatment efficiency and in situ bioremediation often takes longer than other treatment options, such as excavation and removal of soil or incineration
Potential application to part or all of a given site	Acceptable performance criteria and end-points for bioremediation treatments can be difficult to define from a regulatory perspective

organic contaminants (e.g. certain organic pesticides and herbicides, polychlorinated biphenyls and high-molecular weight polycyclic aromatic hydrocarbons (PAHs)) are relatively resistant to microbial attack and are biodegraded very slowly or not at all [5].

4 Bioremediation of Petroleum Hydrocarbons and Chlorinated Aliphatic Hydrocarbons

4.1 Context

Petroleum hydrocarbons and CAHs are important and widespread soil and groundwater contaminants [34–37]. These chemicals pose potential hazards to human health, water resources and the environment [38–40], which is reflected in highly stringent water quality standards that apply for such contaminants.

Petroleum hydrocarbons may arise in releases from a wide range of sites including, but not limited to, crude oil production sites, pipelines, refineries, distribution depots and filling stations, industrial facilities, coal gasification plants, and waste processing and disposal operations. A diverse mixture of compounds, potentially including alkanes, alkenes, aromatics (including PAHs) and heterocyclic compounds, will normally be present in a release. The relative proportions and molecular weight of the components present, and their physical and chemical properties, will vary significantly between different petroleum and related products. Moreover, they will exhibit variable potential for biodegradation by different processes, which must be understood and carefully exploited to ensure successful bioremediation [3].

CAHs have been widely used in industry as solvents in manufacturing (e.g. in the chemical industry) and in degreasing operations, for example, in the automotive and aerospace industries, electronics manufacture and the dry cleaning of textiles and clothing [35, 38]. Commonly encountered examples include tetrachloroethene (perchloroethene; PCE), trichloroethene (TCE), dichloroethene (DCE), tetrachloroethane (TeCA), trichloroethane (TCA) and chlorinated methanes, such as carbon tetrachloride (CT), chloroform (CF) and dichloromethane (DCM) [36]. Vinyl chloride (VC; monochloroethene) is a gas under ambient conditions, but is an important intermediate in the anaerobic biodegradation of PCE, TCE and DCE.

In general, petroleum hydrocarbons are less dense than water (they are LNAPLs); they will float on the groundwater table if present in the ground as a separate organic phase. CAHs as a group are denser than water (DNAPL) and sink below the water table when present as a separate organic phase. However, it is possible for significant proportions of hydrocarbons to be present in a DNAPL, examples include many coal tars and waste chlorinated solvents that have been used for degreasing; conversely, it is

equally possible that CAHs may be present within an LNAPL mixture when they form only a small proportion of the mix.

For both LNAPL and DNAPL, a proportion of the organic liquid will remain trapped in pore spaces above and below the groundwater table as a residual mass. Depending upon volatility, a proportion may also enter the vapour phase in the unsaturated zone [29, 41]. Constituents will dissolve from LNAPL and DNAPL into infiltration percolating through the unsaturated zone and/or groundwater to form dissolved phase plumes of mixed organic chemicals in aquifers, according to their properties (e.g. solubility, volatility and hydrophobicity) and relative concentration within the mixture. Taking the example of gasoline, it is the more water-soluble mono-aromatic hydrocarbons, such as benzene, toluene, ethylbenzene and xylenes (BTEX), that tend to form the most dominant contaminants in groundwater plumes [29, 34].

4.2 Biodegradation of Hydrocarbons

Biodegradation of hydrocarbon compounds in groundwater occurs by aerobic and anaerobic pathways, coupled to the reduction of dissolved electron acceptors (oxygen, nitrate, sulphate and carbon dioxide) and mineral oxidants (manganese and iron oxides) on the aquifer sediment [34, 42]. In most cases, biodegradation of these compounds occurs through primary metabolism, in which the hydrocarbon is used as a growth substrate by the microorganisms [36]. The microorganisms responsible include bacteria (most important), yeast and fungi (reviewed by Das and Chandran [43]). For illustrative purposes in this chapter, we will only discuss the biodegradation of aromatic hydrocarbons in any detail.

Aerobic bacteria can only use molecular oxygen as the terminal electron acceptor for respiration, whereas anaerobic oxidation of hydrocarbons can be undertaken by both facultative and obligate anaerobic microorganisms [34]. The biodegradative microorganisms obtain this energy by coupling the oxidation of an electron donor (the hydrocarbon in this case) with the reduction of an electron acceptor in a redox reaction – the specific energy yield of a redox reaction is quantified using the Gibbs free energy of the reaction (ΔG_r^o). Calculated values of ΔG_r^o for selected electron acceptor and organic electron donor half-cell reactions are shown in Table 4 and expressed per mole e^- transferred. Coupled redox reactions will generally occur in order of their thermodynamic energy yield (i.e. the highest first), provided the microorganisms responsible are present and there is adequate supply of organic carbon and electron acceptors [42, 44]. For a given redox condition and availability of electron acceptors, this implies a preferred order of biodegradation in mixtures of organic compounds. For example, aerobic respiration of BTEX would theoretically occur in the order B > T = E > X, although in reality other factors may affect this sequence.

In hydrocarbon-contaminated aerobic groundwater, these microbially mediated reactions create a characteristic sequence of

Table 4
Gibbs free energy of reaction for electron donor and electron acceptor half cell reactions

Species	Electron donor half-cell reaction	ΔG_r^o (kJ/mol e^-)	Species	Electron acceptor half-cell reaction	ΔG_r^o (kJ/mol e^-)
Carbohydrate	$CH_2O + H_2O > CO_2 + 4H^+ + 4e^-$	−41.84	Tetrachloroethene	$2e^- + H^+ + C_2Cl_4(g) > C_2HCl_3 + Cl^-$	−41.42
Acetic acid	$CH_3COOH + 2H_2O > 2CO_2 + 8H^+ + 8e^-$	−33.48	Trichloroethene	$2e^- + H^+ + C_2HCl_3 > C_2H_2Cl_2 + Cl^-$	−40.17
Benzene	$C_6H_6 + 12H_2O > 6CO_2 + 30H^+ + 30e^-$	−29.44	Dichloroethene	$2e^- + H^+ + C_2H_2Cl_2 > C_2H_3Cl + Cl^-$	−30.12
Toluene	$C_7H_8 + 14H_2O > 7CO_2 + 36H^+ + 36e^-$	−28.91	Trichloroethane	$2e^- + H^+ + C_2H_3Cl_3 > C_2H_4Cl_2 + Cl^-$	−43.09
Ethylbenzene	$C_8H_{10} + 16H_2O > 8CO_2 + 42H^+ + 42e^-$	−28.91	Dichloroethane	$2e^- + H^+ + C_2H_4Cl_2 > C_2H_5Cl + Cl^-$	−37.66
m-Xylene	$C_6H_4(CH_3)_2 + 16H_2O > 8CO_2 + 42H^+ + 42e^-$	−28.62	Vinyl chloride	$2e^- + H^+ + C_2H_3Cl > C_2H_4 + Cl^-$	−36.81
1,2,4-trimethylbenzene	$C_6H_3(CH_3)_3 + 18H_2O > 9CO_2 + 48H^+ + 48e^-$	−28.43	Hexachlorobenzene	$2e^- + H^+ + C_6Cl_6 > C_6HCl_5 + Cl^-$	−39.33
Phenol	$C_6H_6O + 11H_2O > 6CO_2 + 28H^+ + 28e^-$	−30.81	Tetrachlorobenzene	$2e^- + H^+ + C_6H_2Cl_4 > C_6H_3Cl_3 + Cl^-$	−36.40
Naphthalene	$C_{10}H_8 + 20H_2O > 10CO_2 + 48H^+ + 48e^-$	−28.82	Trichlorobenzene	$2e^- + H^+ + C_6H_3Cl_3 > C_6H_4Cl_2 + Cl^-$	−34.73
Tetrachlorobenzene	$C_6H_2Cl_4 + 12H_2O > 6CO_2 + 26H^+ + 4Cl^- + 22e^-$	−51.86	Aerobic respiration	$4e^- + 4H^+ + O_2 > 2H_2O$	−77.40
Trichlorobenzene	$C_6H_3Cl_3 + 12H_2O > 6CO_2 + 27H^+ + 3Cl^- + 24e^-$	−44.49	Denitrification	$5e^- + 6H^+ + NO_3^- > N_2 + 3H_2O$	−70.71
Dichlorobenzene	$C_6H_4Cl_2 + 12H_2O > 6CO_2 + 28H^+ + 2Cl^- + 26e^-$	−38.41	Mn(IV)-reduction	$2e^- + 4H^+ + MnO_{2(s)} > Mn^{2+} + 2H_2O$	−36.03
Chlorobenzene	$C_6H_5Cl + 12H_2O > 6CO_2 + 29H^+ + Cl^- + 28e^-$	−33.51	Fe(III)-reduction	$e^- + 3H^+ + FeOOH > Fe^{2+} + 2H_2O$	−64.62
Dichloroethene	$C_2H_2Cl_2 + 4H_2O > 2CO_2 + 10H^+ + 2Cl^- + 8e^-$	−67.49	Sulphate reduction	$8e^- + 8H^+ + SO_4^{2-} > S^{2-} + 4H_2O$	22.45
Vinyl chloride	$C_2H_3Cl + 4H_2O > 2CO_2 + 11H^+ + Cl^- + 10e^-$	−47.74	Methanogenesis	$8e^- + 8H^+ + CO_{2(g)} > CH_{4(g)} + 2H_2O$	24.95

spatially and temporally dynamic redox processes, comprising successive zones of predominantly aerobic respiration, denitrification, Mn/Fe-reduction, sulphate reduction and methanogenesis along the flow path from the plume source [34, 37, 42, 45]. The development of these redox zones, sustained by the supply of organic compounds from the plume source and the availability of both dissolved and mineral-based electron acceptors in the aquifer, is critical for the in situ bioremediation and natural attenuation of many organic contaminants that may only be biodegraded under specific redox conditions in groundwater [36, 46–49]. Moreover, biodegradation using dissolved electron acceptors is potentially limited by the relatively low solubility of oxygen or rate of mass transfer of oxygen, nitrate and sulphate into the plume by dispersion [50–52]. Conversely, while aquifer sediments typically have a significant reservoir of mineral-bound electron acceptors as Mn and Fe-oxides, only the easily reducible amorphous forms will contribute oxidation capacity for biodegradation of organic compounds in groundwater [34, 36, 46, 47].

Aromatic components are common in hydrocarbon mixtures and are biodegraded to intermediates by central metabolic routes, dioxygenation and ring scission [43,53]. The mineralisation of these compounds to carbon dioxide and water under aerobic conditions involves the use of oxygen as a co-substrate during the initial stages of hydrocarbon metabolism and later use of oxygen as a terminal electron acceptor [36, 54]. Their aerobic biodegradation is mediated by mono- and dioxygenase enzymes, which use oxygen for dihydroxylation of the ring structure and then oxidative cleavage of the resulting catechol, the key intermediate in many pathways. In this respect cytochrome P450 enzyme systems are known to also play an important role in mediating aromatic hydrocarbon biodegradation [43]. Eukaryotic organisms produce catechols from single ring aromatic compounds via an epoxide and a *trans*-diol using a monooxygenase, whereas prokaryotes introduce the entire oxygen molecule by a dioxygenase reaction forming first a *cis*-diol. In both cases, the aromatic ring of the catechol is opened by a further dioxygenase reaction, by either an *ortho*- or *meta*-fission [54]. Ring cleavage can occur in the *ortho* position (between the two –OH groups) or more often the *meta* positions (next to one of the –OH groups), illustrated for benzene oxidation in Fig. 1. The resulting product, often a dicarboxylic acid or semialdehyde, is further metabolised to produce intermediates that can be included in the intermediary metabolism of bacterial cells. Intermediates of these central metabolic routes are partly oxidised to carbon dioxide and water and partly converted into biomass for growth of the microorganisms [53]. The enzymes related to the *meta* cleavage pathway typically have broad substrate specificity, enabling co-metabolism of other contaminants, while the *ortho* pathway supports faster growth of these open ring products, via metabolism through the Krebs cycle [3].

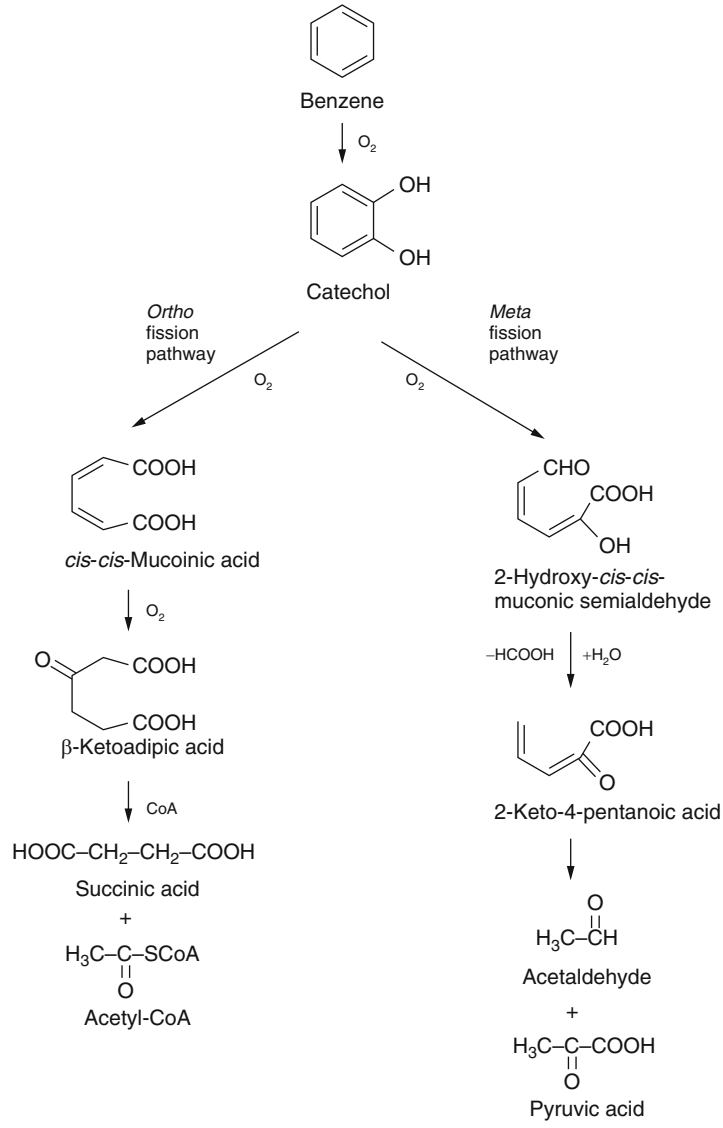

Fig. 1 Aerobic biodegradation of aromatic hydrocarbons by the ortho and meta fission pathways, using benzene as an example [3]

Oxidation of alkybenzenes is more complex in terms of the initial steps and subsequent pathways mediated by different bacteria. This is illustrated in Fig. 2, which shows different biodegradation pathways linked to oxidation of toluene at the methyl group (TOL), addition of two oxygen atoms to the benzene ring (TOD), formation of *o*-cresol via catabolism using *ortho*-monooxygenase (TOM), *m*-cresol using *meta*-monooxygenase (TBU) and *p*-cresol using *para*-monooxygenase (T4MO).

Due to the low aqueous solubility of oxygen, the typically high concentration of oxidisable organic compounds and their high

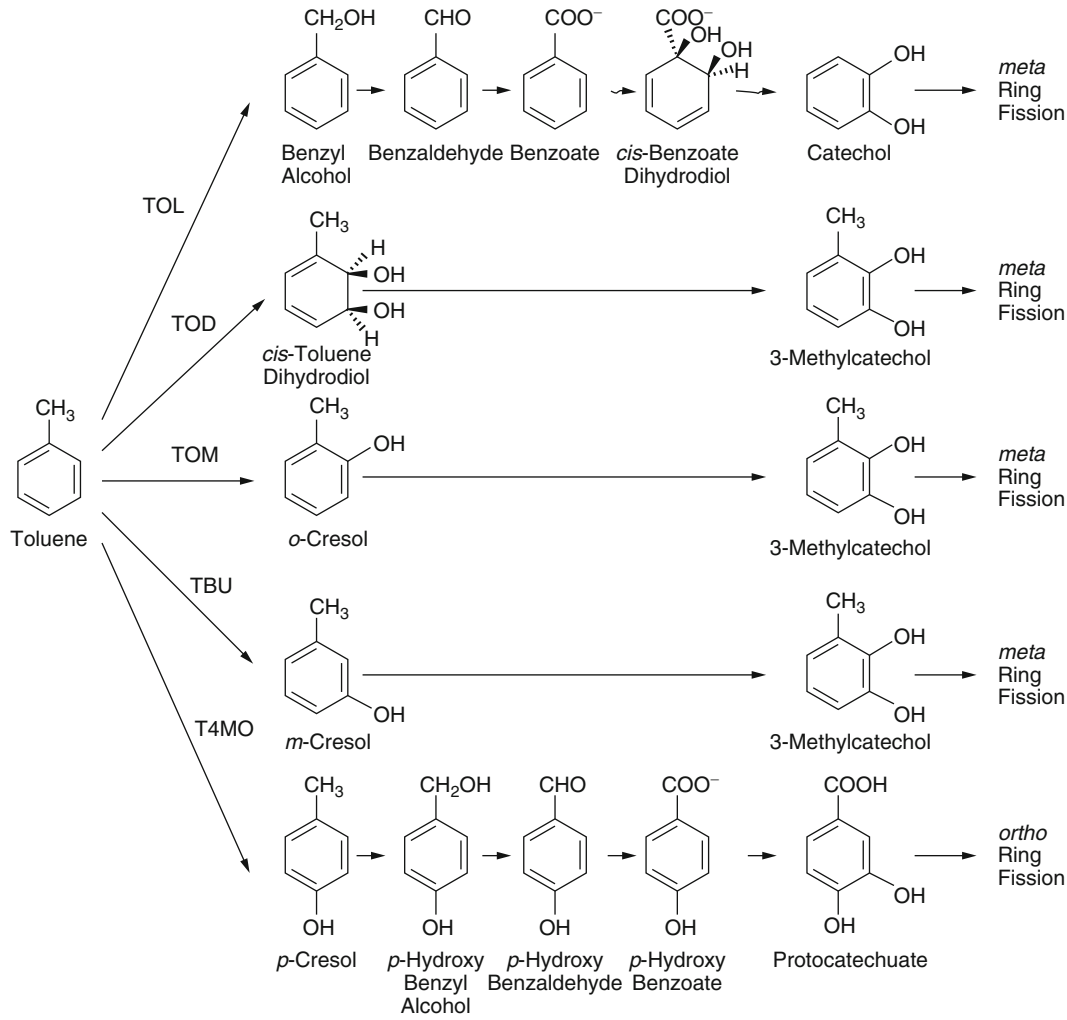

Fig. 2 Variation in pathways for aerobic biodegradation of toluene, linked to oxidation at the methyl group (TOL), addition of two oxygen atoms to the benzene ring (TOD), formation of *o*-cresol via catabolism using *ortho*-monoxygenase (TOM), *m*-cresol using *meta*-monooxygenase (TBU) and *p*-cresol using *para*-monoxygenase (T4MO) [3]

biodegradation rates, aquifers contaminated with hydrocarbons quickly become depleted in oxygen and further biodegradation occurs via anaerobic pathways [36, 42, 44]. Anaerobic biodegradation of aromatic hydrocarbons generally occurs by carboxylation and transformation of the benzene ring to CoA-esters, for example, benzoyl-CoA. This intermediate can then ultimately be hydrolysed to form benzoate prior to ring reduction, hydroxylation with water and ring cleavage along different pathways [43, 54] (Fig. 3).

In contrast to other anaerobic biodegradation processes, oxidation of aromatic hydrocarbons via methanogenesis occurs in two steps. The first step involves fermentation of the hydrocarbons by fermentative bacteria to intermediates such as acetate and

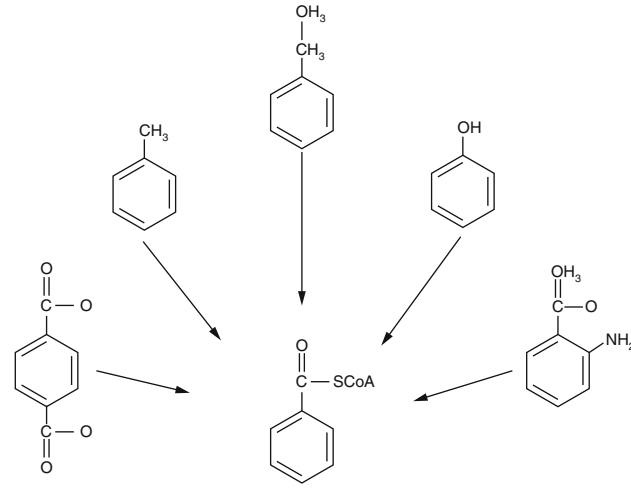

Fig. 3 Anaerobic metabolism of substituted aromatic hydrocarbons via benzoyl-CoA intermediate [3]

hydrogen. These are then metabolised by fermentative and respiratory microorganisms to methane, carbon dioxide and water. This occurs via pathways that include acetoclastic methanogenesis and reduction of carbon dioxide, in which either acetate or carbon dioxide is the terminal electron acceptor [36, 42].

A wide range of microorganisms can mediate hydrocarbon biodegradation in the environment. This in part reflects the presence of hydrocarbons in naturally occurring mixtures of organic compounds and extensive adaption of indigenous microbiota to utilise these as growth substrates. While populations of hydrocarbon utilising microorganisms may account for 0.1% of the population in unpolluted environments, this may increase to 100% under selective pressure following prolonged exposure, returning to background levels after removal of the contaminant [52]. However, rates of anaerobic biodegradation can be slow, often with longer lag phases, compared with aerobic respiration but may be more important for contaminant mass removal in plumes, due to solubility and mass transfer limits on O_2 supply [44, 46, 47, 51].

In general, the low- to moderate-molecular weight aromatic hydrocarbons are most easily biodegraded. Biodegradation potential of aromatic hydrocarbons decreases with the number of benzene rings in the structure, increasing molecular weight and extent of substitution [34, 36]. This trend also reflects their physical–chemical properties and a relative decrease in the bioavailability of these compounds due to increased sorption in soils and lower solubility in groundwater. Substituted aromatic compounds appear to be more easily biodegraded under anaerobic conditions than non-substituted compounds, depending on the functional group and terminal electron acceptor present [54].

4.3 Biodegradation of Chlorinated Aliphatic Hydrocarbons

Biodegradation of CAHs can occur by five mechanisms: direct aerobic oxidation (i.e. the utilisation of the CAH as a carbon and energy source); cometabolic aerobic oxidation, direct anaerobic oxidation, dehalorespiration (also known as 'organohalide respiration'); and cometabolic anaerobic reductive dechlorination. These processes involve substitution and elimination of chloride atoms, coupled with removal or addition of electrons from/to the CAH structure [35, 54–57].

In general, highly chlorinated CAHs (e.g. PCE and TeCA) are biodegraded primarily by reductive reactions, while less chlorinated compounds can be biodegraded either by oxidation or reductive reactions [35, 54, 57]. Highly chlorinated CAHs are reduced relatively easily because their carbon atoms are highly oxidised, by virtue of the presence of Cl atoms in the molecular structure [36, 56]. The presence of the electrophilic Cl atom in the CAH structure restricts the insertion of oxygen and limits aerobic biodegradation to the less chlorinated homologues [35]. In direct reactions the CAH is the carbon and energy source for growth [54, 58]; in the case of dehalorespiration, the chlorinated solvent acts as the terminal electron acceptor [36].

Under strongly reducing conditions dehalorespiration or cometabolic reductive dechlorination will predominate [35, 36, 38, 54, 56]. These cause the sequential replacement of a chlorine atom in the CAH molecule with a hydrogen atom, the chlorine atoms being released as chloride ions. This process creates a series of intermediate metabolites that define the specific biodegradation pathway involved, although in most cases the chlorinated dehalogenation intermediates are transient and either ultimately converted to fully dechlorinated end-products (ethene and ethane) or utilised as primary growth substrates to yield carbon dioxide. Figure 4 shows the reaction sequence for chlorinated ethenes. PCE and TCE are almost exclusively dechlorinated via *cis*-1,2-DCE (cDCE) and the presence of this compound in environmental samples can often be a clear of this process [3].

Dehalorespiration ('organohalide respiration') is normally the most important reductive biodegradation process in the field [36] and involves the CAH being used as the terminal electron acceptor for respiration, so yielding energy as the chlorine atoms are replaced with hydrogen [36, 54, 59–61]. The hydrogen used as the electron donor in the reaction typically originates indirectly from fermentation of co-existing organic compounds, including co-contaminants (e.g. volatile fatty acids in landfill leachate and aromatic hydrocarbons) and natural dissolved or particulate organic carbon in the aquifer.

Dehalorespiration of chlorinated ethenes (PCE, TCE, DCE and VC) and some chlorinated ethanes (TCA, 1,2-DCA) has been observed; some chlorinated aromatics can also be substrates (e.g. [62–64]). Generally, the process occurs more rapidly for highly chlorinated compounds (e.g. PCE and TCE) than less

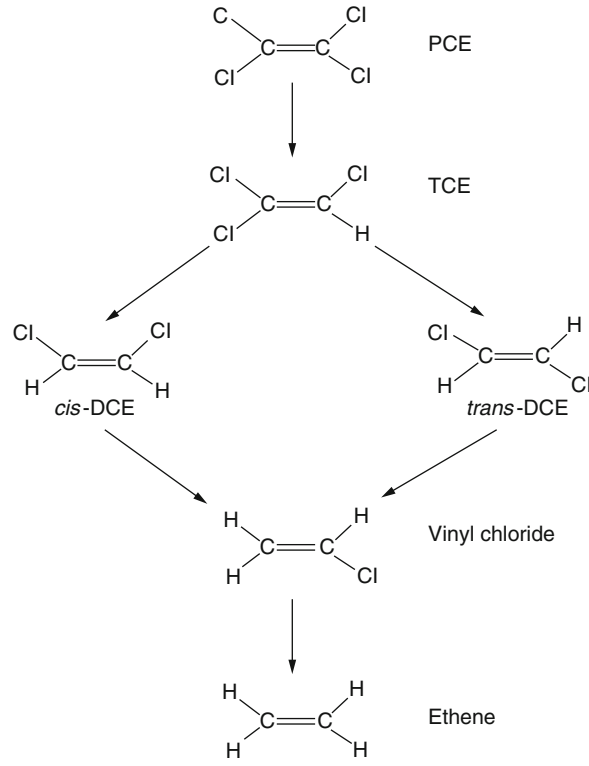

Fig. 4 Reductive dechlorination of chlorinated ethenes via hydrogenolysis [3]

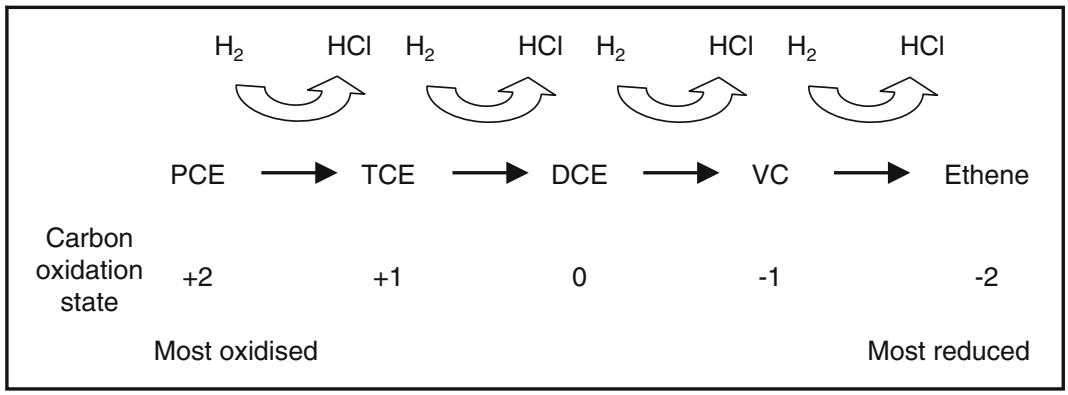

Fig. 5 Anaerobic reductive dechlorination of PCE, showing relative oxidation state of carbon in reaction products

chlorinated ones (e.g. DCE and VC), which may accumulate under anaerobic conditions depending upon which dehalorespiring bacteria are active [36]. This general behaviour is related in terms of chemical thermodynamics to the relative oxidation state of carbon in the respective CAH structures and subsequent theoretical energy yield from the redox reaction (Fig. 5). This can be appreciated by comparing the Gibbs free energy for each dechlorination step.

These are: PCE to TCE, −171.8 kJ/mol; TCE to DCE: −166.1 kJ/mol; DCE to VC: −144.8 kJ/mol and VC to ethene: −154.5 kJ/mol, respectively, which shows that initial PCE dechlorination provides the highest energy yield [56]. Moreover, the energy yield for reductive dechlorination is high relative to anaerobic processes such as nitrate reduction, sulphate reduction and methanogenesis (−112, −38 and −33 KJ/mol of substrate consumed, respectively), implying the potential net benefit of dehalorespiration under the appropriate in situ conditions [56].

To illustrate the range of CAH dehalorespiration activity, examples of bacteria with this metabolic capability are presented in Table 5. Dehalorespiration is associated with diverse bacterial phyla but there is no correlation between phylogenetic affiliation and chlorinated substrate specificities [65]. Some microorganisms, such as *Dehalobacter restrictus* strain TEA and *Dehalococcoides*

Table 5
Examples of bacterial species capable of dehalorespiration of chlorinated aliphatic hydrocarbons

Species	Phylum	Principal dechlorination reactions performed
Dehalobacter restrictus	Firmicutes	PCE to *cis*-DCE
Dehalococcoides mccartyi	Chloroflexi	PCE to ethene
Dehalococcoides mccartyi BAV	Chloroflexi	PCE to VC
Dehalococcoides mccartyi CBDB1	Chloroflexi	PCE to *trans*-DCE
Dehalogenimonas lykanthroporepellens	Chloroflexi	1,2-dichloroethane to ethene 1,2-dichloropropane to propene 1,1,2-trichloroethane to VC 1,1,2,2-tetrachloroethane to *cis*- and *trans*-DCE
Dehalogenimonas alkenigignens	Chloroflexi	1,2-dichloroethane to ethene 1,2-dichloropropane to propene 1,1,2-trichloroethane to VC 1,1,2,2-tetrachloroethane to *cis*- and *trans*-DCE
Desulfitobacterium dichloroeliminans	Firmicutes	1,2-dichloroethane to ethene
Desulfitobacterium hafniense	Firmicutes	PCE to *cis*-DCE
Desulfitobacterium sp. PCE1	Firmicutes	PCE to TCE
Desulfuromonas chloroethenica	Proteobacteria	PCE to *cis*-DCE
Desulfuromonas michiganensis	Proteobacteria	PCE to *cis*-DCE
Geobacter lovleyi	Proteobacteria	PCE to *cis*-DCE
Sulfospirillum multivorans	Proteobacteria	PCE to *cis*-DCE
Sulfurospirillum halorespirans	Proteobacteria	PCE to *cis*-DCE

mccartyi strain 195, only couple reduction of chlorinated compounds to growth on hydrogen. Others, such as *Sulfurospirillum multivorans* and some strains of *Desulfitobacterium*, can use a wider range of electron donors [56]. An important observation is that *Dehalococcoides* is the only genus known to include organisms that can dehalorespire DCE and VC to ethene [38, 66], which means that these will play a critical role in effective anaerobic bioremediation of CAHs.

In cometabolic reductive dechlorination the CAH is fortuitously biodegraded by an enzyme or cofactor produced during metabolism of a co-existing organic compound using another electron acceptor to generate energy [36, 56, 58]. The biodegradation of the CAH does not yield any energy or growth benefit for the microorganism mediating the reaction and only a small fraction of the reducing equivalents obtained from oxidation of the electron donor is used to reduce the CAH [67]. However, cometabolic reductive dechlorination is an important biodegradation process for both chlorinated ethenes and ethanes [36, 58]. Because the CAH is a secondary substrate in this reduction process, there is no lower limit to the final concentration of the CAH and complete biodegradation is possible, provided sufficient primary substrate is available [35]. As with dehalorespiration, the relative rate of biodegradation via this mechanism decreases with decreasing number of chlorine substituents [35, 58]. Many types of microorganism can facilitate this reaction, including methanogens such as *Methanosarcina mazei*, acetogens such as *Acetobacterium woodii* and *Sporomusa ovata* and sulphate-reducing bacteria [56]. While reductive dechlorination can theoretically occur under most anaerobic conditions (including denitrification), it is most effective under sulphate-reducing and methanogenic conditions, and for PCE, TCE, DCE, VC, DCA and carbon tetrachloride [22, 54].

Under less highly reducing conditions, anaerobic oxidation of VC, in particular, can take place coupled to the microbial reduction of iron, manganese or humic acids [38, 68–72]. Considering the theoretical maximum redox potential for transformation, anaerobic reductive dehalogenation is thermodynamically possible [57] but its efficiency is limited and competition from microorganisms that use hydrogen as a substrate for anaerobic biodegradation (e.g. nitrate-reducing, sulphate-reducing and methanogenic bacteria) may also limit dehalorespiration. Hence, anaerobic oxidation can often be a more significant contributor to contaminant mass removal under moderately reducing conditions than dehalorespiration [15, 30, 73].

Under aerobic conditions less chlorinated (mono- and dichlorinated) CAHs (e.g. DCE, DCA, VC, dichloromethane) can be directly oxidised to carbon dioxide, water and chloride, linked to the reduction of molecular oxygen [35, 58, 68, 73, 74]. Microorganisms which can grow on these CAHs as the sole carbon source

includes aerobic bacteria such as *Actinomycetales* sp., *Mycobacterium* sp., *Polaromonas* sp. JS666, *Rhodococcus* sp., *Pseudomonas* sp. and *Nocardioides* sp. [56]. However, at field-scale, direct aerobic biodegradation is usually limited by preferential consumption (and exhaustion) of dissolved oxygen in groundwater, which may restrict the process to the fringe of the plume where oxygenated groundwater mixes with that containing CAH substrates [38, 56].

The fifth potential CAH biodegradation mechanism is aerobic cometabolic oxidation. Many chlorinated solvents (although not PCE) can ultimately be converted to carbon dioxide, water and chloride via the cometabolic reactions brought about by microorganisms utilising electron donors such as methane, ethane, ethene, propane, butane, some aromatic hydrocarbons (such as toluene), phenol and ammonia as growth substrates [36, 38, 54, 68, 73, 75]. The reaction is mediated by a monooxygenase enzyme (e.g. methane monooxygenase in the case of methanotrophic bacteria). Methane oxidation is particularly important for co-metabolism of CAHs as the methane monooxygenase has a broad substrate range that enables biotransformation of many compounds [54]. Generally, biodegradation of CAHs by direct oxidation mechanisms is faster than by cometabolic mechanisms [35, 58], with several other advantages over co-metabolism – no additional carbon or energy sources are required, substrate competition for the same enzyme is not important and there are usually less problems with toxic metabolites [38].

The different mechanisms that have been observed for the biodegradation of common CAH contaminants in groundwater are summarised in Table 6. Direct aerobic and anaerobic oxidation, dehalorespiration and, to a lesser extent, cometabolic anaerobic reductive dehalogenation can be important processes in aquifers under appropriate redox conditions and form the basis of performance assessment monitoring undertaken for in situ bioremediation and natural attenuation of these compounds [3, 36]. These processes also provide the basis to enhance natural rates via the addition of suitable electron donors, electron acceptors or nutrients to stimulate biodegradation under specific conditions, in isolation or in sequential anaerobic–aerobic treatment systems [3, 35]. In contrast, aerobic cometabolic oxidation is a relatively minor process on the plume-scale and is difficult to operate effectively as an engineered bioremediation process.

5 Characterisation and Performance Assessment of Bioremediation in Groundwater

Performance assessment of bioremediation aims to (1) characterise the nature and spatial extent of biodegradation processes used, (2) quantify contaminant removal and (3) confirm that the treatment is occurring at rates which will reduce environmental risk, protect identified receptors and achieve the remediation objectives. It also

Table 6
Biodegradation mechanisms for bioremediation of chlorinate aliphatic hydrocarbons

	Oxidation			Reductive dechlorination	
CAH	Direct aerobic	Cometabolic aerobic	Direct anaerobic	Dehalorespiration	Cometabolic
Chlorinated methanes					
CT	N	N	N	N	Y
CF	N	Y	N	Y	Y
DCM	Y	Y	Y[a]	?[a]	Y[a]
Chloromethane	Y	Y	Y	?	Y
Chlorinated ethanes					
TCA	N	Y	N	Y	Y
DCA	(Y)	Y	N	Y	Y
Chloroethane	Y	Y	?	Y	Y
Chlorinated ethenes					
PCE	N	N	N	Y	Y
TCE	N	Y	N	Y	Y
DCE (all isomers)	(Y)	Y	(Y)	Y	Y
VC	Y	Y	Y	Y	Y

The term "direct" is used in this table to mean utilisation of the CAH as the electron donor and carbon source for growth
N not observed, *Y* commonly observed, *(Y)* variable, dependent on hydrochemical conditions and microbial community composition, *?* uncertain
[a]DCM is readily fermented under anaerobic conditions, which will be the preferential metabolic pathway

identifies the need to adjust process operating conditions and, in the worst case, implement additional measures if the remediation requirements will not be successfully fulfilled by bioremediation alone. Current best practice for the performance assessment of in situ bioremediation may involve the integrated analysis of hydrochemical, isotopic, microbiological and other data using a wide range of qualitative and quantitative techniques (reviewed in Bombach et al. [45] and Hunkeler and Aravena [76]) at different scales (Table 7). This analysis is formalised within the 'lines of evidence' framework outlined above.

5.1 Hydrochemical Analysis

Primary and secondary lines of evidence are obtained from the distribution of dissolved reactants along the plume flow path, using groundwater samples collected from monitoring boreholes in the plume source area, uncontaminated and contaminated sections of the aquifer [3, 36]. While similar hydrochemical indicator species are used to verify bioremediation of hydrocarbons and CAHs (e.g. electron acceptors, inorganic products of redox reactions and metabolic gases), analysis will differ in the organic metabolites and inorganic compounds (e.g. daughter products and chloride for CAHs) needed to deduce specific biodegradation pathways.

Table 7
Typical indicators used for performance assessment of bioremediation in groundwater

Indicator or measurement	Basis for performance assessment
Hydrochemical-based	
Decreased electron acceptor concentration	Utilisation by microorganisms during biodegradation of target organic chemicals
Increased inorganic carbon concentration	Production from biodegradation and mineralisation of organic chemicals
Stoichiometry and mass balance between reactants and products	Balanced consumption of electron acceptors and organic compounds during biodegradation under specific redox conditions
Increased concentrations of intermediate-stage and final products	Identification of specific biodegradation pathways and transformation
Increased ratio of transformation products to parent compounds	Progressive biodegradation of parent compound to metabolite (e.g. daughter compound for CAH) over space and time
Decreased ratio of reactant to inert tracer	Preferential biodegradation of reactive species relative to a non-reactive species in the same mixture, accounting for decreases due to abiotic processes
Relative rates of transformation of different contaminants consistent with laboratory data	Independent confirmation of biodegradation potential at field-scale based on controlled laboratory studies (considering scale-up effects)
Stable isotope-based	
Changes in carbon, chloride and hydrogen isotope ratios of specific organic contaminants (depending on respective chemical group), and isotope ratios of electron acceptors and inorganic carbon in CO_2 and CH_4	Biodegradation of specific organic compounds (if based on compound-specific isotope analysis, CSIA), biodegradation of non-specific compounds (if based on total dissolved carbon isotope ratios) and specific redox processes (if based on isotope ratios of electron acceptors)
Microbiological-based	
Increased number of (live/active) bacteria in treatment zone	Increase in size of viable microbial community for biodegradation
Increase in relative abundance of known degraders in treatment zone	Increased contribution of specific microorganisms within community with capability for biodegradation of contaminants
Increase in abundance of genes associated with biodegradation in treatment zone	Development of metabolic pathways in microorganisms which enable biodegradation of contaminants
Hydraulic/hydrodynamic-based	
Indicators of liquid/gas flow field consistent with technology	Treatment fluids have been successfully delivered to the contaminant area

Visual methods used to interpret these data may include isopleth (concentration contour) maps in plan or section view, which provide a 2-D spatiotemporal plot of changes in solute concentrations and plume development. These maps will show the plume size, shape and general distribution of contaminant and indicator species in the plume, but the detail provided and resolution of solute distributions are limited by the design of the monitoring well network and their screened intervals [49]. Additional visual tests include time-series graphical or regression analyses (with supporting statistical interpretation), trends in solute concentration and ratio plots of reactive species (e.g. electron acceptors, inorganic by-products of redox processes and metabolites/daughter compounds) versus non-reactive species (e.g. internal tracers) along the plume 'centreline' [3, 34, 36]. These allow preferential biodegradation or attenuation and the long-term behaviour of a plume to be deduced, and can provide the necessary inputs for simple transport modelling [3, 34, 77].

A key objective is to estimate a plume-scale contaminant biodegradation rate, often assumed to follow first-order kinetics [78, 79]. However, this analysis is difficult to use when plumes are slow-moving, have low biodegradation rates, have heterogeneous source histories or show temporal variability in flow direction, or the assessment is made using inferior monitoring well networks which fail to delineate the plume centreline [49–51]. In fractured rock or dual porosity aquifers it is likely that multi-level sampling devices will be needed to monitor contaminant distributions and transport in the fracture network [80, 81].

An alternative approach is to use changes in contaminant mass flux through a series of monitoring wells (flux planes) set transverse to the plume flow path to estimate mass loss and bioremediation performance [3, 6, 15, 49, 82]. Flux planes can capture spatially and temporally variable contaminant distribution, as well as estimate biodegradation rates using different methods, such as the sampling of a dense monitoring array [48, 82], control plane pumping of a more sparse array [83] or a passive flux metre approach [84]. As such, flux-based methods provide a superior analysis of bioremediation performance compared with methods based on assumed centrelines of plumes, by integrating results from many monitoring wells and accommodating plume heterogeneity in estimates of mass loss [49].

Chemical mass balances which show that decreases in parent contaminant and/or electron acceptor/donor concentrations are related directly to increases in metabolic by-products and/or daughter compounds are the most reliable method to quantitatively demonstrate contaminant mass loss and estimate biodegradation rates (Table 7). However, the presence of multiple potential electron donors in organic contaminant mixtures which can contribute to electron acceptor consumption in plumes means that

hydrogeochemical data may not easily discriminate the turnover of a single contaminant from electron donor/electron acceptor balances [45]. More sophisticated mass balance methods integrate hydrochemical and stable isotope measurements of carbon species and electron acceptor consumption in groundwater to assess the performance of engineered bioremediation in contaminated aquifers [85]. A novel methodology developed by Thornton et al. [51] enables plume-scale mass balances to be undertaken for oxidisable organic contaminants using basic site investigation and groundwater chemistry data. The approach uses a simple box model to calculate a plume-scale mass balance for contaminated aquifers based on fluxes of electron acceptors and electron donors contributed by all organic and inorganic compounds. It couples the transport of electron acceptors and electron donors into a plume via infiltration from the source and groundwater advection and dispersion, balancing these with residual masses from biodegradation processes in situ. The methodology was developed to interpret the bioremediation of oxidisable organic contaminants only, but can estimate the plume source term, plume length at steady-state, global mass loss, contaminant biodegradation rate and deduce the plume status (i.e. shrinking, stable or expanding). This methodology has been developed into a suite of public-domain screening models for the performance assessment of bioremediation in groundwater (www.sheffield.ac.uk/gprg/technology/corona screen).

Other approaches based on hydrochemical analysis include the application of in situ tracer tests and in situ microcosms. The former involve the injection of a test solution containing a conservative tracer and contaminant of interest as a reactive tracer [86], followed by either continuous monitoring of the tracer distribution along the plume flow path or during retrieval of the tracer solution from the aquifer [87, 88]. The reactive tracers can include isotopically labelled and deuterated analogues of the contaminant of interest to provide a highly specific assessment of biodegradation potential, transformation products and metabolic pathways. In situ microcosms isolate an undisturbed volume of aquifer within which the contaminant(s) of interest and (in some cases) microorganisms are injected and sampled over time to observe microbial processes, fate of introduced microorganisms and obtain field-scale estimates of biodegradation rates [45, 89–91]. More recently, the use of labelled substrates has been integrated with stable isotope and molecular microbial analysis to develop this methodology as a powerful tool for the interpretation of in situ biodegradation pathways at a microbial community level in contaminated groundwater [92, 93].

5.2 Stable Isotope Analysis

Measurements of hydrochemical species in groundwater will not always provide conclusive proof of organic contaminant biodegradation for bioremediation assessments [34, 76]. For example,

changes in redox-sensitive species in plumes can result from the biodegradation of organic compounds (including natural substances) other than the contaminants of concern [45]. Similarly, biodegradation of specific organic chemicals in mixtures can be difficult to deduce if common pathways exist, when organic metabolites are similar to potential parent compounds (e.g. CAH daughter products), or if analytes are present at trace levels that are difficult to quantify [3, 94].

Stable isotope analysis of H, C, N, O, S and Cl has emerged as a powerful tool to address some of these problems. The basis for the technique is that molecules containing the lighter isotopes are metabolised more rapidly because the heavier isotopes have a stronger bond, which requires a higher activation energy to break [95, 96]. As the heavier isotope of each element is present in very small amounts, preferential metabolism of the lighter isotope results in progressive enrichment of the residual compound in the heavier isotope as biodegradation proceeds. This leads to an increase in the ratio (or fractionation) between the heavy and light isotope in each case ($^{2}H/^{1}H$, $^{13}C/^{12}C$, $^{15}N/^{14}N$, $^{18}O/^{16}O$, $^{34}S/^{32}S$ and $^{37}Cl/^{35}Cl$).

The kinetic isotope effect (KIE) arising from this results in a fractionation factor, α, which can be used quantitatively to interpret specific processes [96, 97]. In this way the measurement of relative variations in stable isotope concentrations rather than absolute abundances is used to evaluate the extent of the biodegradation or biotransformation process responsible. Measured isotope ratios are compared to international reference standards and reported in terms of their deviation from the standards in parts per thousand (‰; per mil), using the δ notation as $\delta = (R_s/R_{std} - 1) \times 1{,}000$ ‰, where R_s and R_{std} are the isotope ratios of the sample and reference standard, respectively [38, 96]. Typically the isotopic enrichment factor, ε, (given by $\varepsilon = (\alpha - 1) \times 1{,}000$) is used to interpret biodegradation processes [94, 96]. A negative value of ε ($\alpha < 1$) indicates enrichment of the residual compound in the heavier isotope, which is the observed trend for microbial metabolism [38]. Larger negative values of ε indicate greater degrees of fractionation by the specific process. Significantly, purely physical processes that only affect the concentration of contaminants (e.g. dissolution, sorption, volatilization and dilution) show very minimal isotopic fractionation [95, 96, 98–100]. With respect to the bioremediation of hydrocarbons and CAHs in groundwater, stable isotope compositions of the respective elements in NO_3^-, SO_4^{2-}, CO_2, CH_4 and the individual organic contaminants can be used to: (1) identify the source(s) of compounds in a given environment; (2) identify the origin of a compound *formed* in a given environment; (3) deduce specific pathways and redox processes for biodegradation; (4) assess the contribution of biotic and abiotic transformation processes affecting contaminants during subsurface transport or treatment; (5) quantify the extent of biodegradation; (6) verify

reactive transport models for prediction of environmental impacts, remediation system design and treatment timescales and (7) understand element cycling in natural and contaminated systems using isotope-labelled compounds [45, 96, 101]. Commonly, values of ε obtained from laboratory studies are used to interpret contaminant fate at field-scale, assuming operation of the same biodegradation processes and consistency of ε in the field [3, 96, 102].

Stable isotope fractionation and the development of methods as tools to interpret in situ biodegradation processes have been extensively studied for aromatic hydrocarbons and CAHs, given their widespread importance as contaminants in groundwater [76, 96]. Attention has mainly focused on understanding the reaction mechanisms and enzymes involved [98–100, 103–105], the metabolic pathways that may be operating [103, 104, 106–108], characterising the physiological groups of microorganisms facilitating biotransformation [94, 100, 103, 109–111], deducing the dominant redox processes supporting biodegradation [52, 80, 112, 113] and quantifying biodegradation at field-scale [76, 80, 96, 102, 108, 114–118]. Experimentally determined values of ε for aerobic and anaerobic biodegradation of mono-aromatic hydrocarbons, PAHs and CAHs obtained under different conditions are available for this analysis [3, 96, 97]. Further advances include the development of dual-isotope analysis of C/H and C/Cl to interpret the biodegradation and fate of CAHs in environmental systems at field-scale [103, 119–122]. These methods offer improved discrimination of contaminant sources, reaction mechanisms and biodegradation pathways.

5.3 Microbiological Analysis

The analysis of a microbial community provides a wealth of information on both its structure and function. Fundamental issues that can be addressed include the type and number of organisms present, whether they are dormant or active, their role in the biodegradation of contaminants present, the response of the microbial community over time to interventions or changes in environmental conditions, and limits on microbial growth and activity. Methods used to characterise microorganisms and microbial communities in environmental systems are outlined below, with regard to their principles of analysis and specific application in studies of contaminated groundwater and in situ bioremediation.

In essence, the analysis of microbial communities comprises two approaches: culture-dependent methods where organisms are grown on selective media and culture-independent methods where analysis is performed on samples taken directly from the environment. These approaches are complementary and typically both will be used to develop an understanding of microbial community structure and function. Figure 6 is an overview of methods but the boundaries between culture-dependent and independent methods is becoming increasingly blurred. For example, many of the

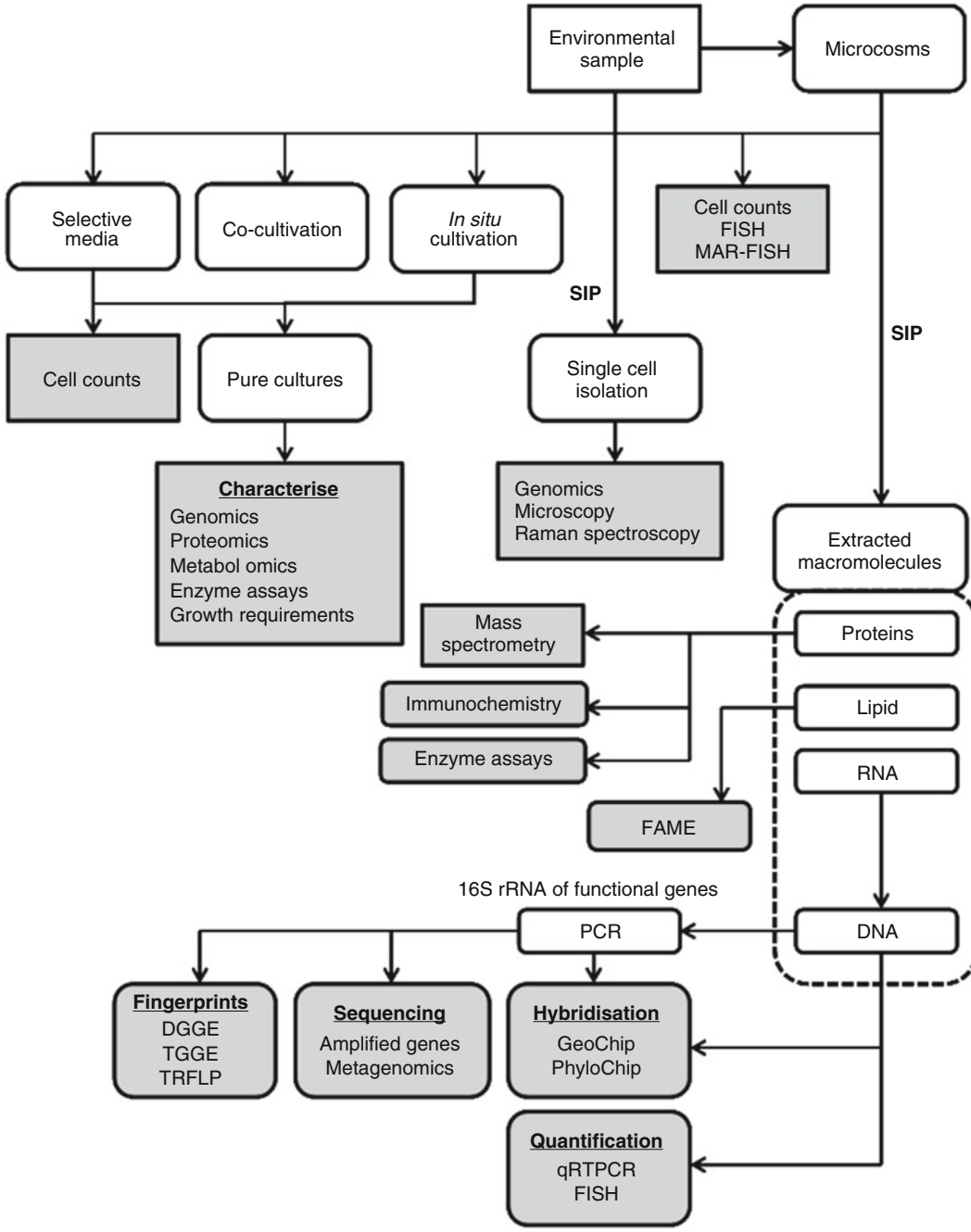

Fig. 6 Overview of methods used to characterise microbial communities in environmental samples. SIP refers to stable isotope profiling

techniques applied to samples collected from field sites are directly relevant to the study of microcosms. Likewise, advances in the technologies available to study single cells can be applied across a range of experimental systems [123].

5.3.1 Sampling for Microbiological Analysis

One of the greatest challenges arises in obtaining representative samples. This is usually straightforward in shallow contaminated soils or marine sediments, but access to deeper sites such as polluted aquifers requires boreholes with an attendant set of problems.

The initial cored drilling of boreholes is vital as this allows direct sampling of the aquifer material. This is expensive and so the relative cost–benefit is a case-specific decision. Care is needed to ensure the core is not contaminated with drilling fluids so fluorescent dyes, or fluorescently labelled microspheres, may be added to identify unsuitable core sections. Longer term monitoring of aquifer conditions requires suitable sampling devices and methods. Planktonic communities can be sampled from different depths over a plume profile using multi-level samplers, with groundwater pumped to the surface for processing and analysis. With any sampling campaign, consideration must be given to the temporal and spatial variation in conditions. Sampling ports must be placed at intervals appropriate to capture vertical gradients in chemical concentrations and redox conditions within the plume, which are typically steep (e.g. [124, 125]). Local variation in hydraulic conductivity (K_s) may result in the over-estimation of microbes in regions of high K_s compared with neighbouring low K_s. regions. The position of the water table may vary seasonally and long-term sampling is needed to develop an understanding of how natural attenuation is proceeding and the impact of interventions such as 'pump and treat' systems [52].

Obtaining sediment samples at regular time and depth intervals is considerably more challenging. However, this is of great importance, as over 90% of microbes in contaminated aquifers are attached to the substratum [126, 127], and the planktonic and attached microbial communities differ significantly in terms of composition and diversity. Material can be incubated in situ within screened boreholes, in situ microcosms (Sect. 5.1) or other similar sampling devices (e.g. 'BACTRAPS') for extended periods to allow a native community to develop, for subsequent characterisation [45, 92]. In highly stratified systems there is a risk that the borehole may disrupt local gradients, as was seen in an analysis of a sandstone/shale aquifer at Cerro Negro. Samples obtained from suspended sandbags did not reflect the stratified community seen in core samples, but still provided insight into the diversity of organisms present, if not their precise locations [128].

5.3.2 Culture-Based Methods

The growth of microbes in culture using hydrocarbons as sole carbon sources has proven invaluable in developing our understanding of the biological mechanisms underlying bioremediation. However, methods based on growth of microorganisms are limited as only a small proportion of the viable microbial community may be culturable under a given set of conditions. This leads to the 'great plate count anomaly' with typically only 1–5% of the

microbes in a sample able to grow on an agar plate. However, these limitations may not be as great with samples from eutrophic environments where, with the use of appropriate media, up to 80% may grow [129]. Culture-based methods form the basis of many industry-standard tests for microbial contamination. For example, the MicroMonitor^2 system is used in the ASTM Standard Method D7978 – '*Determination of the viable aerobic microbial content of fuel and associated water – Thixotropic Gel Culture Method*' to detect microbial contamination in aircraft fuel tanks. Also, with a better understanding of microbial metabolism, insights from metagenomics analysis, the use of co-cultures and micro-cultivation methods, many previously 'unculturable' microbes may be grown in the laboratory greatly, facilitating their study [130]. Even simple adjustments such as autoclaving agar and phosphate separately can have a huge impact on the numbers and diversity of microbes cultured [131].

The recognition that the growth of many microorganisms depends on growth factors released from other community members has led to the use of co-cultivation methods and development of micro-fabricated devices such as the iChip [132]. Environmental samples are diluted so that single cells are captured in individual channels that are sealed with semipermeable membranes. Viable cells proliferate within the channels when the device is incubated in situ, providing sufficient material for subsequent analysis. With the development of improved analytical tools, it is now possible to analyse single cells at the molecular level. For example, Rinke et al. [133] isolated 9,600 individual cells from different environments, including a terephthalate-degrading anaerobic bioreactor, and completed 201 genome assemblies representing many previously uncultured bacteria and Archaea. Although technically challenging, these approaches will undoubtedly allow the exploration of the so-called microbial dark matter that represents the majority of microbial diversity.

5.3.3 Cell Counts

Direct cell counts of microbial communities are routinely used to quantify total cell numbers, avoiding the need for culturing. Cells can be captured on membranes and counted using epifluorescence microscopy with dyes such as DAPI, acridine orange and SYTO9. Dye combinations such as SYTO9 and propidium iodide that form the basis of live/dead cell assays can be used to assess viability [134]. Alternatively, high-throughput counting of microbes in solution is possible using flow cytometry. These methods are straightforward for planktonic bacteria although, even then, care must be taken that sampling methods do not introduce bias. For example, ultra-small organisms such as Archaeal Richmond Mine Acidophilic Nanoorganisms (ARMANs) may pass through the 0.2 μm filters typically used for these analyses [135] and

filamentous fungi can form tangled masses that are difficult to quantify. Enumeration of microbes attached to a substratum is more difficult. Cells may be removed by washing, sonication or treatment with mild detergents but this may result in lysing of fragile species. The use of confocal laser scanning microscopy (CLSM) allows individual cells attached to a substratum to be resolved and counted or quantified as a 'biovolume', with automated systems greatly increasing throughput [136]. Cell numbers can also be quantified using proxies such as total extractable nucleic acid and the quantitative analysis of gene copy number (e.g. genes encoding 16S rRNA).

5.3.4 Extraction and Analysis of Macromolecules

The extraction and analysis of macromolecules directly from environmental samples allow the microbial community to be characterised in varying levels of detail – from a simple 'fingerprint' of microbial diversity through to a detailed mechanistic understanding of enzyme activity. Although microbes produce numerous macromolecules, nucleic acids (DNA and RNA), phospholipid derived fatty acids (PLFA) and proteins are most commonly studied.

Stable Isotope Profiling

A major challenge in the analysis of microbial communities is the identification of organisms that are responsible for key degradative steps. Incubation of samples with stable isotope-labelled substrates provides a means to address this. Uptake of the substrate and incorporation of the heavy isotope into cellular components offer a means to link substrate utilisation and microorganisms, albeit with some limitations [137]. For example, the first steps in the biodegradation of aromatic compounds such as benzene or phenol involve hydroxylation reactions, forming catechol with subsequent ring cleavage. These initial steps do not involve the acquisition of carbon and metabolic intermediates may be taken up by other community members (the so-called cross-feeding). The same is true for the first steps in the biodegradation of halogenated hydrocarbons, in which halogen groups are removed but no carbon acquired. Despite these limitations, stable isotope profiling (SIP) has been used many times to demonstrate that the most important microbial community members in biodegradation are not necessarily the most abundant.

Lipids

One of the most common analyses (before the widespread adoption of nucleic acid approaches) used to be the characterisation of PLFAs by conversion to their Fatty Acid Methyl Esters (FAME) and measurement via GC-MS. FAME analyses can quickly provide a fingerprint of a sample to indicate the diversity of microbes present and their relative abundance. It provides a measure of total

abundance, generally similar to that obtained by other approaches, and a useful measure of the fungal/bacterial ratio. In the case of organisms such as sulphate-reducing bacteria or methanotrophs, the approach can also provide limited information on identity. There are a number of pitfalls with the approach, as PLFAs may persist in the environment after death of an organism and the PLFA content of a cell may be affected by changes in environmental conditions [138]. In addition, as Archaeal lipids are linked via esters not acids, additional analyses are required to provide a complete characterisation of the microbial community. Despite the advent of alternative approaches, it is still widely used and the relative ease with which stable isotopes can be detected makes it an attractive approach for studying the biodegradation of organic contaminants such as PAHs, PCBs, PCPs, toluene as a component of BTEX and herbicides (*see* [139]).

Proteins

Whilst the analysis of proteins from pure isolates has been a mainstay of classical biochemistry for decades, the advent of proteomics allows the sequence of many proteins to be determined from a single sample. Proteomic analysis is complementary to transcriptomic (RNA) and genomic (DNA) analysis, providing an integrated view of microbial function. Metabolomics (metabolites) completes the picture and sophisticated integrated models of organism function have been developed for a limited number of species [140]. As technologies develop these approaches can be applied to complex, mixed environmental samples as metagenomics, metaproteomics, etc. Environmental metaproteomics applied to bioremediation is in its infancy [141], but has been used to characterise chlorobenzene-contaminated groundwater in a bioreactor, identifying all of the proteins associated with the degradation pathway, with *Acidovorax* and *Pseudomonads* matching best [142].

5.3.5 Nucleic Acids

The analysis of nucleic acids provides a powerful tool with which to analyse microbial community structure and function. The ease with which DNA can be extracted and preserved from the environment makes this the most commonly used approach but DNA can persist in dead cells and even free in the environment so RNA provides a better indicator of living and active cell diversity. Molecular analyses based on extracted nucleic acids fall into two broad classes – those that seek to characterise the microbial population at a taxonomic level and those that quantify the number or expression of functional genes associated with specific biodegradation pathways. Taxonomic analyses typically characterise 16S rRNA (prokaryote) or 18S rRNA (eukaryote) genes, although other 'house-keeping' genes can be used, placing organisms into groups known as 'operational taxonomic units' (OTUs). Functional analysis amplifies fragments of genes associated with specific biodegradation pathways to provide

information on which genes are present, their abundance in the microbial community and potentially (via RNA analysis or stable isotope profiling) whether they are active.

First Generation, Next Generation, Third Generation Sequencing

Rapid advances in sequencing technologies are revolutionising the field and how nucleic acid sequences can be utilised. The development of dideoxy (Sanger) sequencing provides sequencing technologies in an affordable albeit slow manner. The advent of highly parallel, next generation sequencing increased throughput and reduced costs by orders of magnitude. A multitude of technologies exist with rapid developments in the marketplace generating improvements in throughput that are not being matched by development in the computational power necessary to analyse them. Current mature technologies include 454 pyrosequencing, Illumina/Solexa, SOLiD and Ion Torrent sequencing, which vary in the length of sequence produced, throughput and amount of starting material required [143, 144]. All these approaches require multiple copies of the target sequence but therefore suffer from biases and errors introduced during amplification. However, third generation technologies such as PacBio promise single molecule sequencing should provide a more representative picture of the metagenome of a sample.

Analysis of 16S rRNA Sequences

Analysis of variations in 16S rRNA sequence is an essential tool to study microbial diversity. Extensive databases of 16S rRNA sequences have been created, such as Silva, Greengenes and RDP [145–147], with sophisticated tools available for searching and analysis. Degenerate primers against conserved regions in the 16S rRNA genes are designed, allowing amplification by Polymerase Chain Reaction (PCR) from nanogram (or less) amounts of environmental DNA. If real-time, quantitative PCR (qRTPCR) is used, a direct quantification of gene copy number is possible. A key challenge of this approach is the design of appropriate primers. Of necessity, primers are designed against known 16S rRNA gene sequences, which can lead to a systematic exclusion of some groups of organisms (the microbial 'dark matter' referred to above). However, as metagenomic approaches are used more widely the representation of these organisms in the databases will increase, with concomitant improvement in primer design. In turn this leads to more challenges as it will become difficult to compare results using different primer pairs.

Fingerprints of the most abundant community members can be quickly obtained using methods such as Denaturing Gradient Gel Electrophoresis (DGGE) or Thermal Gradient Gel Electrophoresis (TGGE). Major bands can be excised and sequenced to confirm the identity of microbes. These approaches suffer the limitation that establishing reproducible gradient systems is difficult, and so the

comparison of samples run at different times or in different laboratories is challenging. The use of fluorescently labelled primers and subsequent restriction enzyme digestion produces labelled fragments of different sizes, which can be quantified by capillary electrophoresis (Terminal Restriction Fragment Length Polymorphisms – TRFLPs) [148]. This approach is highly reproducible and the length of the fragments can be related to sequence information obtained by other methods to provide an opportunity for microbe identification. These approaches are useful to characterise and compare numerous samples before identifying those to be analysed in more depth, although the continuing decrease in sequencing costs means that sequencing may become the first port of call in the future. Amplified 16S rRNA sequences can be hybridised to microarrays such as the PhyloChip that contains oligonucleotides against 8,471 individual taxa [149], or sequenced directly using technologies such as Illumina. For example, an MiSeq machine can generate ~8 GB of sequence from paired end reads (2×250 bp) in a single run. During the amplification process unique sequences (barcodes) are introduced into the primers, allowing multiple samples to be analysed simultaneously and separated using bioinformatic post-processing.

Once 16S rRNA sequences are obtained, they can be used in many ways. Programs such as mothur and Qiime provide a convenient means to analyse community structure, diversity and differences between samples in a statistically robust manner [150, 151]. One criticism of 16S rRNA sequence analysis is that it tells us '*who is there*?' but not necessarily '*what are they doing*?'. If RNA rather than DNA is isolated and reverse transcribed to cDNA before sequencing, the output is more representative of the activity community than DNA. Similarly, in microcosm studies ^{13}C-labelled substrates can be introduced into the system. Active degraders will incorporate a proportion of the stable isotope into nucleic acid (and lipid). Separation of heavy DNA or RNA by differential centrifugation prior to analysis provides a better indication of community members that are actively degrading the labelled substrate. For example, [152] found that introduction of ^{13}C-labelled phenol into an aerobic bioreactor led to the labelling of RNA from *Thauera* sp., in contrast to conventional culture-independent methods that suggested that γ-*Proteobacteria* and *Cytophaga–Flavobacterium* were the dominant degraders. Jeon et al. [153] used ^{13}C naphthalene and incubations of sediment in situ to identify bacteria and degradative genes associated with coal-tar biodegradation. In some cases the presence of an organism is indicative of function, regardless of the likely use of terminal electron acceptors or degradative capacity. For example, *Geobacter* sp. are indicative of iron-reducing environments, Deltaproteobacteria such as *Desulfobacterales, Desulfovibrionales* or Firmicutes such as *Desulfotomaculum* and

Desulfosporomusa are sulphate-reducing bacteria. Genomic studies have shown that members of the *Dehalobacter* and *Dehalococcoides* are obligate organohalide respiring bacteria (OHRBs), although other OHRBs may be generalists capable of multiple respiratory activities [154, 155]. Attempts are being made to link microbial identity to function using phylogenetic approaches [156].

Once an organism of interest has been identified at the sequence level, specific PCR primers can be designed to quantify its abundance in many samples using qRTPCR. Also, fluorescently labelled oligonucleotides can be synthesised and hybridised to environmental samples (Fluorescence In Situ Hybridisation – FISH), allowing the spatial distribution to be characterised. FISH can be combined with incubations with radioactively labelled substrates and detected using MicroAutoRadiography (MAR-FISH), and signal intensities can be improved using CAtalysed Reporter Deposition (CARD-FISH) [157].

In combination these approaches allow a picture of the microbial community to be developed. For example, [158] examined a microbial consortium that reductively dechlorinated TCE using a combination of 16S rRNA clone sequencing, TRFLP to identify *Dehalococcoides* sp., *Desulfovibrio* sp. and members of the Clostridiaceae as dominant community members, then FISH to examine their relative abundances. Freeborn et al. [159] used 16S rRNA sequencing, qPCR and TRFLP analysis to assess the relative effectiveness of enrichment with various electron donors on TCE dechlorination, but could not establish correlations between the quantities of *Dehalococcoides* cells and rates of solvent degradation.

Analysis of Functional Gene Sequences

Many of the approaches that have been developed for the analysis of 16S rRNA sequences can also be employed for the analysis of functional genes. Typically, our understanding of functional genes is developed by studying pure isolates in culture – knowledge that can then be transferred to environmental samples. Increasingly, whole genome sequencing, whether from isolated organisms or via metagenomes isolated directly from the environment, also provides invaluable insight into degradative pathways. The main challenge is the diversity of functional genes associated with biodegradation processes and the limited sequence homology that makes primer design for their amplification challenging. The development of databases of known sequences, such as those for *dsrAB* [160], and the increasing breadth of knowledge from metagenomics studies will greatly facilitate these approaches.

The presence of specific functional genes in an environmental sample indicates potential for biodegradation and their enrichment in the population as a whole (or expression as determined by RNA analysis) is indicative of function. Maphosa et al. [154] list 111 genes encoding reductive dehydrogenases (RdhA) that have been

identified with diverse substrate specificities including chlorophenol oxidases (cprA), trichloroethene reductases (tceA) and vinyl reductases (vcrA, bvcA). Phylogenetic analysis places these genes into clades, which provides information on substrate specificity at one level and the organisms containing these genes when sequences are compared more closely. Silva et al. [161] extracted high-molecular weight DNA from sludge samples from a wastewater treatment plant at a petroleum refinery. Large DNA fragments (25–50 kb) were used to form metagenomic libraries that were expressed in *E. coli* and tested for phenol biodegradation in a functional assay, and for known phenol degradation genes (phenol hydroxylase) in a PCR-based screen. Whilst 26 of their 13,200 clones were positive in the PCR screen, 413 were positive in the functional screen, showing the utility of functional screening but also limitations of sequence analysis.

Functional genes can be quantified by qRTPCR, localised using FISH, fingerprints derived using DGGE/TGGE/TRFLP, sequenced using next generation technologies and hybridised to microarrays such as GeoChip. The microarray approach offers advantages in that as little as 500 ng of environmental DNA is required for hybridisation, removing the need for amplification in many cases [162]. This might be obtained directly from environments where microbes are abundant, or amplified using whole community genome amplification [163]. Functional Gene Arrays (FGAs), such as GeoChips, have been extensively used in bioremediation studies. For example, studies of the Deepwater Horizon oil spill in the Gulf of Mexico found that genes involved in aerobic and anaerobic hydrocarbon biodegradation became enriched in the plume. The abundance of *alkA* genes from a number of species that encode alkane 1-monooxygenase and *nahA* naphthalene 1,2-dioxygenase genes increased in the plume. Genes associated with N and S inputs from the plume, such as nitrate (*nasA*) and nitrite (*nir*) genes for assimilatory nitrate reduction, also increased. Liang et al. [164] used a similar approach to study the impact of increasing oil contamination on functional gene expression in soils. Whilst the abundance of many genes associated with the metabolism of organic compounds decreased, those associated specifically with biodegradation of contaminant metabolites increased. Statistical analysis showed that both oil concentration and available soil nitrogen influenced community structure, providing insight into potential remediation interventions. The increasing affordability of metagenomic approaches will undoubtedly lead to their widespread adoption in bioremediation [165]. Many studies have used this methodology to investigate systems as diverse as diesel-contaminated soils in the Arctic [166] to oil-polluted mangroves [167].

5.3.6 Putting It All Together

Any molecular technique on its own has severe limitations in providing insight into community structure and function. However, in combination they can provide a deep insight into mechanisms. Carreón-Diazconti et al. [110] used a combination of isotopic, geochemical and molecular approaches to demonstrate reductive dechlorination of CAHs in groundwater. The presence of known biodegraders, such as *Dehalococcoides* sp. and *Desulfuromonas* sp., together with sequences for reductive dehalogenases *pceA*, *tceA*, *bvcA* and *vcrA* indicated bioremediation potential, which was demonstrated using microcosm systems. The combination of analysis of 16S rRNA genes and functional genes addresses one of the main limitations of 16S rRNA analysis – that of linking identity to function. Whilst the presence of these species indicates CAH biodegradation potential, organisms with closely related 16S rRNA sequences may differ in their ability to biodegrade specific CAHs. Of course, the presence of a microorganism (or DNA) does not indicate that it is functional. Analysis of environmental RNA can address this issue, but is technically challenging as microbial RNAs turnover within minutes – a period significantly longer than that required for sample collection in most cases. However, such approaches have been used to show that the expression of the naphthalene dioxygenase *nahA* gene involved in the aerobic degradation of naphthalene was positively correlated with rates of naphthalene biodegradation in contaminated soil [168]. The use of substrates labelled with stable isotopes provides a direct link between the incorporation of the substrate into cellular macromolecules and the identity of an organism.

Huang et al. [169] combined several techniques to identify and characterise naphthalene-degrading microorganisms from a former manufactured gas plant. Groundwater samples from the contaminated site were incubated in microcosms with ^{13}C labelled-naphthalene. Biodegradation was monitored by measuring the production of salicylate, a degradation intermediate, and samples collected when biodegradation was maximal. Three naphthalene degraders were isolated from these samples (two *Pseudomonas putida* strains and a *P. fluorescens*), but analysis of ^{13}C labelled-RNA indicated the presence of an uncultured Acidovorax sp. with a high-affinity for naphthalene biodegradation. PCR analysis identified a *Comamonas*-type naphthalene dioxygenase (NDO) gene in samples incubated in low concentrations of naphthalene, whilst the *Pseudomonas*-type NDO genes were only present in samples incubated with high concentrations. These results were interpreted as indicating the presence of two populations of naphthalene degraders in this environment – an *Acidovorax* group capable of high-affinity biodegradation and a *Pseudomonas* group operating with low affinity. A direct connection between ^{13}C-naphthalene utilisation and identity was confirmed using RAMAN-FISH [170]. Incorporation of the heavy C atoms leads to a shift in the

vibrational energies of macromolecules that can be detected at the single cell level using a Raman microspectroscope. Hybridisation of these samples with a fluorescent in situ hybridisation (FISH) probes, specific for the *Pseudomonas* and *Acidovorax* species, allowed the labelled cells to be identified.

5.3.7 The Undiscovered Country...

Despite decades of studies of microbial systems, we know that we understand little of the true biodiversity and even less about the diversity of functional genes in the environment. Our understanding of contaminated sites is probably greater than that of pristine environments because of the research focus on bioremediation, but only represents a fraction of the total. Metagenomics provides a window into this 'undiscovered country', but only by using a multidisciplinary approach. From genomics through geochemistry and ecology will we truly develop a system-wide understanding of contaminated systems and develop a systematic understanding of how the biology of a system can be manipulated to enhance bioremediation.

6 Engineering Scale-Up of Bioremediation

6.1 Scale-Related Controls on Bioremediation

A major objective of bioremediation in practice is to achieve risk reduction targets in the shortest possible time in the most cost-effective and sustainable manner. For engineered bioremediation conditions in the subsurface are optimised to maximise biodegradation rates and treatment performance, which can only be achieved by understanding the factors which limit bioremediation activity and then designing practical full-scale methods to overcome these.

In situ biodegradation rates are controlled by scale-dependent phenomena. Using the definitions proposed by Sturman et al. [33], these include (Table 8): *microscale* factors, which occur at the scale of the microbial cell (10^{-6}–10^{-5} m) where chemical and microbiological species and reactions can be characterised independently of any transport phenomena; *mesoscale* factors, which occur at a scale (10^{-5}–10^{-2} m) when transport phenomena and system geometry become important and *macroscale* factors, which occur at a scale (10^{-2}–>10^{2} m) when transport processes and spatial heterogeneity in geological, hydrogeological and geochemical properties become important, particularly for in situ bioremediation technologies.

Despite the wide range of methods available to characterise factors affecting bioremediation at different scales, observations made at the micro- or mesoscale may not necessarily apply at the macro-scale [33, 171]. For example, field-scale estimates of half-lives for contaminant biodegradation are typically orders of

Table 8
Factors which may influence engineering scale-up of bioremediation and corresponding assessment method

Scale	Category	Typical method of characterisation
Microscale		
Composition of microbial consortia	Microbiological	Total or viable count methods; molecular biological techniques
Growth until critical biomass is reached		Static microcosm studies
Mutation and horizontal gene transfer		Static microcosm studies; molecular biological techniques
Enzyme induction		Enzyme assay; molecular biological techniques
Enrichment of the capable microbial populations		Total or viable count methods; molecular biological techniques
Production of toxic metabolites		Static microcosm studies
Biodegradation pathways		Static microcosm studies
Energy yield, reaction kinetics and stoichiometry		Static or continuous flow microcosm studies; theoretical calculations
Too low concentration of contaminants	Substrate	Static or continuous flow microcosm studies; theoretical calculations
Type and chemical structure of contaminants		Static microcosm studies; theoretical calculations
Contaminant toxicity		Microbial toxicity testing
Substrate inhibition, interactions and competition		Static or continuous flow microcosm studies
Alternative carbon source present		Static microcosm studies
Growth substrate vs co-metabolism		Static microcosm studies
Depletion of preferred substrates	Environmental	Chemical analysis
Availability of electron acceptors and nutrients		Chemical analysis
Inhibitory environmental conditions (e.g. pH) and toxicity effects (e.g. contaminant matrix)		Static microcosm studies
pH and oxidation-reduction (redox) potential		Electrochemical probes
Reactions with soil or aquifer matrix		Abiotic microcosm studies
Sorption (equilibrium)	Physico-chemical bioavailability	Abiotic batch sorption studies
*Mesosc*ale		
(Non-equilibrium) sorption/desorption	Physico-chemical bioavailability	Abiotic batch and continuous flow column sorption studies
Microorganism attachment and detachment		Biofilm studies, borehole sediment incubation studies
Incorporation into humic matter		Batch and/or continuous flow column microcosm studies
Enumeration		Total or viable count methods; molecular biological techniques
Diffusion of contaminants from aggregates		Abiotic batch studies, theoretical calculations
Diffusion of electron acceptors and nutrients	Mass transfer limitations	Theoretical analysis and lab diffusion studies

(continued)

Table 8
(continued)

Scale	Category	Typical method of characterisation
Plugging or filtration		Column studies, pressure drop and flow rate
Oxygen diffusion and solubility		Theoretical analysis and lab diffusion studies
Interphase transport		Multiphase column studies
Contaminant solubility/miscibility in water		Theoretical calculations, laboratory testing
Macroscale		
Advective flow velocity	Mass transfer limitations	Water table elevations, hydraulic tests, tracer studies
Dispersion		Conservative tracer studies, modelling simulations
Spatial heterogeneity		Borehole logs, rock/soil core analysis, porosity and permeability testing
Hydrogeological properties and boundary conditions		As for advective flow and dispersion
Geochemical properties		Rock/soil core analysis

magnitude higher (i.e. slower biodegradation rates) than laboratory-determined values, due to scale-dependent rate limitations (e.g. mass transfer) at the field-scale which cannot be adequately reproduced at the laboratory-scale [171]. Therefore, it is necessary to consider all scale-related effects on in situ biodegradation potential and undertake relevant analyses at a range of scales (Table 8) to determine the feasibility of a bioremediation strategy at a particular site. In this context, experience shows that bioremediation can be enhanced effectively in groundwater at field-scale by modification of several fundamental factors which influence biodegradation, described below. These improvements aim to increase the reliability, cost efficiency and speed of bioremediation.

Where sufficient literature and field-scale evidence is not available to allow process design, the design of engineered in situ bioremediation will need to be supported by laboratory bench-scale and field pilot-scale screening and treatability (e.g. microcosm) studies [5, 28, 31, 35, 53, 172]. Such assessment is recommended to include an evaluation of the type and metabolic activity of indigenous microorganisms, presence of potential inhibitory substances or conditions, with supporting studies to deduce the effect of nutrients, electron donors/acceptors and specialised inocula or other amendments [28, 173]. It is important that such laboratory studies are designed to realistically simulate conditions within the aquifer to allow valid conclusions to be drawn.

6.2 Site Characterisation for Bioremediation

A comprehensive site characterisation must be undertaken to underpin assessment and remediation design [3, 5, 6, 31, 34, 44, 58]. It should deduce, as appropriate for the materials to be treated: (1) the type, quantity and spatial distribution of all contaminant phases

in the source(s) and pathway(s); (2) the subsurface geological conditions (type, spatial relationships and heterogeneity of rock units, including sedimentary structures, bedding, faulting and fracturing); (3) the hydrogeological properties (temporal variation in groundwater flow direction and velocity, range and spatial variation in aquifer hydraulic conductivity, location and connectivity of preferential solute transport paths, such as sand bodies, fractures, faults, or potential barriers to solute migration, such as clay bodies and other low permeability strata); (4) aquifer geochemical properties (distribution of particulate organic matter and metal oxide content) and (5) groundwater hydrochemistry (concentration of all organic and inorganic species within and outside the contaminated zone, including intrinsic supply and consumption rate of electron acceptors, electron donors and nutrients affecting biodegradation). This information is used to formulate conceptual site models that support decision-making on the feasibility of engineered bioremediation, system design and process control, by evaluating the micro- to macroscale controls on up-scaling and implementation [3, 6, 33].

The 'optimum' site conditions to implement engineered in situ bioremediation include a relatively uniform host media (e.g. river delta or floodplain deposits, glacial outwash or sand and gravel aquifer) with pH-neutral groundwater, aquifer hydraulic conductivity of >8 m/day and, in the unsaturated zone, an intrinsic gas permeability of $>8 \times 10^{-5}$ m/day [3, 174, 175]. This reflects the priority to ensure control on fluid and gas transport within the treatment zone for sufficient distribution and delivery of amendments, according to the demand from microorganisms [31]. Attention should be given to the existing redox status of the groundwater with respect to the conditions needed to support or enhance bioremediation of the specific contaminants of concern [34, 35]. The existence of unfavourable site conditions or aquifer settings for engineered bioremediation, such as fractured rock aquifers where it is difficult to control the flow regime and microbial population densities are low, should also be considered. Nevertheless, appropriate engineering design may allow the application of bioremediation in many settings that may initially appear unfavourable.

6.3 Biostimulation

The growth and activity of microorganisms in the subsurface can normally be enhanced using biostimulation, in which essential nutrients, electron acceptors and/or other substrates promoting biodegradation are introduced directly [3, 31, 44, 53, 55, 176, 177].

Most uncontaminated sub-soils and aquifers have low mineral nutrient status, which will limit microbial growth on introduced organic contaminants. Nutrients are therefore often added to support bioaugmentation (*see* below) or amendments of electron acceptors and electron donors to ensure bioremediation is not nutrient-limited [44, 55]. Dissolved or dry fertiliser formulations are generally used, often on the basis of assumed nutritional

requirements, such as C:N of 10:1, C:P of 30:1 or C:N:P of 120:10:1 [3, 8, 31, 43]. However, the influence of inorganic and organic nutrients on in situ biodegradation of hydrocarbons and CAHs varies widely, such that the addition of nutrients into the subsurface environment can be unpredictable and may not always enhance biodegradation rates [31, 36, 43, 178]. Nutrient addition must therefore balance nutrient supply to the specific physiology of the biodegradative population [7]. Furthermore, an excess of nutrients (or other amendments) can promote significant in situ microbial growth, leading to biofouling of remediation wells and/or plugging of the aquifer with biomass or chemical (e.g. Ca, Fe and P) precipitates [3, 53, 58, 174].

For aerobic biodegradation, the addition of oxygen in the gaseous phase to provide a sufficient electron acceptor supply for bioremediation can usually be readily engineered in the unsaturated zone by forced ventilation with air or, occasionally, pure oxygen. For aquifers, air or pure O_2 can be introduced by sparging or recirculation by pre-treated groundwater. However, the dissolved concentration of oxygen in groundwater will be solubility limited to approximately 10 mg/L in the case of air and 50 mg/L in the case of pure oxygen. This is usually much less than the stoichiometric demand for complete biodegradation of the organic contaminant. The mode of gas delivery may also fail to adequately deliver or mix oxygen with the contaminated groundwater, due to mass transport (phase partitioning) limitations, biofouling of injection wells and inadequate dissolution [174]. Consequently, increasing the extent of aerobic biodegradation in plumes is usually restricted and controlled by oxygen supply [44, 50, 51]. To overcome this, hydrogen peroxide (H_2O_2) or ozone (O_3), which decompose to oxygen, have been used to provide much higher dissolved oxygen concentrations. However, these substances are highly reactive and may 'off gas' at the point of injection (blocking the host formation) or prematurely decompose if delivery is not carefully controlled [8, 53]. Furthermore, these supplements can have strong antimicrobial effects at elevated concentrations and are hazardous to handle. Alternative options, such as slow-release proprietary oxygen-releasing formulations based on inorganic peroxide salts, designed to provide a constant rather than pulsed input for more efficient biodegradation, are therefore widely used in preference [3]. No matter what means is used to add oxygen into the subsurface, there is a risk that this will react with reduced species within the matrix (e.g. iron, manganese or sulphur species), which may result in significant precipitation of oxidised products and possible pore blockage, contaminant mobilisation and/or significant loss of oxygen. A good understanding of the redox geochemistry of the subsurface is therefore vital in designing a successful aerobic bioremediation treatment.

For anaerobic biodegradation where the contaminants are to be biodegraded as carbon and energy sources, the addition of NO_3^-or SO_4^{2-}can be considered. Such supplements are not subject to the same solubility and reactivity constraints as oxygen, and create fewer problems with biofouling as they result in lower biomass yields. However, biodegradation rates will often be significantly slower than for aerobic respiration [44]. For enhancing reductive dechlorination of CAHs the indirect supply of hydrogen as an electron donor is the preferred approach [55, 176, 179]. Substrates such as acetate, lactate, methanol and vegetable oils are commonly used as metabolic sources of hydrogen and a range of proprietary hydrogen-releasing formulations are also available [3, 5, 38, 55, 178, 180].

Other factors such as pH or temperature are important to optimise for bioremediation, but are seldom a key issue for engineering scale-up. This is because groundwater temperatures do not vary significantly at a given location (although geographical variations of 25°C exist, with a corresponding approximate doubling of biodegradation rates for each 10°C rise) and groundwater pH is usually 6–8, considered satisfactory for microbial activity [8, 34, 53, 54]. Groundwater pH adjustment may be required if the aquifer is poorly buffered and the biodegradation process results in significant production of acidic metabolites, or if the pH has been modified by other treatments (e.g. in situ chemical oxidation).

6.4 Bioaugmentation

At most sites the intrinsic microbial population will have the necessary capability to biodegrade the contaminants of concern, although there may be cases, particularly for CAHs, where the appropriate population has not developed sufficiently under prevailing conditions [28, 174, 176]. In these circumstances the metabolic capability can be enhanced by bioaugmentation, which is the introduction of catabolically relevant microorganisms into the subsurface [55, 172, 181]. In principle, bioaugmentation can shorten the lag period before active biodegradation develops and stimulate the overall biodegradation rate [182, 183]. Although there have been conceptual proposals to use genetic modification to develop appropriate metabolic profiles, in practice the approach to date has been based on the cultivation of naturally occurring populations with the appropriate metabolic capability, either obtained from the site itself or another location [7, 8, 182]. An alternative strategy that can also lead to successful in situ bioremediation is the combination of different biodegradation abilities into a single bacterial strain or syntrophic consortium, which may offer selective functional advantages in a given environment [172, 184].

Unfortunately, exogenous microorganisms introduced into a new environment may not adapt easily to existing conditions, survive competition with the indigenous bacteria or avoid predation by

protozoa to sustain adequate population levels [172, 181–183, 185, 186]. Reported failures of bioaugmentation often result from lack of consideration given to the fact that the introduced organisms must be functionally active and persistent [175, 183]. There is potential to transfer genes that encode for specific biodegradation pathways from donor strains to indigenous microflora, thus ensuring that biodegradation capability is independent of the survival and propagation of the donor strain. However, this has seldom been demonstrated at field-scale [172]. Furthermore, it can be difficult to evenly re-distribute new microorganisms introduced to the subsurface, where the location and concentration of organic contaminants are heterogeneous and not easily accessed, the bacteria may be adsorbed to, or filtered by, the aquifer matrix and bypass channels (e.g. fractures, fissures or cracks) may exist [175].

As hydrocarbon-biodegrading microorganisms are relatively ubiquitous and robust, low biodegradation rates generally reflect nutrient or electron acceptor deficiencies, or other limiting factors (e.g. mass transport) [44] and it is usually unnecessary to bioaugment [3, 175]. However, bioaugmentation has been widely reported for bioremediation of hydrocarbon-contaminated soils (e.g. [172, 177, 187–191]) and, to a lesser extent, groundwater [178, 186, 192–195].

Of greater demonstrable benefit has been the use of bioaugmentation for CAH remediation in cases where a suitable biodegradative population has not developed due to environmental constraints. Communities isolated from other contaminated sites or available commercially have been effectively applied in this way [176, 178, 180, 186, 190, 192, 196, 197].

Molecular profiling of the in situ microbial community using oligonucleotide microarray analysis, real-time PCR quantification and proteomics can help determine the requirement for, and feasibility of, bioaugmentation [38, 189]. Moreover, only a fraction of total microbial diversity has been explored to date while the genetic resource for biodegradation of more recalcitrant organic chemicals is vast [182, 198]. Hence, a greater focus on the ecology of the native consortia, the relative spatial and temporal abundance of potential source populations and their ability to tolerate in situ conditions, is viewed more likely to reveal robust strains suitable for bioaugmentation than the selection of specific strains with a given biodegradation potential [172, 183].

6.5 Bioavailability of Organic Contaminants

Increased biodegradation rate and system capacity will not lead to higher biodegradation rates when contaminant mass transfer becomes a limiting factor [28]. This is because organic contaminants must be bioavailable for microorganisms to biodegrade them. The rate at which microbial cells can biodegrade contaminants depends on the rate of contaminant uptake and metabolism relative

to the rate of transfer to the cell (mass transfer). Bioavailability and bioaccessibility can therefore affect the engineering scale-up and performance of in situ bioremediation.

Where NAPL is present in the contaminant source, contaminant dissolution will be controlled by the NAPL composition, distribution and the effective solubility of each component, which will result in a contaminant profile in the plume that changes over time [199]. Attempts to increase mass transfer from the NAPL to the aqueous phase in order to accelerate biodegradation of dissolved substrates have focussed mainly on the use of surfactants to enhance NAPL dissolution [28, 177], because organic compounds must be dissolved before they can be taken up by microorganisms [7, 54]. Evidence of direct biodegradation of NAPLs during in situ bioremediation has not been conclusively demonstrated, although nutrient and electron donor amendment has been shown to enhance the dissolution of CAHs from DNAPLs in aquifers [192, 197, 200].

In alluvial aquifers with a relatively high particulate organic matter content, sorption of hydrophobic organic chemicals can reduce their availability for biodegradation, which is then controlled by desorption kinetics [3, 28, 58]. Similarly, the diffusion of contaminants into low permeability materials (e.g. clays) in heterogeneous aquifers or low permeability matrix in fractured rock (e.g. chalk) aquifers can prevent physical access of microorganisms [28, 54, 80, 175]. Biodegradation is then determined by the rate of contaminant back-diffusion into more permeable material containing the microorganisms.

6.6 Engineering Implementation of In Situ Bioremediation

Common design configurations of engineered in situ bioremediation systems for hydrocarbon- and CAH-contaminated groundwater include direct injection, groundwater circulation and permeable reactive barriers [55, 179]. Alternative designs exist for bioremediation of soils and for ex situ applications [5, 174, 201]. Direct injection systems add amendments such as electron acceptors, inorganic nutrients and/or metabolic sources of hydrogen into the aquifer, using the natural groundwater flow to disperse and mix these with the organic contaminants [55, 193]. In groundwater recirculation systems the groundwater is extracted, amended and re-injected upgradient into the contaminated zone within a controlled flow field using injection and extraction (recovery) wells [3, 31]. These wells can be arranged vertically or horizontally and parallel or transverse to the groundwater flow direction, or at different elevations to create a vertical circulation [5, 58, 174]. In general horizontal wells may be more effective for thin plumes, whereas vertical wells are preferred for thick plumes. Amendments can be introduced under continuous or pulsed injection, according to the degree of mixing possible and rate of assimilation by in situ microorganisms. Pulsed alternate injection of amendments helps

reduce well screen biofouling by eliminating optimum growth conditions at the injection point [3, 174]. The critical feature is that the amended groundwater should sweep the entire contaminated zone and the extraction wells must capture the injected groundwater to prevent migration outside the treatment area [31, 55]. Each injection well has several zones of influence according to relative rates of transport and reaction. These are: (1) a hydraulic zone defined by pumping tests that reflects the rate of injected water and aquifer hydraulic properties; and, (2) a second (usually smaller) zone defining the travel distance of amendments (each of which has its own zone of influence according to injection concentration/mass, sorption to the aquifer matrix and rates of reaction and biological reaction). The amendment that limits the biodegradation rate establishes the effective zone of influence for bioremediation and required well spacing. Different configurations of injection–recovery well network are possible, as influenced by the site-specific variation in aquifer hydraulic conductivity, subsurface travel times, sustainable pumping rates, contaminant distribution and subsurface structures [174]. Groundwater flow and transport modelling will be required to design the injection–extraction well network, recirculation system and pumping regime, based on site characterisation and treatability studies [6]. Usually multiple scenarios for system design must be explored to ensure cost-effective treatment performance [58].

Permeable reactive barriers (PRB) are in situ treatment zones which intercept and remediate a contaminant plume [202]. They comprise reactive material placed in the flow path of the plume to abiotically or biologically transform or immobilise contaminants, under either hydraulically passive (existing groundwater flow) conditions or an induced (pumped) flow regime [3]. There are various design configurations (e.g. 'funnel and gate' and continuous wall), with the reactive components for bioremediation ('biobarriers') comprising solid electron acceptor-releasing materials (e.g. inorganic peroxide formulations) or sources of metabolic hydrogen (e.g. proprietary slow-release formulations, compost, sawdust or mulch). Combinations of materials can be used in sequenced aerobic–anaerobic bioremediation to, for example, treat mixed plumes of hydrocarbons and CAHs [5, 202, 203]. PRB performance depends critically on contaminant residence time versus reaction rate within the treatment zone and technical guidelines have been developed for their design [202, 204].

6.7 Process Control Monitoring and Verification

Field monitoring of bioremediation systems will be required to: (1) confirm the performance of the installed configuration in transporting amendments into and within the treatment zone; (2) ensure balanced delivery of amendments for biodegradation according to demand which may vary temporally and spatially as the system develops and (3) demonstrate the achievement of

remedial objectives. For example, demand for electron acceptors and substrates is initially likely to be less than expected for operational conditions while the indigenous microorganisms adapt and increase, after which amendment delivery must increase to maximise biodegradation. Also amendments are likely to be consumed by microbial activity close to the injection point, with this demand propagating outwards as the contaminants are biodegraded at progressively further distances from this location [174]. As bioremediation continues, the amendments must therefore be transported increasingly longer distances across the treatment zone until eventual breakthrough at the extraction/recovery well (if installed). This microbial response can require a considerable time, such that careful control of the pumping regime is needed to minimise loss of excess amendment. All such monitoring and verification of performance must take account of seasonal variations in the groundwater flow regime and the variability in, and resolution of, the monitoring techniques applied. Furthermore, the potential for 'rebound' caused by back-diffusion of sorbed or trapped contaminants must be considered.

There are a large number of bioremediation process types and configurations, which means that the appropriate design, process control and verification techniques will be highly case-dependent. As we have explained in this chapter, a wide range of relevant microbiological and related monitoring techniques are available. These should be selected appropriately and used in combination with appropriate hydrogeological, chemical and other relevant parameters.

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Protocols for Ecological Risk Assessment Using the Triad Approach

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Abstract

An ecological risk assessment of a contaminated site is usually based on a model approach using chemical analyses of soil, sediment or water. These methods take neither the biological availability of contaminants, combination toxicity nor ecological field effects into account. To overcome these limitations, the Triad approach has been developed for sediment by Chapman (Environ Toxicol Chem, 5:957–964, 1986) and adapted for soil by van der Waarde et al. (Effectiviteit van bioassays bij het monitoren en beoordelen van het milieurendement van in situ biorestauratie. NOBIS 96-1-13, 2000; TRIADE benadering voor beoordeling van bodemkwaliteit. NOBIS 98-1-28, 2000). This approach combines chemical data, toxicity testing and ecological data of a site to determine the effect of contamination on the ecosystem. In this protocol, the Triad approach is described for the evaluation of the ecological impact of petroleum hydrocarbons (PH) in soil. It has been shown that no theoretical standard threshold value can be derived for PH above which they will have a negative effect on the ecology. Depending on the type of oil, the composition of the oil mixture, but also environmental characteristics at a certain site, ecological effects can be found at both low and high concentrations. Site-specific research is needed in order to assess the need for remediation.

Keywords: Bioassays, Chemical analyses, Ecological field observations, Ecological risk assessment, Petroleum hydrocarbons (PH), Triad approach

1 Introduction

Owing to past and present anthropogenic activities and natural seeps, petroleum hydrocarbons (PH) have entered the environment where they cause problems such as diminished growth and functioning of (micro)organisms. PH or mineral oil is a generic term for a large group of hydrocarbons with different physical, chemical and toxic characteristics. They therefore each have different ecological effects on the environment. It is not possible to explain or predict the toxicological effects based on the results of standard chemical analyses on total concentration of petroleum hydrocarbons [1]. For instance, the EC_{50} (Effect Concentration at which 50% of the model organisms suffer from the pollutant) of different PH for earthworms varies from 30 to 71,000 mg/kg dw [2]. To what extent

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organisms are affected by PH depends on the characteristics of the compounds and the environment as well as the organism itself. The total concentration of PH at a polluted site does not mean anything about the actual ecological impact and risks. In order to determine the actual ecological impact and ecological risk, it is important to analyse the biological availability of PH. The Triad approach addresses this issue by determining the direct effects of a pollution on the ecosystem [3]. It combines chemical data, toxicity testing and ecological data from a site to give a complete and actual description of ecological effects of environmental contaminants. This approach fits well within current policy developments in the Water Framework Directive and the proposed European Soil Strategy in which remediation based on ecological risks and ecological quality is included.

1.1 The Triad Approach

The ecological risk of a certain pollutant in the environment depends on the characteristics of the pollutant and the characteristics of the ecosystem at risk. An obvious ecological effect of a pollutant is when an organism is completely covered by the pure product, for example, when birds are covered with oil after an oil spill. However, pollutants, if biologically available, can also accumulate in the cells of an organism where it can disrupt its metabolism and/or growth. The main disadvantage of assessing ecological risks based only on chemical analyses is that bioavailability or combination toxicity (effects of the combination of pollutants in the environment, some pollutants can have an antagonistic or synergistic effect) is not taken into account. Another disadvantage is the lack of sufficient (terrestrial) ecotoxicological and ecological field data to perform an ecological risk assessment (ERA) by means of a (computer) model. To make a site-specific ERA, biological parameters need to be analysed.

The practice of ERA of contaminated sites varies around Europe, but is generally following a tiered approach with different stages (or tiers) representing increased details of assessment. The first stage consists in general of rather simplified approaches including comparison of soil concentrations with generic ecotoxicity-based soil screening levels (quality criteria, benchmarks, guideline values, etc.). The successive tiers require more effort and thus more money. This means that the unnecessary use of expensive techniques and analyses can be prevented.

In the following tiers, site-specific bioassays and ecological screening tools are commonly used and a methodology for this purpose that has recently gained interest is the Triad approach [4–7]. The Triad approach is not used to identify risks of pure pollutants but has been developed to identify the ecological quality of polluted surface waters, sediments and soils. Polluted samples always need to be compared with a site-specific clean reference sample, preferably from the same site and with similar soil characteristics (*see* **Note 1**). The total number of samples taken for an

ERA depends on the spatial distribution of the pollutant, the complexity of the pollution situation and the complexity of the site itself [5, 8] (*see* **Note 2**).

This methodology is based on three independent types of assessments, the so-called lines of evidence (LoE), deployed simultaneously. The three LoEs are (1) chemical characterisation (analyses of pollutants and the bioavailable fraction in soil, sediment and/or water and analyses of bioaccumulation in organisms), (2) toxicity characterisation (bioassays and biomarkers for toxicity testing using model organisms in the laboratory) and (3) ecological surveys (field observations of vegetation, macro-, meio- and microfauna) [3, 9]. The tests and analyses that need to be performed depend on the pollution, environmental characteristics, the ecosystem and the requirements and preconditions of the stakeholders [10]. Each of the LoE can comprise multiple analyses or tests.

Finally, the result of the different LoEs is combined to LoE-specific risk estimates, which are further integrated to produce integrated risk estimates (IREs) for each studied soil sample.

2 Methods

2.1 Environmental Chemistry

In the chemical part of the Triad approach, chemical analyses are performed on water, sediment or soil (depending on the environment that is polluted). Ideally not only total concentrations of a contamination are measured but also the bioavailable fraction. Several techniques have been developed for organic compounds to do this, such as persulphate oxidation (poly aromatic hydrocarbons (PAH) degradability and availability) [11], solid phase (micro) extraction (hydrophobic organic compounds (HOC) availability) [11, 12] and polyoxymethylene-solid phase extraction (POM-SME) [13]. The bioavailable fraction of metals can be measured, for example, by extracting soil samples with 0.01 M $CaCl_2$, after which the extract is chemically analysed [14].

The total concentrations of pollutants can be used to calculate the potentially affected fraction of species (or PAF, total fraction of organisms that potentially are affected by the pollution) of a site [15, 16]. The Web-based tool www.risicotoolboxbodem.nl can be used to calculate the PAF (or TP, toxic pressure) for soil (*see* **Note 3**). The PAF or TP expresses, in one value, the fraction of species that is expected to be locally exposed beyond a selected effect level (No Observed Effect Concentration (NOEC), Hazardous Concentration for 50% of the exposed test species (HC50)) due to the mixture of contaminants (total concentrations, normalised with respect to organic matter and clay content) in the sample [17]. It is calculated from species sensitivity distributions (SSDs), for the studied contaminants, based on EC_{50} (Effect Concentration demonstrating 50% effect in a toxicity test) values from the literature, and is obtained

from mixture modelling using models for concentration addition (CA) and response addition (RA) ([18]; for equations see below). The following interpretation of TP intervals is used in Dutch soil legislation: TP < 0.25 indicate no risk; $0.25 <$ TP < 0.50 indicate low risk; and TP > 0.50 indicates high risk.

For freshwater, one of the models used to calculate the PAF is the computer model OMEGA [19]. The model can calculate the PAF for single compounds and multiple compounds based on total concentrations. For a limited number of pollutants, the PAF based on bioavailable fractions can also be calculated. The same model can also perform bioaccumulation calculations in the food chain, using NOEC (No Observed Effect Concentration) and LC_{50} values (the concentration that is estimated to be lethal to 50% of test organisms). It calculates the risk of accumulation of a compound in the food chain by using the measured concentration of that compound in organisms (in most cases, sediment worms or earthworms).

Other (supportive) analyses performed in this part of the Triad approach are, for example, pH, DOC and conductivity as well as the distribution of soil or sediment particles. These parameters are also (qualitatively) taken into account in the final analyses of the Triad results.

2.2 Toxicity

In this part of the Triad approach, bioassays or biomarker tests are performed. Different model organisms and analyses can be used depending on the pollution, environmental characteristics and the ecosystem and soil use. Usually the most sensitive model organism is chosen. It is also important to select representative model organisms for the polluted site under investigation. For example, fish are only used to test aquatic samples and earthworms are only used to test soil samples. A combination of different organisms at different trophic levels is preferred because this gives the best impression of risks at the entire system. Both chronic (long term with endpoints such as growth, reproduction and DNA damage) and acute tests (short term with endpoints such as survival) can be used.

The final choice of tests to be performed in this line of evidence depends on the pollutant, the environmental conditions (*see* **Note 4**) and the type of ecosystem but also on requirements and preconditions of stakeholders.

Table 1 shows a list of some of the possible bioassays to test both acute and chronic effects of pollutants in different environments. These tests are validated and standardised (either OECD or ISO guidelines). Although the use of standardised tests is advisable, also non-standardised tests can be used if validated and proven to have added value.

2.3 Ecology

In the third part of the Triad approach, ecological data is collected from a polluted site. This data is used to assess the effects of a pollution on the site itself. Different organisms can be used for

Table 1
Examples of bioassays in different environments

	Soil	Freshwater sediment	Marine sediment	Freshwater	Seawater
Acute tests	Microtox® (bacteria) solid phase	Microtox® solid phase	Microtox® solid phase	*Brachionus calyciflorus* (rotifer) survival	*Brachionus plicatilis* (rotifer) survival
	C. elegans (nematode) survival	*Ephoron virgo* (mayfly) survival and growth	*Hyalella azteca* (amphipod) survival	*Pseudokirchneriella subcapitata* (algae) growth	*Phaeodactylum tricornutum* (algae) growth
	Folsomia candida (springtail) survival	*Daphnia magna* (water flea) survival	*Corophium volutator* (mud shrimp) survival	*Danio rerio* (zebra fish) survival	*Psammechinus miliaris* (green sea urchin) fertilisation
Chronic tests	*Eisenia andrei* (earthworm) reproduction	*Chironomus riparius* (midge larvae) growth and development	*Hyalella azteca* growth and reproduction	*Daphnia magna* survival and reproduction	*Callinectes sapidus* (crab) growth and development
	Folsomia candida reproduction	*Daphnia magna* survival and reproduction	*Echinocardium cordatum* (sea potato) survival and condition	*Danio rerio* early life stage development	*Cyprinodon variegatus* (sheepshead minnow) early life stage development
	Lolium perenne (grass) growth	*Chydorus Sphaericus* (water flea) survival and reproduction	*Arenicola marina* (lugworm) survival and faeces production	*Pimephales promelas* (fathead minnow) early life stage	*Mysidopsis bahia* (shrimp) reproduction and growth

these analyses. For soil and sediment (both marine and freshwater) nematodes are often used. Nematodes are small soil/sediment worms that are present throughout the entire soil or sediment food web. The composition of the nematode population is an indication of environmental quality. Nematode analyses consist of determining the total number of nematodes per 100 g of soil, as well as the number of different species, number of nematodes per species, number per feeding group (e.g. plant feeding, fungal feeding, bacterial feeding, etc.), number per life strategy group (coloniser-persister (cp) scale) and the maturity index (MI). Colonisers are species with a short-generation cycle and are able to colonise new sites more quickly. Persisters on the other hand prefer stable environments and have a long-generation cycle [20–22]. The maturity index (MI) is the ratio between the number of colonisers and the number of persisters and is a measure for soil health. The use of nematodes and the MI is based on the evidence that colonisers dominate nematode communities in disturbed ecosystems [9]. For instance, in a disturbed ecosystem, the number of persisters will be decreased, which decreases the MI.

Plant and algal community structures can also be analysed as well as bacterial community structures. Another parameter that is often used is the identification of soil and sediment macrofauna (numbers, species and community structures of, for instance, earthworms, oligochaetes, snails, etc.) or soil functions such as nitrification rate (11; 25).

2.4 Combining All Lines of Evidence

By combining and integrating the lines of evidence, the actual ecological risks are assessed. This can be done using numerical methods (*see* calculations for numerical methods in Chap. 3) or non-numerical methods with symbols (*see* Table 2 for an example).

Table 2
Example of non-numerical method for risk assessment [23]

Parameter	No negative effects	Moderate negative effects	Severe negative effects
Chemistry	< Lowest guideline value	> Lowest, < highest guideline value	> Highest guideline value
Microtox[a]	<10%	10–50%	>50%
Plant growth[a]	<10%	10–50%	>50%
Earthworm survival[a]	<10%	10–50%	>50%
Nematode numbers[a]	0–25%	25–50%	>50%
Nematode MI[a]	<5%	5–10%	>10%
Symbol	–	±	+

[a]Compared to results of site-specific reference sample

Different methods to combine and integrate the lines of evidence are described in literature. McDonald et al. [24] describe both numerical and non-numerical methods and give insight into advantages and disadvantages of each method. Rutgers et al. [25] and Mesman et al. [5] describe a numerical method which results in one number (with deviation) that indicates whether ecological risks are present or not.

3 Calculations

In this section, the numerical method that is used in the Netherlands for ecological risk assessment [5, 25] is described in more detail. For all lines of evidence, another method to scale the results of the polluted samples is used. Finally the method for integrating all lines of evidence is described in the final paragraph of this section.

3.1 Calculation LoE Chemistry (Toxic Pressure)

The toxic pressure (TP) can be calculated using the Dutch Web-based Sanscrit model (www.risicotoolboxbodem.nl, *see* notes), which is based on mixture modelling using models for concentration addition (CA) and response addition (RA) [17].

Which model to use depends on the mixture of contaminants and their mode of action in the organisms. The RA model calculates an overall TP (TP_{RA}) of contaminants with different modes of action using the following equation:

$$\begin{aligned} TP_{RA} &= 1 - ((1 - TP_1) \cdot (1 - TP_2) \cdot \ldots (1 - TP_n)) \\ &= 1 - \Pi(1 - TP_n) \end{aligned} \quad (1)$$

where TP_i is the toxic pressure of individual contaminants ($i = 1, 2, \ldots n$) with different modes of action. TP_i can be calculated for every individual compound using

$$TP_i = \frac{1}{1 + e^{\left[\frac{\alpha_i - \log(C_i)}{\beta_i}\right]}} \quad (2)$$

in which C_i is the concentration of a contaminant in the soil (mg/kg d.w.), α_i is a log-transformed value of the toxicity of that compound (e.g. log HC50) and β_i is the value of organism sensitivity scatter, which equals the slope in the SSD [17].

The CA model is used for contaminants which have the same toxic mode of action:

$$HU_j = \frac{C_1}{10^{\alpha_1}} + \frac{C_2}{10^{\alpha_2}} + \ldots \frac{C_n}{10^{\alpha_n}} = \sum_{i=1}^{n} \frac{[C_i]}{10^{\alpha_i}} \quad (3)$$

and

$$\mathrm{TP_{CA}} = \frac{1}{1 + \mathrm{e}^{-\left[\frac{\log\left(\mathrm{HU}_j\right)}{\beta_j}\right]}} \tag{4}$$

where HU is the hazard unit for a group of contaminants j for which the CA model can be used; C_i is the concentration of the contaminants after correction for standard soil (10% organic matter, 25% soil particles smaller than 2 μm); α_i is a log-transformed value of toxicity (based on literature values for the different compounds); and β_j is a joint parameter (representing slope) of the SSD (based on literature values for toxicity).

The RA model (Eq. 1) is then applied to the set of $\mathrm{TP_{CA}}$ values (one value for each toxic mode of action) to calculate the overall TP. This aggregated value corresponds then to the final combined fraction of species potentially affected at the site.

The CA and RA models are integrated in Sanscrit, and the model automatically uses the equations in the right order whenever $C_{i,\mathrm{soil}}$ of different compounds is filled in at the calculation sheet. Other input parameters are organic matter content and percentage of soil particles smaller than 2 μm (to correct for the differences between the standard soil example given in the model and our site-specific conditions).

3.2 Calculation LoE Toxicology

For each test, the results are expressed as deviation from the chosen reference (fraction or percentage). The reference is expressed as 1 or 100%. The effect is calculated as 1-effect. For all tests together, the geometric mean is calculated from the different results:

$$(1 - \mathrm{Effect_{mean}}) = \{(1 - \mathrm{Effect_1}) * (1 - \mathrm{Effect_2}) * \ldots * (1 - \mathrm{Effect}_N)\}^{1/N}$$

By calculating the LoE like this, the more severe negative results have a bigger effect on the LoE.

3.3 Calculations of LoE Ecology

The results from the nematode test are scaled against the values obtained for the reference soil using the following equation:

$$\mathrm{Effect(Nbr\ of\ nematodes)} = \left|1 - \frac{R_{\mathrm{Sample}}}{R_{\mathrm{Ref}}}\right| \tag{5}$$

$$\mathrm{Effect(Nbr\ of\ species)} = \left|1 - \frac{R_{\mathrm{Sample}}}{R_{\mathrm{Ref}}}\right| \tag{6}$$

where R_{Sample} is the result of the measurement endpoint in the test (i.e. numbers of nematodes, numbers of species) and R_{Ref} is the corresponding result for the reference soil.

For the relative effect of the MI, the following equations are used:

$$\mathrm{Effect(MI)} = \frac{\mathrm{MI_{Ref}} - \mathrm{MI_{Sample}}}{\mathrm{MI_{Ref}} - \mathrm{MI_{max}}} \tag{7}$$

where

$$\mathrm{MI}_{\max} = 1 \text{ if } \mathrm{MI}_{\text{sample}} < \mathrm{MI}_{\text{Ref}}$$
$$\mathrm{MI}_{\max} = 5 \text{ if } \mathrm{MI}_{\text{sample}} > \mathrm{MI}_{\text{Ref}}$$

The multiple results from the nematode test are then combined to obtain a final risk estimate for the ecology LoE (LoE_e) using the following equations:

$$R'' = \log(1 - R') \tag{8}$$

$$\mathrm{LoE}_e = 1 - 10^{\frac{\sum_1^n R''}{n}} \tag{9}$$

where n is the number of measurement endpoints and R the result for each test.

3.4 Calculation of Integrated Risk Estimate

For the final judgement, the risk estimates for each LoE (LoE_x) are integrated into IREs (integrated risk estimate) using Eqs. (10) and (11):

$$\mathrm{LoE}_x' = \log(1 - \mathrm{LoE}_x) \tag{10}$$

$$\mathrm{IRE} = 1 - 10^{\frac{\sum_1^3 \mathrm{LoE}_x'}{3}} \tag{11}$$

After the IRE has been calculated, the deviation needs to be calculated (Eq. 12). This is an integrated measure that expresses the differences in the results of the three lines of evidence. A high deviation means that the results of the lines of evidence strongly differ. In that case the Triad needs to be extended to the next research tier in order to decrease the deviation.

$$\text{Deviation} = \left(\mathrm{Stdev}_{3\,\text{TRIAD steps}}\right) * 1.73 \tag{12}$$

4 Examples of Application of the Triad Approach

4.1 Two Examples of Application of the Triad Approach

In the following paragraph, two examples are given of an ecological risk assessment using the Triad approach. Two terrestrial sites are described that were contaminated with PH [23, 26]. At both sites, the physical and chemical characteristics were determined (pollutant concentration, pH, organic matter, fraction soil particles smaller than 2 μm, cation exchange capacity (CEC), results not shown), several acute and chronic bioassays (*see* Tables 3 and 4) were conducted as well as ecological field surveys on nematodes. The soil guideline value for PH for site A was 1,000 mg/kg dw and 15,000 mg/kg dw for site B. This high variation in guideline values is caused by the fact that the Dutch guideline value depends on the organic matter content of a given soil. The organic matter content of site A was 5% and for site B 50%. The results of the tests are shown in Tables 3 (site A) and 4 (site B).

Table 3
Triad example A

Parameter	Sample 1	2	3	4
Site A [23]				
Chemistry				
– PH (mg/kg dw)	3,200	1,200	520	790
Toxicology				
– Algae growth	+	n.d.	n.d.	–
– *Daphnia magna* mobility	+	–	–	–
– Microtox	+	–	–	–
– Plant germination	+	–	–	–
– Plant biomass	+	–	–	–
– Earthworm survival	–	–	–	–
– Springtail survival	+	–	–	–
– Springtail biomass	+	–	–	–
– Springtail reproduction	+	–	–	–
Integrated risk estimate	+	–	–	–

–: No negative effects
±: Moderate negative effects
+: Severe negative effects
n.d.: Not determined
3,200: Above guideline value

For site A, a non-numerical method was used to integrate the results of all Triad tests and analyses in order to follow the effect of bioremediation in time. No reference sample could be taken. For site B, the numerical method according to Mesman et al. [5] was used to calculate the site-specific risks (see section calculations).

For site A, sample 1 is before bioremediation. Sample 2 is a sample during bioremediation as well as sample 3 and sample 4 are taken at the end of bioremediation. Because samples were not taken from the top soil, no ecological observations have been performed.

The results of the analyses performed on site A showed that remediation was necessary, both based on chemical analyses (comparison to guideline value) and on the biological tests. Tests showed a negative effect on bacteria, algae, reproduction and springtails and germination of plants. After performing the ERA by means of the Triad approach, the site was remediated using a technique called bioventing (venting of the soil in combination with aerobic degradation of the PH compounds) at laboratory

Table 4
Triad example B

	Sample						
Site B [26]	1	2	3	4	5	6	7
Chemistry							
– Concentration mineral oil (mg/kg dw)	<25	1,600	2,700	3,600	5,500	9,900	14,000
– PAF[a]	0	0.06	0.11	0.14	0.22	0.40	0.56
Toxicology							
– Microtox	0.00	0.62	0.16	0.40	0.20	0.02	0.11
– Earthworm total[b]	0.00	0.73	0.43	0.32	0.35	0.35	0.50
– Earthworm survival	0.00	0.02	0.00	0.00	0.00	0.00	0.00
– Earthworm growth	0.00	0.00	0.01	0.02	0.01	0.03	0.05
– Earthworm reproduction	0.00	0.98	0.81	0.68	0.73	0.72	0.87
Ecology							
– Nematodes MI	0.00	0.20	0.36	0.36	0.56	0.24	0.40
– Nematodes number of species	0.00	0.19	0.21	0.17	0.23	0.18	0.22
Judgement chemistry	0.00	0.06	0.11	0.14	0.22	0.40	0.56
Judgement toxicology	0.00	0.68	0.31	0.36	0.28	0.20	0.33
Judgement ecology	0.00	0.20	0.29	0.26	0.42	0.21	0.32
Integrated risk estimate (IRE)	0.00	0.38	0.24	0.26	0.31	0.28	0.41
Deviation	0.00	0.59	0.23	0.17	0.17	0.17	0.21

[a]No PAF could be calculated for mineral oil due to the properties of mineral oil. In this case we have calculated a potential risk based on the measured concentration in relation to the Dutch intervention value for this specific site
[b]In case more endpoints are determined from one test (like the different endpoints from the earthworm test), first the results of that test are integrated (so all endpoints of the earthworm test) and after that, the integrated result from that test (in this case the earthworm test) is integrated with the results of the other tests
Value range: 0.00–0.25 indicates no risk; 0.26–0.49 indicates moderate risk; > 0.50 indicates high risk

and field scale (results not shown). Remediation resulted in a large decrease of pollution grade and a shift in fractions; the fraction C_{10}–C_{14} was removed completely and the relative fraction C_{14}–C_{20} and C_{20}–C_{26} increased. These fractions cause less ecological effects because they are less available to organisms. No toxic effects were found in the remediated soils not even in the soils during remediation with higher concentrations than the guideline value.

Site B is a natural environment with peat soil at which a calamity with crude oil had occurred some decades ago. The oil found at the site turned out to be strongly weathered and consisted mainly of branched alkanes with more than 24 carbon atoms, meaning that availability of the oils to organisms was expected to be low. Because it was a peat site, the organic matter content of the soil was high and the pH was low. Owing to the fact that PH bind to organic matter, the contaminants were largely biologically unavailable. This was confirmed using bioassays and ecological field surveys. Only low risks were present at the site and the risks did not correlate to the pollution grade, but more to pH and organic matter (*see* **Note 5**). Remediation of the site was not necessary based on chemical analyses (comparison to guideline value) which were confirmed by the biological tests.

4.2 Resume

The examples described above show that no theoretical standard threshold value can be derived for PH above which they will have a negative effect on the ecosystem. Depending on the type of oil, the composition of the oil mixture, but also environmental characteristics at a certain site, ecological effects can be found at both low and high concentrations. The Triad approach has shown to have advantages compared to the use of only chemical analyses. The approach can be used to assess ecological risks in soils, sediments, freshwater and marine environments. Additionally the Triad can be used to identify remediation or redevelopment options so costly and unnecessary remediation can be prevented (*see* **Note 6**).

5 Notes

1. The reference sample needs to be taken as close as possible to, or on, the location. In case a high heterogeneity of soil characteristics, soil use and pollution concentrations is expected, several reference samples need to be taken. After chemical analyses (pollution, pH, organic matter, clay content), the final reference samples can be chosen.

   If no site-specific clean reference sample can be taken from the site, the alternatives in the following order can be used:

   - The cleanest sample in the concentration gradient
   - A reference site further away from the research site which has comparable soil use and soil characteristics
   - Literature data
   - Databases

2. The total number of samples that is required for Triad research depends on site-specific conditions and the research goal.

Three different strategies have been described in Dutch protocol SIKB 2301 [8]:

- If the pollution is homogeneously distributed, so in a steep gradient from clean to polluted in a clearly defined area (for instance, cover layer, dampened ditches), effects in the polluted area need to be compared to clean samples from outside of the defined area. In this strategy, at least four clean and four (comparably) polluted samples are taken. This strategy can only lead to a decision whether or not to remediate the entire site.
- If the pollution is heterogeneously distributed, the gradient strategy can be used. In this strategy, samples are taken in a gradient from (relatively) clean to (relatively) strongly polluted. Precondition for this strategy is that, based on previous research, gradients in pollution grade are known. Samples need to be taken at relative large distance from each other. At least eight samples are needed in this strategy.
- In complex situations or big locations (>500,000 m^2), more samples are needed. In situations in which two (or more) independent polluted (sub)sites are spatially separated, strategy 2 must be followed for each pollution type or soil type. At least 16–20 samples need to be taken.

3. Risicotoolboxbodem.nl is a Dutch website. This note describes a short manual in order to be able to calculate the TP for soil pollution. First one has to create an account for this website. Click button below "Registreren" and enter first name, family name, organisation and e-mail address. Then click "versturen". An e-mail will be sent with a username and password. In the section "Aan de slag", you can enter this information and click button "Aanmelden". You will enter the website.

   For the calculation, you have to click the button "Nieuw dossier" below the section "Acties". Enter the name of your location or file and a code (can be imaginary as well). Under section "Type Bodemgebruik", you need to tick either "Huidig" or "toekomstig". For the calculations, it does not matter which one is chosen. Then, under section "Stap 1: Ernst verontreiniging", you have to tick "ernstige bodemverontreiniging". Then click button "opslaan".

   You will remain at the same page. But now you can click the red cross next to "Ecologisch" under section "Stap 2: Standaardbeoordeling". This will lead to another page. At this page (Called Standaard ecologische risicobeoordeling), you can click the blue link "spreadsheet". This downloads an excel spreadsheet for the calculation of the toxic pressure of soil pollution.

Save this file on your computer. In row 9 fill in the organic matter content, and in row 10 the percentage of soil particle below 2 μm (callud Lutum) presents in your sample. Standard organic matter is set to 10% and lutum to 25%. Then you can enter the concentrations of pollutants. The sheet automatically calculates the TP (which is called Resultaat msPAF in row 6).

4. Risicotoolboxbodem.nl does not calculate PAF or TP for mineral oil or PH due to its diverse properties. In the examples, we have divided the measured concentration by the normalised intervention values.
5. Tests and analyses are chosen based on site information, environmental and soil characteristics and the pollutant present. Some extreme environmental conditions, for instance, low pH, can have a severe effect on organisms in standardised tests. That means that standardised tests cannot be used under these non-standardised conditions. Adjusting the conditions influences the pollution characteristics and bioavailability and thus the potential effects of the pollution to the tested organisms. This is not favourable. In case of extreme environmental conditions, it might be advisable to use a non-standardised, validated test.
6. Although the use of bioassays in the Triad approach has shown to be effective, some bottlenecks still remain. For example, in the current ecological risk assessment, it is not always clear which pollutant or other stressor is causing negative effects, especially when a site is polluted with a cocktail of pollutants or in case of extreme soil conditions. Although the strength of the Triad is that it takes all present pollutants and stressors into account, in case of remediation or redevelopment, one wants to know at which pollutant remediation efforts have to be directed. Also, traditional tests can be expensive and time-consuming, although biomarkers can resolve parts of those drawbacks. It is expected that in the future genomics-based tools can play an important role in resolving these bottlenecks and that they will add enormous value to the current risk assessments (for instance, by being pollutant specific) [27].

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Field Studies Demonstrating the Efficacy of Bioremediation in Marine Environments

Roger C. Prince, James R. Clark, and Jon E. Lindstrom

Abstract

The ultimate fate of most oil released into the biosphere is biodegradation. Yet oil lacks some of the essential nutrients for microbial life, and its biodegradation can be limited by the availability of such nutrients from the local environment. Bioremediation of oil on shorelines aims to at least partially alleviate this limitation by the judicious application of fertilizers. Bioremediation played a central role in the response to the *Exxon Valdez* spill in Prince William Sound, AK, being applied to more than 120 km of shoreline. This short paper describes the program designed by Exxon, the USEPA, and the Alaska Department of Environmental Conservation to assess the environmental safety and efficacy of bioremediation as it was being carried out in the Sound. It provides a prototype if monitoring shoreline bioremediation needs to be done in the future.

Keywords: Conserved internal markers, Nutrient analysis, Oleophilic fertilizer, Radiorespirometry, Slow-release fertilizer, Toxicity testing

1 Introduction

Bioremediation played a significant role in the cleanup following the 1989 spill from the *Exxon Valdez* in Prince William Sound, AK. Oil was stranded on many miles of shoreline, typically between the mid-intertidal zone and the high-water mark. Under the oversight of federal and state response organizations, Exxon mobilized a massive cleanup operation to wash the beached oil back into the sea where it could be collected with skimmers [1]. At the same time, the USEPA began field experiments to try and stimulate the biodegradation of the beached oil by relieving the nutrient limitation believed to be slowing the process [2], getting several fertilizer products onto the National Contingency Plan list of products that could be used in oil spill response [3]. The application of an oleophilic fertilizer, designed to adhere to oil, was particularly effective in apparently stimulating oil biodegradation (*see* Fig. 3 of [4]), and bioremediation became an important part of cleanup

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operations by August 1989. By the time cleanup operations were suspended in September because of harsh winter weather, more than 120 km of shorelines had received fertilizer [1].

When cleanup efforts resumed in 1990, bioremediation was planned to be a central part of the process, but there were concerns that although the previous year's experiments had been promising, there was a lack of statistically validated "proof" that bioremediation was effective. There were also concerns that the bioremediation protocol might yet have some unexpected adverse environmental impacts, although none had been observed in environmental data collected in 1989. Exxon, the USEPA, and the Alaska Department of Environmental Conservation were charged with jointly addressing these issues; this document describes the experiments performed to address this charge. Our approach provides a useful model if the need to evaluate bioremediation efficacy for large-scale oil spill response arises in the future.

2 Bioremediation of Hydrocarbons on Shorelines

Crude oils are excellent sources of carbon and energy for those organisms that can utilize them, but of course they contain minimal amounts of other biologically useful elements such as nitrogen, phosphorus, iron, etc. Oil biodegradation on an oiled shoreline is thus likely to be limited by the availability of these nutrients from some background source, presumably tidal influx of seawater. The bioremediation protocol initiated as part of the cleanup operations in Prince William Sound used two fertilizers to supplement this natural supply (Fig. 1): an oleophilic liquid product designed to adhere to oil, Inipol EAP22 [5], and a slow-release granular horticultural product, Customblen [2]. Inipol EAP22 was a microemulsion containing an internal phase of concentrated urea in an external oil phase of oleic acid and trilaureth-4-phosphate, cosolubilized by 2-butoxyethanol [6]. It contained 7.4% nitrogen and 0.7% phosphorus by weight. Inipol EAP22 was applied with airless paint sprayers transported on small pontoon catamarans. Customblen was a slow-release horticultural fertilizer consisting primarily of ammonium nitrate, calcium phosphate, and ammonium phosphate, encapsulated in polymerized linseed oil. Customblen contained 28% nitrogen and 3.5% phosphorus by weight. It was applied with broadcast spreaders carried by workers walking the beaches.

Inipol EAP22 was applied where there was surface oil and Customblen where there was subsurface oil, and both were applied where both surface and subsurface oil were present. In 1990 Inipol EAP22 was applied at 0.3 l/m^2, and Customblen was applied at a dose of 15.8 g/m^2 if applied with Inipol EAP22 and 96 g/m^2 if

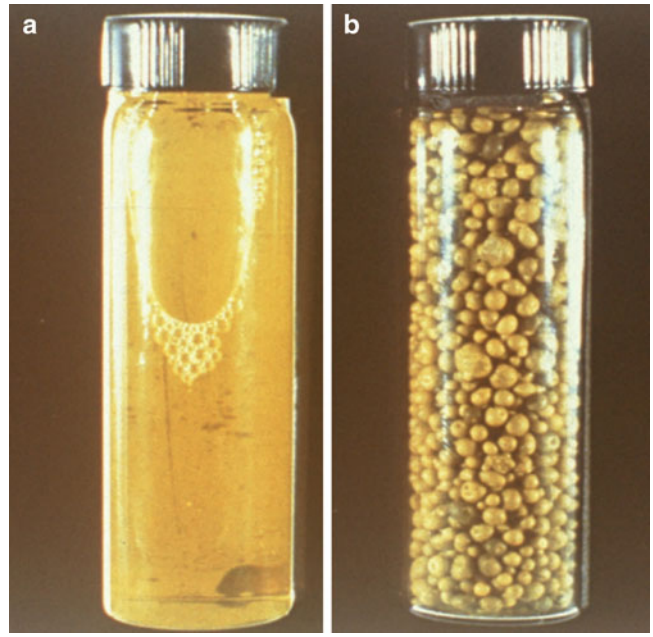

Fig. 1 The two fertilizers used in the bioremediation protocols. (**a**) Inipol EAP22 [5] and (**b**) a slow-release granular horticultural product, Customblen [2]. The products are in 4 dram (15 mL) vials

applied alone. These levels were based on the USEPA calculations of the highest concentrations of ammonia that could be released, with the lowest potential dilution, to still maintain acceptable water quality as the shorelines were flushed with daily tides. Inipol EAP22 could be reapplied after 30 days and Customblen after 15 days, as required [7].

3 Testing the Efficacy of the Bioremediation Protocol

At first glance, this sounds relatively simple; surely one measures the amount of oil in two representative sections of an oiled shoreline, applies fertilizer to one, and returns after some reasonable time to measure the amount of oil remaining. The major reason why this is not trivial is the problem of heterogeneity of the natural world. The amount of oil in a sample of beach material can be measured with high precision, but environmental replicates are extremely heterogeneous and oil residues per area or volume of beach tend to be lognormally distributed [8]. Thus simple statistics are unlikely to measure the oil losses due to enhanced biodegradation predicted to occur in a few months. The lognormality is principally due to the broad range of sizes of particles encountered in beach samples, such as sand, gravel, and cobble, even with careful sampling, but it can also be attributed to the fact that oil rarely arrived on a shoreline in a

uniform manner. The effect of this heterogeneity is that it is inappropriate to assume that differences between the amounts of oil in samples collected some time apart are mainly due to microbial processes that have occurred in that intervening period – the samples may always have been different. As we shall discuss below, understanding the way that biodegradation changes the composition of oil as it is biodegraded provides a way of understanding what has happened to each sample since it was spilled – in effect allowing each sample to act as its own control.

Researchers from Exxon, the USEPA, and the State Department of Environmental Conservation agreed that the way forward was to develop a tiered analytical program that addressed all the component parts of what the bioremediation protocol was meant to deliver [9] and to test for statistical significance at each step. They agreed to follow bioremediation on three distinct, numbered segments of shoreline on the northern end of Knight Island in Prince William Sound: KN132, the mouth of a stream that had light surface oiling; KN135, a sheltered shoreline in the Bay of Isles that had both surface and subsurface oil; and KN211, an exposed beach with substantial cobble armor that had only subsurface oil by 1990 [10, 11]. Each was chosen because it had no obvious input of surface water from the supratidal zone, which might have confounded attempts to enhance nutrient concentrations. A second key attribute for test site selection was a reasonably large extent of what appeared to be similarly oiled shoreline that could be divided into two portions, one to be treated and one to be left as the control (e.g., Fig. 2). All shorelines had received manual cleanup in both 1989 and 1990 to remove any significant tar mats before selection was made [1]. All operations in the monitoring program were carried out in a minimally intrusive way, aiming to leave the shoreline in as natural a state as possible so that data could be reasonably extrapolated to other shorelines. The monitoring program was designed to measure what was expected to happen following nutrient additions:

3.1 Fertilizer Was Applied to the Beach Surface: Did It Indeed Penetrate into the Full Subsurface Oiled Zone?

Sampling wells (5 cm diameter, 70 cm long) were installed into the shorelines: three on the area to be fertilized and three on the control plot. They were driven in by "gentle" hammering with a sledge hammer (Fig. 3); digging a hole in the shoreline would obviously have altered the hydraulic flow in the beach. The well was capped when not being sampled. Water samples were collected by bringing a battery-powered pump to the sites as the tide fell, and collecting water from the bottom of each well. At least two well volumes were discarded before samples were collected. For these experiments, the samples were frozen and shipped to a remote laboratory for precise nutrient measurements; we have subsequently found that simple colorimetric tests for nitrate, ammonium, and phosphate (http://www.chemetrics.com/) are quite

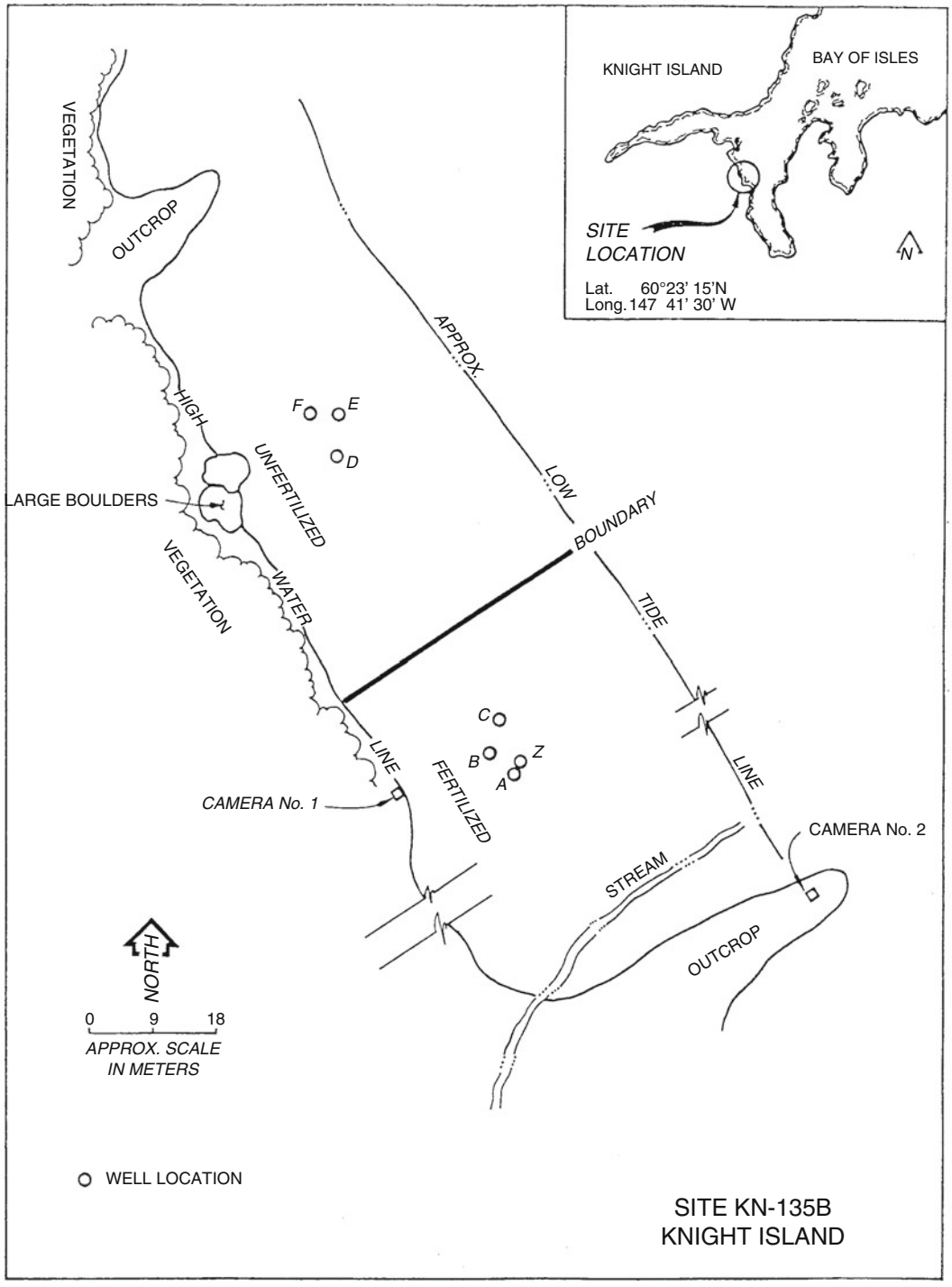

Fig. 2 Schematic of shoreline segment KN135B. The fertilized portion was the southern end of the segment. Chart from Final Report submitted to the Federal On-Scene Coordinator in December 1990

Fig. 3 Installing a sampling well on a shoreline in Prince William Sound. A slotted steel well is being driven into the sediment so that water flow within the shoreline material is only minimally affected. Note the use of safety glasses

adequate for such measurements, and these can be done at remote field sites or using local facilities within an hour of sampling, obviating the need for the complexities and expense of freezing and shipping [12].

The results demonstrated that nitrogen from the fertilizer did indeed penetrate to the depth of subsurface oiling and that it washed out over the course of 30 days or so, and the reapplication strategy described above was appropriate [10, 11]. Phosphate was not measurably increased by the fertilizers – it was likely precipitated by divalent cations in seawater.

The conductivity of the water was also measured, with a portable meter, to look for unexpected freshwater intrusions from the land – none were found.

3.2 Did Fertilizer Application Increase Microbial Activity?

Since the expectation was that the shorelines would be aerobic to at least the depth of tidal influence, an increase in microbial activity might be recognized by a decrease in the level of dissolved oxygen in the beach interstitial water. This was measured with simple colorimetric assays (http://www.chemetrics.com/) on the beaches as soon as water was collected from the well. Obviously this required care in the collection so that the water was not aerated during collection. The predicted decrease in oxygen concentration, from about 7 ppm prior to fertilizer application to 3 ppm dissolved oxygen within several days or a week of treatment, was indeed

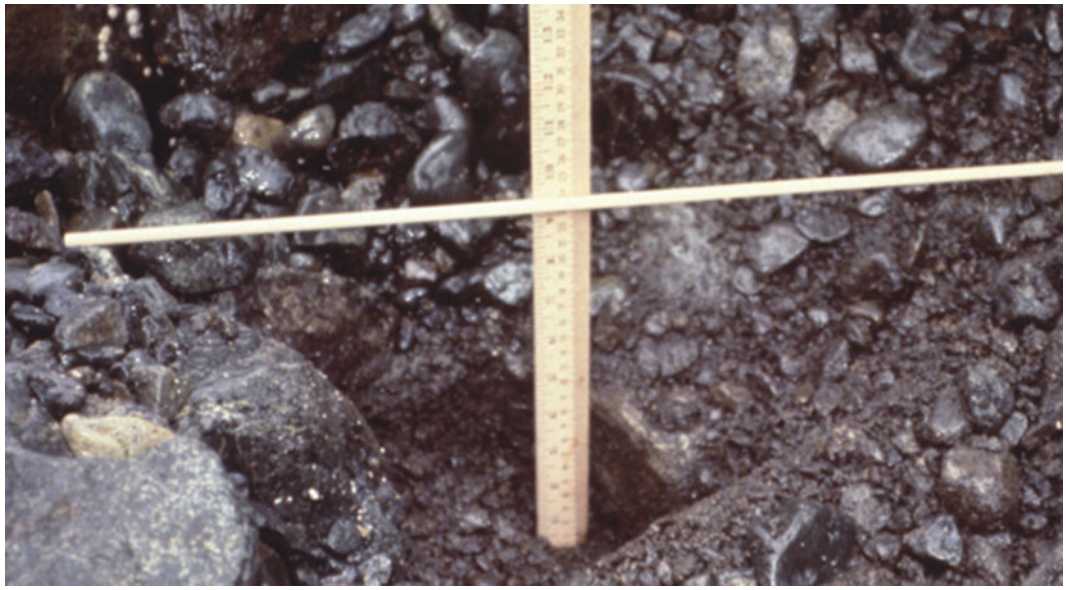

Fig. 4 Simple device for assuring samples were collected at the correct depth. A dowel laid on the beach surface is used as the datum for determining the depth of the hole so that samples are collected from the prescribed depth

established, and the oxygen depletion decreased as fertilizer levels fell. It reappeared following fertilizer reapplication [11].

Microbial enumeration was also performed [13]. It had already been shown that there were more oil-degrading microbes on shorelines that had received fertilizer treatment in 1989 than on those that had not [14]. Sediment and substrate samples were collected from the surface gravel and from a depth of 20–30 cm in shorelines with subsurface oil (KN135 and KN211, Fig. 4). Care was taken during sampling to ensure that the site was disturbed as little as possible and that all samples were taken from previously undisturbed sediment. The monitoring wells served as spatial benchmarks for sediment sampling, and three samples were collected in areas adjacent to each well at each sampling event. At each location, the surface sample was collected before the subsurface sediments, and the material excavated to get to the subsurface samples was placed in a bucket rather than on the beach surface so that it could be replaced in the hole after sampling (Fig. 5). Each sampling site was marked with surveyor's tape so that it could be recognized and avoided in subsequent samplings (Fig. 6). Sediment samples were collected for microbial experiments (Fig. 7) and oil extraction (see below).

The microbial counts were undoubtedly influenced by the sediment heterogeneity discussed above, and despite 5–9 replicates for each sample, statistically significant differences between fertilized and unfertilized plots were only observed in the subsurface.

Fig. 5 Subsurface sampling – material removed to get to the appropriate depth was temporarily stored in a bucket to prevent contamination of surface areas to be sampled later and marked with surveyor's tape so that the area would not be resampled later

Fig. 6 Sampling area toward the end of the monitoring program. Samples had been collected around the sampling well and marked with surveyor's tape to prevent resampling

On the other hand, radiorespirometry provided convincing evidence that hydrocarbon biodegradation was stimulated in the fertilized shorelines [13]. In brief, sediment samples were shaken in sterile seawater, and the supernatant amended with crude oil

Fig. 7 A sediment sample for microbial analysis. A robust sample-labeling system was an important part of assuring that a reliable chain of custody was maintained

spiked with either 1-^{14}C-hexadecane or 9-^{14}C-phenanthrene in sealed vials with enough air-filled headspace that the vials remained aerobic during the assay. Samples were incubated for 48 or 96 h, respectively, and then injected with strong base to stop bacterial activity and trap the CO_2 produced by metabolism. The CO_2 was subsequently removed and counted for radioactivity [13]. Both hexadecane and phenanthrene mineralization were enhanced severalfold by fertilizer treatments, on the surface and in the subsurface, and when fertilizer was eventually applied to the reference plots at the request of the Federal On-Scene Coordinator, the differences collapsed.

3.3 Did Microbial Activity Preferentially Remove the More Degradable Components of the Oil First?

It has been known since at least the 1960s that microorganisms display clear preferences as they degrade hydrocarbon mixtures, the most well known probably being their preference to degrade *n*-alkanes before *iso*-alkanes, for example, heptadecane before pristane [15]. Decreases in the ratio of heptadecane to pristane are an excellent indicator that biodegradation has begun, but pristane is itself soon degraded, and the ratio becomes unreliable. In searching for a more robust conserved marker, we chose 17α(H),21β(H)-hopane, a saturated pentacyclic biomarker, which is readily measured as the $m/z = 191$ ion in GC–MS [16]. Plotting the ratios of other analytes to hopane as the bioremediation proceeded demonstrated that the fertilizer applications stimulated biodegradation up to fivefold, both on the surface and in the subsurface [10], and this biodegradation extended to all the hydrocarbons measured, including the total GC-detectable hydrocarbons, the alkanes, and specific individual polycyclic aromatic compounds. As noted above, the success of the monitoring program after 70 days led the Federal On-Scene Coordinator to insist that fertilizer be applied to the unfertilized plots on the monitored beaches, and enhanced biodegradation then began on those plots too [10, 11].

3.4 Did the Bioremediation Protocol Lead to Any Adverse Environmental Impacts?

The principal acute concern for shoreline bioremediation is that the fertilizer would wash off the beach at the first tide and deliver a relatively concentrated dose of nitrogen in the form of ammonia and/or nitrates that would be toxic to nearshore biota. Secondary concerns included the possibility (1) that birds or mammals would come onto the exposed shoreline at low tide and have direct contact with the fertilizers, perhaps consuming the slow-release granules; (2) that increased microbial activity might produce surfactants that might make oil leave the shoreline and be transported elsewhere rather than be biodegraded in place; and/or (3) that the fertilizers washing out of the beaches might stimulate nearshore algal blooms. All of these were examined in the monitoring program by collecting water over the sampling areas at high tide ([11], Fig. 8). The potential for acute toxicity was assessed using *Mysidopsis* (now *Americamysis*) *bahia* as a surrogate for indigenous nearshore species in 96 h tests [17]. A total of 30 tests from the three beaches were run, in triplicate, and survival in undiluted seawater collected over the fertilized shorelines at high tide indicated no acute toxicity. Time-lapse photography indicated no animals visited the shorelines, at least during daylight hours, with the exception of a brief visit by a single crow. Fluorescence analysis showed no oil release from any of the shorelines, and routine monitoring of chlorophyll in nearshore waters off the fertilized beaches and remote unfertilized ones provided no evidence for any algal bloom associated with fertilizer application [11].

Fig. 8 Collecting water samples at high tide when the water over the monitoring area was about 1 m deep

4 Safety Aspects

Working in the field requires careful attention to the safety of all personnel involved. In this case, all access to the monitored shorelines was by small inflatable dinghies, which required some care and the wearing of life jackets or flotation suits. The shorelines were slippery, and care was essential, especially when carrying sampling equipment and samples in coolers. Safety glasses are essential for hammering sampling wells.

5 Concluding Remarks

The efficacy of bioremediation in Prince William Sound was demonstrated using a tiered approach designed to yield results that could be used by the Federal On-Scene Coordinator in a timely fashion to enhance oil spill cleanup. Bioremediation had support from the response community, but alternative approaches might be needed if it was not effective. Thus field measurements, and their timing, were planned to provide statistically rigorous data as soon as possible, and measurements were timed to ensure they were likely to be informative, giving natural processes time to progress. Care was taken to collect background data before fertilizer application began so that subsequent data could be put into perspective [10, 11]. After bioremediation was begun, early measurements focused on whether the surface application of fertilizer was indeed delivering nutrients to the oiled surface and subsurface

areas and that microbial activity was thereby enhanced. Oil chemistry measurements, which provide the most compelling evidence of biodegradation [9], were not expected to reveal biodegradation until at least several weeks into the program, and therefore they were not executed in the early days of the program except for analysis of the samples collected before the fertilizer was applied. Potential acute toxicity was measured as quickly as possible following treatments in case unexpected problems were occurring. The focus was on statistical tests that addressed simple comparisons of fertilized and unfertilized plots over time and allowed responders to assess whether bioremediation should continue to be a central part of cleanup operations. Only later, when all the data had been collected, could sophisticated statistical models give insight into the fundamental controlling factors of biodegradation – not surprisingly it was available nitrogen in the porewater of the shorelines [11].

The program demonstrated that the bioremediation protocol designed for Prince William Sound in 1990 was working, and the Federal On-Scene Coordinator ordered it to proceed apace. A total of 112,040 L of Inipol EAP22 were applied in 1990, along with 50.85 tons of Customblen, and a further 29,150 L of Inipol EAP22 and 4.09 tons of Customblen were applied as operations wound down in 1991 [7].

In retrospect, it is clear that shipping samples for remote measurements, such as done for the nutrient analyses here, meant that data were not available for several days. The use of simple colorimetric tests now seems adequate for such time critical/operationally important measurements [12], and data could be available before the next low tide window for subsequent operational plans. Fertilizer application rates could be thus adjusted to ensure adequate delivery of nutrients to the subsurface if initial applications had failed; reasonably sustained delivery of 100 μM biologically available nitrogen proved to be an effective goal [11, 18].

Also in retrospect, it would be worthwhile to consider monitoring CO_2 evolution directly from oiled shorelines; portable IR spectrometers available for field deployment can measure evolution in minutes [12, 19] and provide speedy confirmation of enhanced biodegradation if it is indeed occurring.

Similar tiered approaches to demonstrating the efficacy of bioremediation have been used on a small application following the 1996 Sea Empress spill [20] and on experimental spills on the Delaware Estuary [21] and on Svalbard [12]. The approach described here provides a useful model if the need to evaluate bioremediation efficacy for large-scale oil spill response arises in the future.

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Biostimulation of Marine Crude Oil Spills Using Dispersants

Roger C. Prince

Abstract

Dispersants provide a bioremediation option for oil spills at sea for both surface and subsea releases, as demonstrated in the *Deepwater Horizon* response. By decreasing the interfacial tension between oil and water, dispersants substantially reduce the amount of energy required to disperse oil as tiny droplets (<70 μm) in the water column. Such droplets are essentially neutrally buoyant, so with minimal turbulence they stay in the water column and diffuse apart rather than coalesce as a slick. Since biodegradation of minimally soluble oil components is likely a surface phenomenon, this increase in surface area leads to more rapid biodegradation. This paper describes the tests mandated by regulators to test the efficacy of dispersants for registration on National Contingency Plans and microbiological studies aiming at understanding the longer-term fate of dispersed oil. These two categories have essentially no overlap – the conditions for one set of tests are inappropriate for the other.

Keywords Corexit, Dispersants, Environmentally-relevant conditions, Regulatory tests

1 Introduction

Oil spill dispersants play an important role in oil spill response; they have been used on a large scale in many responses, notably to the 1993 *Braer* spill in Shetland Islands [1], the 1996 *Sea Empress* spill in South Wales [2], and the 2010 *Deepwater Horizon* blowout in the Gulf of Mexico [3], and substantial stocks are available around the world in case they are needed [4, 5]. Floating oil is a very real hazard to diving birds, reptiles, and mammals (e.g., [6]), and ameliorating this hazard was a primary impetus for the initial development of oil spill dispersants in the 1970s [7]. Modern dispersants are complex mixtures of anionic and neutral surfactants in a hydrocarbon solvent (e.g., [8]) that lower the interfacial tension between oil and water so that minimal wave action and turbulence can disperse the oil into tiny droplets (<70 μm) in the water column. Such droplets are essentially neutrally buoyant, so with minimal turbulence they stay in the water column and diffuse apart. Concentrations of dispersed oil may be ≈1,000 ppm in the first minutes after dispersion, but they fall to a few ppm, in a correspondingly larger volume, within hours,

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and to sub-ppm levels within a day [3, 9]. Furthermore, even these concentrations are only found in the top few meters of the sea. This diffusive dilution means that although the levels of nutrients in the sea are relatively low [10], it does not take long for oil concentrations to fall so that even those low levels are adequate for significant and rapid biodegradation. Hazen et al. [11] measured "half-lives" of *n*-alkanes of a few days in the dilute dispersed submarine plume (<1 ppm hydrocarbon) from the *Deepwater Horizon* at 1,100–1,220 m (and 5°C), and very similar results were reported for a broad array of individual hydrocarbons at low concentrations in New Jersey seawater at 8°C [12] and in Norwegian waters [13, 14]. The approximate biodegradation half-life of the total measurable hydrocarbons was 11–14 days (initial oil concentration of 2.5 ppm).

Unfortunately there is a large amount of misinformation about dispersants in the popular literature. A persistent misunderstanding involves dispersant toxicity; of course all compounds are toxic at some finite dose, and dispersants are no exception, but the acute toxicity of Corexit 9500®, the major dispersant used in the 2010 *Deepwater Horizon* response, is indistinguishable from common dish soaps and shampoos [15], including those used for cleaning oiled seabirds [16, 17]. Little is known of any potentially chronic effects of dispersants, but "all of the ingredients contained in Nalco's dispersants are safe and found in common household products, such as food, packaging, cosmetics, and household cleaners" [8]. Corexit 9500 does not contain any compounds that might act as endocrine disruptors [18].

On the other hand, dispersed oil is measurably acutely toxic, due to a general narcosis as dissolved hydrocarbons move to the lipids of exposed organisms [19]. This is undoubtedly due to the fact that in effectively dispersing the oil and thereby increasing the surface to volume ratio of the oil, more hydrocarbons become available to be toxic. The question is how significant these effects might be in the field. Canonical acute toxicity tests involve constant exposures for 48 or 96 h [20], but in a response at sea, the concentrations of oil will be dropping rapidly due to dilution by diffusion (e.g., [3, 9]).

Another misconception is that dispersants make oil sink, or, conversely, work only transiently; neither is true in oil spill response. Most oils in commerce float in seawater, including the vast majority of so-called "heavy" oils (API gravity <22.3); only "extra heavy crudes" or bitumens, with API gravity <8, will sink in seawater [21]. Clearly dispersants cannot change this density, or the fact that at equilibrium an oil will float unless it binds to dense mineral particles [22]. Dispersants decrease the interfacial tension between oil and water, and this allows even small waves to break the oil into tiny droplets and move them into the water column. Stokes' law describes the frictional forces operating on droplets, and if oil droplets are smaller than about 70 μm, the forces pushing the oil to float are not enough

to overcome the frictional inertia that keeps the droplets entrained in the water column. If droplets coalesce, their effective buoyancy increases [23], so an essential part of dispersant effectiveness is diffusion so that the droplets do not encounter each other [24]. Such diffusion is very hard to mimic in the laboratory, although it is readily achieved in large tanks such as OHMSETT, a wave tank in New Jersey that is 200 m long, 20 m wide, and 2.5 m deep [25].

Laboratory investigations with dispersants fall into two categories: short-term effectiveness tests to compare different formulations and toxicities to qualify dispersants for listing in National Contingency Plans and microbiological studies aiming at understanding the long-term fate of dispersed oil. These two categories have essentially no overlap – the conditions for one set of tests are inappropriate for the other.

2 Testing for Dispersant Effectiveness

In the USA the USEPA maintains the *National Oil and Hazardous Substances Pollution Contingency Plan Product Schedule* [26] as part of the *National Contingency Plan* and lists chemical dispersants that may be authorized for use. Dispersants on the *Product Schedule* have demonstrated effectiveness; they can disperse at least 45% of Prudhoe Bay or South Louisiana crude oil in a standard "swirling flask" test [27].

The test is relatively simple [27], but requires specific 125-mL glass Erlenmeyer flasks that have been modified to include an attachment of an external glass side spout that extends from the bottom of the flask upward to the neck region (Fig. 1a). One hundred and twenty milliliter of artificial seawater is placed in each of four replicate flasks, followed by 100 μL of oil-dispersant mixture (1 part dispersant to 10 parts oil). The flasks are then placed on an orbital shaker table and shaken at 150 rpm. After 20 min of shaking, the flask is removed and allowed to settle for 10 min. Water is then decanted using the side spout that extends to the neck; the first 2 mL are discarded, and the next 30 mL collected and analyzed for dispersed oil content. To be listed on the National Contingency Plan list [26], a dispersant must disperse at least 45% of Prudhoe Bay and South Louisiana crude oils in this test.

USEPA has recently offered a revised test for comment [28]. This test, the baffled flask test [29], replaces the modified Erlenmeyer flasks with a baffled flask with a stopcock at the bottom (Fig. 1b) and uses different oils (Alaska North Slope crude oil and an IFO-120 fuel oil). As befits a regulatory test, it has been subjected to a range of control studies, and its reproducibility has been well defined at both 5 and 25°C [30, 31]. Because this test uses slightly higher energies for dispersion and has significantly improved precision, USEPA [28] proposes a much higher "pass"

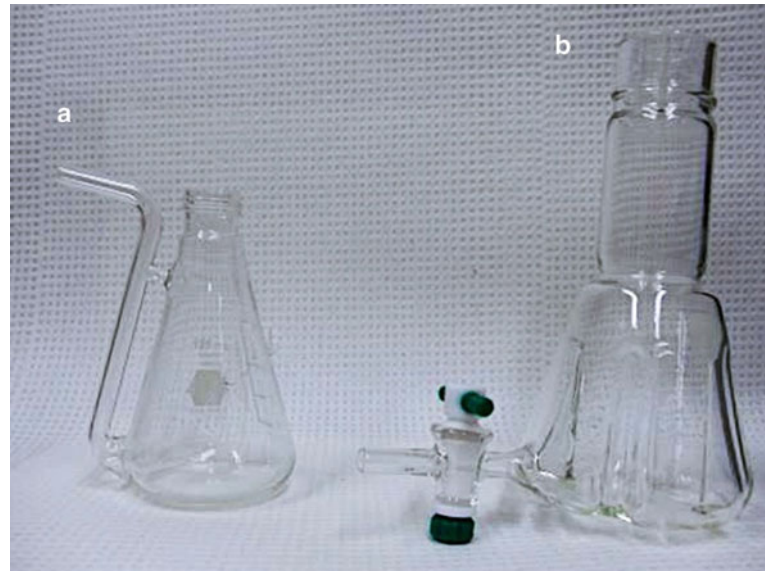

Fig. 1 "Swirling" flasks for USEPA dispersant testing. (**a**). The classic swirling flask (USEPA 2006) without baffles and with the drain tube extended to above the top of the flask. (**b**) The baffled flask of the recent USEPA proposal (USEPA 2015), with vertical baffles and a stopcock at the bottom. In use this flask sits level

mark – 70 and 75% of Alaska North Slope crude oil at 5 and 25°C and 55–65% of the IFO-120 fuel oil.

Other countries have different requirements. For example, Norway mandates that dispersants must be compared using the IFP (Institut Français du Pétrole) test [32, 33], which uses an oscillating ring immersed in seawater to generate the energy for dispersion, and the ability to disperse weathered oil be assessed using the MNS (Mackay–Nadeau–Steelman) test [34]. Both these tests require specialized equipment, and interested readers should consult the original papers for directions. It should be noted that the Norwegian regulations do not specify a "pass rate," recognizing that effectiveness will vary from one oil to another [35].

The "swirling," IFP, MNS, and baffled swirling flask tests are just four among many tests designed to discriminate between dispersants with different efficacies at the laboratory scale (Fingas et al. [36] list 35 that had been developed prior to 1989!). They do this well, but unfortunately the "passing grade" of 45% in the USEPA swirling flask test has often been assumed to indicate expected field performance. In fact the test dramatically underestimates efficacy in the field, primarily due to the amount of energy it imparts to the floating oil and the volume of seawater available for diffusion of the dispersed droplets when agitation ceases. Tests in the OHMSETT facility, a wave tank in New Jersey that is 200 m long, 20 m wide, and 2.5 m deep, routinely measure

dispersant efficiencies >95%, even at low temperatures with ice in the water [25].

3 Testing for Dispersant Toxicity

A second requirement for listing on the USEPA product schedule [26] is that the acute toxicity of the dispersant to two reference species (silverside fish, *Menidia beryllina* [96 h], and mysid shrimp, *Americamysis bahia* [48 h], [37]) be reported. These tests use "water-accommodated" and "chemically-enhanced water-accommodated fractions" [38].

As noted by the USEPA [20], Hemmer et al. [37], and Word et al. [15], the appropriate toxicity tests for registration under the *National Contingency Plan* are with dispersant alone – the toxicity of oil + dispersant is essentially another estimation of dispersant effectiveness, because in most cases [37] the toxicity emanates from the dispersed oil, not the dispersant itself. The Norwegian regulations acknowledge this [35] and only require the testing of the dispersant alone, using ISO 10253:2006 [39], an algal toxicity test. In spill response, the potential toxicity of the dispersed oil is weighed against the potential environmental impacts of non-dispersed oil in a "Net Environmental Benefit Analysis" [40] by oil spill response coordinators [41–44].

4 Testing for Enhanced Biodegradation

Although dispersants were originally developed principally to protect birds, reptiles, and mammals from floating oil slicks, it has always been recognized that increasing the oil's surface area available for microbial colonization ought to stimulate biodegradation [7]. Unfortunately this has been a tricky thing to test, and at first glance the literature appears equivocal about whether it really occurs [45]. The problem comes from the difficulty of mimicking oil spill response in the laboratory and a misunderstanding of what effects might be expected; dispersants encourage the formation of small droplets of oil in the water column, and if this happens naturally, there is little reason to expect that dispersants would have any dramatic additional effect.

While an initially dispersed oil slick may have a concentration of ≈1,000 ppm in the first minutes after dispersion, this falls to a few ppm, in a correspondingly larger volume, within hours, and to sub-ppm levels in a yet larger volume within a day [3, 9]. Microbes undoubtedly begin to respond to the dispersed oil within hours, but significant growth, and biodegradation, takes several days to become apparent [12]. Experiments aimed at understanding this biodegradation should thus work with a dilution appropriate to at

least a day after the dispersion. We have found that 2.5-ppm oil gives reliably reproducible results [12, 45], but requires 4 L of seawater per sample to allow the recovery of enough oil for GC-MS analysis. We note that at these concentrations, seawater from both Sandy Hook, NJ, and Barrow, AK, had enough nutrients for substantial biodegradation to occur – higher concentrations of oil would undoubtedly run into nutrient limitations and the unknown effects on natural microbial succession that might occur with added nutrients.

The "problem" with doing experiments at 2.5-ppm oil is that crude oils effectively disperse with or without dispersant at such a low concentration, and added dispersants have little effect on biodegradation [12, 46]. This highlights an obvious issue with oil spill response – dispersants are only used when there are substantial slicks on the sea surface or oil is emanating from a broken subsea installation. But if one tries to emulate the dispersion of a floating slick in the laboratory without adequate volume for dilution, as in the dispersant effectiveness testing described above (e.g., [29]), the dispersion is relatively short lived because of droplet coalescence, and the amount of oil is far too high for significant biodegradation to occur, even if nutrients are added.

We have developed a protocol to maintain a small amount of oil (in our case 10 μL) as a floating slick by corralling it in a small glass boom. The glass booms of Fig. 2, kept erect by a flotation collar of ethylene vinyl acetate foam, each enclose a surface area of 28 mm^2, so 10 μL of oil results in a slick of 350 μm nominal thickness, although of course there is a meniscus around the edge [47]. Oil floating as a constrained slick provides an appropriate control sample for oils dispersed with dispersant. We have had the most reproducible results by adding oil to the booms and comparing this

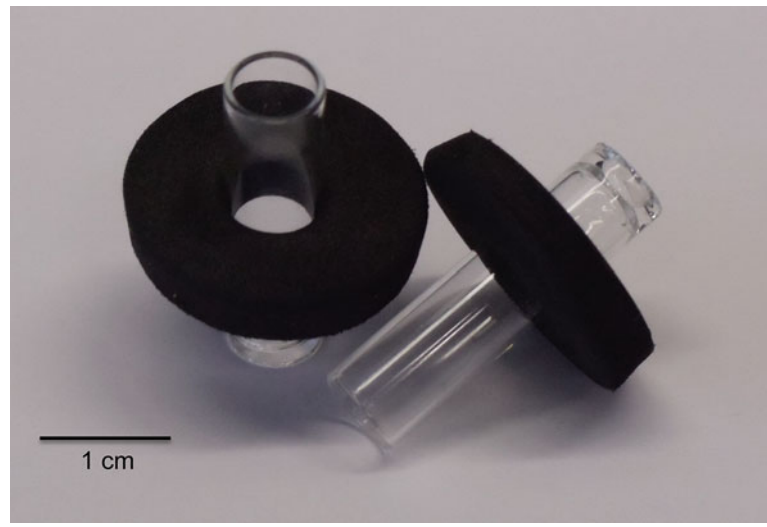

Fig. 2 Glass "booms" to maintain small volumes of oil as a floating slick. The thin glass tubing is held upright by the closed cell ethylene vinyl acetate foam

to premixed oil + dispersant added directly to the water in the dispersant flasks, although if the glass "apron" below the water surface is small enough and the vortex in the flask is aggressive enough, oil + dispersant added into the glass boom will disperse out of the boom almost completely. Under these conditions, the "half-life" of biodegradation of 2.5-ppm dispersed oil is about 7 days, while the same amount of oil constrained as a slick, with the same microbial inoculum (indigenous seawater organisms) and indigenous nutrients, had lost only 40% in 40 days [47].

5 Experimental Notes and Considerations

As discussed more fully in Prince et al. [38], it is best to use positive displacement pipettes for dispensing crude oils and dispersants, and it is good practice to check reproducibility by repeatedly dispensing small volumes onto a balance before beginning experiments. Any experiments involving solvent extraction of oils should avoid plastic equipment (except PTFE), and glass is usually best.

Experiments mandated for the registration of new dispersants are required to be done under conditions designed for specific regulatory requirements, and they have little to teach about the biological fate and effects of oil and dispersants in spill response. To address this latter need, we believe it is essential to keep environmental conditions in mind. The one most often ignored is the rapid dilution of dispersed oil that occurs after successful dispersion [48] – experiments at oil concentrations much over a few ppm, dispersant to oil ratios much higher than 1:20, or nutrient levels much above natural levels are unlikely to inform oil spill response. Dispersants are unlikely to be used on refined products such as diesel.

Another misapprehension concerns dispersants and shoreline materials. Dispersants are only applied in deep water far from shore [5], so experiments with significant amounts of dispersant or dispersed oil in contact with shoreline material or terrestrial flotsam have little environmental relevance. Dispersants are not currently registered for use in freshwater and most are optimized for marine use.

Whole and partially "weathered" crude oils contain volatile compounds, and it is important that they be stored in full glass or metal containers with PTFE seals.

6 Safety Considerations

Many fresh crude oils have flash points $<38^{\circ}C$ and are classified as flammable liquids in the USA; they require careful shipping, storage, and handling. Fortunately most biological experiments involve only small quantities of hydrocarbons, so providing stocks are kept

suitably low, and risks can be minimal with careful planning. Protective gloves and safety glasses with splash shields should be worn when handling hydrocarbons.

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Bioaugmentation

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Abstract

The ability of microorganisms to transform pollutants is well documented. However, in many cases microbial communities with the desired capabilities may develop too slowly or may not be sustained. In these cases, manipulation of the microbial composition may be advantageous. Bioremediation has been established as an environmental friendly treatment capable of improving the removal of the contaminants in natural and environmentally systems by circumventing insufficient response time and initiating the removal with a minimal lag phase. Bioremediation exploits the microbial ability to transform contaminants into less harmful compounds. Bioremediation techniques encompass natural attenuation, biostimulation, and bioaugmentation. While natural attenuation and biostimulation by indigenous microorganisms might work for certain applications, bioaugmentation using microbial populations with specialized capabilities for degrading the contaminants is often advantageous, and will be the focus of this chapter.

Bioaugmentation has been widely applied to assist bioremediation, but it has also frequently been associated with significant challenges and limited success, which is most likely due to lack of information leading to inappropriate application strategies.

Keywords: Bioaugmentation, Delivery limitations, Immobilization of bioaugmentation strains, Survival of bioaugmentation strains

1 Introduction

Bioaugmentation works by manipulating the genetic composition in order to improve the biodegradation capability. This can be accomplished through amendment of specialized microbial strains or enriched microbial consortia. The inoculated cells may be directly responsible for the degradation of the pollutant or work indirectly by supplying the indigenous population with partial degradation or important metabolites for increased activity. Understanding of these scenarios relative to non-bioaugmented controls has resulted in limited well-characterized field applications. Numerous laboratory- and demonstration-scale applications have been described and reviewed [e.g., 1]. In this chapter we will describe frequent occurring challenges associated with bioaugmentation and possible solutions.

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2 Delivery Limitations in Heterogeneous Soils

The nature and physical conditions of the contaminated environment might complicate the application of bioaugmentation. Contaminated groundwater in subsurface soil and similar porous matrices with high levels of heterogeneity pose challenges to distribution of augmented materials, whereas aqueous environments allow for better mixing and distribution of the amended cultures. Amendments to subsurface soils are usually mediated by pumping bacterial suspensions into the groundwater. Silted and clay soils with low pore size might require significant pressure to ensure a proper distribution with cells reaching microfractures and interstitial pore water. However, extensive pressure could result in cell lysis and might pose a large problem for the successful bioremediation performance.

An efficient full-scale delivery system in subsurface saturated soils employs biocurtains or permeable reactive barriers in which a row of strategically placed and closely spaced injection wells are installed in the path of the pollution plume migration [e.g., 2]. The natural flow of contaminated groundwater passes through the biocurtain, reducing the need for hydraulic controls or bioaugmentation over a large spatial area. To ensure remediation success, careful system monitoring partnered with time for system "rest" to account for diffusion limitations.

3 Parameters Limiting the Survival of Bioaugmented Bacteria

The ability of bioaugmentation cultures to survive and function over time depends on a complex interaction of multiple variables. Among the variables receiving current attention are attachment to surfaces and substrate availability.

A bacterial culture's ability to incorporate into a biofilm or to attach to a solid surface can improve survival by offering protection from harsh chemical conditions in the environment, from loss due to grazing by protozoa, and from washout when growth rates are slower than hydraulic retention times. These phenomena have been studied predominately in reactor systems, but are of universal importance for all bioaugmentation applications. A common bioaugmentation approach involves amendment of bacterial suspensions as planktonic cells. The bulk of the cells may have difficulty adsorbing to surfaces and are thus subject to washout and protozoan grazing activity. In fact, many protozoa specifically prey on laboratory culture strains used for bioaugmentation [3, 4]. Amendment together with selective inhibitory compounds such as nystatin and cycloheximide targeting rotifers and other protozoa can effectively reduce protozoan activity [5]. However, it still remains to be

demonstrated that concomitant amendment of protozoan inhibitory compounds is economically feasible on a larger scale. An alternative is to choose microorganisms capable of adsorbing to surfaces or even producing protective biofilms and thereby become inaccessible for the protozoa. Several attempts have therefore been made to improve adsorption by immobilization of the augmentation cultures, for example, by introducing a starvation period prior to augmentation to enhance cell surface hydrophobicity [e.g., 6]. Additionally, amendment of microorganisms grown as self-forming dense aggregates (granules) may improve survival. Growth in granules results in compact microbial structures with increased resistance to toxins, reduced grazing activity and, when operated with a settling step, promotes a hydraulic selection pressure supporting long-term survival in suspended systems [e.g., 7]. In aqueous systems, implementation of membrane bioreactors (MBR) might withhold the bioaugmented cells and eliminate wash out effects of planktonic cells [8]. Augmentation of aggregated consortia or cells added simultaneous with the addition of nutrients has been shown to improve the growth and activity of the microbes [9].

Immobilization improves the longevity of the process [10] as it allows the cells to settle and buffer the augmented cells against suboptimal environmental conditions, protozoa, competing indigenous microbes and viruses [4]. Immobilization of the augmentation cultures can be obtained by mixing with various carriers such as porous materials (e.g., gel beads, lignite, isolite, and charcoal) or by encapsulation in gel matrices such as agar, alginate, or polyurethane [e.g., 11]. The capsule matrix can be combined with addition of electron donors and acceptors, as well as surfactants and nutrients to improve initiating activity. Maintaining a hydrophobic cellular surface during the isolation may also improve attachment of the cells to various surfaces and thereby reduced washout [12]. Several studies have suggested that microorganisms deriving from the same ecological niche as the polluted area have better chances of surviving in the environment after augmentation [e.g., 13, 14]. This hypothesis is substantiated by the species-dependent survival among various augmentation strains observed in multiple studies [e.g., 15], and stress the importance of knowing the augmentation strains as well as the ecological niche. *See* Table 1 for delivery methods associated with bioaugmentation.

Loss of activity due to insufficient substrate availability also constitutes a problem, especially since most bioaugmentation cultures derive from highly artificial laboratory environments with high substrate availabilities. Application of bioaugmentation strains with zymogenous or low substrate affinities (high K_m) such as r-strategists with fast substrate turnover (high V_{max}) is an important feature for good augmentation cells [21]. Furthermore, the ability to rapidly shift between dormancy and active stages supports the selection of a successful bioaugmentation culture. The bacteria

Table 1
Examples of delivery methods used for bioaugmentation

Delivery method	Benefits and limitations	Example references
Direct injection into in situ soil	Minimal culture manipulation is needed; however, large portions of the injected culture may be lost due to cell lysis due to pumping pressure or to predation of unprotected cells	[2–4]
Encapsulation in alginate matrices	Provides protection from dehydration, osmotic shock, extreme temperature, and oxidative damage. Reduces activity and dispersion through porous media (e.g., soils)	[11, 16]
Biogranulation	Only works with bacteria that are capable of flocculation. Initiated by introducing starvation, which also reduces cell activity, and reduces dispersion through porous media	[7, 9, 17]
Biocurtains/biobarriers	Bacteria are incorporated into a fixed zone of soil, coined a "curtain," which is easy to replenish after depletion. Requires that groundwater flow through the "curtains"	[2]
Membrane bioreactors	Ex situ treatment. Requires sufficient growth in reactor. Increases retention time, which increases protozoan growth	[8]
Inhibition of protozoa activity	Inhibitors of the protozoa are added to reduce bioaugmentation loss due to predation. Requires injection of chemicals and might lead to resistance by the protozoa	[5, 18]
Augmentation through rhizoremediation	The root zone acts as a habitat for bioaugmentation. Only works on root colonizers, and on contaminants near the root zone	[19]
Gene bioaugmentation	Genes are augmented as DNA fragments. Degradation of DNA segments occurs and recombination efficiencies are low. Expensive	[20]

must be able to degrade the contaminant with favorable kinetics in order to result in high removal efficiencies. Augmentation cultures should be able to maintain the ability to degrade the pollutant of interest even after periods without exposure to contaminants, which could occur periodically during growth prior to or after augmentation. Augmentation cultures should also be able to grow using readily available carbon sources while maintaining the ability to degrade the pollutant [22].

Genetic modification can enhance a cell's ability to degrade contaminants. However, genetic modified organisms are susceptible to lose the genetic elements coding for the degradation ability especially when present in mobile genetic units such as plasmids. In fact, only very few examples of amendment of such modified organisms have turned out to perform better than natural and non-modified organisms (reviewed by Cases and de Lorenzo [23]). Legal regulations on releasing genetically modified organisms to the environment can limit their use. However, loss of the augmented bacteria due to the lack of knowledge about microbial

ecophysiology is an even larger barrier for application [23]. Lack of long-term survival often requires regular resupplementation, but improved understanding of the factors influencing longevity and adaptations to an ecosystem may lead to future improvements. Further study into the survival of genetically modified organisms has been initiated [1], and it has been hypothesized that a few strains possess exceptional catabolic and survival abilities which makes them better suited for bioaugmentation [24]. These microorganisms, also denoted as Heirloom superbugs, have evolved over years in laboratory transformations and possess high resistance properties and can easily be cultivated. Experiences from full-scale applications still remain to be explored.

4 Documenting Bioaugmentation Performance

Following bioaugmentation, the removal of the contaminant should be accompanied by measuring the presence and activity of the amended cells. It has been proposed to follow amendment by labeling cells by staining or gfp-labeling. This allows visual tracking of the strain in the given environment [e.g., 25, 26]. Other studies of the survival of non-native strains have typically applied qPCR or RT-(q)PCR targeting phylogenetic or functional markers [e.g., 27–29].

A more sophisticated approach to monitor the survival and activity of individual bioaugmentation strains has been demonstrated through a case study on degradation of aromatic hydrocarbons in activated sludge using *Pseudomonas monteilii* [26]. This multiphasic approach involved genome sequencing to establish highly specific qPCR and RT-qPCR tools for in situ cell enumerations and quantifications of transcripts from functional genes, stable isotope probing to follow growth on the amended target compounds, and gfp-tagging to visualize the cells directly in the sample. The study revealed that the planktonic cells were quickly washed out and only a minor part (3%) of the added cells were present after a few days. However, the remaining cells continued to actively degrade the aromatic hydrocarbons and to actively incorporate carbon into its biomass.

5 Interactions with Host Community

Bioaugmentation performance is frequently impaired by the lack of knowledge about the indigenous microbial populations and about the microbe's ability to survive in the new ecosystem. Instead, most studies have focused on the ability of the augmentation culture to degrade specific contaminants with less attention to phenotypic properties that might improve its adaptation. Understanding the

composition of the indigenous communities and how these might interact with the augmented strain will most likely provide important knowledge to further improve their survival. Empirical knowledge on the competitiveness of the amended strain relative to the receiving environment might therefore reveal which strains have greater survival abilities. Choosing the best strains should be based on both the ability to interact and survive in the environment as well as the ability to degrade specific pollutants.

6 Bioaugmentation Applications Beyond Soil and Groundwater Remediation

While bioaugmentation is mostly associated as a bioremediation strategy, it is also applicable to many other fields of environmental biotechnology. Examples of recent bioaugmentation studies shown in Table 2 reveal the wide range of current applications. Much of the recent work has focused on improved biological treatment of industrial wastewater and municipal wastewater. Industrial wastewater has its own set of treatment challenges including high organic loadings, salinity, pH, recalcitrance, and color. Here, similar to approaches used for bioremediation, bioaugmented microorganisms are isolated (or consortia are enriched) with highly specific metabolisms. The targeted contaminants can be similar to contaminants bioremediated in soil or groundwater (e.g., naphthalene [8]), but can also be highly specific to the industry producing the waste (e.g., tetrahydrofuran [36]; tannery waste [37]; pharmaceutical wastewater [38], and tobacco wastewater [39]). An important difference for industrial wastewater is that treatment generally occurs in engineered reactor systems rather than in the subsurface. However, an interesting recent development has been the use of constructed wetlands for industrial wastewater treatment. Readers are referred to the recent review on use of constructed wetlands for industrial wastewater treatment for a discussion on the role of bioaugmentation in these systems [40].

Bioaugmentation efforts for domestic municipal wastewater have focused on enhancing start-up. Because the function of the community is less specialized (i.e., removal of chemical oxygen demand and nitrogen species can be mediated by a wider range of bacteria) the approach has differed from other areas of bioaugmentation. Overcoming the challenges of start-up in cold climates, which have the particular challenges of temperature-related slower metabolic reactions, has been approached both by using cold-adapted consortia enriched for nitrogen oxidizing activity [31] and using bacterial strains isolated from cold habitats [30].

A less-studied application of bioaugmentation for improved domestic municipal wastewater treatment has focused on the emerging issue of trace-level organic compounds (TOrCs) removal. Several unique challenges exist for this treatment process. Unlike

Table 2
Examples of recent bioaugmentation studies on improved biological treatment of industrial wastewater and municipal wastewater

Application	Contaminant/scale	Augmentation culture	Conclusion	Reference
Secondary wastewater treatment (WWT)	Psychrophilic WWT at start-up Reactor study (aerobic dynamic membrane bioreactor)	Deep-sea psychrotolerant strains	Shortened start-up Improved treatment Dehydrogenase activity enhanced	[30]
Secondary wastewater treatment (WWT)	Treatment of synthetic and municipal WW with high ammonia at low temperature Sequencing Batch Reactor (SBR) study	Cold-adapted ammonia- and nitrite-oxidizing bacteria from WW	Accelerated start-up Increased nitrification in synthetic WW Minor long-term effects for municipal WW	[31]
Removal of emerging contaminants during wastewater treatment	Bisphenol A and triclosan removal during secondary (activated sludge) treatment	Continuous bioaugmentation from a side-reactor	Model prediction for improved removal with minimal impacts to other WWT function	[32]
Constructed wetlands for wastewater treatment	Domestic WW and polluted river water. Constructed wetland microcosms augmented with denitrifying bacteria	Six denitrifying bacteria isolated from rhizosphere soil	Bioaugmentation effective in short-term but should be repeated weekly	[33]
Green waste composting	Addition of nitrifying activated sludge on composting of household wastes Reactor-scale	Nitrifying sludge containing *Nitrosomonas europaea/eutropha* and *Nitrosomonas nitrosa*-like AOB	Augmented bacteria did not persist and had no effect on emissions	[34]
Green waste composting	Composting tomato plants and pine chips. Field-scale	30-Member co-culture isolated from compost	Improved composting as determined by reducing sugar content, hemicellulose, and lignin	[35]

industrial wastewater treatment, bacterial degradation activity must occur at extremely low contaminant concentrations and unlike in bioremediation in soils and groundwater, the bacteria must be able to degrade the contaminants while in a nutrient-rich habitat and in the presence of high concentrations of readily available substrates. Many bacteria have been identified with capabilities for degrading TOrCs in isolation [e.g., 41–44]. However, most have not been tested for their suitability to the conditions encountered in municipal wastewater secondary treatment. In the study by Zhou and coworkers [22] isolated bacteria were specifically targeted for their applicability to degrade TOrCs in the complex wastewater treatment habitat. Modeling suggests that routine small bioaugmentation doses have high potential for mitigating impacts from this emerging contaminant class [32].

Biogas production during anaerobic digestion is another area that has benefited from bioaugmentation. The complexity of community interactions of anaerobic communities [45], sensitivity of anaerobic digestion to perturbation [e.g., 46], and known influence of community structure on system performance [e.g., 47] make these systems ideal for bioaugmentation. Multiple examples of bioaugmentation for the initial transformation of cellulosic/lignocellulosic substrates, with a fungus [48], a *Clostridium* sp. [49], a proprietary cellulolytic bioculture [50], and a mixture of hydrolytic bacteria [51], demonstrate the potential for use of bioaugmentation to increase availability of feedstocks often considered recalcitrant in anaerobic digestion. Bioaugmentation also holds promise for overcoming ammonia inhibition – a common complication during anaerobic digestion – through addition of ammonia-oxidizing *Clostridium* sp. [52]. These reports suggest that with further study many of the complications associated with anaerobic digestion and biogas production could be tackled through use of bioaugmentation.

The challenges of bioaugmentation are often unique to the targeted habitat and treatment goal. For some bioaugmentation applications it is not necessary to sustain a stable culture in the system, and instead, it is sufficient to use pulse-dosing in response to dynamic conditions, e.g., in water treatment with irregular loadings. In such environments it is important that augmentation is followed by high activity to overcome temporal accumulations or periods with high loadings. However, in most other applications selection of the right organism for the environment is pivotal for successful bioaugmentation. Application of highly specific and sensitive molecular tools to detect and measure the performance of various strains in the environment is a critical research need and will improve our understanding of bacterial survival and adaptation. In the future, these tools, along with mathematical modeling, will provide a platform to improve the assessment of the augmentation performance and bacterial survival resulting in a wider range of bioaugmentation applications.

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The Use of Multiple Lines of Evidence to Substantiate Anaerobic BTEX Degradation in Groundwater

Janneke Wittebol and Inez Dinkla

Abstract

Aromatic compounds are nowadays still of major environmental concern. These compounds have been proven to be biodegradable under both aerobic and anaerobic conditions. Under anaerobic conditions several biodegradation pathways are proposed, but the bacteria and specific genes involved remain largely unknown. The detection of the actual biological degradation potential and expected kinetics of degradation in the field are therefore a challenge. Usually, a combination of different lines of evidence is used to determine and predict the biodegradation of BTEX under anaerobic conditions in the field. These include compound-specific monitoring of pollutants and intermediates in groundwater, laboratory degradation tests, stable isotope probing and application of BACTRAPs and/or microcosms. Each of these methods provides part but indirect evidence for the actual in situ biodegradation kinetics. Molecular monitoring of biodegradation of aromatic compounds in the field is not commonly used but can provide important additional evidence, especially when directed to target RNA.

Molecular analysis of functional genes involved, in combination with other lines of evidence, can be used to directly and accurately determine the degradation potential. The protocol described in this chapter allows for the accurate assessment of the BTEX biodegradation potential on-site following four steps:

1) Groundwater sampling using conventional, dialysis or microcosm sampling
2) Groundwater characterisation
3) Sampling protocol for molecular analyses
4) Molecular analyses of functional genes

Keywords: Anaerobic BTEX degradation, Aromatic compounds, Bioremediation, Extra line of evidence, Functional genes, Molecular tool, qPCR, Site characterisation

1 Introduction

1.1 General Introduction

Aromatic compounds are nowadays still of major environmental concern. Aromatics can be present in aquifers which are known for their anaerobic conditions [1]. These compounds have been proven to be biodegradable under both aerobic and anaerobic conditions. Under anaerobic conditions these compounds are

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highly persistent, which is in turn of great concern for industries and site owners. A fast and reliable monitoring tool is therefore necessary.

Biodegradation pathways have been investigated intensively, and several pathways are proposed [2], but the pathways, bacteria and specific genes involved remain largely unknown. There are several well-established techniques to investigate biodegradation. The issue now is that each of these methods provides part but indirect evidence for the actual in situ biodegradation kinetics.

Since biodegradation cannot be assigned to single species, molecular monitoring of functional genes in the biodegradation pathways of aromatic compounds can provide important additional evidence, especially when directed to target RNA.

1.2 Functional Genes

In the literature several functional genes have been associated with anaerobic biodegradation of aromatic compounds. These genes have been described by Lueders & Von Netzer in the chapter 'Functional Genes for Anaerobic Hydrocarbon Degrading Microbes' in more detail [3]. In short, the first step in the degradation of the hydrocarbons toluene, *o*-xylene and *p*-xylene comprises the addition of fumarate by the enzyme benzylsuccinate synthase (*see* Fig. 1) which is present in nitrate-, iron- and sulphate-reducing bacteria as well as in fermenting bacteria [4]. The enzyme ethylbenzene dehydrogenase is involved in the first step in ethylbenzene biodegradation [5, 6]. This pathway is found specifically in denitrifying and sulphate-reducing bacteria. The pathway for naphthalene degradation is proposed via octahydro-2-naphthoyl-CoA, in which the enzyme 2-naphthoyl-CoA reductase is involved [7]. Benzene on the other hand is a challenge for bacteria due to its ring structure and stability. It is assumed that a consortium of syntrophic microorganisms is involved in the biodegradation [8]. Sulphate- [9] and nitrate-reducing bacteria but also iron-reducing bacteria such as *Clostridium* have been identified to be involved in the process [6]. The functional genes benzoyl-CoA reductase and 6-oxocyclohex-1-ene-1-carbonyl-CoA hydrolase are proposed to be involved in ring dearomatisation. Both enzymes are involved in a different pathway; the first is found in

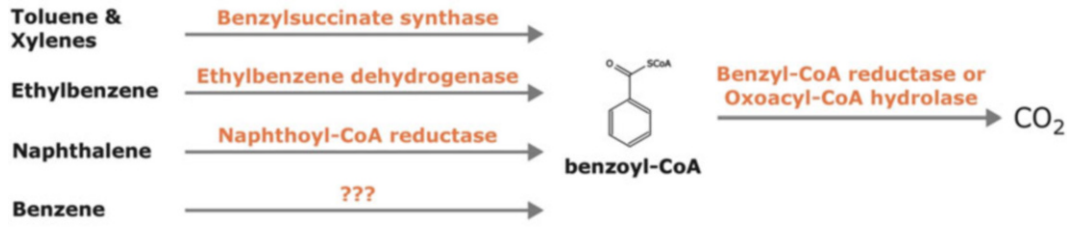

Fig. 1 Schematic view of functional genes involved in anaerobic BTEX and naphthalene degradation

facultative anaerobes such as denitrifying bacteria. The latter is present in fermenting and obligate anaerobic bacteria, such as iron- and sulphate-reducing bacteria [10]. Both methanogenic bacteria and nitrifying bacteria are detected in benzene-degrading microcosms [6, 11]. Detection of these genes in the field can be used as an additional line of evidence for the identification of biological degradation of BTEX.

The knowledge on the complete process of anaerobic BTEX degradation is still limited [12]. However, there are several tools and methods that can be used as evidence for anaerobic BTEX degradation. Usually, a combination of different lines of evidence is used to determine and predict the biodegradation of BTEX under anaerobic conditions in the field.

1.3 Current Methods to Detect the Biodegradation Potential

To investigate the feasibility of biological degradation, several lines of evidence can be applied. Three lines of evidence were set by the US EPA in 1999 [13] and are also adopted in Europe via the Water Framework Directive [14]:

- Primary line of evidence: trend analyses of historical chemical data to determine whether the contamination is shrinking, stable or expanding.
- Secondary line of evidence: interpretation of site-specific data is used to determine the likelihood of occurrence of specific biological processes. For example, if no dissolved oxygen, nitrate, iron or sulphate are present and the concentration methane is relatively high, a site can be considered highly reduced and suitable for methanogenic conversions. The occurrence of biodegradation can be determined by measuring the levels of the redox, establishing the correlation between the BTEX concentrations and the electron acceptors and reduced products. Alkalinity can be used as well to determine the produced CO_2 in situ, as a measure of the microbial activity. Analyses for specific degradation products or compound-specific stable isotopes analyses are also considered as a second line of evidence.
- Tertiary line of evidence: data from field or laboratory microcosm studies that directly demonstrate biological activity in the sediment or aquifer in relation to degradation of the compound of concern. Detection of specific microorganisms or functions is used as a third line of evidence.

Within this third line of evidence, molecular monitoring of biodegradation of aromatic compounds in the field is not commonly used but can provide important additional evidence, especially when directed to target RNA. Reluctant use of molecular techniques might be due to the lack of experienced commercial labs, lack of long-term experience with biodegradation and therefore impaired interpretation of the data, or the costs. Nowadays

research institutes and universities investigate molecular techniques to monitor biodegradation of aromatics [15, 16]. However, authorities responsible for the cleanup of sites are in need of more guidance for monitoring microbial contaminant removal such as the determination of the proposed degradation rate under specific anaerobic conditions or target genes that can be used for monitoring biodegradation (potential).

Because not all pathways and microorganisms involved are known, the literature provides only partial starting points for monitoring anaerobic BTEX [17]. Further research using metabolomics approaches could and should aid in revealing more relevant molecular targets. Even so, it is important to combine the available techniques, knowledge and experience for the best possible answer. Most of these lines of evidence do not identify specific biological degradation potentials or are indirect. The first line of evidence is based on chemical data. Too often historical chemical data of the compounds of concern are not available. Measuring intermediates formed from aromatic compounds in situ is difficult since many of the aromatic compounds but also cresols, phenylacetate and benzoate are transformed to the common intermediate benzoyl-CoA. Benzoyl-CoA can also be formed from carboxylation of phenolic compounds and possibly benzene/naphthalene or the anaerobic hydroxylation of aliphatic side chain compounds like amino acids [10]. This compound has a half-life of several hours in soil; therefore, it will not accumulate or be present after weeks or months of biodegradation. Monitoring of metabolites remains a challenge since, e.g. benzoates are only detected if initial concentrations of intermediates are higher than several hundreds of µg/l [18].

Several methods are available as secondary line of evidence such as stable isotope analyses and site characterisation. In Table 1 advantages and disadvantages from practical experience of several available techniques are represented. These techniques are based on sampling both contaminated wells and (a) reference well(s).

Isotope analyses are conducted to assess predominant biodegradation pathways in contaminated groundwater systems. Compound-specific isotope analysis (CSIA) is an appropriate quantitative method to assess contaminant biodegradation in aquifers, independent of the decrease in contaminant concentration. This method is based on the principle that ^{12}C substrates are preferentially degraded in biological processes. Heavy isotope enrichment provides evidence for biodegradation. The advantage is that only a few samples will provide enough evidence for the extent of natural attenuation within the aquifer. Sampling locations with a shift to the heavier isotope values (e.g. ^{13}C) support the assumption for natural attenuation [19, 20]. The disadvantage for field application, however, is that at the site indications for natural attenuation must

Table 1
Advantages and disadvantages from practical experience of various techniques used for detection of microbial degradation potential

Technique	Advantage	Disadvantage
Isotope analyses	Predominant degradation route is known. Signs for biodegradation are determined. Little sampling material required	Prior analyses indications for biodegradation must be assessed. Costly
Degradation tests	Site-specific, molecular analyses possible, measure metabolites. Test for enhanced remediation strategies	Period of minimum 24 weeks. Discrepancy field: lab. Soil and groundwater from location required. Not always possible due to costs/ infrastructure
BACTRAPs	Trap microorganisms of concern, compound-specific analysis. Both isotope and microbial analyses can be performed. Microorganisms attach strongly to activated carbon. Sufficient concentration to do CSIA analyses (when low in groundwater). Better detection of intermediates	Prior analyses indications for biodegradation must be assessed. Period of several months. Active absorption of compound of concern
Microcosm	No soil from location required. Location-specific conditions. Soil microbial analyses possible. 'Naturally' occurring bacterial community	Period of minimum 6 weeks. Groundwater sampled separately

be demonstrated in advance, since isotopic analysis are costly and widespread monitoring is therefore often not a feasible option. Additionally, some insight into the biodegradation processes is required. If both aerobic and anaerobic biodegradation occur for a specific compound, a distinction amongst those processes might not be possible. Even though this technique is elaborate, specific and valuable for identifying natural attenuation in situ, additional microbiological analyses might be required for better differentiation between processes [15, 21].

Detection of biodegradation metabolites like (alkyl)phenols, benzoates and benzylsuccinate might indicate the occurrence of anaerobic biodegradation. However, in case these compounds are also part of the original contamination, the increase in, for example, benzoates will not indicate biodegradation. Therefore, this might not be the optimal indicator for biodegradation of BTEX [22].

In BTEX biodegradation, anaerobic microorganisms depend on electron acceptors like nitrate or sulphate. During biodegradation, electron acceptors are reduced, thereby decreasing electron acceptor availability and increasing reductant concentrations (e.g. iron(II), sulphide, methane). Comparison of the redox conditions

from a contaminated site with an uncontaminated site can provide evidence for the occurrence of degradation through reduction processes.

For the third line of evidence, laboratory degradation tests, BACTRAPs or in situ microcosms are used which can be further complemented with molecular analyses.

Laboratory degradation tests or laboratory microcosms are degradation tests in which soil and groundwater from a contaminated site are incubated in the lab over a period of time (for anaerobic tests, for instance, 24 weeks). This technique is applied to establish site-specific data about biodegradation, biodegradation rates or accumulation of intermediates and physical/chemical influences [21, 23]. In addition molecular analysis can be performed to gain insight in the microbial processes occurring [24]. However, this is limited by the current knowledge on specific genes and microorganisms involved. The advantage is that several tests can be performed simultaneously to answer different questions. For example, the effects of nutrient additions or bioaugmentation could be investigated [18]. The disadvantages are the long time-span for incubations and the discrepancy between in situ and lab conditions. Furthermore, soil and groundwater need to be obtained from the site, which is not always possible (financially or due to infrastructure). This makes the technique, depending on the urgency and site-specific conditions in certain circumstances, unfavourable.

In situ biodegradation can be directly monitored using BACTRAPs that are loaded with contaminants labelled with stable isotopes (^{13}C). A BACTRAP® (Isodetect GmbH) is an in situ microcosm containing ^{13}C-labelled substrate in order to trap bacteria. The BACTRAPs are deployed in monitoring wells over a period of time, often several months. Similar to the isotopic analyses, knowledge on biodegradation potential is required for monitoring well selection. The advantage to sole isotopic analyses is the option for identification of microorganisms involved [16, 25, 26]. The BACTRAP material consists of activated carbon which exhibits two stable isotopes and provides an appropriate surface for colonisation by microorganisms. The activated carbon has a nonpolar surface and will preferentially adsorb nonpolar substances like benzene and tetrachloromethane. The nonpolar substances will be actively adsorbed from the environment and therefore give an overestimation of the compound concentration. As a result the microbial consortia on the polar material might give an altered representation of the natural consortia.

In situ *microcosms* are used to validate and determine the on-site biodegradation rate of aerobic and anaerobic biodegradation processes [24, 27]. A microcosm is a porous HDPE sample vial filled with (industrial) soil and placed at filter height in the monitoring well; see Fig. 2. It is known from previous research [21, 28] (Bioclear, unpublished data) that the bacteria carrying the genes

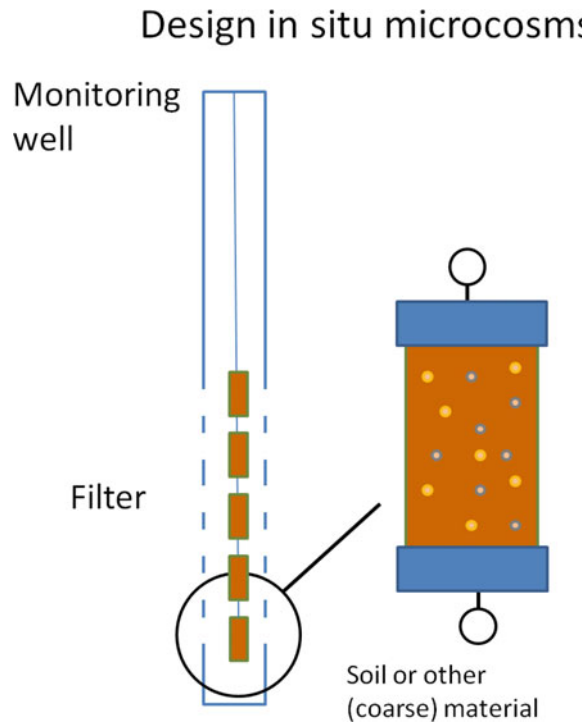

Fig. 2 Schematic view of porous HDPE microcosm sample vial in a monitoring well. The vial is filled with sand and is permanently in contact with the groundwater. The total content of the vial is used for molecular analyses

responsible for anaerobic aromatic degradation or bacterial consortia involved in anaerobic degradation under highly reducing conditions are more prone to adhere to sediment surfaces than being present in the water phase. The advantage of in situ microcosms is that industrial sand can be used, so no expensive soil samples need to be collected from the site. In addition the industrial sand is known to have adhering capacities that are similar to clay or sandy soils. The disadvantage is that the minimal period of deployment is 6—10 weeks. After this period maximal colonisation is expected/was determined [24].

1.4 Novel Tools for Site Monitoring of Aromatic Compounds

Molecular analyses such as qPCR on DNA extracts can generate quantitative information on biodegradation capacities. The DNA samples can either be obtained from the monitoring wells by traditional sampling or by a novel dialyser method [29]. The dialyser method is a sampling method that uses a relatively large ultrafiltration membrane, allowing the filtering of larger quantities of groundwater and therefore microorganisms. Backwashing of the filter allows the concentration of the organisms in a small volume. Both of these sampling methods might not be sufficient/adequate for the intended purpose since only organisms from the water phase

are included. Therefore, molecular analyses on the soil matrix in in situ microcosms can provide valuable (additional) information on the biodiversity of microbes involved. The molecular analytics used at Bioclear are based on previous work [30] and have been optimised over the last 15 years [31–33].

Analysis of DNA can provide information on the presence of specific species or (functional) genes. Analysis of RNA can also provide information on activity of (functional) genes or species. Using the qPCR method, quantitative data can be obtained about the species or genes involved in BTEX degradation. However, using qPCR you need to have upfront knowledge of the genes or species you expect to be involved.

A promising, relatively new tool to identify species or genes that are transcribed is the use of next-generation sequencing (NGS). Using NGS makes it possible to analyse all the genetic material in a sample, even though drawbacks need to be considered and investigated [34]. NGS can be performed on DNA but also on RNA, which makes it possible to show all the genes that have been actively transcribed in a sample. From this information all the genes or processes that are involved in BTEX degradation can be identified, including new yet unknown species or processes. Using the NGS data also new qPCR analysis can be developed to quantify this newly identified gene or species. An additional advantage of the NGS/qPCR combination approach is that specific qPCR analysis can be developed based on-site-/location-specific information.

All the data generated from the different techniques available gives an elaborated estimation of the degradation potential. This is a step forward to clarify the processes and microorganisms involved.

2 How to Assess the Potential?

In order to determine the biodegradation capacity on a location, several field samples have to be taken. These samples should be selected on the basis of, for example, contaminant concentrations, redox conditions, soil type and/or other contaminants. In order to maintain the intrinsic redox conditions and the corresponding microbial population, samples should be taken in an anaerobic way. To avoid irregularities, for instance, due to intrusion of air [6], or improper sample conservation, the following sampling protocol is proposed. However other methods have also been described and can also be used.

Issues considering sampling and preservation:

- Step one: groundwater sampling – conventional, dialyser or microcosm sampling
- Step two: groundwater characterisation
- Step three: sampling protocol for molecular analyses
- Step four: molecular analyses on functional genes

3 Materials

3.1 Groundwater Sampling Materials

- Dialyser (Fresenius Polysulfone® High-Flux, Fresenius Medical Care Deutschland GmbH)
- Microcosm HDPE sample vials (4 ml) and steel wires
- Sand or sediment (industrial or local) to fill the microcosm vials
- Cable ties
- Pump
- Tubing

3.2 Sampling Materials for Molecular Analysis

- Sampling containers/flasks for chemical analyses.
- Sample container for groundwater, non-groundwater or slurries: 100 ml containing + 60 ml of specific Bioclear preservation solution. But also other methods can be used for sample preservation, like cooling and freezing on-site with liquid nitrogen or ethanol.
- Sampling bottle for sediment:

  18 ml sampling containers with 10 ml preservation solution
- Sterile scalpels
- Sterile spoons
- Sterile gloves

3.3 Sampling Materials for Microcosm Analyses

- Materials microcosm: 18 ml sampling containers with 6 ml specific preservation solution. But also other methods can be used for sample preservation, like cooling and freezing on-site with liquid nitrogen or ethanol.
- Sterile tweezers.
- Sterile gloves.

4 Methods

4.1 Sampling

4.1.1 Groundwater Sampling

The standard Dutch groundwater sampling protocol (according to Dutch regulation BRL SIKB 2000 and VKB protocol 2002 'taking groundwater samples' [35]), using slow purge, can be used in any situation. However if previous results indicate that small quantities of microorganisms are expected, the dialyser method or microcosms are advised. The method for taking the reference sample should be equal to the 'contaminated' samples. With the dialyser method a large volume of groundwater is sampled and concentrated [29], thus increasing the chance of detecting specific genes/bacteria that may be present in low numbers.

Activities Groundwater Sampling

For each sample a new and clean sampling container and sampling material must be used. Furthermore it is important to take the sample as 'cleanly' as possible:

1. Wear sterile gloves.
2. Fill the sampling container completely with unfiltrated water. Do not allow the sample container to overflow to prevent dilution of the preservative.
3. Close the sampling container securely and shake the solution mildly.
4. Mark the sampling container.
5. Samples do not have to be kept cool after sampling (when using a preservation solution). Store the samples in a refrigerator if the samples are kept longer than one month. When using no preservation solution, keep samples cool during transport and storage.

4.1.2 Sediment Samples

Sediment samples could be taken with a coring tube. The air is completely replaced by the sediment, therefore maintaining reduced (anaerobic) conditions.

Activities Sediment Sampling

1. Wear the sterile gloves.
2. Take a sterile scalpel, spoon or tweezers.
3. Take a sample of the material to be analysed.
4. Place the end of the scalpel, spoon or tweezers in the sample container and remove sample by shaking in the fixative fluid.
5. Close the sampling container securely and shake the solution mildly so the entire sample is covered by the preservation solution.
6. Mark the sampling container.
7. Samples do not have to be kept cool after sampling when using a preservation solution. Store the samples in a refrigerator if the samples are kept longer than one month.

4.1.3 Microcosms

The microcosms are small containers which are placed with a steel wire in the monitoring well. The containers are attached to the wire with cable ties. The microcosm vials are placed inside the well at the filter depth and attached with the steel wire at the top of the well. The advantage of the dialyser method or the microcosms sampling is that it is cost effective. In one single monitoring round redox conditions, contaminant concentrations and biodegradation potential are determined and can provide evidence for biodegradation.

Activities Microcosms

1. Wear sterile gloves.
2. Take a pair of sterile tweezers.
3. Remove the microcosm from the steel wire by cutting the cable tie.

4. Place the microcosm upside down in the sampling container and shake container to dissolve sample in the fixative.
5. Close the sampling container securely and shake the solution mildly so that the entire sample is covered by the fixative.
6. Mark the sampling container.
7. Samples do not have to be kept cool after sampling. Store the samples in a refrigerator if the samples are kept longer than one month.

The protocol encompasses only free living microorganisms, and the presence of attached bacteria may be underestimated.

4.2 Groundwater Characterisation

During groundwater sampling an anaerobic flow through cell (Royal Eijkelkamp BV) is used to measure pH, temperature, electric conductivity, redox potential and oxygen content. In addition, samples are collected for (total) iron, nitrate, sulphate, sulphide, methane, carbon (as nonpurgeable organic carbon, NPOC) and alkalinity analysis; methods used are shown in Table 2. Care should be taken that samples are taken (and stored) in an anaerobic way before further analysis. Depending on the analysis preservation,

Table 2
Overview of sampling volumes and methods for groundwater analyses

Analyses	Method	Flask/volume	Sample	Filtration	Preservative
Methane	Gas chromatography	500 ml glass flask	200 ml	No	2 ml 2.5 M (5 N) H_2SO_4
NPOC[a]	Ultraviolet method	100 ml brown glass flask	Full	No	2 ml 2.5 M (5 N) H_2SO_4
Nitrate, sulphate	Ion chromatography analysis as described by Lovley et al. 1995 [9]	100 ml plastic tube	Full	No	–
Iron total	Mass spectrometry	50 ml plastic tube	Full	No	0.5 M HNO_3
Iron 2+[b]	Mass spectrometry	250 ml glass flask	Full	Yes	–
Sulphide (S^{2-})[b]	Spectrophotometric analysis as described by Lovley et al. 1995 [9]	250 ml glass flask	Full	Yes	–
BTEX(N)	Gas chromatography	100 ml brown glass flask	Full	No	2 ml 2.5 M (5 N) H_2SO_4
Alkalinity	Titrimetric	250 ml glass flask	Full	Yes	–

[a]NPOC: Nonpurgeable organic carbon
[b]Must be transferred to the lab within 24 h

additives are used that stop any oxidation after sampling. Fill bottle according to instructions of the labs or fill to the top, as described in Table 2.

4.3 Molecular Analysis

To safeguard the quality of the molecular analyses, samples must be taken using clean and sterile sampling material, and preferably all microbial activity should be inactivated as soon as possible to avoid RNA or DNA degradation. One can use cooling systems (e.g. using liquid nitrogen or dry ice) in the field and during transport and store samples in the fridge (4°C) or freezer (−20°C or −80°C). A more easy-to-use way is to add a preservative that inactivates the cells, for example, ethanol.

For the protocol of DNA extraction and qPCR method we refer to the chapter of Lueders and Von Netzer. In the chapter of this Handbook describing "Functional genes for anaerobic hydrocarbon degrading microbes" by Lueders & Von Netzer [3], the qPCR method is described in more detail.

We recommend the use of (mixes) of dedicated primers rather than degenerated ones and the use of very stringent conditions for primer attachment. The design of primers is crucial for a reliable quantitative result. Since the amount of known functional genes (e.g in the NCBI database) is growing every day we recommend the design to be made based on the latest information possible. Designs can be made using publically available software (e.g. NCBI primer design tool, Primer3 or ARB primer design tool) or by contracting a professional organisation.

5 Interpretation

Based on the different lines of evidence, an overall estimation can be made of the occurrence of natural attenuation. With regard to the molecular analysis, we first look at whether or not any positive qPCR results have been obtained. The numbers of target copies found differ per location; this strongly depends on the site. The number in the polluted sample should be significantly higher (10 fold or more) than in the reference samples. Otherwise the amount would only represent natural background concentration that cannot be linked to degradation of the pollutants. Positive results for multiple targets strengthen the result, whereas only single results just above the criteria are only regarded as positive evidence in conjecture with positive results on decreasing pollutant levels.

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Protocols for Harvesting a Microbial Community Directly as a Biofilm for the Remediation of Oil Sands Process Water

Joe Lemire, Marc Demeter, and Raymond J. Turner

Abstract

The prevalence of inorganic pollutants co-contaminating sites with multiple organic pollutants complicates bioremediation efforts. For this reason, new methods are needed for bioremediation of co-contaminated sites. One strategy being explored is the use of microbial community biofilms. Biofilms offer advantages in bioremediation that their planktonic counterparts don't. These advantages include: (1) the biofilm matrix provides protection from the rapid diffusion and penetration of toxins; (2) biofilms exist as a community with diverse metabolic potentials, increasing their ability to degrade a variety of xenobiotics; and (3) biofilm formation is an effective way to retain biomass in a bioreactor.

Here, we describe a robust method for harvesting and applying environmentally derived mixed-species biofilms for the remediation of contaminants – namely, naphthenic acids – from Oil sands process water (OSPW). OSPW is an alkaline mixture of clay, sand, and residual hydrocarbons. In addition, OSPW is rife with acutely and chronically toxic levels of heavy metals, polyaromatic hydrocarbons, and naphthenic acids.

Currently, we have established facile methods for harvesting a microbial mixed-species biofilm in a high-throughput device – the Calgary Biofilm Device (CBD) – and on various wastewater treatment support materials using a modified CBD. We have observed that the established biofilm can then be used to inoculate an ex situ bioreactor. To date, we have established that our biofilm-inoculated bioreactor maintains the capacity to degrade a mixture of commercially available naphthenic acids at concentrations exceeding those found in OSPW over a 30-day period.

Altogether, this chapter will provide a template for an easy and effective example of how biofilms can be used to remediate organic pollutants in co-contaminated sites.

Keywords: Biofilms, Bioreactor, Bioremediation, Naphthenic acids, Oil sands process water

1 Introduction

Biological degradation of hydrocarbon pollutants is a naturally occurring process undertaken by ubiquitous microorganisms found in soil and aquatic environments [1–4]. Two modern examples where indigenous microbial communities aided in the cleanup of large-scale hydrocarbon pollution are the Exxon Valdez (1989) and Deepwater Horizon (2010) oil spills [5–8]. Moreover, the

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innate capacity of bacteria to metabolize diverse carbon sources has demonstrated utility for the remediation of halogenated hydrocarbons [9–11], polyaromatic hydrocarbons [12, 13], naphthenic acids [14, 15], BTEX (benzene, toluene, ethylene, and xylene) [16, 17], and phthalates [18, 19]. Though much success has been realized with bioremediation of organic pollutants, most sites contaminated with them are also co-contaminated with heavy metals, which inhibit their degradation [20–22]. Hence, for bioremediation to be successful, strategies are needed to harvest a microbial community that can survive both the organic and inorganic pollutants present in a contaminated site.

A novel co-contamination challenge in wastewater remediation has been realized in the tailings of the Alberta bitumen mining process. Following the bitumen extraction process from oil sands, tailings are collected in mine tailings ponds. Oil sand processed water (OSPW) is the upper layer of the tailings found in the mine tailings ponds following the flocculation and settling of mature fine tailings [23]. As of 2011, an area covering 182 km^2 of northern Alberta, Canada, has been used to collect and store OSPW. OSPW contains a mixture of naphthenic acids (NAs) – a complex mixture of cyclic and acyclic carboxylic acids found naturally in bitumen ore [14] – polyaromatic hydrocarbons (PAHs), heavy metals, and other residual hydrocarbons [14, 23], the former of which is managed under a zero discharge policy due to its potential toxicity to mammalian and aquatic life [14, 24]. Certainly, remediation of OSPW is a multifaceted co-contamination issue. Thus, any solution needs to address these complexities. In recent years we've witnessed some varying success in bioremediating contaminants from OSPW. Phytoremediation [25], remediation using algae [26], and microbial remediation [15, 27–29] have been experimentally demonstrated to be effective methods for degrading NAs. Indeed, many studies have also demonstrated the ability of microorganisms to degrade residual hydrocarbons and PAHs and be metal tolerant [30–36]. However, no studies thus far have demonstrated the capacity for bioremediation of OSPW contaminants in the presence of both organic and inorganic pollutants.

One strategy that shows potential for bioremediating co-contaminated sites, such as OSPW, is employing a bacterial biofilm. Biofilms are – biotic or abiotic – surface-attached microorganisms that typically produce an extracellular polymeric substance (EPS) composed of proteins, sugars, and extracellular DNA. The biofilm mode of life is typically characterized by a sessile population of microorganisms that have a unique physiology, gene expression, and cellular signaling profile. For a comprehensive read on biofilms refer to reference [37]. Unlike their planktonic counterpart, the biofilm mode of life offers three main advantages: (1) the presence of an extracellular matrix – composed of polar and nonpolar polymers – slows the penetration of chemicals [38], reacts with metals

[39, 40], offers protection from mechanical stress [41, 42], and increases toxicant resistance [43]; (2) the establishment of a multispecies bacterial community allows for the co-metabolism and syntrophic metabolism of xenobiotics [44–46] as well as an enhanced resistance to toxicants [46, 47]; and (3) the biofilm environment allows for the harvesting of a more environmentally representative microbial population, a method to culture the "unculturables" [48–50]. Altogether, these properties make biofilms the ideal option for bioremediating organic xenobiotics both in situ and ex situ. It is not surprising then that biofilms have been used in both a laboratory and applied setting for bioremediation. Routinely, biofilm-based bioreactors – the biofilm fluidized bed reactor and the rotating biological disk reactor – are used for water treatment in municipal wastewater plants [51, 52]. Additional industrial utilities include using biofilms to treat ammonium-rich waters, remove heavy metals from wastewaters, and remediate volatile organic compounds [53–56]. Indeed, the lab-based, experimental capacity of biofilms to remediate a variety of soil and aquatic pollutants has been widely demonstrated.

In this chapter, we define two methods for harvesting an environmentally derived multispecies microbial biofilm for:

1. Use in a laboratory setting, where high-throughput experimental testing is needed to test the variable conditions one would encounter in a contaminated site. Specifically, we will describe our work using the Calgary Biofilm Device (CBD) to test the ability of an OSPW-derived mixed-species biofilm community to degrade commercially available NAs.
2. Use in a simple bioreactor, where an upscaled proof-of-principle analysis is needed to validate the infield bioremediation potential of the chosen microbe or microbial community. We will detail our efforts using conventional wastewater support materials to fabricate a modified CBD. The modified CBD was used to grow a mature OSPW-derived mixed-species biofilm community that was subsequently transferred to a small-volume bioreactor. This biofilm reactor also demonstrated the capacity to degrade commercially available NAs.

Altogether, this chapter will provide an overview of how to harvest a multispecies biofilm for use in the remediation of a variety of industrial wastewaters using contaminants from OSPW and NAs as a proof of concept. Our logic is that the multispecies biofilm has a wide array of metabolic potential and participates in syntrophic or co-metabolic processes to degrade the pollutant to completion, where the single-species biofilm, consisting of one microorganism, may only have the potential to degrade or modify the pollutant partially, due to a lack of metabolic potential (Fig. 1).

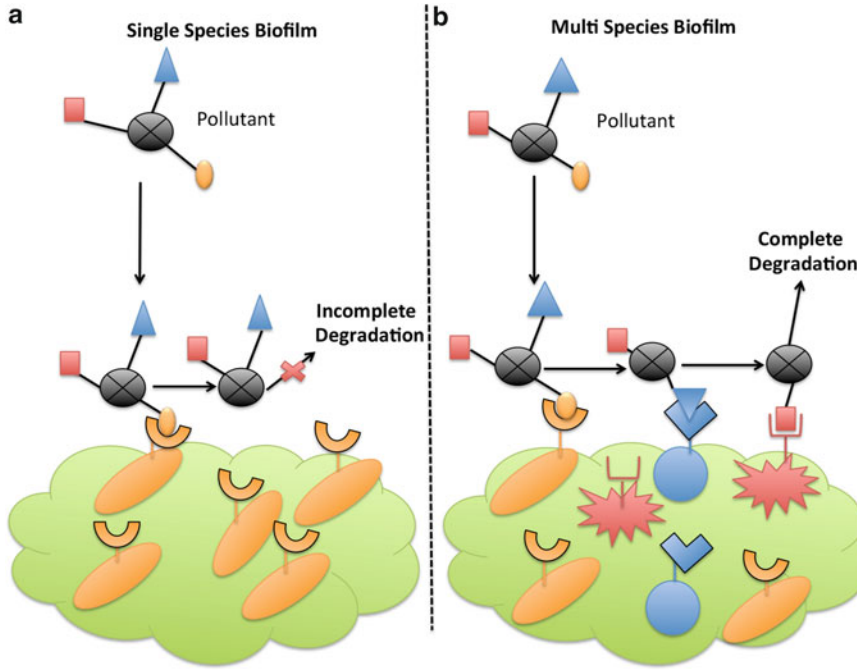

Fig. 1 The advantage of a multispecies biofilm for pollutant degradation. A pollutant with various functional groups (represented by the shapes: *triangle*, *circle*, and *square*) is introduced to (**a**) a single-species and (**b**) a multispecies biofilm. The single-species biofilm consisting of one microorganism may only have the potential to degrade or modify the pollutant partially, due to a lack of metabolic potential. On the other hand, the multispecies biofilm has a wide array of metabolic potential and participates in syntrophic or co-metabolic processes to degrade the pollutant to completion

2 Materials (*See* Note 1)

2.1 Growing a Mixed-Species Biofilm Community from an Environmental Source in the CBD

1. *The Inoculant*:
   (a) *Mixed-Species Biofilm*: An environmentally derived, mixed-species inoculant isolated from the source contaminated water (*see* **Note 2**).
   (b) *Single-Species Biofilm*: Single-species bacteria from a select number of culturable isolates (*see* **Note 3**).
2. *Growth Media*:
   (a) Trypticase Soy Broth (VWR International, https://ca.vwr.com/).
   (b) Modified Bushnell-Haas minimal salts media [1.0 g/L KH_2PO_4, 1.0 g/L Na_2HPO_4, 0.5 g/L NH_4NO_3, 0.5 g/L $(NH_4)_2SO_4$, 0.2 g/L $MnSO_4 \cdot 7H_2O$, 0.02 g/L $CaCl_2 \cdot 2H_2O$, 0.002 g/L $FeCl_3$, and 0.002 g/L $MnSO_4 \cdot 2H_2O$ at pH 7.0] (all available from Sigma-Aldrich, https://www.sigmaaldrich.com/Sigma-Aldrich/home.html).

3. *Biofilm Culturing Device*:
   (a) *Lids*: MBEC-assays™ device also referred to as the Calgary Biofilm Device (CBD) (Innovotech Inc. http://www.innovotech.ca/products_mbec.php) (*see* **Note 4**).
   (b) *Plates*: Nunclon 96-well sterile microtiter plates (VWR, https://ca.vwr.com/).

2.2 Exposing the Biofilm to the NA Mixture

1. *Naphthenic Acid Mixture*: hexanoic acid ($C_5H_{11}COOH$), cyclopentane carboxylic acid ($C_5H_9CO_2H$), cyclohexane carboxylic acid ($C_7H_{12}O_2$), *cis*-3-methyl-1-cyclohexane carboxylic acid ($CH_3C_6H_{10}CO_2H$), *trans*-3-methyl-1-cyclohexane carboxylic acid ($CH_3C_6H_{10}CO_2H$), cyclohexane acetic acid ($C_6H_{11}CH_2CO_2H$), decanoic acid [$CH_3(CH_2)_8COOH$], cyclohexane butyric acid [$C_6H_{11}(CH_2)_3CO_2H$], and 1-adamantane carboxylic acid ($C_{11}H_{16}O_2$). All obtained from Sigma-Aldrich, https://www.sigmaaldrich.com/Sigma-Aldrich/home.html.
2. *Sterile Reagent Reservoirs*: Corning, 100 mL sterile reagent reservoirs (VWR international, https://ca.vwr.com/).

2.3 Evaluating the Growth of the Biofilm by Confocal Laser Scanning Microscopy

1. 0.9% *Isotonic Saline*: Sodium chloride (NaCl) (Sigma-Aldrich, https://www.sigmaaldrich.com/Sigma-Aldrich/home.html).
2. *Microscopy Fixative*: Electron microscopy grade, 25% glutaraldehyde (Cedarlane, https://www.cedarlanelabs.com/).
3. *Nucleic Acid Dye*: Syto® Red 62 nucleic acid stain (Invitrogen™ Life Technologies, http://www.lifetechnologies.com/ca/en/home/brands/invitrogen.html).

2.4 Semiquantitative Analysis of Biofilm and Planktonic Cell Growth Using qPCR

1. 0.9% *Isotonic Saline*: Sodium chloride (NaCl) (Sigma-Aldrich, https://www.sigmaaldrich.com/Sigma-Aldrich/home.html).
2. *Genomic DNA Isolation Kit*: FastDNA® Spin Kit for soil and FastDNA® Spin Kit (MP Biomedical, http://www.mpbio.com/).
3. *Microfuge Tubes*: Eppendorf 1.5 mL microcentrifuge tubes (VWR International, https://ca.vwr.com/).
4. *Lysozyme*: Molecular biology grade lysozyme in a lyophilized powder (Sigma-Aldrich, https://www.sigmaaldrich.com/Sigma-Aldrich/home.html).
5. *Proteinase K*: Molecular biology grade proteinase K from *Tritirachium album* as a lyophilized powder (Sigma-Aldrich, https://www.sigmaaldrich.com/Sigma-Aldrich/home.html).
6. *Primers*: For 16S rDNA amplification, 926f (5°-AAACTYAAAKGAATTGRCGG-3°) and 1392r (5°-ACGGGCGGTGTGTRC-3°).
7. *SYBR Green Mix*: IQ™ SYBR® Green Supermix (Bio-Rad, http://www.bio-rad.com/en-us/sku/170-8880-iq-sybr-green-supermix).

2.5 Quantifying Viable Planktonic and Biofilm Cells by Spot Plating

1. 0.9% *Isotonic Saline*: Sodium chloride (NaCl) (Sigma-Aldrich, https://www.sigmaaldrich.com/Sigma-Aldrich/home.html).
2. *Media*: Lysogeny Broth or Luria-Bertani Broth (VWR International, https://ca.vwr.com/). To prepare the solid media, 1.0% Bacto agar is added to the solution. The media solution is then autoclaved and poured into petri dishes.
3. *Tween®-20 Detergent*: Tween®-20 viscous liquid (Sigma-Aldrich, https://www.sigmaaldrich.com/Sigma-Aldrich/home.html).
4. Universal Neutralizer: 1.0 g/L of cysteine, 1.0 g/L L-histidine, and 2.0 g/L of reduced glutathione (Sigma-Aldrich, https://www.sigmaaldrich.com/Sigma-Aldrich/home.html).
5. *Petri Dishes*: VWR, 100 × 15 mm, semi-stackable petri dishes (VWR international, https://ca.vwr.com/).

2.6 Quantifying the Extracellular Polymeric Substance (EPS) Portion of the Biofilm

1. *Crystal Violet Dye*: Crystal violet, Hardy Diagnostics (VWR international, https://ca.vwr.com/).
2. *Methanol*: 99.8% anhydrous methanol (Sigma-Aldrich, https://www.sigmaaldrich.com/Sigma-Aldrich/home.html).
3. *Plates*: Nunclon 96-well sterile microtiter plates (VWR, https://ca.vwr.com/).

2.7 NA Degradation Analysis

1. *Microfuge Tubes*: Eppendorf 1.5 mL microcentrifuge tubes (VWR International, https://ca.vwr.com/).
2. Internal Standard for GC-FID: 99% 4-phenyl butyric acid (PBA) (Sigma-Aldrich, https://www.sigmaaldrich.com/Sigma-Aldrich/home.html).
3. *Glass Vials*: Borosilicate 2 dram vials with poly-seal screw cap lids (VWR International, https://ca.vwr.com/).
4. *Dichloromethane*: 99.8% anhydrous dichloromethane (Sigma-Aldrich, https://www.sigmaaldrich.com/Sigma-Aldrich/home.html).
5. *Phase Separation Filter Paper*: Whatman, water-repellent phase-separating paper (VWR International, https://ca.vwr.com/).
6. *Chromatography Vials*: Agilent autosampler screw top 1.5 mL vials (VWR, https://ca.vwr.com/).
7. *Derivatization Reagent*: 99% N,O – bis(trimethylsilyl)trifluoroacetamide (Sigma-Aldrich, https://www.sigmaaldrich.com/Sigma-Aldrich/home.html).

2.8 Growth of an Environmentally Sourced Mixed-Species Biofilm Community on Wastewater Support Materials in a Modified CBD

1. *Wastewater Support Materials* ("*Grapes*"): K1, K3, K4, and K5 polyethylene terephthalate (PET) plastic wastewater supports (Veolia Water Solutions, http://veoliawatertechnologies.com/en/).
2. *Microtiter Plates*: Falcon®, 6- and 24-well sterile multiwell plates with lids, Corning (VWR, https://ca.vwr.com/) (*see* **Note 5**).
3. *Growth Media*:
   (a) Trypticase Soy Broth (VWR International, https://ca.vwr.com/).
   (b) Bushnell-Haas minimal salts media (*see* Sect 2.1).

2.9 Evaluating the Growth of Biofilm and Planktonic Populations in the Modified CBD

1. 0.9% *Isotonic Saline*: Sodium chloride (NaCl) [Sigma-Aldrich, https://www.sigmaaldrich.com/Sigma-Aldrich/home.html].
2. *Tween®-20 Detergent*: Tween®-20 viscous liquid (Sigma-Aldrich, https://www.sigmaaldrich.com/Sigma-Aldrich/home.html).
3. *Conical Tubes*: 15 mL Falcon® sterile, polystyrene centrifuge tube, Corning (VWR, https://ca.vwr.com/).
4. *Cell Storage Buffer*: 50 mM Tris–HCl, 1 mM phenylmethylsulphonylfluoride, 1 mM dithiothreitol, and 250 mM sucrose. All available from Sigma-Aldrich, https://www.sigmaaldrich.com/Sigma-Aldrich/home.html.
5. *Plates*: Nunclon 96-well sterile microtiter plates (VWR, https://ca.vwr.com/).
6. *Bradford Reagent*: Bradford reagent dye (Bio-Rad, http://www.bio-rad.com/).
7. *Bovine Serum Albumin*: 98% crystallized, lyophilized powder BSA (Sigma-Aldrich, https://www.sigmaaldrich.com/Sigma-Aldrich/home.html).

2.10 Transferring the Biofilm from the Modified CBD to a Bioreactor

1. *Wastewater*: OSPW from an oil sand tailings pond (Suncor Pond 6).
2. *Wastewater Support Materials* ("*Grapes*"): K1, K3, K4, and K5 polyethylene terephthalate (PET) plastic wastewater supports (Veolia Water Solutions, http://veoliawatertechnologies.com/en/).
3. *Model Bioreactor*: 3 L round-bottom borosilicate flasks.

2.11 Evaluating the Growth of the Biofilm by Scanning Electron Microscopy

1. 0.9% *Isotonic Saline*: Sodium chloride (NaCl) [Sigma-Aldrich, https://www.sigmaaldrich.com/Sigma-Aldrich/home.html].
2. *Microscopy Fixative*: 2.5% glutaraldehyde in sodium cacodylate buffer (Cedarlane, https://www.cedarlanelabs.com/).

3. 0.1 M *Cacodylate*: Sodium cacodylate trihydrate (Cedarlane, https://www.cedarlanelabs.com/).
4. 70% *Ethanol*: 99% anhydrous ethanol (Sigma-Aldrich, https://www.sigmaaldrich.com/Sigma-Aldrich/home.html).
5. *SEM Mounts*: Aluminum mounts, 9.6 × 3.1 mm (Cedarlane, https://www.cedarlanelabs.com/).

2.12 Exposing the Biofilm-Inoculated Bioreactor to Commercially Available NAs

See sections 2.1 and 2.2.

2.13 NA Degradation Analysis

See section 2.7.

3 Methods

The following protocol provides a descriptive method for growing a mixed-species bacterial community, as a biofilm, in the CBD for high-throughput testing (Sect. 3.1) and on wastewater support materials for utility in a bioreactor (Sect. 3.2). The establishment of a biofilm is pertinent for OSPW remediation because of their increased resistance to heavy metals and synergistic metabolic capabilities [14]. In addition, a biofilm is an effective method of retaining microbial biomass. The procedure will detail how to inoculate a biofilm and assess growth and biofilm production in different media conditions. In addition, this procedure will outline how to test the effectiveness of the biofilm system at bioremediating xenobiotics – in this case commercially available NAs.

3.1 Harnessing a Mixed-Species Biofilm for Toxin Degradation Using the Calgary Biofilm Device

3.1.1 Growing a Mixed-Species Biofilm Community from an Environmental Source in the CBD

Biofilms can be harvested from an environmental source as a mixed-species or as single-species isolates (Fig. 2) (*see* **Notes 2** and **3**).

1. Inoculate a multispecies biofilm by pipetting a 1:1 mixture of OSPW: Twofold concentrated tryptic soy broth (TSB) or Bushnell-Haas (BH) minimal salts media (*see* **Note 6**) to each well of the CBD received to a final volume of 150 µL [14]. A single-species (*see* **Note 3**) biofilm was inoculated by pipetting a 1:30 dilution of a 1.0 McFarland standard (3.0×10^8 CFU/mL) into 150 µL of BH media into each well of the CBD [14].
2. Place the inoculated CBD at 25°C on a gyro rotary shaker at 125 rpm under aerobic conditions for 2 days to establish the biofilm (*see* **Note 7**).

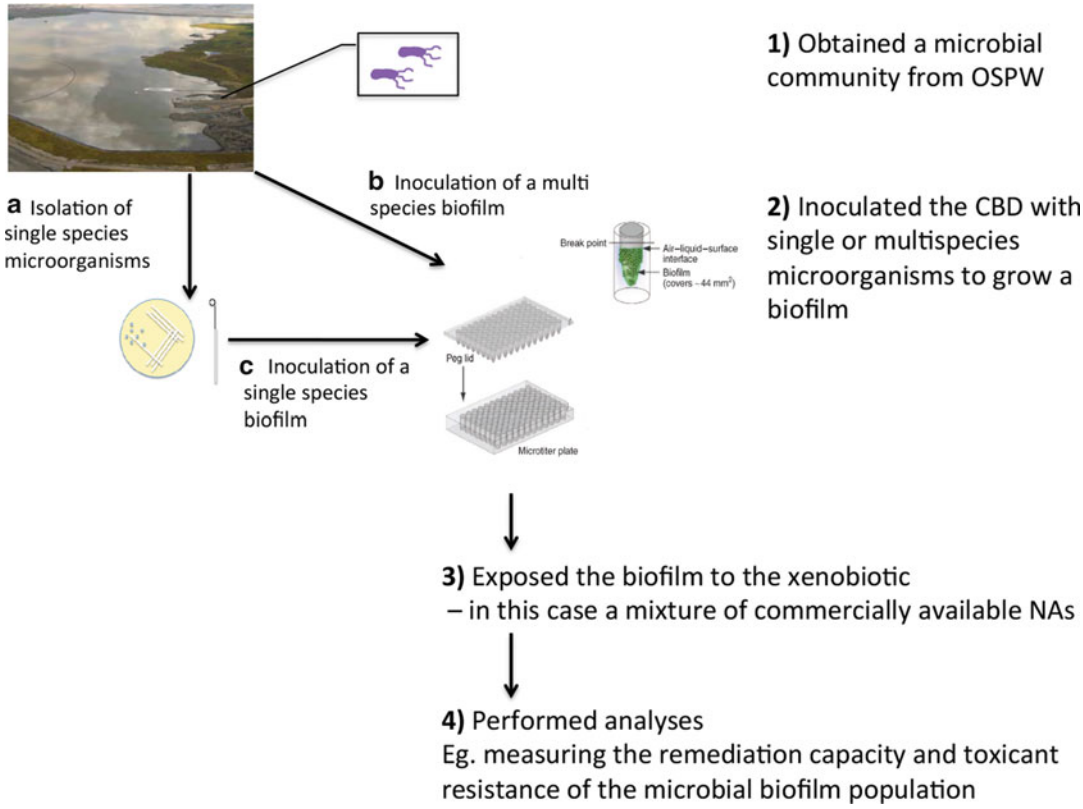

Fig. 2 Harvesting a microbial biofilm from an environmental source. *1*: microorganisms were isolated from an oil sand processed water (OSPW) source. *2*: (*A*) single-species microorganisms were isolated using conventional microbiology techniques. (*B*) Multispecies and (*C*) single-species biofilms were cultured using the Calgary Biofilm Device (CBD). *3*: The cultured biofilms were then exposed to xenobiotics – naphthenic acids (NAs). *4*: Various analyses were then performed to monitor the remediation efficacy or pollutant resistance of the microbial biofilms

3.1.2 Exposing the Biofilm to the NA Mixture

The following procedure outlines the exposure of biofilms to commercially available NAs. However, a similar process could conceivably be used to test the biofilm remediation capacity for a variety of natural organic and xenobiotic pollutants.

1. Prepare a 10× stock of a 200 mg/L mixture of eight commercially available NAs as sodium salt naphthenates in distilled and deionized water (ddH_2O) at an equimolar concentration of 1.7 mM for each NA (*see* **Notes 8** and **9**).
2. Add 200 µL of the 10× NA into 1,800 µL of media and place into a sterile reagent reservoir.
3. To expose the biofilms to the NA challenge, place 150 µL of the NA mixture into each well of the CBD that contained a biofilm from Sect. 3.1.1. The exposure concentration of

NAs – 200 mg/L – is above the levels found in OSPW (40–120 mg/L) [57].

4. Incubate the NA-exposed biofilms for 14 days at 25°C on a gyrorotary shaker set at 125 rpm.

3.1.3 Evaluating the Growth of the Biofilm by Confocal Laser Scanning Microscopy

The biofilm was both qualified and quantified following exposure to NA by performing confocal laser scanning microscopy (CLSM). This step is performed to confirm growth of a biofilm and to monitor how it is affected by NA exposure [50, 58].

1. Rinse the planktonic cells from the CBD pegs using 200 μL of 0.9% saline.
2. Place the pegs in 200 μL of 5% glutaraldehyde for 30 min to fix the biofilm.
3. Stain the biofilms with 10 μM Syto® Red 62 nucleic acid stain for 15 min [50].
4. Visualize the biofilm via CLSM (Leica DM IRE2; Leica, USA) using a Texas Red filter (excitation 550 nm, emission 600 nm) with a 64× water immersion objective.

3.1.4 Semiquantitative Analysis of Biofilm and Planktonic Cell Growth Using qPCR

As a semiquantitative measure of microbial growth, quantitative polymerase chain reaction (qPCR) for 16S rDNA was performed on the biofilm and planktonic bacterial populations following their exposure to the NAs [14].

Biofilm Cells

1. Following the NA exposure period, rinse the peg lid of the CBD twice in 200 μL of 0.9% saline for 1 min.
2. Remove two identical pegs per sample – aseptically with sterilized pliers – into the Lysing Matrix E tube from a FastDNA® Spin Kit for Soil from MPbio.
3. Subject the biofilm-containing pegs to a bead-beating extraction method with a FastDNA® Spin Kit for Soil following the manufacturers' instructions [14, 47, 50].

Planktonic Cells

1. Following the NA exposure, 1.2 mL of media (eight wells or eight replicates) is combined into a 1.5 mL microcentrifuge tube and centrifuged for 10 min at 5,000*g*.
2. Decant the supernatant and remove excess media using a 200 μL pipette.
3. Add 200 μL of lysozyme (20 mg/mL) to each cell pellet and incubate on a shaker at 37°C for 30 min.

4. Concurrently, pipette 20 μL of proteinase K and 880 μL of CLS-TC buffer onto one lysing matrix A (per sample) from an MP Bio FastDNA® Spin Kit.
5. Vortex the cell pellet to resuspend any precipitated cells and pipette each sample onto a lysing matrix A tube setup in **step 4**.
6. Subject the planktonic cells to a bead-beating extraction method with a FastDNA® Spin Kit following the manufacturers' instructions [14, 47].

Genomic DNA from Biofilms and Planktonic Cells

1. Suspend the isolated genomic DNA from the biofilm and planktonic samples in 100 μL of DNase- and pyrogen-free H_2O. This can be stored at −20°C until needed.
2. Make a qPCR master mix (per sample) consisting of 0.5 μL each of the 16S rDNA primers [forward = 926 (20pmol/μL), reverse = 1392r (20pmol/μL)], 9 μL molecular grade H_2O, and 12.5 μL of IQ™ SYBR® Green super mix from Bio-Rad (*see* **Note 10**).
3. Add 2.5 μL of genomic DNA to 22.5 μL of qPCR master mix.
4. Run the qPCR using a Rotor-Gene qPCR thermocycler with the following cycle conditions: (a) 95°C for 5 min, (b) 95°C for 30 s, (c) 55°C for 45 s, and (d) 72°C for 90s × 40 cycles.
5. Resolve the gene copy number per peg or well of the CBD for biofilms and planktonic populations using the formula: $X =$ [(gene copy number) · (vol. DNA extracted (μL))]/[(2.5 μL DNA per rxn) · (2 pegs or 1,800 μL)] [50, 59].

3.1.5 Quantifying Viable Planktonic and Biofilm Cells by Spot Plating

Quantification using qPCR provides an indication of the number of total cells in the biofilm and planktonic populations – alive and dead. To quantify the number of viable cells, the biofilm and planktonic populations are recovered and spotted onto rich media agar.

Biofilm Recovery

1. Following the exposure to NA, wash the pegs of the CBD twice by placing the lid into a 96-well plate containing 200 μL of 0.9% saline for 1 min.
2. Remove the CBD lid from the rinse plate and place it in a 96-well plate containing 196 μL of Lysogeny Broth (LB), 0.1% Tween 20, and 4 μL of universal neutralizer (UN).
3. Sonicate the biofilms off the pegs using a model 250 T sonicator (VWR international) for 10 min.
4. Pipette 75 μL of each row of the sonication plate into the first row of a new 96 well (per row of the CBD).
5. Serially dilute 20 μL of the sonicated biofilm media into 180 μL of 0.9% saline.

Planktonic Recovery

1. Following the exposure to NA, pipette 40 μL of the media from each row of wells from the CBD into the first row of a new 96 well (per row of the CBD) containing 9 μL of saline and 1 μL of UN.
2. Serially dilute 20 μL of the planktonic cell media into 180 μL of 0.9% saline.

Spot Plating the Biofilm and Planktonic Cells

1. Pipette a 20 μL spot of each serially diluted bacterial cell mixture onto LB agar plates [50, 60, 61].
2. Incubate the plates for 24 h at 21°C.
3. Perform colony counting to determine the number of viable planktonic and biofilm cells.

3.1.6 Quantifying the Extracellular Polymeric Substance (EPS) Portion of the Biofilm

In addition to determining the amount of cells in the biofilm, it is informative to quantify the amount of EPS present. A facile method for determining the quantity of EPS in the biofilm is using an adapted version of the crystal violet method developed by the O'Toole research group [62].

1. Rinse the pegs of the CBD in 200 μL of ddH_2O twice to remove nonadherent biomass.
2. Place the peg lid from the CBD into a 1% crystal violet solution for 10–15 min at room temperature.
3. Rinse the peg lid 3–4 times in 200 μL of ddH_2O to remove excess crystal violet.
4. Let the plate lid dry for 3 h or overnight.
5. Place the peg lid into 200 μL of 100% methanol in a 96-well plate for 15 min to solubilize the crystal violet stain.
6. Transfer 125 μL of the solubilized crystal violet solution into a new 96-well microtiter dish.
7. Measure the absorbance at 550 nm using a microtiter plate reader (PerkinElmer EnSpire®) using 100% methanol as the blank, to quantify relative biomass compared to an uninoculated peg.

3.1.7 NA Degradation Analysis

This section describes the procedure for monitoring the capacity of the biofilm population to degrade NAs. NAs are separated via an organic extraction protocol and subsequently injected into a gas chromatographer coupled to a flame ionization detector (GC-FID) (*see* **Note 11**). The samples chosen for analysis can be extracted daily or following a given incubation (e.g., 14 or 30 days).

1. Following the incubation period with the NAS, remove eight identical microtiter wells or 1.2 mL of spent media from the CBD and place it into a 1.5 mL microcentrifuge tube.

2. Add 100 μL of a previously prepared internal standard of 4-phenyl butyric acid (PBA) at a concentration of 1.3 g/L into each 1.5 mL sample microcentrifuge tube (*see* **Note 12**).
3. Acidify the samples to a pH of 2 with 5.2 M HCl to reduce the naphthenates to naphthenic acids – decreasing their polarity.
4. Transfer the acidified solutions into 2 dram glass vial with Teflon-lined lids.
5. Add two volumes of dichloromethane to generate a phase separation.
6. Collect the organic phase containing the NAs by filtering the solution through phase-separating filter paper; thoroughly rinse the glass vial and filter paper with dichloromethane.
7. Condense the organic phase using a rotary evaporator.
8. Remove the condensed organic phase with a Pasteur pipette into 1.5 mL Agilent autosampler chromatography vials.
9. Derivatize the carboxyl group on the NAs with trimethylsilylates by adding 150 μL of N,O-bis(trimethylsilyl) trifluoroacetamide (BSFTA) using a 1 mL syringe with a slip-tip needle [14] (*see* **Note 13**).
10. Incubate the vials in a water bath at 60°C for 10 min.
11. Load the samples into the autosampler of a GC-FID.
12. Use the following parameters to detect NAs: an Agilent HP-5 30 m column, operating with an injection volume of 4 μL, an injector split ratio of 2:1, and the following oven temperature program, 2 min at 70°C and followed by an increase at a ramp rate of 5°C/min until reaching 230°C (held for 2 min).
13. Determine the NA abundance and quantity of degradation following the incubation period relative to the initial concentration using the Agilent ChemStation data analysis software package.

3.2 Transferring a Mixed-Species Biofilm Community to Biofilm Supports for Wastewater Remediation

Where Sect. 3.1 focused on the cultivation of a biofilm for high-throughput work, Sect. 3.2 aims to detail the procedure for harnessing an environmentally derived biofilm on wastewater support materials for use in a bioreactor. The procedure follows the harnessing of a mature biofilm on wastewater support materials in a modified CBD. The wastewater support containing the established, mature biofilm was then transferred to a bioreactor where it successfully founded new biofilm colonies on sterile supports.

3.2.1 Growth of an Environmentally Sourced Mixed-Species Biofilm Community on Wastewater Support Materials in a Modified CBD

1. Fix K1 (Fig. 3) wastewater support materials ("grapes") onto 24-well microtiter plates using hot glue to form a modified CBD.
2. Fix K3, K4, and K5 (Fig. 3) grapes onto 6-well plates using hot glue to form a modified CBD.

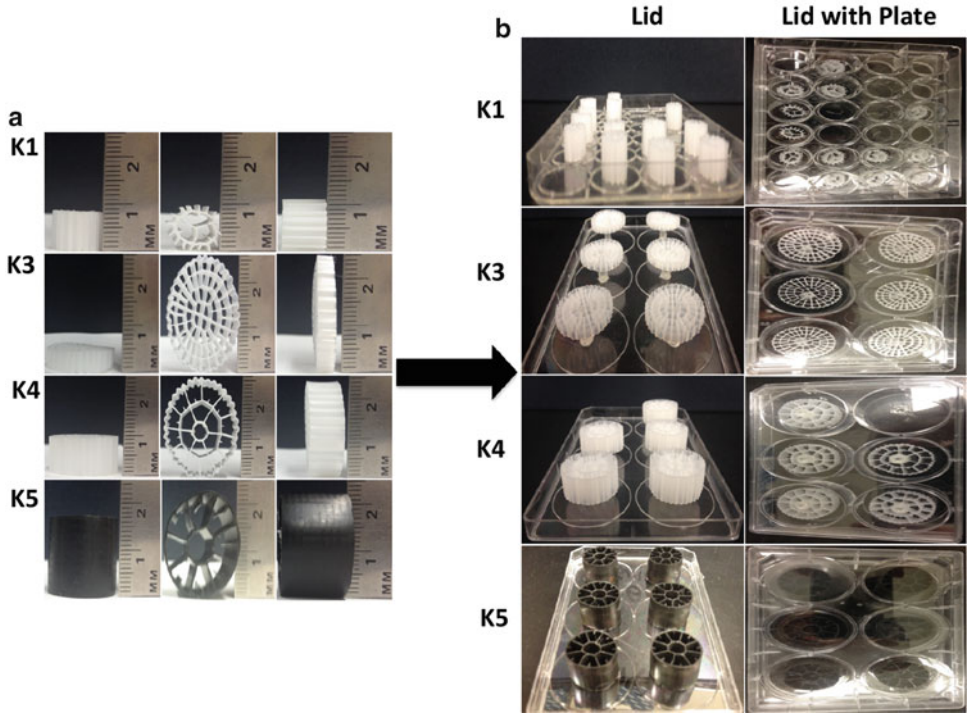

Fig. 3 Fabricating a modified Calgary Biofilm Device from conventional wastewater support materials. (**a**) K1 (0.8 × 1.0 × 1.1 cm), K3 (0.5 × 2.6 × 2.5 cm), K4 (1.0 × 2.5 × 2.4 cm), and K5 (1.7 × 2.3 × 2.2 cm) polyethylene terephthalate plastic support materials or "grapes" from Veolia Waters were fixed to the lid of (**b**) 24-well (K1) and 6-well (K3, K4, and K5) plates. **Note*: the K-generation nomenclature is assigned by the company – Veolia Waters

3. Inoculate the modified CBD similarly to the CBD (Sect. 3.1.1) by pipetting a 1:1 mixture of OSPW – twofold concentrated TSB or BH minimal salts media (*see* **Note 6**).
4. Place the inoculated modified CBD at 21°C on a gyro rotary shaker at 125 rpm under aerobic conditions for up to 14 days to establish the biofilm (*see* **Notes 7** and **14**).

3.2.2 Evaluating the Growth of Biofilm and Planktonic Populations in the Modified CBD

For optimal transfer of the biofilm population from a grape grown in the CBD to other sterile grapes in a bioreactor, it was important to establish when the biofilm reached maturity – its stationary growth phase. A rapid, easy method measured protein production via a modified protein assay [63].

1. Remove a grape from the CBD at various time points (e.g., daily) and place it in a minimal volume of 0.9% saline with 0.1% Tween 20.
2. Sonicate the biofilm from the grape using a model 250 T sonicator (VWR international) for 30 min.

3. Place the sonicated media containing the biofilm cells into a 15 mL conical tube.
4. Extract 2 mL of media from the wells of the modified CBD to obtain planktonic cells and place it in a 15 mL conical tube.
5. Pellet the biofilm and planktonic cells for 10 min at 10,000 g in a Thermo Scientific Sorvall Legend RT centrifuge.
6. Resuspend the cell pellet in a minimal volume (100 μL) of cell storage buffer.
7. The resuspended cells can then be placed at −20°C for a period of 1 month.
8. Boil the samples for 10 min to solubilize the proteins (*see* **Note 15**).
9. Prepare your Bradford protein assay in a 96-well plate by adding, in triplicate, (a) 20 μL of Bradford reagent (prepared according to the manufacturers' recommendations) into each well that will receive sample and (b) 2 μL of the protein sample and (c) adjust volume to 100 μL with ddH_2O. The samples will be compared to a standard curve from 1 to 100 μg of bovine serum albumin (200 μg/mL stock).
10. Gently aspirate the samples with a multichannel pipettor to mix the solution – being careful to avoid bubble formation.
11. Allow 5 min for the Bradford reagent to bind to the protein.
12. Measure the absorbance at 595 nm using a PerkinElmer plate reader.
13. Determine protein concentration in the samples using the standard curve and incorporating all dilution steps.

3.2.3 Transferring the Biofilm from the Modified CBD to a Bioreactor

This section describes the procedure for transferring the established biofilm population grown on the grapes in the CBD to other sterile grapes in a bioreactor. The following protocol also details how to measure transfer rates from the inoculant grapes to the sterile grapes.

1. Prepare the bioreactor, in this case a 3 L round-bottom flask, by filling it with 1 L of autoclaved wastewater. For this procedure the OSPW was the wastewater used.
2. Place 20 sterile grapes into the bioreactor containing the wastewater (*see* **Note 16**).
3. At the stationary phase of growth of the biofilm on the grape (when a mature biofilm has grown) in the CBD, remove the grape from the modified CBD using sterile pliers.
4. Carefully place the grape containing the biofilm into the sterile bioreactor.

5. Place the inoculated bioreactor(s) into a New Brunswick gyro-rotary incubator at 125 rpm and 21°C.
6. At various time points, grapes and media can be removed from the bioreactor to sample transfer efficiency (measured by protein production) from the inoculant grape to the sterile ones and planktonic growth in the wastewater using the Bradford protein assay as outlined in Sect. 3.2.2.

3.2.4 Evaluating the Growth of the Biofilm by Confocal Laser Scanning Microscopy and Scanning Electron Microscopy

Again, the biofilm was both qualified and quantified during its growth in the modified CBD and following the inoculation process of the bioreactor, using confocal laser scanning microscopy (CLSM) and scanning electron microscopy (SEM). This step is performed to confirm growth of a biofilm on the grapes and to monitor how it is affected during incubation in the bioreactor.

CLSM

1. *See* Sect. 3.1.3 and follow the outlined procedure with the exception that a volume of 2 mL is needed for all reagents to accommodate the larger size of the grapes.

SEM

1. Remove the grapes using sterile pliers and rinse it in 2 mL of sterile 0.9% saline.
2. Fix the biofilm to the grapes by placing it in 2 mL of freshly prepared 2.5% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.2) for 3 h at room temperature.
3. Rinse the grapes in 2 mL of 0.1 M cacodylate buffer for 10 min.
4. Rinse the grapes in 2 mL of ddH_2O for 10 min.
5. Dehydrate the grape by placing it in 2 mL of 70% ethanol for 20 min and let it air dry for 24 h.
6. Mount the grape on an SEM mount using mounting tape.
7. Powder-coat the grapes with a platinum/gold coating.
8. Perform SEM using a Philips ESEM XL-30 microscope.

3.2.5 Exposing the Biofilm-Inoculated Bioreactor to Commercially Available NAs

Similar to Sect. 3.1.2, the following procedure outlines the exposure of biofilms to commercially available NAs. However, this procedure focuses on testing the proof of principle that a biofilm-inoculated bioreactor can be used to degrade NAs in OSPW.

1. Prepare a 10× stock of a 200 mg/L mixture of eight commercially available NAs as sodium salt naphthenates in distilled and deionized water (ddH_2O) at an equimolar concentration of 1.7 mM for each NA (*see* **Notes 8** and **9**).
2. Add 200 mL of the 10× NA stock into 1,800 mL of BH media and place it to a 3 L round-bottom flask (model bioreactor).

3. Inoculate the bioreactor as outlined in Sect. 3.2.3.
4. Incubate the NA-exposed biofilms for up to 30 days at 25°C on a gyrorotary shaker set at 125 rpm.

3.2.6 NA Degradation Analysis

This section summarizes the procedure for monitoring the degradation of eight commercially available NAs in a biofilm-inoculated bioreactor. Similar to Sect. 3.1.7, NAs are separated via an organic extraction and subsequently injected into a GC-FID. The samples chosen for analysis can be extracted daily or following a given incubation (e.g., 14 or 30 days).

1. Extract 0.2 mL of the media from the bioreactor(s) using a serological pipette daily for 30 days.
2. Follow the procedure outlined in Sect. 3.1.7 to extract and analyze NA degradation.

4 Notes

1. In the Materials (Sect. 2), we list the vendors that we purchased our reagents from. However, this does not imply that we endorse these vendors. We've included the URLs to the vendor sites, which can be used to purchase and review the products.
2. The goal was to remediate organic contaminants – specifically NAs – from OSPW. So, the inoculant was obtained from the water layer of an oil sand tailings pond where the OSPW is located.
3. Six single-species isolates were culturable from the OSPW using conventional microbiological techniques; a lawn was formed by placing 100 μL of OSPW onto minimal media agar plates (Bushnell-Haas media amended with either glucose or yeast extract at 1 g/L) and incubated for 21 days at 25°C under aerobic conditions. The isolates obtained from this procedure were identified to the genus level using unidirectional sequencing of the 16S rRNA gene [14].
4. The Calgary Biofilm Device (CBD) can be used for the rapid and highly reproducible production of biofilms [64]. This is advantageous for testing biofilms against many, variable conditions that may be encountered in a given environment. Indeed, biofilms can be formed in the well of a 96-well microtiter plate. However, for continuity between the experiments, the pegs on the CBD are more representative of the wastewater supports attached to the lid of the modified CBD.
5. The choice of which microtiter plate to use was dependent on the type of wastewater support used. For K1 supports, 24-well plates were preferred as the wells accommodated the size of the supports and maximized replicate numbers. Meanwhile, 6-well

plates were favored for K3, K4, and K5 supports for the same reason (Fig. 3). The modified CBD functions in the same manner with which the CBD works (*see* **Note 4**).

6. The Bushnell-Haas minimal salts media was amended with various carbon sources at different concentrations to analyze which would (a) grow the "best" biofilm and (b) work best for degrading the NAs. The amendments include no carbon source addition, 0.001–1 g/L of molasses (from the local grocer), peptone (VWR), yeast extract (VWR), and glucose (VWR).
7. Depending on which microorganism you're growing, anaerobic or microaerophilic conditions can be used to grow a biofilm. In addition, different growth periods may be needed to form a biofilm depending on the microorganism being grown.
8. Commercially available naphthenic acids (NA) from different families were chosen for testing based on their availability. The stock solution is made at a total NA concentration of 2 g/L, whereas the working concentration is 200 mg/L.
9. Solid sodium hydroxide (NaOH) helps in dissolving the naphthenic acids at a pH = 10–11 to sodium naphthenates.
10. A standard curve can be generated using genomic DNA from *Pseudomonas fluorescens* Pf – 5 (ATCC 13525). Standard curve [DNA]: range = 9×10^7 copies/rxn – 9×10^2 copies/rxn.
11. Several analytical techniques (and variations thereof) have been used to identify and quantify NAs. For example, Fourier transform infrared spectroscopy (FTIR) is employed for quantifying total NA content by measuring the spectra from the carboxyl groups [65]; this method is not capable of distinguishing the structural or compositional characteristics between NAs. Both gas chromatographic (GC) and liquid chromatographic (LC) methods are capable of elucidating a certain level of structural differences between classical and nonclassical NAs. Moreover, advanced analytical techniques such as GC-MS/MS and HPLC/HRMS can enhance resolution of NAs beyond that of basic GC-FID, GC-MS, and LC-MS [66–70]. For the purpose of resolving and identifying model NAs, GC-FID provides a fast, effective, high-throughput, and reproducible method.
12. 4-Phenyl butyric acid (PBA) is detectable using GC-FID and is used as an internal standard in the protocol to monitor the extraction and separation efficiency of the NAs. A 1.3 g/L PBA stock is prepared in ddH_2O for easy addition to individual samples.
13. NAs are derivatized prior to analysis by GC-FID in order to produce volatile, stabile structures capable of enhanced selectivity and reproducible detection by GC. If stored in sealed autosampler vials (non-punctured septa), the tri-methylsilylated NAs can be stored long term at room temperature.

14. At first the experiments were run for 14 days. However, once we determined that the biofilms reached maturity within 3–7 days (depending on the grape), the biofilms were grown over shorter periods.
15. If the sample is difficult to solubilize, boiling in the presence of 0.5 N sodium hydroxide (NaOH) can be used to improve solubilization.
16. We found that 20 sterile supports and 1 inoculation support were the ideal ratio for inoculating this particular 1 L model bioreactor. Depending on the parameters – the size of the reactor, the microorganisms, or the wastewater – other ratios of inoculant grape to sterile grapes may work better.

5 Summary

Here we demonstrate a facile method for harvesting an environmentally derived microbial population as a mixed-species biofilm. The advantages of our method are (1) the use of a biofilm instead of a planktonic population of microorganisms and (2) a multispecies community is employed as opposed to a single species or superbug. As a basis for comparison, the capability of the single-species biofilm to degrade the NAs was assessed alongside that of the multispecies biofilm early in our studies [14]. Our working model is that the community has greater functionality against the pollutant as there is greater genetic diversity, thus greater biochemical potential to degrade different chemical structures (Fig. 1). We observed that our single-species isolates could not degrade the NAs as effectively as the multispecies biofilm [14]. The ease of execution of this method gives it utility not only for the remediation of NAs but possibly also other sources of polluted industrial and wastewaters.

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Methods to Assess the Fate and Impacts of Biofuels in Aquifer Systems

Marcio Luis Busi da Silva, Jie Ma, and Pedro J.J. Alvarez

Abstract

Soil and groundwater contamination from accidental or incidental releases of biofuel blends is a growing concern in many countries. Improved understanding of how different biofuel releases behave in the environment and affect the fate and transport of priority pollutants in aquifers is critical for long-term management strategies. Different experimental approaches have been used to advance our understandings of the fate and impacts of biofuel releases in aquifer systems, to develop improved monitoring and remediation approaches, and to validate mathematical fate and transport models. This chapter summarizes currently used experimental approaches, including bench-scale batch tests, laboratory scale column, pilot-scale aquifer tank, and field-scale controlled releases. Physical-chemical analyses commonly used to monitor fate of biofuels and petroleum-based contaminants in groundwater and molecular biomarkers used to quantify catabolic genes associated with the biodegradation of benzene, toluene, ethylbenzene, and xylenes (BTEX) were also summarized in this chapter.

Keywords: Biodegradation, Biofuels, Experimental approaches, Hydrocarbons

1 Introduction

Biofuel is a generic term that refers to a wide range of fuels derived from a variety of renewable feedstock, as replacement of (or supplement to) petroleum-derived gasoline or diesel. Bioethanol produced from corn or starch-rich crops (e.g., sugar cane, maize, or wheat) as well as microalgae is the most recognized biofuel worldwide. Biodiesel is another very well-known biofuel in the market used to replace petroleum-based diesel. Biodiesel is made from algae, vegetable oils, animal fats, or recycled greases.

Soil and groundwater contamination from environmental releases of biofuels has been a concern in many countries where significant consumptions of these fuels occur. For instance, whereas direct exposure to fuel ethanol in drinking water has minimal adverse impacts on human health, ethanol may increase the potential exposure to toxic fuel constituents such as benzene, toluene,

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ethyl benzene, and the isomers of xylene (collectively known as BTEX). This can be the result of enhancing BTEX dissolution and migration through cosolvency (at aqueous ethanol concentrations greater than 10%) or hindering BTEX biodegradation, which also contributes to increasing plume length and thus exposure potential. The inhibitory effects of ethanol (and biodiesel to some extent) on BTEX and polyaromatic hydrocarbons (PAH) biodegradation can occur through different mechanisms, including: (1) *Depletion of dissolved oxygen and other electron acceptors*: the preferential degradation of the biofuel ethanol or biodiesel may deplete available O_2 and other electron acceptors that would otherwise be available for BTEX and PAH degradation [1–4]. (2) *Catabolic gene repression*: ethanol is metabolized by microorganism's constitutive enzymes through central metabolic pathways, while the initial step of BTEX degradation is usually catalyzed by inducible enzymes that may not be needed while the bacteria are feeding on ethanol. Hence, the presence of ethanol could repress the synthesis of inductive enzymes required for BTEX degradation, thus hindering BTEX degradation at the transcription level [5, 6]. (3) *Metabolic flux dilution*: the specific utilization rate (i.e., degradation rate per cell) of any substrate in a mixture decreases in direct proportion to its relative contribution to the assimilable total organic carbon in the mixture [6, 7]. Thus, as the fraction of ethanol in groundwater increases, the fraction of BTEX decreases and so does the corresponding specific degradation rate. (4) *Thermodynamic inhibition*: the buildup of ethanol-derived acetate (and possibly H_2 under fermentative methanogenic conditions) could affect the thermodynamic feasibility of benzene degradation under methanogenic- [1] and sulfate-reducing [8] conditions. (5) *Ethanol toxicity*: high concentrations of ethanol in water are known to be inhibitory to microorganisms with partial inhibitory concentration threshold of 3.4–10 g/L and complete inhibitory concentration threshold of >24–47 g/L [9]. (6) *Cell physiology*: in poorly buffered aquifers, ethanol-derived volatile fatty acids (VFAs) could significantly decrease groundwater pH (<pH 5 in the core of the plume) [10], thus adversely affecting anaerobic BTEX degradation. (7) *Genotypic dilution*: more microbial species can feed on ethanol than on BTEX, which is conducive to a greater proliferation of commensal microorganisms and a decrease in relative abundance of BTEX degraders (genotypic dilution) [2]. Concentrations of dissolved ethanol in groundwater impacted by ethanol-blended fuel are usually lower than 10,000 mg/L [1, 11–14]. Because ethanol and biodiesel are easily and preferentially biodegradable, the contamination plume is usually short-lived in comparison to more persistent BTEX and PAH hydrocarbons. As mentioned above, one of the major risks associated with the use of ethanol or biodiesel blend fuels releases is that these can mobilize and expand BTEX and PAH contamination plume.

As higher ethanol blends are introduced, the greater it is the interest in investigating potential environmental impacts from ethanol degradation byproducts [9]. Under anaerobic conditions, which are commonly observed near source zone, ethanol can be fermented to VFAs (e.g., acetic, propionic, and butyric acids), butanol, methane, and carbon dioxide [15, 16]. Some of these metabolites could impact public safety, groundwater quality, or natural attenuation processes. For example, high generation of methane entering a confined space may pose an explosion hazard or enhance BTEX vapor intrusion [17–23]. Butanol is a regulated compound in drinking water in several states in the United States of America [24]. VFAs (particularly butyric acid) have odors that could compromise groundwater esthetic quality [25]. The accumulation of VFAs may also decrease groundwater pH, possibly facilitating heavy metal dissolution into groundwater [26].

Overall, groundwater contamination by biofuel-blended petroleum products such as gasoline and diesel is an emerging concern associated with the increased use of biofuels to improve air quality and reliance on renewable resources. Consequently, improved understanding and modeling of how different biofuel releases behave in the environment and how they may affect the fate and transport of priority pollutants in aquifers (and related monitoring and remediation activities) are needed before a widespread changeover occurs. In particular, there is a need to address critical knowledge gaps associated with multiphase fluid flow and subsurface microbiological processes that influence natural attenuation and bioremediation. Also, there is an increased interest to understand how biofuels can affect vapor intrusion pathways of volatile contaminants. For example, the formation of biofuels-derived methane could limit the attenuation of BTEX in the unsaturated zone (owing to oxygen depletion by methanotrophic bacteria) and enhance BTEX vapor intrusion in aboveground enclosed spaces. The expected increase in biofuels-derived methane could also pose a potential explosion risk aboveground when ignitable conditions exist. Metagenomic tools and stable isotope probing are currently being used by researchers to advance quantitative understanding of the dynamics and functional diversity of impacted microbial communities and degradation pathways. Such environmental forensic tools may eventually become more widely used by the practitioner community to improve the characterization and assess the performance of biogeochemical processes that attenuate such releases and discern the associated microbial adaptation mechanisms and metabolic niches. Different experimental protocols can be utilized to advance fundamental understanding of the potential impacts of biofuel releases, to develop improved monitoring and remediation approaches, and to validate mathematical fate and transport models. This chapter presents various experimental approaches to accomplish this and include (in order of increasing complexity

and scale) laboratory scale microcosms through more complex field-scale studies. Such experimental approaches can be easily adapted to also investigate the fate and transport of other priority pollutants in aquifer systems, to calibrate and validate mathematical models, and to conduct techno-economic assessment of alternative remediation approaches.

2 Materials

2.1 Microcosm Batch Reactors

- Hollow-stem auger and polybutyrate Shelby tubes or similar.
- Serum-stopper glass bottles of different volumes.
- Teflon-coated butyl rubber stoppers. Alternatively Mininert™ valves can be used.
- Aluminum caps.
- Aluminum cap crimper tool.
- Sterile Millipore syringe filters (0.22 μm).
- Glass flasks.
- Sorted gas-tight syringes.
- Compressed gases cylinders.
- Sorted connection and fittings.
- Anaerobic chamber.
- Reagents.

2.2 Column Studies

- Columns are conventionally made of either glass or stainless steel to resist corrosion and avoid contaminant losses through absorption and inner diffusion.
- Syringe pumps (Harvard Apparatus model 22 or equivalent).
- Sorted gas-tight syringes.
- Peristaltic pumps (Masterflex model 7519-15). Alternatively high-pressure liquid chromatography (HPLC) pumps can be used (Agilent Technologies 1200 series or equivalent).
- Two- and three-way port valves (Mininert™ valves Sigma-Aldrich).
- Masterflex neoprene tubings for peristaltic pump.
- Tubing and fittings made of Teflon, Tygon, or Fluran® are needed when working with contaminants to minimize sorption and/or volatilization (Cole-Parmer; Vernon Hills, IL).
- Teflon end plates to cover both ends of the columns.
- Viton O-rings for the Teflon end plates.
- Stainless steel mash (100 μm).
- Sterile Millipore syringe filters (22 μm).

- Autoclave.
- Large carboy containers.
- Magnetic stirrers and stirrer bars.
- Autoclavable flasks and/or carboy containers.
- Reagents to prepare culturing media or synthetic groundwater.
- Compressed gas, cylinders, connections, and fittings.

2.3 Pilot-Aquifer Tank Experiments

- Dumpster-like metal tanks or similar.
- Different diameter stainless steel tubing (0.6 and 1.3 cm internal diameter) to be used as injection and sampling wells.
- Stainless steel mash screens (100 μm).
- Syringe pumps (Harvard Apparatus model 22 or equivalent).
- Sorted gas-tight syringes.
- Peristaltic pumps (Masterflex model 7519-15 or equivalent).
- Masterflex neoprene tubings for peristaltic pump.
- Tubing and fittings made of Teflon, Tygon, or Fluran® are needed when working with contaminants to minimize sorption and/or volatilization (Cole-Parmer; Vernon Hills, IL).
- Teflon-coated butyl rubber stoppers. Alternatively Mininert™ valves can be used.
- Aluminum caps.
- Aluminum cap crimper tool.
- Glass flasks.
- Two- and three-way port valves (Mininert™ valves Sigma-Aldrich).
- Autoclave.
- High-density polyethylene (HDPE) sampling tubes (Fisher Scientific; Pittsburgh, PA).
- Reagents to prepare synthetic groundwater.
- Sorted connections and fittings.
- Transparent acrylic sheet (50×50 cm^2; 0.7 cm thick).
- Water flow mechanical flow controllers (Gilmont GF-8521-1606).
- 50-L carboys.
- Reagents.
- Granular activated carbon.
- Perspex flow cells with a quartz glass plate.
- Quartz sand (212–300 μm).
- Fluorescent tracer.

- Charge-coupled-device camera (color KP-D581; Hitachi Kokusai, Leeds, UK) with a UV long-pass filter.

2.4 Field Scale

- Well-drilling equipment/hollow-stem auger drilling.
- Polyethylene tubing (3/16″ ID) for construction of multilevel monitoring wells (MW).
- Expendable point (Geoprobe® type or similar).
- Stainless steel mash screens 200.
- Fine and coarse sand.
- Wellhead cap.
- PVC pipes (1 in ID) to be used as well casing.
- Peristaltic pumps (Masterflex model 7519-15 or equivalent).
- Tubing and fittings made of Teflon, Tygon, or Fluran® are needed when working with contaminants to minimize sorption and/or volatilization (Cole-Parmer; Vernon Hills, IL).
- Small filters (sintered bronze silencers, Norgren, UK).
- High-density polyethylene (HDPE) sampling tubes (Fisher Scientific; Pittsburgh, PA).
- Reagents.

3 Methods

3.1 Batch Reactors

Contaminated or uncontaminated groundwater is collected from site-specific monitoring wells in aerobic or anaerobic zones in the contamination plume boundaries. Samples are placed in glass bottles, capped tight without headspace and then shipped on ice to laboratory. Saturated soil is taken at specific depths below the water table using a hollow-stem auger and polybutyrate Shelby tubes using hydraulic pushing systems or similar. Cores are sealed in both ends and shipped on ice to laboratory for preparing batch reactors. Physical-chemical characteristics of the soil or sediment are determined by standard methods. These include soil particle size, organic fraction (f_{oc}), pH, metals, type, and concentration of contaminants (if using contaminated sediment).

Serum-stopper glass bottles are filled with known volumes of groundwater and headspace. If preparing batch reactors with soil slurry sediments, add a known mass of aquifer core material. Aerobic batch reactors are prepared by flushing the medium with sterile compressed air (filtered through 0.22 μm sterile filter). Flasks are then crimp sealed with aluminum caps and Teflon-coated butyl rubber stoppers (alternatively, Mininert™ valves can be used). Anaerobic batch reactors are prepared similarly, but the medium are flushed with oxygen-free gas. In this case, N_2, He, or a mixture

of gases (80% N_2; 10% CO_2; 10% H_2) is used. Alternatively, anaerobic batch reactors can be prepared and kept inside anaerobic chambers with specific atmospheric gas compositions. Resazurin dye (4 mg/L) is added to batch reactors as indicator of groundwater oxidation-reduction potential (ORP) and presence of aerobic condition (blue in the presence of dissolved oxygen, pink at ORP above −100 mV, and colorless at ORP of −200 mV and below).

Sterile or negative controls are prepared by autoclaving the serum bottles then adding biocides to inhibit microorganism's growth (in this case 1% sodium azide, Kathon® biocide for aerobic and mercuric chloride for anaerobic tests).

Anaerobic batch reactors can be amended with NO_3^-, amorphous $Fe(OH)_3$, or SO_4^{2-}, to stimulate nitrate-, iron- and sulfate-reducing conditions, respectively. Batch reactors containing no electron acceptors are designed to be representative of methanogenic conditions. In this case, the use of CO_2- and H_2-enriched headspace may be necessary.

Hydrocarbon compounds (BTEX, PAH), ethanol, and/or biodiesel is added directly into batch reactors using previously prepared stock solutions. Batch reactors are kept at room temperature (22–24°) or at any particular temperature that best mimics a particular site-specific condition. Samples are taken over time with gas-tight syringes and analyzed for dissolved constituents (BTEX, PAH, ethanol, esthers of biodiesel, pH, ORP, volatile fatty acids, dissolved oxygen, NO_3^-, Fe(II), SO_4^{2-}) and headspace gases (H_2, CH_4, CO_2, H_2S) (Table 1). Groundwater and/or sediment samples are subject to microbial analyses. Determining changes in microbial community structures over time can be achieved through the use of conventional plating methods or through more sophisticated molecular-based metagenomics. Specific 16S rRNA and catabolic genes involved in the biodegradation of the contaminants BTEX and PAH can be quantified through PCR-based methods. Several PCR-based primers have been used to target specific aerobic [35–38] and anaerobic [39–41] catabolic genes involved in BTEX biodegradation. Figure 1 shows a schematic drawing of a batch reactor.

First-order biodegradation kinetic rate coefficients (k) can be estimated from the removal of a compound over time using the equation

$$k = \frac{\ln\left(\frac{C_0}{C}\right)}{t_0 - t} \tag{1}$$

where C_0 and C are the concentrations of a specific compound at times t_0 and t, respectively. Although first-order kinetics usually best represents the removal of contaminants in these studies, different kinetic orders may also apply [42, 43].

Table 1
Physical-chemical analyses commonly used to monitor fate of biofuels and petroleum-based contaminants in groundwater

Parameter	General comments	Method	Reference
Oxidation-reduction potential	Indicator of electron-accepting conditions	Eh/ORP meter	Standard methods [27]
Dissolved oxygen	Indicator of aerobic conditions	DO meter	Standard methods [27]
pH	Indicator of the potential active hydrogen ion in aqueous solution. Too low or too high pH can have adverse effects on water esthetics characteristics as well as detrimental effects on contaminants biodegradation	Ion selective electrode	Standard methods [27]
Bromide	Used as a tracer for hydraulic characterization of the system	Ion chromatography	EPA method 300.1 [28]
Nitrate	Highly soluble electron acceptor that can also be used as nutrient. High oxidation potential [E_h^0(W) + 0.42 V for NO_3^- to N_2] High assimilative capacity ($5e^-$ equiv. accepted per mol of NO_3^- reduced to N_2). Can also be used as a nutrient. Nitrate is a regulated pollutant (MCL 10 ppm). Permeability decreases due to N_2 (g) formation	Ion chromatography	EPA method 300.1 [28]
Nitrite	Metabolite from denitrification. Could be an undesirable byproduct (methemoglobinemia, bacteriostatic at ≥100 mg/L)	Ion chromatography	EPA method 300.1 [28]
Iron (II)	Insoluble at neutral or basic pH under aerobic conditions. Clogging due to oxide precipitation. Low assimilative capacity [only $1e^-$ accepted per mol of Fe(III) reduced to Fe(II)]. High oxidation potential [E_h^0 (W) + 0.36 V for $FeOOH_{(s)}$ to $FeCO_{3(s)}$]	Spectrophotometer, phenanthroline method	Standard methods [27]

Sulfate	High assimilative capacity (8 e^- equiv. accepted per mol of SO_4^{2-} reduced to H_2S). Potential laxative effect if present in drinking water at 250 mg/L. Low oxidation potential [E_h^0 (W)-0.22 V for SO_4^{2-} to HS^-]	Ion chromatography	EPA method 300.1 [28]
H_2S	Byproduct of sulfate-reducing bioprocess. Highly soluble H_2S can be bacteriostatic at concentrations >200 mg/L. Esthetic issues associated with odors. H_2S could complex and precipitate with inhibitory heavy metals	Thermal conductivity detector (TCD) equipped gas chromatography	ASTM D1945 – 14 [29]
H_2	Byproduct of fermentative bioprocesses. Serves for methane production by hydrogenotrophic archaea. Its accumulation can hinder thermodynamic feasibility of BTEX biodegradation under anaerobic methanogenic conditions	Thermal conductivity detector (TCD) equipped gas chromatography	ASTM D1945 – 14 [29]
CO_2	Product of aerobic microorganism's respiration. Can be utilized as electron donor by many types of methanogens to produce methane. Its accumulation can alter carbonate system equilibrium, hence acidifying groundwater pH	Thermal conductivity detector (TCD) equipped gas chromatography	ASTM D1945 – 14 [29]
Methane	Byproduct of methanogenesis; concentrations (in air) above 5% (v:v) can pose explosion hazard. Monoaromatic hydrocarbon biodegradation in methanogenic conditions is not always observed	Thermal conductivity detector (TCD) equipped gas chromatography	ASTM D1945 – 14 [29]
BTEX (benzene, toluene, ethylbenzene, and xylene isomers)	The most soluble and toxic compounds present in petro fuels. Among BTEX, benzene is a known carcinogenic compound which drives the needs for corrective remediation action	Flame ionization detector (FID) equipped gas chromatography	EPA method 815C [30]
PAH (polyaromatic hydrocarbons)	Toxic aromatic hydrocarbons with two or more fused benzene rings that can pose detrimental biological effects including mutagenecity and carcinogenicity. With low aqueous solubility and high absorption to solid particles, these	Flame ionization detector (FID) equipped gas chromatography/mass spectroscopy	EPA method 8100 [31]

(continued)

Table 1
(continued)

Parameter	General comments	Method	Reference
	compounds are recalcitrant to biodegradation. However, several studies reported its biodegradation into less complex metabolites under both aerobic and anaerobic conditions		
Ethanol	Used as gasoline oxygenate in percentages varying from E5 up to E85 (5–85% anhydrous ethanol mixed to gasoline; volume basis). Hydrous E100 is used as a sole source of fuel in Brazil. High concentrations are inhibitory to microorganisms with partial inhibitory concentration threshold of 3.4–10 g/L and complete inhibitory concentration threshold of >24–47 g/L. Its presence in groundwater influences BTEX biodegradation as described earlier in this chapter	Flame ionization detector (FID) equipped gas chromatography	ASTM 4815/D5599 [32]
Esthers of biodiesel	Biodiesel is used to formulate a range of mixtures from B2 (2% biodiesel mixed with 98% fossil diesel; v:v) up to B100 (100% v:v biodiesel). With twice of the biochemical oxygen demand (BOD) exerted by ethanol, its preferential biodegradation can result in the development of methanogenic conditions which are not conducive for faster benzene degradation	Flame ionization detector (FID) equipped gas chromatography	ASTM D6751 [33]
Volatile fatty acids	Byproduct from the biodegradation of hydrocarbons particularly from ethanol and biodiesel decreases groundwater pH and stimulates anaerobic methanogenic conditions that are mostly not conducive for benzene degradation. Present a potential esthetic problem due to odors (e.g., butyrate)	Flame ionization detector (FID) equipped gas chromatography	Manni and Caron [34]

3.2 Column Studies

Aquifer columns are either filled with uncontaminated or contaminated sediment depending on the purpose of the study. Physical-chemical characteristics of the soil or sediment are important. These include soil particle size, organic fraction (f_{oc}), pH, metals concentrations, type, and concentration of organic contaminants (if using contaminated sediment). Each column is capped with Teflon end plates and sealed with Viton O-rings. Fine and coarse stainless steel mesh screens can be used at each end to separate the end plates from the packed column material. This aids to prevent the loss of sediment from the column. A coarse screen can be placed against the end plate to assist the end-plate groove pattern in distributing influent solutions uniformly across the base of the column. Columns are filled with soil under saturated conditions to prevent air bubbles entrapment and the formation of channels that can lead to preferential flow [44, 45]. Natural or synthetic groundwater [46] is used to saturate the soil during the process of filling the columns with soil.

Columns operate in upflow mode to minimize the formation of void spaces and preferential flow-through channeling. Alternatively, flow-through system can also be operated upright with horizontal flow [47]. All tubing and fittings should be autoclaved prior to the beginning of the experiments to prevent cross-contamination. Millipore syringe filter is placed in line prior to columns inlet to avoid the entrance of exogenous microorganisms and/or contamination of the feeding solution by chemotaxis or backflow (please *see* Sect. 4). Effluent from the column is collected in large carboy container(s) for latter treatment before discharge.

The hydraulic characteristics of the columns can be determined from tracer studies. In this case a known concentration of a tracer (e.g., potassium bromide or tritium) is spiked into the feeding solution that is continuously pumped into the column inlet using either a peristaltic or HPLC pump. Column's effluent samples are then collected over time and analyzed for bromide concentrations. Bromide breakthrough curve is used to estimate effective porosity (η_e), dispersion coefficient (D), and retardation factors (R_f) [2] by fitting the breakthrough data to one-dimensional advection-dispersion equation [48] (Eq. 2):

$$C = \left(\frac{C_0}{2}\right) \operatorname{erfc}\left[\frac{\left(Rfx - \left(\frac{Q}{A\eta e}\right)t\right)}{2\sqrt{DRft}}\right] + \exp\left[\frac{\left(\frac{Q}{A\eta e}\right)x}{D}\right] \operatorname{erfc}\left[\frac{\left(Rfx + \left(\frac{Q}{A\eta e}\right)t\right)}{2\sqrt{DRft}}\right] \tag{2}$$

where C = effluent bromide concentration, C_0 = influent bromide concentration, x = column inlet distance, t = elapsed time, Q = flow rate, A = cross-sectional area, η_e = effective porosity,

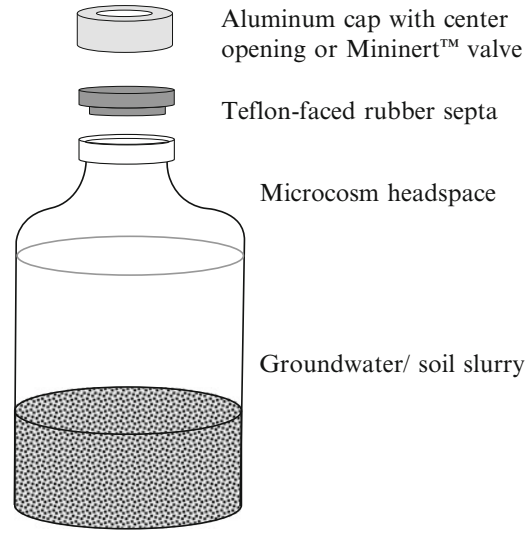

Fig. 1 Schematic drawing of a typical microcosm batch reactor

D = dispersion, R_f = retardation factor ($R_f = 1$ for bromide), and erfc = complementary error function.

Sampling ports are installed along the column length for determination of concentration profiles, which facilitate estimating degradation rates and discerning potential spatial changes in contaminants gradient along the columns length. These lateral ports are also used to collect sediment cores for microbiological analyses. When working with synthetic groundwater, it is recommended that the media be autoclaved prior to the beginning of the experiments. A solution containing neat or diluted hydrocarbons, ethanol or biodiesel compounds can be injected directly into columns influent "Teed" into main line (*see* Fig. 1 for details) thus minimizing the chances of microbial growth in the stock groundwater feeding solution. The intended concentration of contaminants entering the columns can be set by adjusting the flow ratio between the syringe (containing the contaminants) and peristaltic (containing the nutrient growth solution) pumps. Groundwater stock feeding solution can be constantly purged with different gas composition according to the experimental goals. For instance, purging the feeding groundwater solution with a gas mixture composed of N_2 (80%), CO_2 (10%), and H_2 (10%) helps to maintain anoxic conditions and the proliferation of anaerobic/methanogenic which are conditions typically encountered near the contamination source zone [49, 50]. Columns are kept under dark conditions and at controlled temperature to simulate site-specific groundwater temperature. It is important to note that microorganisms may take weeks to months to acclimate before system steady-state conditions are met.

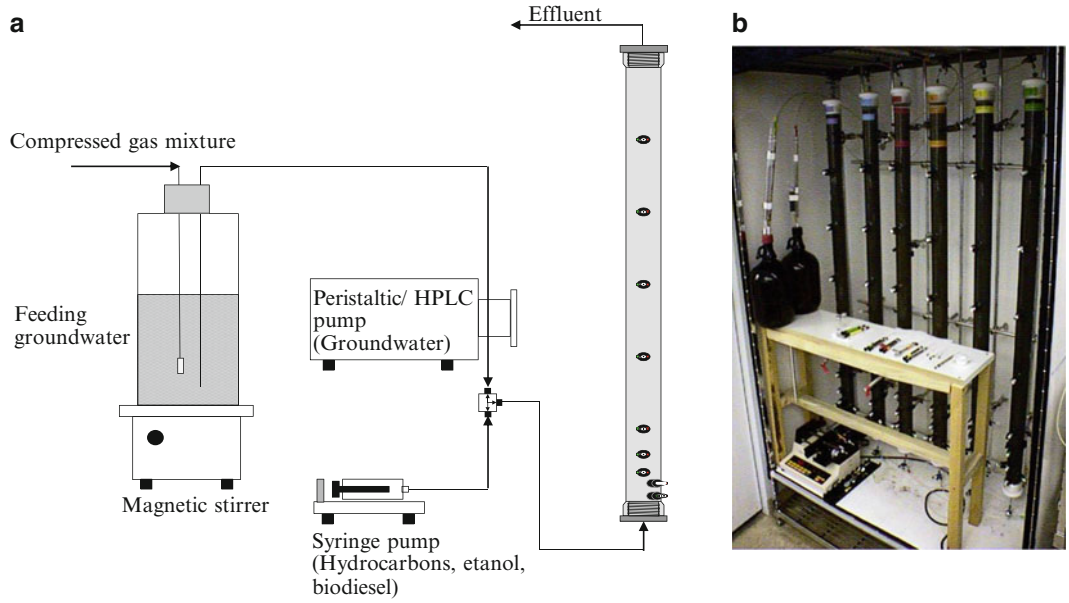

Fig. 2 Schematic drawing (**a**) and picture of column experimental setup (**b**). *Source*: [2]

Samples are taken directly from the columns' port vials over time using gas-tight syringes and analyzed for dissolved constituents (BTEX, PAH, ethanol, esthers of biodiesel, pH, ORP, dissolved oxygen, NO_3^-, Fe(II), SO_4^{2-}) and headspace gases (H_2, CH_4, CO_2, H_2S). Groundwater and/or sediment samples are subject to microbial analyses (for details on how to collect sediment, please *see* Sect. 4). Determining changes in microbial community structures over time can be achieved through the use of conventional plating methods or through more sophisticated molecular-based metagenomics. Specific 16S rRNA catabolic genes involved in the biodegradation of the contaminants BTEX and PAH can be quantified through quantitative real-time PCR (qPCR)-based methods [11, 51–53]. Figure 2 shows a schematic drawing and picture of columns experiment.

A sterile control column is usually run in parallel to distinguish biodegradation from potential abiotic losses (e.g., volatilization). The control column is filled with autoclaved soil and fed groundwater stock solution spiked with biocides (please *see* Sect. 4: notes).

3.3 Pilot Scale

A pilot-scale continuous-flow aquifer tank is used to conduct a quantitative biofuel release study. These experiments serve to characterize source zone generation and behavior and to calculate mass balances in support of fate and transport assessments at the near-field scale. Neat biofuel or a petroleum-based amended biofuel is spilled in the experimental tank. These tanks are usually made of dumpster-like metal containers (approximately 4–10 m, 0.7–2 m, 0.2–1.5 m, in length, wide, depth, respectively; Fig. 3) [55, 56].

Fig. 3 Two metal tanks (5.5 m long × 2.1 m wide × 1.8 m high) filled with a non-contaminated sandy soil. Multiple internal sampling or injection points (0.6 and 1.3 cm ID, respectively) were installed using stainless steel tubing during tank packing. *Source*: [54]

The tank can either be placed indoors or outdoors under a canopy. Tanks can be completely covered with a nonreactive membrane cover to account for volatilization losses; however, this type of monitoring increases the level of complexity. When desirable, an acrylic-made window can be installed in the tank side wall to allow observation of contaminant behavior at the injection point near the water table interface.

The tank is usually filled with low organic-, high-porosity soils such as sand to minimize the effects of contaminant plume retardation as well as system clogging. Soil physical-chemical analyses should be conducted to determine organic, nutrient (nitrogen and phosphorus), alkalinity, electron acceptor, and mineral contents as well as porosity and conductivity. The soil is evenly spread inside the tank using shovels and rakes followed by manual compaction using tampers. Simultaneously, the tank is slowly filled with water from the bottom. This sand-water saturation strategy is designed to enhance distribution of the sand and to minimize mounding, channeling, and other heterogeneities that can occur during soil packing. The water is then drained to the desired water table level from the base of tank with a desirable vadose zone and an initial capillary fringe height above the water table. This is a depth of approximately midway in the tank sideway window, so that both the saturated and unsaturated zones could be observed. A continuous flow of tap water (or synthetic groundwater that best mimics local groundwater characteristics) unceasingly runs through the

tank using inlet pipes within a screened well extending a couple centimeters below the water table. A steady flow rate (within groundwater flow range) is achieved by controlling flow out of the head driver on the system. The effluent of the tank is collected in reservoirs containing activated carbon canister for treatment prior to discharge to the sewer system. The level between the inlet reservoir tanks coupled with the outlet overflow level across the entire tank determines the hydraulic gradient. Several sampling ports can be installed downstream from the injection wells (or source zone) to monitor the spatial and temporal changes in groundwater contaminant plume concentrations. These wells are made of different diameter sizing stainless steel tubing. A piece of stainless steel mash is placed at the tubing bottom end to avoid sediment entering the tubing causing clogging. Effluent lines are placed at the bottom of the tank and in both sides to minimize preferential flow and channeling. Outflow lines generally use larger diameter tubing (1 in.) to prevent clogging.

For the hydraulic characterization, a bromide tracer test is conducted. A solution with known concentration of bromide is continuously injected through injection ports and at constant flow rate over a time period. Aqueous samples are then collected at the effluent sampling ports in sterile HDPE field sampling bottles. Samples are collected over time for the duration of the test and analyzed for bromide concentration (Table 1). Analysis of the breakthrough curves using advection-dispersion equation (as described above for the columns experiments) estimates seepage velocity. The obtained values should be representative of reported groundwater velocities found in natural systems. The hydraulic conductivity and average hydraulic retention times are also estimated.

The source zone is established by injecting neat biofuel or petro-based amended biofuels continuously at the water table interface through stainless steel injection wells. Peristaltic pumps using Viton or Fluran® tubing are used to deliver the fuel into the tank. The inlet end of the Viton tubing pulls the fuel from a feed container. The outlet end of each tube is connected by a hose clamp to a stainless steel well inserted to the water table. Acrylic walls can be inserted down to the water table on either side of the source addition wells to fix the initial width of the spill and achieve conditions that would yield phase separation of nonaqueous phase liquid (NAPL) near the point of injection.

Aqueous samples are collected either directly from the tank effluent or sampling wells located along the tank length. Prior to sampling each Viton tubing sample port should be purged with a minimum of 1.5 line volumes. Samples are collected in glass vials, leaving no headspace, using gas-tight syringes (Fisher Scientific; Pittsburgh, PA). The capped samples are stored on ice and taken to laboratory for further analyses. Physical-chemical and biological

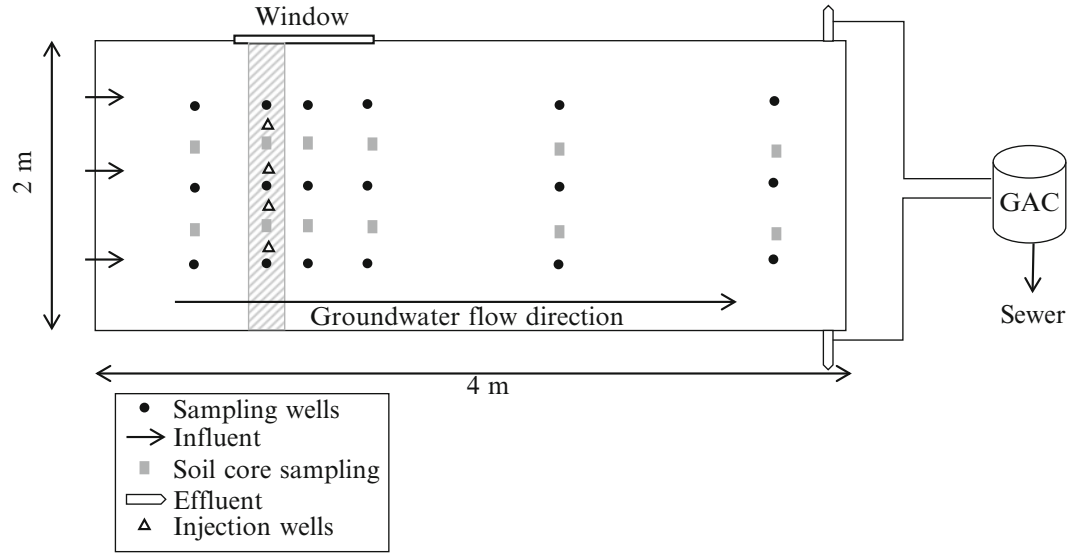

Fig. 4 Plan view of an experimental pilot-scale tank showing injection and sampling well locations. *Cross-hatched rectangle* represents the source zone. Granular activated carbon (GAC)

characteristics of the sample are then performed (Table 1). Sand cores can also be collected over time for analyses. The core samples are taken from the sand surface to the water table at different transect along the tank. The cores are collected in sterile HDPE cylinders, sealed, and stored on ice prior to analysis (Table 1) (Fig. 4).

Small flow cells containing quartz sand colonized with biofilm can also be utilized as physical model aquifers allowing degrading contaminant plumes to be formed and monitored from a point source [57, 58]. A noninvasive fluorescent tracer technique is combined with chemical and biological sampling in order to quantify transport and biodegradation processes. A perspex flow cell (internal dimensions of 156–200 by 100–120 by 3–5 mm) with a quartz glass plate on the front side for visualization is filled with the inoculum established as a quartz sand/mineral medium slurry through an inlet pipe located on the back of the flow cell. Slurry is mixed inside the flow cell to prevent layering. Sterile stainless steel mesh filter is used to prevent loss of the quartz sand through the outlet pipes. Porosity of the matrix is determined from the reactor volume and density and mass of quartz sand. A point source injection pipe (internal diameter of 3 mm) with perforations in the flow direction is placed centrally through the internal space of the flow cell, 20 mm from the top. Septa ports are arranged in the back of the flow cell to allow for aqueous sampling both across a transect and along the plume in the flow direction. A constant flow rate is applied using a peristaltic pump and a syringe pump to inject groundwater and/or nutrient solution at a constant flow rate. Flow cell experiments are disinfected with ethanol prior to construction and experiments

conducted in the dark to minimize interference by external sources of light. Similar studies using a larger two-dimensional flow cell (56 × 44 × 1 cm) were described [59].

3.4 Field Scale

When releases of ethanol-blended gasoline reach the groundwater, a light nonaqueous phase liquid (LNAPL) typically forms at the water table interphase. The LNAPL can serve as a source for sustained groundwater contamination by BTEX, which slowly leaches into the aqueous phase. In contrast, ethanol readily partitions into the groundwater, and aqueous ethanol concentrations above 10% may exert a cosolvent effect that enhances BTEX dissolution resulting in higher BTEX concentrations [60]. However, ethanol is biodegraded relatively fast compared to the released hydrocarbons and/or migrates away from the source zone, eventually becoming unavailable to accelerate LNAPL dissolution. As discussed above, preferential ethanol biodegradation may consume electron acceptors and nutrients that would otherwise be available for hydrocarbon biodegradation, thereby hindering their intrinsic bioremediation and promoting longer BTEX plumes. Assessing the net effect of biofuels on BTEX natural attenuation is also confounded by compound-specific differences in transport, retardation, dilution, and fate following changes in electron-accepting conditions. Furthermore, the rate and extent of hydrocarbon biodegradation in aquifers are compound specific and vary with redox transitions. Thus, it is very difficult to characterize the dynamics of natural attenuation of ethanol-blend releases in the laboratory, which underscores the need for long-term natural attenuation studies at the field scale.

The long-term nature of field-scale experiments permits the observation of important transient processes that occur over various time scales [1]. In these experiments, a series of monitoring wells (MW) is installed upgradient and downgradient from the contamination source zone (Fig. 5). The locations for the installment of MW are determined primarily by the groundwater flow direction and seepage velocity through tracer tests. MW can be multilevel tubing made of a bundle of 3/16″ ID polyethylene tubing with each tube cut to length corresponding to a particular depth below soil surface. The end of each tube is thermally bonded to a narrow diameter (20 cm long) 200 mesh stainless steel wire-cloth screen containing an expendable point in the end (Fig. 6). The set of multilevel tubings is attached to the outside of a 1 in ID PVC pipe which is then placed inside the aquifer hole previously made by conventional drilling process. The annular space between the samplers and the aquifer is filled with fine sand to prevent channeling. Low-flow groundwater sampling is conducted using a peristaltic pump connected individually to each of the MW (Fig. 7). High-resolution (down to 3 cm) multilevel sampling wells can be used to determine the geochemical gradients at the fringe of a

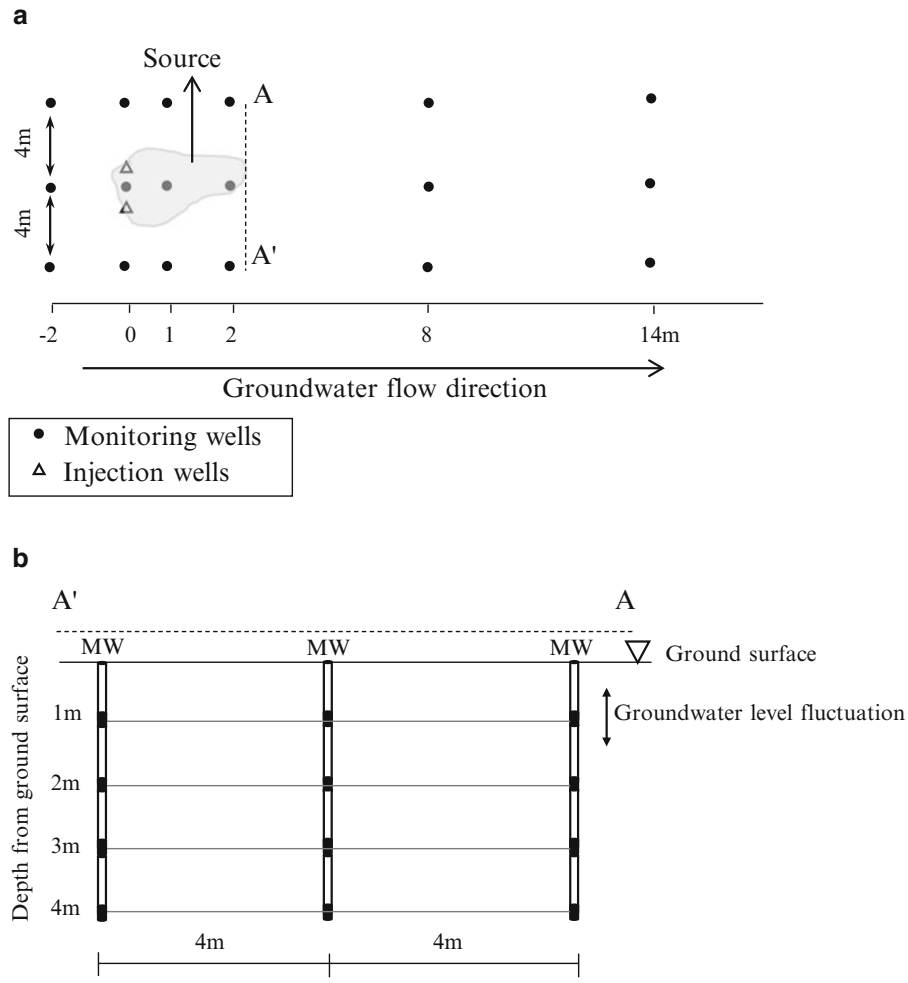

Fig. 5 Experimental area plan view showing the MW distribution (**a**) and (**b**) cross-section from A to A′. Well cluster screens are shown as black-shaded areas in the cross-section. *Source*: adapted from [1]

contamination plume [62]. The multilevel wells are assembled in several single units, each consisting of a high-density polyethylene (HDPE) tube of, for example, 1 m length and 15 cm outer diameter. At predetermined depths, small filter units (sintered bronze silencers, Norgren, UK) are inserted at distances of 3 cm for high resolution or above for middle resolution (10 cm) or low resolution (30 cm) from each other. Each filter unit is connected to a peristaltic pump via a stainless steel capillary (1 mm inner diameter) that is inserted tightly through a rubber stopper into the filter port. Hollow-stem auger drilling was applied prior to installation of the high-resolution multilevel wells. After insertion of the well and removal of the supporting steel pipes, the sediment is allowed to collapse for embedding the filter ports in the aquifer sediments. Groundwater is then collected from the multilevel wells over time

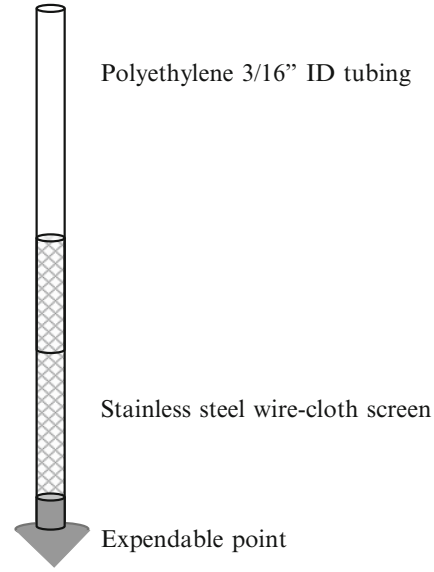

Fig. 6 Monitoring well (MW) construction in detail

Fig. 7 Groundwater is sampled directly into HDPE flasks using a low-flow peristaltic pump and Masterflex Tygon tubing. *Source*: [61]

usually after a couple months (e.g., 3 months) after acclimation. For sampling, the selected steel capillaries are connected to multi-channel peristaltic pumps using 2-stopper Fluran HCA tubes. Aqueous sample is then collected in HDPE flasks and stored in ice for further analyses in the laboratory (Table 1).

For tracer tests, a known mass of tracer solution (e.g., potassium bromide) is released constantly or in one pulse via injection

wells directly into groundwater. Groundwater samples are then collected downgradient from the tracer injection wells over time and analyzed for bromide. Conventionally, MWs are installed to form grids that cover the entire contamination plume.

The contamination source can be made by releasing a known volume of biofuel formulation into aquifer ([1]; please obey local environmental regulations) or by the injection of biofuel and BTEX solution directly and continuously into injection wells [63].

To discern the effects of biofuels on petroleum-based groundwater contaminants (BTEX, PAHs), a parallel (control) field experiment can be conducted. In this case, the same volume of the fuel (with the same physical-chemical characteristics but without the addition of biofuel) would be released.

The mass flux approach [64, 65] method can be used to estimate BTEX and biofuel source weathering. For field studies a simplified mass balance procedure can be accomplished by using the differences in total contaminant mass flux across perpendicular lines of MWs located downgradient from the contaminant source [1]. The mass flux (F_i) associated with the monitoring wells is calculated as

$$F_i = C_i \cdot A_i \cdot v_x \tag{3}$$

where C_i is the contaminant concentration at a designated polygon i, A_i is the area of the designated polygon (influence area), and v_x is the groundwater flow velocity. A mass flux first-order decay model is then used to estimate BTEX attenuation rates:

$$F = F_0 \cdot \exp(-k \cdot t) \tag{4}$$

where F is the total mass flux through the MW line at a distance x from the source in time t, F_0 is the initial mass flux through the MW line near the source, and k is the first-order attenuation rate coefficient. The mass distribution over the wells is given by Thiessen's Polygon technique [64].

There are several alternative approaches to estimate contaminant removal first-order rate coefficients (k). One approach is to consider contaminant concentration data from the entire network of MW. In this case k is used as a fitting parameter to a fate and transport model, using nonlinear regression techniques. The accuracy of this approach is, however, significantly impaired by uncertainties regarding other model parameters.

Field experiments also allow the determination of k based on mass balance approach [66]. The mass of a contaminant present in groundwater is monitored over time by interpolation and integration of data collected through several MW installed in the experimental site. The k value (Eq. 1) is related to the percentage of contaminant removal per period of time as shown in Eq. 5:

$$k = -(dM/dt)/M = \ln(M_0/M)/\Delta t \quad (5)$$

where M_0 is the initial and M is the remaining contaminant mass after a time interval of Δt. The use of mass balance to estimate contaminant removal does not account for other potential mechanisms such as sorption, volatilization, etc., so k represents an overall attenuation (rather than biodegradation) rate coefficient. The relatively high costs associated with monitoring network as well as analytical complextities makes application of this method suitable for research purposes only [66]. Another possible limitation to this method occurs when the contaminant plume reaches a steady state; i.e., the rate of dissolution from a continuous source is equivalent to the rate of downgradient transport and biodegradation. In this case the removal rate cannot be determined as the dissolved mass of contaminants remains constant. Sites with pulse releases of contaminants can still take advantage of this method, however.

Additional methodologies to describe the rate of a contaminant removal can be utilized besides first-order kinetics (Eq. 1 above). This is the case of Buscheck and Alcantar [67] (and its extended 3-D dimensional plume [68]), the normalization of contaminant concentrations to the concentration of relatively recalcitrant co-contaminant present in the initial release such as trimethylbenzene or tetramethylbenzene [69], and the use of in situ microcosms. The latter approach is particularly useful to directly measure biodegradation rates because natural conditions are difficult to replicate in laboratory microcosms studies, potentially leading to misrepresentative estimates [70–73]. In situ microcosms are made of stainless steel cylinders that once installed in the aquifer collects groundwater and sediments that can be spiked with known concentrations of contaminants. Microcosms are installed in the aquifer using drilling rigs. Changes in contaminant removal are monitored over time under real site conditions.

Compound-specific isotope analysis (CSIA) is a very useful and powerful analytical method used to assess organic contaminant sources, identifying and quantifying contaminant transformation processes in situ as well as helping in elucidating biodegradation metabolic pathways [73–77]. This is done through multielement isotope analysis. This is possible because every contaminant compound has unique isotopic ratio. Therefore, isotopes of a given element such as carbon, hydrogen, and chlorine have the same number of protons and electrons but a different number of neutrons and thus different atomic mass. Carbon, for instance, has most abundant isotope ^{12}C and one or more less abundant isotopes such as ^{13}C that contains one or more extra neutrons. The ratio between ^{13}C and ^{12}C changes as a result of abiotic or biological transformations. These ratios are then measured by direct coupling of gas (GC) or liquid (LC) chromatography to isotope ratio mass spectrometers with or without pre-concentration using purge and

trap or GC/quadrupole mass spectrometry (GC/qMS) [75, 78, 79]. Biodegradation of organic compounds in groundwater can significantly exert a high biological oxygen demand and depletion of available anaerobic electron acceptors leading to methane production in contaminated sites. These phenomena are even more pronounced when ethanol and/or biodiesel which are easily and preferentially biodegraded are present in the contamination plume (please *see* Sect. 1). The produced methane can escape from soil and be entrapped in indoor spaces posing a potential explosion hazard. Isotope fractioning can be used to distinguish between methane produced from gasoline and biofuel biodegradation as well as to assess the occurrence of methane in ethanol fuel-contaminated sites [14]. Associated with signature metabolite diagnostic of anaerobic hydrocarbon metabolism and molecular biology methods, CSIA can provide strong evidences to support in situ biodegradation of hydrocarbons in gasoline-amended biofuels [80].

4 Notes

- Filters installed at the column's influent feeding and outlet lines require regular replacement or flow problems can occur due to filter obstruction and clogging.
- Kathon® or sodium azide and mercuric chloride are examples of biocides used to inhibit the growth of aerobic and anaerobic bacteria, respectively, in negative control experiments.
- Two procedures are commonly used to obtain soil samples from tank- and field-scale experiments. Soil core can be obtained by split spoon sampling at different depths. The cores are then stored on ice in sterile flasks and transferred to laboratory for further analyses. Alternatively, soil core samples can be collected using a Geoprobe system (Geoprobe Systems, Salina, Kans). In this case, a coring tube is pounded to different depths and retrieved. A sterile acrylic sleeve is placed in the tube. The tube is reinserted into the ground and driven in an additional depth. The acrylic sleeve is then removed, capped, sealed with duct tape, and stored on ice for transport to the laboratory for analyses. The use of a PVC tubing (2.5 cm $\times$ 1.83 m) can also be used. In this case, the PVC tube is vertically introduced into soil surface and pulled. The tube containing sediment material is then cut into sections and the soil material from inside the sections collected [53, 81, 82]. For microcosm's experiments, about 0.25–1 g of soil is enough for DNA analyses using several commercially available DNA/RNA extraction kits. These relatively small amounts of sediment taken usually do not cause major soil disturbance [83]. Depending on the size of columns, the whole experimental setup can be temporarily transferred

into an anaerobic chamber to avoid oxygen intrusion during sampling. This is particularly important when experiments are simulating anaerobic conditions. To collect samples, Mininert port vials (or similar) are unscrew and sediment taken using a small sterile spoon. Samples are then placed into sterile vials, on ice. Microbial analyses from sediment can also be conducted by filtering groundwater. 1-L groundwater samples (the higher the microbial biomass, the less volume is needed to obtain enough DNA) are vacuum filtered using a 0.22 mm filter (Osmonics Inc., Minnetonka, MN) [11, 84, 85]. The filter is then used as a matrix for DNA extraction using the MoBio Power Soil kit (Carlsbad, CA) according to the manufacturer's protocol. This method is particularly useful to minimize soil disturbance that can potentially affect hydraulic characteristics of system.

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Protocol for Biopile Construction Treating Contaminated Soils with Petroleum Hydrocarbons

Guozhong Wu and Frédéric Coulon

Abstract

When investigating the treatment of contaminated soils, the application of biotreatment is growing rapidly. Factors influencing this rapid growth include that the bioremediation processes are cost-efficient, safe, and nature-based. In the past, thermal, chemical, and physical treatment methods have failed to eliminate the pollution problem because those methods only shift the environmental pollutants to a new environmental phase such as air and water. Bioremediation technology, which leads to degradation of pollutants, may be a lucrative and environmentally beneficial alternative. Two major groups of bioremediation treatment techniques are used: in situ and ex situ remediation. While in situ remediation is more cost-effective, the thoroughness of this method is less effective than the ex situ remediation. Ex situ remediation is less cost-effective but is a more thorough remediation method. This paper presents biopile design settings and example of calculation for design.

Keywords: Biopile, Bioremediation, Composting, Petroleum hydrocarbons

1 Introduction

Over the past 25 years, the development and use of remediation technologies have progressed and a large number of clean-up alternatives have evolved and improved. Details of individual technologies are available in US EPA reports [1–15], Federal Remediation Technologies Roundtable reports [16–31], and published papers [32–35].

Remediation technologies can be grouped into categories based on their treatment process including thermal, physicochemical, and biological. The development of sustainable remediation requires using these technologies to destroy, transform, or immobilise environmental contaminants to protect potential sensitive receptors [36]. In the past, thermal, chemical, and physical treatment methods have failed to eliminate the pollution problem because those methods only shift the pollution to a new phase such as air or water. In contrast, bioremediation is a natural process

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which relies on bacteria, fungi, and plants to alter contaminants as these organisms carry out their normal life functions. Metabolic processes of these organisms are capable of using chemical contaminants as an energy source, rendering the contaminants harmless or less toxic products in most cases. Bioremediation technology exploits various naturally occurring mitigation processes: natural attenuation, biostimulation, and bioaugmentation. Bioremediation which occurs without human intervention other than monitoring is often called monitored natural attenuation (MNA). This natural attenuation relies on natural conditions and behaviour of soil microorganisms that are indigenous to soil. Biostimulation consists of adding nutrients and other substances to soil to enhance natural attenuation processes, while bioaugmentation consists of introducing indigenous microorganisms (sourced from the contaminated site) or exogenous microorganisms (outside the soil environment) capable of detoxifying a particular contaminant, sometimes employing genetically altered microorganisms [37]. Bioremediation can be carried out in situ or ex situ with different degrees of success. Compared with in situ bioremediation, the ex situ bioremediation is relatively more complicated as it requires more bioengineering control and management and requires large amount of land. However, it offers the possibility to take the bulk of contaminants away before they can further leach or diffuse in the environment and allows a better control of the remediation process and efficiency within a short period of time (generally less than 6 months) [38–40]. Land-based ex situ bioremediation technology includes land farming, composting, and biopile. Land farming is a simple technique in which contaminated soil is excavated and spread over a pre-pared bed and periodically tilled until pollutants are degraded [38]. Composting is a technique that involves the addition of non-hazardous organic amendments (e.g. manure, straw, saw dust, wood chips) into the contaminated soils and utilises the rich microbial population and nutrients in the organic amendments to enhance the biodegradation of contaminants in soils [41]. Biopile is a hybrid of land farming and composting, which provides a favourable environment for indigenous microorganisms through aeration and amendment of moisture and nutrient in a constructed pile [42]. Typically, it is a refined version of land farming when used to treat surface contamination with petroleum hydrocarbons. It is also called composting if organic amendments are applied into the biopile [41].

Here, we describe the pilot scale protocol for biopile construction, which could also be applied for composting if organic amendment is required. It does not provide details about the screening and selection of biopile technology or the civil engineering construction details which are site-specific and therefore should be referred to local engineering guidance.

2 Materials

The protocol described below provides the description of the construction of a field-scale biopile with 382 m^3 size as recommended by von Fahnestock et al. [42], which is large enough to process a significant volume of soil and small enough to allow two workers to apply and remove the biopile cover (*see* **Notes 1** and **2**).

2.1 Base Construction

- Grid screen shaker
- Wood beam (10 cm × 10 cm) × 4
- Wood beam (5 cm × 10 cm × 15 m) × 2
- Wood beam (5 cm × 10 cm × 18 m) × 2
- 2 cm × 15 cm lag bolt with washer and nut
- Connecting brackets to join 10 cm × 10 cm beams
- High-density polyethylene (HDPE) liner (15 m × 18 m, thickness ≥60 mm)
- 10 mm × 76 mm hex head sheet screw w/washer × 100
- Clean clay soil (~55 m^3)
- Contaminated soils (~400 m^3)

2.2 Biopile Construction

- PVC pipe (Ø 10 cm × 3 m length) × 3
- PVC pipe (Ø 5 cm × 25 m length) × 1
- PVC pipe (Ø 2.5 cm × 15 m length) × 1
- Perforated plastic drainage pipe (Ø 10 cm × 12 m) × 3
- End caps (Ø 10 cm) × 3
- Rubber union × 3
- Brass gate valves (Ø 10 cm) × 3
- PVC threaded/slip coupling (10 cm) × 6
- PVC reducing bushing (Ø 10 cm to Ø 5 cm) × 3
- PVC slip tee (5 cm) × 2
- PVC slip elbow (5 cm) × 2
- Water knockout tank with automatic level control (20 L) × 1
- Water tank (2,000 L) × 1
- 1 kw blower ×1
- 0.5 kw centrifugal pump for ×1
- 210 L granular activated carbon canister ×1
- HDPE top cover (23 m × 23 m, thickness ≥20 mm) × 1
- 5 cm × 10 cm × 182 cm treated wood slat for securing cover × 30

- Ø 0.8 cm × 10 cm hex head sheet metal screw × 100
- Nylon rope (120 m) × 1
- 8 mm × 76 mm eye-screw × 12

2.3 Nutrient and Moisture Addition

- Fertiliser with C:N:P ratio of 100:15:1 (*see* **Notes 3** and **4**)
- Water hoses with nozzles
- Scoop for nutrient addition
- 4.5 L bucket for holding/distributing nutrients

2.4 Monitoring Point

- Nylon tubing for monitoring points (6 mm)
- Monitoring point screen ×10
- Brass quick-disconnect coupling set for monitoring points (6 mm) ×13
- Thermocouple wire with plug (12 m length) ×2
- K-type thermocouple wire with plug (6 m length) ×2
- Shovel ×2
- Duct tape ×1
- Health and safety equipment

2.5 Sampling

- Soil sampler ×2
- Brass sleeve ×2
- Air sampling bag (1 L) ×10

3 Methods

3.1 Pre-treatment of Contaminated Soils

- Excavate approximately 400 m^3 of soils from contaminated sites and transport to the biopile area.
- Sieve the soil with a 65 mm grid screen shaker to remove stones, lumps of concrete, oil containers, and asphalt that may cause mechanical problems during mixing.
- Add dry fertiliser into the contaminated soils at a rate of approximately 1 kg of fertiliser per cubic metre of soil (*see* **Note 5**).

3.2 Biopile Construction

- Clean the biopile base by removing the brush, debris, and other obstacles.
- As shown in Fig. 1, construct a berm using one layer of 10 cm × 10 cm wood beam topped with a layer of 5 cm × 10 cm wood beam (*see* **Note 6**).
- Cover the base using a 60 mm-thick HDPE bottom liner (15 m × 18 m).

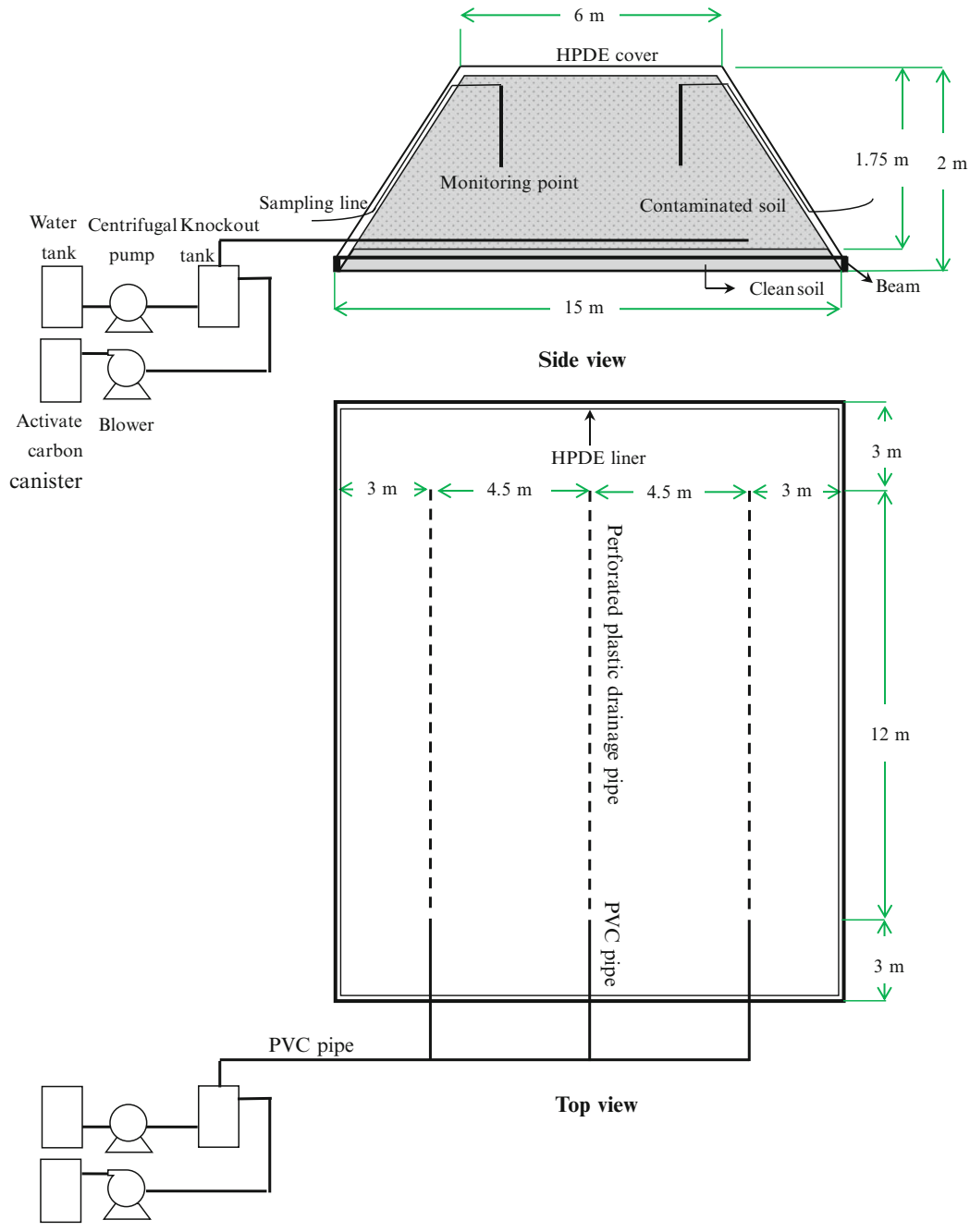


Fig. 1 Schematic of the biopile construction

- Fasten the liner to the berm using lag screws with washers.
- Load 25 cm-thick clean soil over the liner.
- Construct aeration pipes by connecting a 3 m-long smooth PVC pipe with a 12 m-long perforated plastic drainage pipe using a rubber union.
- Place 3 parallel aeration pipes with 3 m intervals on the clean soil.
- Connect the aeration pipes to a manifold header via a gate valve.
- Connect the header to a 40 L water knockout tank followed by a 2,000 L water collection tank.
- Connect a blower to the water knockout tank (*see* **Note 7**).
- Load the pre-treated contaminated soils in rows with a height of 1.5–2.4 m.
- Add water via a dripline irrigation system installed across the top of the biopile until the moisture content reaches 70–95% of field capacity (FC) (determine FC using ASTM D 2365 or ASTM 3152).
- Construct monitoring point by filling a suction strainer (2.5 cm diameter × 15 cm length) with gravel and connect it to a nylon tube.
- Install thermocouples and moisture sensors with the monitoring point (*see* **Notes 8** and **9**).
- Install between 6 and 8 monitoring points at about 0.9–1.2 m below the top of the pile and 0.5 m above the clean soil (*see* **Note 10**).
- Cover the biopile using a 20 mm-thick waterproof HDPE cover (23 m × 23 m) (*see* **Note 11**).
- Tie the cover across the surface of the biopile using nylon ropes.

3.3 Soil Sampling

- Collect soil samples at the start-up of the biopile construction and then determine the constituent of degradation and biodegradation (CO_2, O_2, CH_4, H_2S, and VOCs) in the air extracted or collected from the biopile weekly during the first 3 months and then monthly or quarterly.
- Determine the sampling grid and identify the sampling locations with a sampling frequency of 1 sample per 38 m^3.
- Remove the cover and bore a hole to approximately 0.3 m above the desired sampling depth using hand auger.
- Collect a core sample using a slide hammer-type hand sampler assembled with a brass sleeve at one end.
- Remove the brass sleeve from the sampler, cap both ends of the sleeve with Teflon inert caps, label the sleeve containing soil sample, and place the sample in a cooler chilled with ice.

- Cover the biopile and transport samples to the lab for analysis of physicochemical properties (e.g. pH (*see* **Note 12**), moisture, loss on ignition, total carbon, organic carbon of soil, available phosphorus, total phosphorus, total nitrogen, and particle size distribution), contaminant concentration (e.g. total petroleum hydrocarbons and heavy metals), and microbial biomass.

3.4 Soil Gas Monitoring

- Collect soil gas samples at the start-up of the biopile construction, 1 week after start-up and then monthly.
- Connect the constant flow vacuum pump to the quick-connect coupling at the monitoring probe.
- Place a trap between the pump and the monitoring probe to collect the water that might be pulled from the monitoring probe.
- Connect the pump outflow to a 1 L air sampling bag (e.g. Tedlar™ bag).
- Open valve on the bag, turn on the pump, and fill the bag with soil gas.
- Flush the bag with soil gas twice and collect the final soil gas sample.
- Close the valve on the bag and disconnect the bag from the pump.
- Analyse the soil gas in the bag for O_2 (*see* **Note 13**), CO_2, and volatile hydrocarbons (i.e. benzene, toluene, ethylbenzene, and xylenes (*see* **Note 14**).

4 Notes

1. A laboratory-scale biopile can be constructed using polyethylene container (e.g. size: 27 × 24 × 13 cm). Briefly, remove the plant residues in the contaminated soil and mix the soil thoroughly after adding nutrients and water. Place 5 kg soil in each mesocosm and incubate it in the dark under aerobic conditions for 6 months. Water and turn the mesocosm twice a month to maintain humidity and aerate the soil. Collect the surface soil for microbiological, chemical, and toxicity analysis at the beginning of the experiment and then after monthly.
2. Treatability studies should be performed before designing a field-scale biopile to assess whether the physicochemical and microbiological parameters are either within the appropriate

range or need to be optimised. Key parameters that should be assessed include:

(i) **Soil characterisation**: moisture content (60–80%), soil temperature (10–45°C); soil texture (for heavily textured soil, windrow turning might be more effective than biopiling because the resultant soil will be more friable [40]); microbial count of the indigenous population in typical soil ranging between 10^3 and 10^7 CFU g^{-1} dry soil (plate counts lower than 10^3 CFU g^{-1} dry soil could indicate the presence of toxic compounds); nutrient levels (C:N:P = 100:10:1–100:1:0.5), pH (6–8) (values in parentheses are the optimal range for an effective biopile). If some parameters are not optimal, then the biopile can be engineered to be potentially effective for any combination of site conditions and petroleum products. For example, adjust the pH by soil amendments or tune the temperature by heated or cooled air injection.

(ii) **Contaminants characterisation**: identify the type of contaminants (e.g. total petroleum hydrocarbons, heavy metals, chlorinated or recalcitrant organic compounds) and determine the initial concentration for each contaminant group (i.e. quantify the fractions of petroleum hydrocarbons using sequential ultrasonic extraction method [43]). Biopiling technology may be unsuitable when exceptionally high contaminant concentrations are present (i.e. TPH > 5,000 mg/kg^{-1}; toxic metals > 2,500 mg kg^{-1} soil).

(iii) **Biodegradation in lab-scale experiments**: set up microcosms to evaluate the biodegradation efficiency by quantifying the petroleum concentration changes during incubation at different scenarios. For instance, load various concentration of nutrients to determine the optimum C:N:P ratio, inoculate the microcosms with hydrocarbon-degrading microorganisms to determine if biodegradation can be improved by inoculation, and sterilise the microcosm to measure the abiotic degradation rates over time.

3. The indigenous microbial community may be sufficient for bioremediation, making it unnecessary to add microbes to the system. However, there are several commercially available bioaugmentation, nutrient, and surfactant products that may work with the indigenous microbes to augment remedial efforts.

4. Fertiliser application is usually based on the theoretical C:N:P ratio 100:10:1 of a microbial cell but rarely really takes into account the fact that excessive fertiliser application can be inhibitory due to toxic or osmotic effects. When applying and

optimising fertiliser application, it is important to determine the C:N:P ratio based on the resulting concentration in soil solution rather than the theoretical N and P demand [44]. Oxygen uptake monitoring using a differential manometry Gilson respirometer, for example, can be used as a rapid measurement method to optimise the application of fertiliser [44].

5. The nutrient can also be dissolved in water before adding them into the soil, but we recommend adding them as dry powder or granules because the nutrient solution soaked into the soil may leave the soil as runoff.
6. Ideally the foundation for the biopile should be smooth with approximately 1 to 2 degree slope. If the site contains an asphalt or concrete surface, it could serve as the foundation for the soil storage area instead of the compacted soils [42].
7. The blower is used to push or pull the air through the biopile. Ensure the cover is properly secured when the air injection mode is selected.
8. Temperature – Temperature also affects biodegradation rate. Microbial activity slows when the temperature decreases, and very high temperatures can essentially sterilise soil, creating an adverse environment for certain microbial activity. When microbes mineralise hydrocarbons, heat is generated within the soil piles, often raising the soil pile temperature between 5 and 10°C. Monitor the temperature using thermocouples vertically embedded in the biopile at 0.9–1.2 m depths. Use a portable LCD thermometer to collect the temperature data. Increase the monitoring frequency during biopile thawing and thermal stabilisation, while reducing the frequency after reaching relative thermal equilibrium. Sustain the temperature by covering the biopile with a waterproof HDPE cover. If the heat loss is high in the biopile, hot air can be supplied to the biopile to maintain the temperature for a more efficient degradation. Generally, optimum biopile temperatures range from 10 to 38°C for a permanent concrete facility.
9. Water – Water is essential for microbial activity. While low moisture content will inhibit microbial growth and mobility, excessive moisture will clog soil pores, thereby restricting necessary airflow. Soil moisture is normally maintained in the pile at 60–80% of the soil field capacity, while site-specific conditions should be considered and therefore testing should be carried out at lab scale for optimisation.
10. The monitoring points should be placed in two diagonally opposed corners, one in the centre, one over an aeration line, one between the aeration lines, and one or two close to the pile edge.

11. The HDPE cover should be black or some opaque colour in order to minimise the reflection of sunlight radiation. Ensure there are no sharp objects on the top of the biopile or low points in the centre of the biopile which may rip the cover or retain water during incubation. Time-zero sampling of soil and gas should be carried out before covering the biopile.
12. pH – Most hydrocarbon-degrading bacteria grow best at a neutral to slightly alkaline pH; thus, pH levels are maintained near 7, but definitely within the 5 to 9 range. Where concentrations of aromatic compounds are present and where soils have low alkalinity, liming may be necessary. The pH in soil samples can be measured in a suspension of 1 g of soil in 9 mL of distilled water using an Orion pH-metre.
13. Oxygen – For aerobic degradation of fuel contaminants, a suitably high oxygen level must be maintained in the soil pile. Oxygen is supplied by injecting air via a piping network in the soil pile. The system is closely monitored to maintain the soil oxygen content above 15% (>0.2 mg L^{-1} dissolved oxygen, > 10% air filled pore space for aerobic degradation) [45].
14. It is optional to install activated carbon canisters to remove volatile organic compounds (VOCs) if the exhaust gas needs to be treated before release into the environment.

Design Biopile Calculation Example for 286 m^3 Gasoline-Contaminated Soil with Low Organic Content

1. **Moisture for biopile**

   Assume:

   - Porosity, $\varphi = 30\%$ and initial saturation, $S = 20\%$
   - Desired water content = 25–85%, use 60%

   Therefore:

   - The water needed = $286 \times 0.30 \times (0.6–0.2) = 34.3\ m^3 = 34{,}300\ L$

2. **Nutrient requirement for biopile**

   - 158 kg spill of gasoline (C_7H_{16})
   - Nutrient sources: Ammonium sulphate ($(NH_4)_2SO_4$); trisodium phosphate ($Na_3PO_4 \cdot 12H_2O$)

     MW of gasoline = $7 \times 12 + 1 \times 16 = 100\ g\ mol^{-1}$

     Moles of gasoline = $158 \times 10^3/100 = 1{,}580$ mol

     Moles of C = $7 \times 1{,}580$ mol = 1.106×10^4 mol

     Molar ratio C:N:P = 120:10:1

     Moles of N needed = $10/120 \times 1.1 \times 10^4 = 917$ mol

Moles of $((NH_4)_2SO_4)$ needed $= 917/2 = 458$ mol

MW of $((NH4)_2SO_4) = (14 + 4) \times 2 + 32 + 4 \times 16 = 132$ g mol^{-1}

Mass of $((NH_4)2SO_4)$ needed $= 132 \times 460 = 6.1 \times 10^4$ g $= 61$ kg

By similar calculation:

Mass of $(Na_3PO_4 \cdot 12H_2O)$ needed $= 35$ kg

3. **Oxygen requirement for biopile**

$$C_7H_{16} + 22O_2 \rightarrow 7CO_2 + 8H_2O$$

1 mol (100 g) gasoline requires 22 mol ($16 \times 2 \times 22 = 704$ g) O_2

Oxygen content of air = 21% by volume = 210,000 ppmv

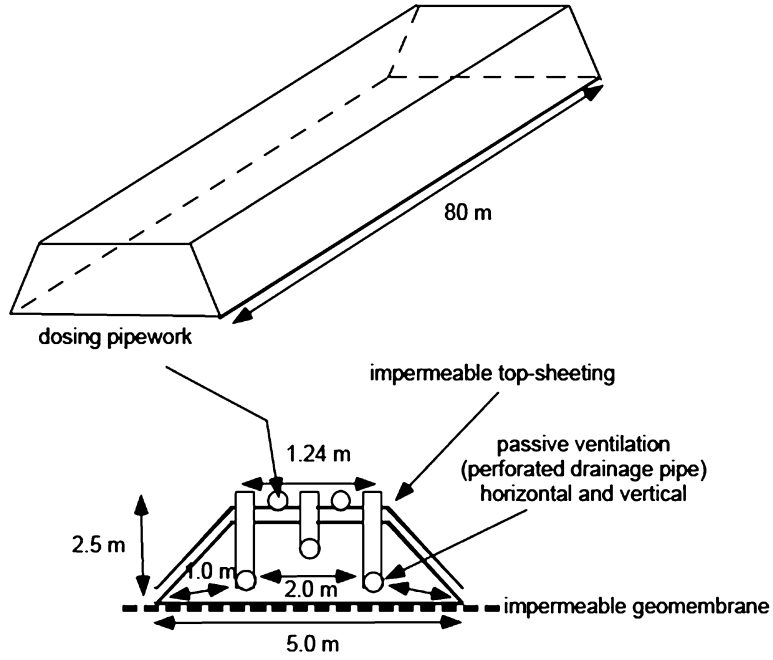
mg L^{-1} to ppmv:

$$\text{ppmv} = \frac{\text{mg}}{\text{L}} \times 10^3 \times \frac{1}{\text{MW}_{\text{contaminant}}\left[\text{g mol}^{-1}\right]} \times 8.314 \left[\frac{\text{L kPa}}{\text{mol K}}\right] \times T_{\text{air}}[K] \times \frac{1}{P_{\text{air}}[\text{kPa}]}$$

Oxygen needed for 158 kg spill of gasoline ($\cong C_7H_{16}$)

- 100 g gasoline needs ~704 g O_2.
- 158 kg gasoline $\times$ 7 = 1,106 kg $O_2 = 1.1 \times 10^6$ g O_2.
- Water in pile $= 286$ m$^3 \times 0.30 \times 0.6 = 51.5$ m$^3 = 51{,}500$ L.
- At saturation at 20°C and 1 atm (101.325 KPa), dissolved oxygen $= 9.2$ mg L^{-1}.
- Mass of oxygen in soil moisture $= 51{,}500$ L $\times$ 9.2 mg L^{-1} $\times$ 0.001 g mg$^{-1} = 473.6$ g O_2.
- 473 g O_2 in soil moisture is much less than 1.1×10^6 g O_2 required.
- At 0.28 g L^{-1} air, air requirement is 1.1×10^6 g/0.28 g L$^{-1} = 3.93 \times 10^6$ L = 3,930 m^3.
- Daily air requirement for a duration of 3 months is 3,930 m^3/90 day = 43.7 m^3/day.
- Air void volume in pile $= 286$ m$^3 \times 0.30 \times 0.4 = 34$ m^3.
- Need to daily exchange $43.7/34 = 1.3$ void volumes to fulfil oxygen requirement (Note: this rate should be compared to passive aeration and check if the latter is sufficient. In the case that it is not, the pump should provide at least twice the air flow as not all the air will be absorbed by the biopile).

Example of calculation of the volumetric composition of a biopile [39]



Height: 2.5 m

Upper width: 1.24 m

Lower width: 5 m

Length: 80 m

Total volume: 624 m^3

Total mass: 7.49×10^5 kg

Area: $((5 + 1.24) \times 2.5)/2 = 7.8\ m^2$

Volume: $7.8 \times 80 = 624\ m^3$

(i) Total volume: 624 m^3

(ii) Total mass: 7.49×10^5 kg = 749 tonnes (assuming a bulk density of 1,200 kg m^{-3})

1. Water: 80% of field capacity (v/v), field capacity of sandy loam (SL) soil: 20%

   Water volume = 624 $m^3 \times 0.16 = 99.84\ m^3$

2. At 50% porosity, water + air = 50%

   Air volume = 312–99.84 $m^3 = 212.16\ m^3$

3. 2% organic matter, 48% inorganic matter

   Volume of 2% organic matter = 624 $m^3 \times 0.02 = 12.48\ m^3$

   Volume of 48% inorganic matter = 624 $m^3 \times 0.48 = 299.52\ m^3$

4. $TPH_{t=0}$: 80,000 mg oil/kg soil = 0.08 kg oil/kg soil,

Density of heavy oil (NAPL): 0.97 g/cm^3,

NAPL volume = 0.08 kg oil/kg soil = 61.72 m^3

Assume NAPL shares air volume with air

Air volume = 212.16–61.72 m^3 = 150.44 m^3

So overall:

- Volume of soil = 312 m^3
- Volume of water 100 m^3
- Volume NAPL = 62 m^3
- Volume of air = 150 m^3

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Ex Situ Bioremediation Treatment (Landfarming)

Maria Nikolopoulou and Nicolas Kalogerakis

Abstract

Landfarming provides a platform where soil conditions (pH, nutrient, moisture, and tilling) can be optimized to promote microbial activities and thus the desired degradation of soil pollutants can be achieved. The factors under which landfarming is applicable and leads to increased effectiveness are reviewed and design parameters for successful landfarming applications are provided.

Keywords: Biochemical processes, Landfarming design, Land treatment unit, Soil bioremediation

1 Introduction

Landfarming that has been routinely applied to remediate refinery petroleum sludges belongs to the bioremediation technologies that have been also applied to treat crude oil contaminated marine beach sand and sediments [1].

Landfarming is a very simple and straightforward soil remediation technology, also known as land treatment or land application. Contaminated soil that principally is excavated and spread into a thin layer above ground surface serves both as the substratum and substrate for microbial activities that stimulate biodegradation processes. Pollutant is mainly removed through volatilization and biodegradation (Fig. 1). Biochemical processes within the treated soil can be controlled and enhanced by optimizing parameters (aeration, addition of minerals, nutrients, and moisture) that favor microbial activities [2]. Optimization of the above parameters can be easily achieved with commonly used landfarming equipments (tractors, rotary tillers, chisel plows, soaker hoses, rotary sprinklers, etc.) that are available in the agriculture market. The nature of the technology is such that requires open large areas which should be prepared for drainage management, operation equipment access, and materials management. Landfarming has been successfully exercised for over 100 years to treat a variety of pollutants but mostly for hydrocarbon polluted soils. More volatile fractions of

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Fig. 1 Typical landfarming treatment unit

petroleum (i.e., gasoline) tend to evaporate whereas denser and heavier fractions (i.e., diesel fuel, lubricating oil) are mainly removed due to biodegradation [3].

Nonetheless it has become more attractive than other soil remediation methods because it has low cost, energy consumption, risk of contaminant migration, and low environmental impact, but most importantly landfarming complies with government regulations and is very versatile to any climate and location [4]. Major benefits and drawbacks of landfarming are summarized in Table 1. Landfarming can be in situ or ex situ; for shallow polluted soils (i.e., <1 m below ground surface), as long as the contaminant cannot migrate to the aquifer, no excavation is needed and contaminated soil can be treated locally, whereas contaminated soils at depth greater than 1.7 m need excavation and reapplication on the ground surface [2]. Soil is treated until the contaminant concentrations are below or at acceptable limits as established by environmental control agencies.

2 Technology Requirements

As mentioned earlier, landfarming is a simple technique which although takes advantage of several physicochemical mechanisms of contaminant loss (volatilization, photooxidation, or humification), still its primary goal is to optimize the biological mechanisms through which the removal of most organic contaminants is succeeded.

Thus, successful landfarming requires the maximization of biochemical processes through supplementation with essential nutrients nitrogen (N) and phosphorus (P) in addition to other growth-limiting substrates as well as oxygen, which is provided by regular

Table 1
Advantages and disadvantages of landfarming

Advantages[a]	Disadvantages
Technology is relatively easy to design and to put into practice	Difficult to accomplish complete removal of the pollutant (concentration depletion < 95%)
Short treatment times (6–24 months under optimal conditions)	Effectiveness can be limited if pollutant's concentration exceeds 80,000 ppm in total petroleum hydrocarbons
Initial capital and operation costs are extremely low	Applicable only to biodegradable pollutants
Large soil volumes can be treated	Large treatment area is required
It is applicable also ex situ	Volatilization rather than biodegradation of lighter pollutants
Efficient with slow metabolizing organic pollutants	Potential risk from pollutant exposure
Environmentally safe and friendly	Contaminants bioavailability can be reduced by the presence of clay or humic substances into the soil matrix and thus decrease pollutants biodegradation rate
Energy saving	Cost increases considerably if excavation is required

[a]Adapted from US EPA [2] and Maila and Cloete [5]

Table 2
Major factors influencing landfarming performance [2]

Soil characteristics	Contaminant characteristics	Climatic conditions
Microbial population density	Volatility	Ambient temperature
pH	Chemical structure	Rainfall
Moisture Temperature Oxygen Nutrient concentrations Texture	Concentration	Wind

tiling, and water to maintain the moisture at the optimal levels [6]. Several studies also recommend to augment the contaminated area with specialized cultures of allochthonouscontaminant-degrading prokaryotes at the beginning of the landfarming treatment [7]. Overall, the effectiveness of landfarming depends on a number of factors (Table 2) which influence biodegradation, the main mechanism of contaminants removal and are grouped into three categories: soil characteristics, contaminant characteristics, and climatic conditions. These factors are usually monitored or/and controlled and evaluated before implementation of a land treatment unit (LTU) [2].

2.1 Soil Characteristics

2.1.1 Microbial Population Density

Soils usually contain microorganisms in the range from 10^4 to 10^7 CFU/g of soil. According to US EPA [2] in order for landfarming to be successful heterotrophs population shouldn't be lower than 10^3 CFU/g. Population densities below this evidences the presence of toxic concentrations of organic or inorganic (e.g., metals) compounds, still though landfarming may be effective as long as soil is amended to increase the bacterial population or is supplemented with suitable consortia. If the contaminated soil lacks a significant population of degraders it is possible to use bioaugmentation. The presence of heterotrophic as well as hydrocarbon degrading bacteria should be monitored in soil by plate count, most probable number (MPN) technique, phospholipid fatty acid (PLFA) analysis, or denaturing gradient gel electrophoresis (DGGE) [8].

2.1.2 Soil pH

Desired soil pH values to sustain microbial growth should fall between 6 to 8 ranges, with a value of about 7 (neutral) to be considered the optimum. When soil pH values fall outside this range they can be adjusted through the addition of lime for acidic soils or through the addition of elemental sulfur, aluminum sulfate, ferrous sulfate for alkaline soils preferably prior to landfarming operation [2].

2.1.3 Moisture Content

Too much water in the soil will hinder the supply of oxygen and as a result will decrease microbial activities and subsequently the rate of biodegradation. On the other hand, too little water will inhibit microbial activities. The optimal soil moisture range for supporting the microbes is between 40 and 85% of the water-holding capacity (field capacity) which results in soil water content between 12 and 30% by weight [2].

2.1.4 Temperature

Biological activity is promoted when soil temperature is kept within the range of 10–45°C [2, 9]. Microbial activity generally decreases at temperatures below 10°C or greater than 45°C and hence, special temperature-controlled enclosures or special bacteria required for areas with extreme temperatures should be incorporated.

2.1.5 Oxygen

Aeration of landfarmed soils is critical since sufficient amounts of oxygen favor microbial activity and thus degradation of pollutants, however simultaneously it should be reasonably low to avoid uncontrolled evaporation of highly volatile compounds, such as BTEX [10]. Moreover, aeration creates a more homogenous distribution of contaminants, nutrients, water, air and microorganisms and increases biodegradation rates. On the contrary when aeration through tilling is applied to a highly moistened or saturated soil then soil structure is dismantled and hence, oxygen and water infiltration are decreased and thus, biological activities

are suppressed. Typically aeration of LTUs can simply be achieved through regular tilling, ploughing, or turning the material to increase oxygen intake. The use of a tractor-mounted rotary tiller is suggested to ensure satisfying mixing and aeration of the treated soil [9].

2.1.6 Nutrient Concentrations

Nutrients such as nitrogen and phosphorus are essential to favor microbial activity and thus promote biodegradation processes. The optimal nutrients ratio (C:N:P) so that biodegradation is successful should be between 100:10:1 and 100:1:0.5 depending on the site's specific contaminant characteristics and potential microbial community associated to the biodegradation process [2]. Fertilizers may be added in pellet form or dissolved in the irrigation water added to the landfarm. The amount and frequency of fertilizer addition depends on site's particular characteristics and conditions [11].

2.1.7 Soil Texture

Soil texture affects the permeability, water content, and bulk density of the soil. Clayey soils that glue together are extremely hard to aerate, which consequently contain lower oxygen concentrations, and hinder the delivery of nutrients as well as moisture within their structure. Clayey soils tend to retain water for prolonged periods whereas coarse grained soils do not, however both are considered unsuitable for landfarming. Moreover volatile compounds tend to evaporate in coarse soils instead of fine grained soils. Bulking agents (woodchips, sawdust, straw) can be applied to reduce bulk density, improve soil structure and thus favor oxygen and water intake of clumpy soils [2, 10].

2.2 Contaminant Characteristics

Volatile compounds present in the contaminant are usually released to the atmosphere during soil tilling and plowing and their emission can be controlled by utilizing covers (plastic sheet) and structural enclosures (greenhouse or plastic tunnel). The VOC emissions can be captured and treated later through activated carbon filters or any other possible treatment process that complies with national regulations for air quality standards [2, 10].

2.2.1 Volatility

2.2.2 Chemical Structure

The chemical structure of the constituents to be treated by landfarming determines their potential biodegradation rate. Typically, almost all compounds present in petroleum products are susceptible to biodegradation but at different rates; so the higher the molecular weight and complexity of compounds structure (polynuclear aromatic hydrocarbons) the lower the biodegradation rate. Thus the evaluation (biotreatability studies) of the chemical structure of the pollutants suggested for degradation through landfarming dictates which compounds will be the most degradable and to what extent [2].

2.2.3 Concentration and Toxicity of Contaminant

High concentrations of total petroleum hydrocarbons (TPH > 80 g/kg or 8%) or heavy metals (>2.5 g/kg) in contaminated soils can be toxic to microorganisms and thus hinder biodegradation processes in the LTUs. Moreover, concentrations of petroleum product up to 25% by weight of soil could be treated by mixing with clean or less contaminated soils to dilute the contaminants concentrations to desirable ranges [11]. The achievable TPH levels highly depend on the site conditions, the pollutant's amount and properties. For instance, a highly weathered heavy petroleum product that contains high molecular weight hydrocarbons which are resistant to biodegradation is not susceptible to bioremediation.

2.3 Climatic Conditions

Exposure to nature elements (rain, snow, wind, temperature) is typical for ex situ uncovered landfarms.

2.3.1 Ambient Temperature

The ambient temperature is significant since it is directly related to soil temperature. Favorable temperatures for microbial growth and sustainability and thus biodegradation are in the range 10–45°C.

2.3.2 Rainfall

Rainfall can increase the water content of the soil to saturation level and excess water can erode the treatment area. Landfarming is not suitable in areas where the annual rainfall exceeds 750 mm. In general, water management systems for control of runon and runoff should be installed. Runon can be managed through the installation of berms or ditches that deflect and divert the stormwater flow, while runoff can be leaded and diverted to a retention pond by simply grading the treatment site (slope <5%). A leachate collection/treatment system at the retention pond is considered obligatory to manage, control emissions, and also recycle leachates for irrigation purposes [2].

2.3.3 Wind

Soil erosion is a common trait in landfarming mostly due to weather conditions (wind) and tilling operations. However it can be controlled/prevented if the soil is organized into windrows and is sprayed systematically to minimize dust [2].

3 LTU Design and Construction

3.1 Site Preparation

3.1.1 Soil Preparation

Usually a wide range of wastes (rocks, roots, wood, plastic and metal) that may interfere with the process can be found in the polluted soil. For that reason the excavated contaminated soil should be screened to remove such wastes (greater than about 2.5 cm diameter) with, for instance, portable screening buckets, as the ones used in road construction, quarries and mines, and then is laid on top of a porous sublayer (sand or soil). Modern tillage equipment can till until about 65 cm depth. Depending on the

tillage equipment capabilities specialized equipment can reach at depth of 1 m or more [11, 12].

3.1.2 Size Estimation of Land Treatment Area

In general the volume of soil due to fluffing and soil disturbance throughout excavation increases approximately 1.25 and 1.4 times its initial volume [13]. For example, if the estimated volume to be excavated is 535.7 m^3, then the increased volume (V) due to "fluffing" is $1.4 \times 535.7\ m^3 = 750\ m^3$.

The depth of the in question treatment zone is generally 15–50 cm and is a function of the soil depth that can be adequately tilled and treated [12–14].

For our example, if the soil depth (d) is decided to be 0.3 m, then the resulting needed surface area is A = soil volume (V)/soil depth (d) = $750\ m^3/0.3\ m = 2{,}500\ m^2$.

In reality, most of the time the surface area available for land treatment dictates the size of the LTU to be constructed.

3.1.3 Liners–Leachate Control system

Initially an impermeable layer to prevent infiltration and cross contamination of the lower soil layers and aquifer from leachates is constructed. The LTU base is prepared by removing excess debris that could tear apart the liners placed on top of it. Sloping is also crucial for controlling excess runon and runoff from the LTU [15].

The liner system which mainly depends on the adapted national regulations and design criteria is constructed to prevent leachates penetration to the aquifer or lower uncontaminated soil layers below the treatment zone. The liner system also serves as a collection-controlling system for leachates towards a retention pond [12].

Liners/barriers are mandatory at sites with less than 5 m of native underlying soil or if the hydraulic conductivity is $>1 \times 10^{-6}$ cm/s [14].

A conservative liner-leachate design typically used is a double liner-leachate collection system. The first layer of the liner system should be between 30 and 60 cm thick and usually consists of compacted, low-permeability ($l0^{-7} \cdot$ cm/s) clay material. The clay liner is oriented with 2% slope towards the gravel drain, located along the central axis of the LTU and also graded (1%) to a collection sump located at one end of the LTU. A second optional layer, a membrane liner, can be set above the compacted clay layer. The membrane liners can be made from different materials, however the most popular and recommended for this purpose is 1 mm high-density polyethylene (HDPE) due to its endurance and chemical resistance with petroleum leachates [12].

A drainage system which is necessary to collect leachates due to irrigation and/or precipitation is installed on top of the liners. The leachate collection system consists of two drainage layers that lie on top of the HDPE liner to divert leachates into the collection sump. A geotextile, filter fabric (15 cm) is placed beneath the gravel layer to

prevent clogging of the leachate collection system from particles. Drainage network consists of perforated HDPE pipes between 10 and 15 cm in diameter that are covered with geotextile and run lengthwise the LTU towards the ditch. The gravel layer (<13 mm rounded gravel) protects the HDPE liner from the heavy machinery but also leads drainage towards the pipes that direct flow to a gravel sump located at the very end of the LTU. Leachates from there can either be removed or recirculated [12]. The pipes size of the drainage system depends on the expected infiltration. Designing parameters such as pipe's diameter and distance between are calculated based on the leachates flow rate and are presented in detail in Sect. 3.3.

Finally a protective layer for sand or soil, with thickness between 0.6 and 1.2 m lies on top the liner(s) and drainage system to prevent any damage due to heavy machinery [15]. The minimum thickness though is normally about 0.3–0.6 m. Ditches or berms of at least 0.8 m high and 0.5 m thick are installed at the perimeter of the LTU so that any run-on is prevented and runoff is contained. The system suggested and shown in Fig. 2a, b should be regarded as a common practice, however depending on site characteristics and national regulations different liners-drainage system designs can be applied to effectively capture leachate within treatment zone.

3.2 Optimization of Soil Properties

The LTU constitutes the action field where soil properties and conditions (pH, nutrient, moisture, and tilling) can be optimized to stimulate microbial activities and should be constantly monitored and controlled.

3.2.1 Soil pH

The desired soil pH ranges from 6 to 8 for ideal biodegradation. Soil's pH greatly influences microbial activity, nutrients availability, metals immobilization, contaminants abiotic removal rate, and soil structure. For instance at high pH (>8.5) values, ammonia becomes more volatile and tends to be released into the atmosphere while at lower pH (6.5) values phosphorous solubility is maximized [11, 12, 16].

There are several chemical reagents that can be used to adjust soil pH at the optimal range for biodegradation processes. Usually most soils are characterized as acidic than liming agents (calcium or magnesium containing reagents) like calcium oxide (CaO), calcium hydroxide, magnesium carbonate and agriculture lime ($CaCO_3$) may be used to increase pH. On the other hand, if a soil is alkaline, then aluminum sulfate, ferrous sulfate, elemental sulfur, or inorganic acids (sulfuric, phosphoric) may be used to lower the pH [9, 12] (Tables 3 and 4).

3.2.2 Tilling-Oxygen Supply

Tillage equipment mounted on farm tractors is widely used in agriculture and can easily be found in the market. The rotary tiller is mostly used and recommended since it ensures satisfying

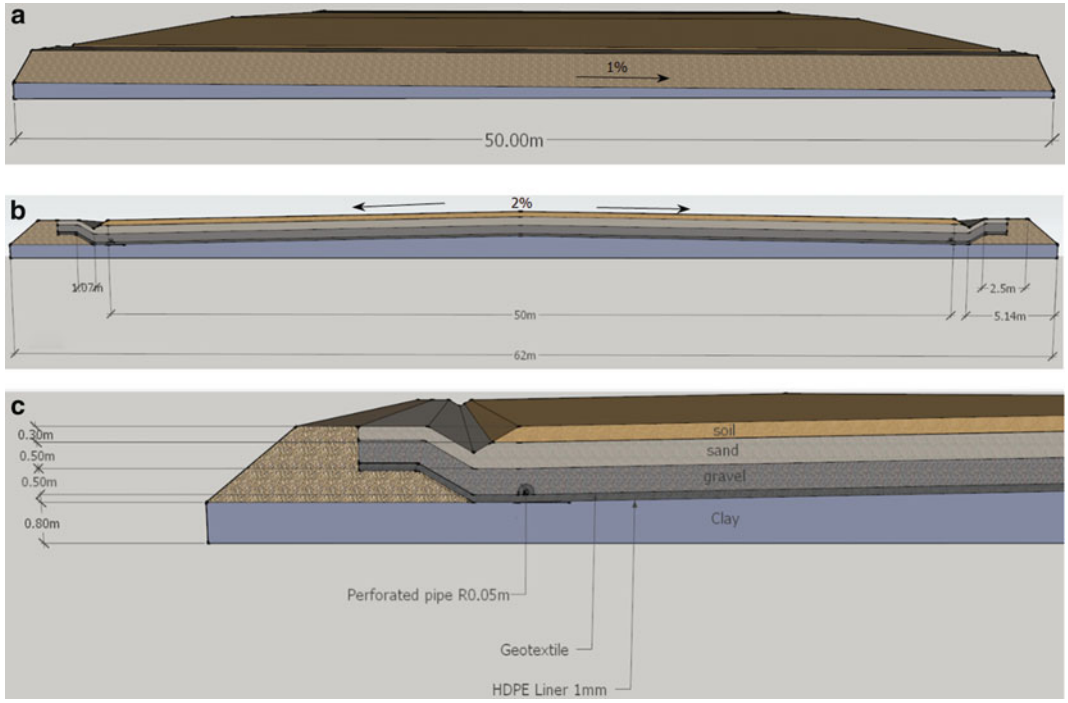


Fig. 2 Design details of landfarming units (**a**) overall diagram – length view, (**b**) overall diagram – width view and (**c**) berm details

Table 3
Amount of sulfur needed to lower soil pH by 1

Material	pH Change	kg/100 m^2
Sulfur	7.5 to 6.5	7.3
	8.0 to 6.5	17.1
	8.5 to 6.5	19.5
Iron sulfate	7.5 to 6.5	61.0
	8.0 to 6.5	14.2
	8.5 to 6.5	16.2

1. Effective only on soils without free lime
2. Higher rates will be required on fine-textured clayey soils and soils with pH > 7.3
Source: http://www.ext.colostate.edu/mg/gardennotes/222.html

mixture and aeration of the soil. Moreover other farm equipment and accessories (chisel plows, disks, flotation tires, spreaders, storage tanks, etc.) that are necessary or can be used supplementary to aid the landfarming process are available in the agriculture market [12].

Table 4
Lime application rates to raise soil pH to approximately 7.0

Existing soil pH	Lime application rate (kg/100 m^2)		
	Sandy	Loamy	Clayey
5.5–6.0	9.76	12.2	17.1
5.0–5.5	14.6	19.6	24.4
3.4–5.0	19.5	26.8	39.1
3.5–4.5	24.4	34.2	39.1

Lime application rates shown in this table are typically for dolomite and assume a soil organic matter <2%. In case soils contain 4–5% organic matter, limestone (grounded or pelletized) application rate should be increased by 20%
Source: http://www.ext.colostate.edu/mg/gardennotes/222.html

Tilling is most effective in terms of maximum and more homogeneous mixing if it is performed in every direction (i.e., cross length and width and diagonally). Tilling frequency is likewise a crucial parameter depending on soil type and irrigation frequency. It is suggested to tile the soil at its lower moisture range. Tilling wet soils close to their saturation level affects soil consistency and altogether reduces oxygen and water transfer and consequently reduces biodegradation rates. For that reason it is recommended to tile after 24 h following irrigation or a significant rainfall event [11].

3.2.3 Moisture Demand-Control

Specifying important soil parameters in order to determine the application water volume needed in the first irrigation of the LTU.

Determination of Soil Moisture Content

Water Holding Capacity

The water-holding capacity of the soil can be determined by placing duplicate 20 g field-moist soil samples in funnels fitted with folded Whatman 2 V filter paper on the inside and mounted on preweighed 250 ml flasks as described by Forster [17]. Percentage water-holding capacity is calculated with the following formula:

$$\%\,\text{Water holding capacity} = \frac{(100 - W_p) + W_i}{d_{\text{wt}}} \times 100, \quad (1)$$

where W_p is the weight of the percolated water in grams, W_i is the initial amount of water in grams contained in the sample, and d_{wt} is the soil dry weight in grams [17].

Soil Gravimetric Water Content and Soil Dry Mass

Water content in sand samples can be determined gravimetrically after desiccation at 105°C overnight. The differences in masses before and after drying are a measure for the water content of soils. The water content is calculated on gravimetric (*g* water/*g* soil) or on volumetric basis (cm^3 water/cm^3 soil) [18].

The dry mass content (W_{dm}) or water content (W_{H_2O}) on a dry mass basis expressed as percentages by mass to an accuracy of 0.1% (w/w) is calculated using the following equations:

$$W_{dm} = \frac{m_2 - m_0}{m_1 - m_0} \times 100 \tag{2}$$

$$W_{H_2O} = \frac{m_1 - m_2}{m_2 - m_0} \times 100 \tag{3}$$

where m_0 = mass of the empty container (*g*), m_1 = mass of the container with field-moist soil (*g*), and m_2 = mass of the container plus oven-dried soil (*g*).

The soil used in this study is classified as sandy and its estimated water-holding capacity for the soil was 33.73%. The optimal soil moisture range for supporting the microbes is between 40 and 85% of the water-holding capacity (field capacity) and accordingly the estimated optimal soil moisture content should be between 13.5 and 28.7%.

Determination of the Water Addition Requirement for Landfarming

Moisture Requirement [19]

The following formula can be used to determine the volume of water needed for bioremediation.

$$V_w = (V_s)(\theta_f - \theta_i) = (V_s)[(\eta)(S_f - S_i)]$$

where θ_i = initial soil moisture content, θ_f = desired soil moisture content, η = porosity of soil, S_i = initial degree of saturation, and S_f = desired degree of saturation. In order to determine the amount of water needed (V_w) for the first spray applied to the excavated $V_s = 750$ m^3 oil-contaminated soil we use the following measured data: soil porosity η is 43.7% and initial saturation S_i is 20%.

Solution:
The optimum moisture content range for landfarming as already mentioned is between 40 and 85% of the water-holding capacity. A middle value of this range, 60%, is selected, however an optimization study should be carried out. So the needed water is $V_w = 750 \times 0.437 \times (60-20\%) = 131.1$ m^3

The irrigation frequency highly depends heavily on the specific treatment site's environmental conditions.

Soil moisture is usually applied via sprinkler systems via pressurized water installations. In remote locations, water can be supplied from nearby groundwater or surface water bodies.

3.2.4 Nutrient Demands

To sustain microbial growth nutrients should be applied following the optimal C:N:P molar basis ratio of 100:10:1. Prior landfarming treatment, a feasibility study should be performed in order among others to establish the site's optimum nutrient ratio. In the case no further data are provided then the aforementioned ratio should be used. Usually it is more convenient to use water soluble nutrients that can be easily irrigated or sprayed onto the soil.

To determine the nutrient requirements, two different procedures can be followed as presented in the preceding examples and the needed information is:

- The mass or concentration of the contaminants
- The chemical formula of the contaminants or % w/w carbon content in contaminants
- The optimal C:N:P ratio
- The chemical formula of the nutrients

Case 1. Determine the nutrient requirement for landfarming when % w/w carbon content in contaminants is known

We are going to estimate the amount of nutrients needed to remediate the contaminated soil aforementioned and thus we are going to need the following data in our calculation:

1. Volume of excavated soil $V_s = 750\,\mathrm{m}^3$
2. Initial concentration of oil $C_i = 1{,}000\,\mathrm{mg/kg}$
3. Soil porosity $\eta = 0.437$
4. Soil bulk density $\rho_b = 1.04\,\mathrm{g/cm}^3 \left(1{,}040\,\mathrm{kg/m}^3\right)$
5. The background concentration levels of N and P present in the excavated soil are negligible
6. Dihydrogen potassium phosphate (KH_2PO_4) as the P source
7. Sodium nitrate ($NaNO_3$) as the N source
8. Assuming optimal C:N:P ratio (100:10:1) unless it is estimated
9. Only one application

Solution 1

The elemental composition of petroleum products varies considerably and depends on the origin of crude oil. Petroleum is a complex mixture of five main elements: carbon (82–87% w/w), hydrogen (11–15% w/w), sulfur (0–8% w/w), nitrogen (0–1% w/w) and oxygen (0–0.5% w/w) [20]. Depending on the carbon content estimated in the petroleum product, the amounts of essential nutrients (N, P) needed for optimal biodegradation can be calculated. However, when the carbon content in the petroleum is not known, the mass of carbon available for biodegradation can be approximated by the measured total mass of hydrocarbons in the soil (TPH). This assumption is reasonable since the carbon content

found in most petroleum products is usually more than 80% carbon by weight.

1.a. Determine the mass of contaminated soil. Contaminated soil is equal to its volume and bulk density:

Soil mass $M_S = 750\ \text{m}^3 \times 1{,}040\ \text{kg/m}^3 = 7.8 \times 10^5\ \text{kg}$

1.b. Determine the mass of the contaminant (and carbon), which is equal to the product of the mass of contaminated soil, the average oil concentration in the contaminated soil and the percentage of carbon content (approximately 85% carbon) in the oil:

Contaminant mass (Carbon) $M_C = 7.8 \times 10^5\ \text{kg} \times 1{,}000\ \text{mg/kg} \times 85\% = 663\ \text{kg}$

1.c. Determine the mass of N needed according to the optimal C:N:P ratio.

Mass of N needed $= (10/100) \times 663 = 66.3\ \text{kg}$

Sodium nitrate molecular weight ($NaNO_3$) $= 23 + 14 + (16 \times 3) = 85$

Nitrogen molar ratio in $NaNO_3 = 14/85$ (each mole of sodium nitrate contains one mole of N).

Amount of $NaNO_3$ needed $= (85/14) \times 66.3 = 402.5$ kg.

1.d. Determine the mass of P needed according to the optimal C:N:P ratio.

Mass of P needed $= (1/100) \times 663 = 6.63$ kg

Dihydrogen potassium phosphate molecular weight (KH_2PO_4) $= 39 + (1 \times 2) + 31 + (16 \times 4) = 136$

Phosphorous molar ratio in $KH_2PO_4 = 31/136$.

Amount of KH_2PO_4 needed $= (136/31) \times 6.63 = 29.1$ kg.

Case 2. Determine the nutrient requirement for landfarming when contaminants formula is known

Let's assume that the excavated soil is contaminated with kerosene. We are going to estimate the amount of nutrients needed to remediate the kerosene-contaminated soil so we are going to need the following data in our calculation:

1. Volume of excavated soil $V_s = 750\,\text{m}^3$
2. Initial mass of kerosene $M_K = 204\,\text{kg}$
3. Soil porosity $\eta = 0.437$
4. Formula of kerosene (assumed) $= C_{12}H_{26}$
5. The background concentration levels of N and P present in the excavated soil are negligible
6. Dihydrogen potassium phosphate (KH_2PO_4) as the P source

7. Sodium nitrate ($NaNO_3$) as the N source
8. Assuming optimal C:N:P ratio (100:10:1) unless it is estimated
9. Only one application

Solution 2

2.a. Determine the number of moles of kerosene in soil. Kerosene molecular weight $(C_{12}H_{26}) = 12 \times 12 + 1 \times 26 = 170$, dividing the kerosene mass by its molecular weight then the moles of kerosene = 204/170 = 1.2 kg-mole.

2.b. Determine the number of moles of C with respect to the total moles of kerosene present in the soil. Each mole of kerosene according to its formula ($C_{12}H_{26}$) contains 12 carbon atoms, then total moles of C in soil = 1.2× 12 = 14.4 kg-mole

2.c. Determine the number of moles of N needed taking into account the optimal C:N:P ratio.

Mole of N needed = (10/100) × 14.4 = 1.44 kg-mole

Mole of $NaNO_3$ needed = 1.44/1 = 1.44 kg-mole (each mole of sodium nitrate contains one mole of N).

Amount of $NaNO_3$ needed is obtained by multiplying the calculated moles of fertilizer required by its molecular weight = 1.44 × (23 + 14 + 16 × 3) = 122.4 kg.

2.d. Determine the number of moles of P needed taking into account the optimal C:N:P ratio.

Mole of P needed = (1/100) × 14.4 = 0.144 kg-mole

Mole of KH_2PO_4 needed = 0.144 kg-mole.

Amount of KH_2PO_4 needed is obtained by multiplying the calculated moles of fertilizer required by its molecular weight = 0.144 × [39 + (1 × 2) + 31 + (16 × 4)] = 19.6 kg.

However, if ones need to be more precise a third more accurate option would be to measure total organic carbon and nitrogen in soil by Dry Combustion Method ("Elemental Analysis") using a CHNS Analyzer. Combustion technique calculates all the carbon in a sample.

3.3 Irrigation Requirements

The irrigation system should be programmed to apply no less than 25, 5 mm of water within 10–12 h. The amount and frequency of the irrigated water should not exceed soil's absorbance capacity or else the resulting excess runoff could erode the LTU zone considerably. Overall, coarser (sandy or loamy) soils water infiltration rate is faster than finer soils (clay or clay loam). In general the suggested irrigation rate shouldn't exceed 13 mm/h of water and can easily be controlled through the installation of a water meter [11].

Irrigation rate depends on soil properties, which correspond to certain infiltration capacity. Soil infiltration can be estimated through either Horton's or Green-Ampt's method [21].

3.3.1 Horton's Method

Infiltration rate: $f(t) = f_c + (f_0 - f_c)e^{-kt}$
Cumulative infiltration: $F(t) = f_c t + \frac{f_0 - f_c}{k}(1 - e^{-kt})$

where f_0 = initial infiltration rate, f_c = constant infiltration rate, and k = decay constant.

3.3.2 Green-Ampt's Method

Infiltration rate: $f = K\left(\frac{\Psi\,\Delta\theta}{F(t)} + 1\right)$
Cumulative infiltration: $F(t) = K\,t + \Psi\,\Delta\theta \ln\left(1 + \frac{F(t)}{\Psi\Delta\theta}\right)$

where K is the hydraulic conductivity, Ψ is the wetting front soil suction head, and $\Delta\theta$ is the change in moisture content when the wetting front passes. The latter can be obtained from

$$\Delta\theta = \eta - \theta_i = (1 - S_e)\,\theta_e$$

- where effective saturation, S_e, is the ratio of the available moisture to the maximum possible available moisture content, i.e., $S_e = \frac{\theta - \theta_r}{\eta - \theta_r}$,
- θ_i = initial moisture content
- θ_r = residual moisture content
- η = porosity
- $\theta_e = \eta - \theta_r$ = effective porosity

According to Brooks–Corey equation, Ψ can be expressed as a logarithmic function of the effective saturation: $S_e = \left[\frac{\Psi_b}{\Psi}\right]^{\lambda}$, where Ψ_b, λ are constants obtained by soil draining tests.

During a rainfall, water accumulates in ponds on the surface only if the rainfall intensity is greater than the infiltration capacity of the soil. The ponding time t_p is the elapsed time between the time rainfall begins and the time water begins to pond on the soil surface. The cumulative infiltration during the ponding time t_p is given by $F_p = i \times t_p$, where i is the infiltration rate and thus, ponding time t_p can be calculated:

$$t_p = \frac{K\,\Psi\,\Delta\theta}{i\,(i - K)}$$

Estimate the time needed for the excavated oil contaminated soil to become saturated and thus the suitable irrigation rate. The oil contaminated soil is classified as sand thus according to Table 5 the infiltration parameters are: $K = 11.78$ cm/h, $\Psi = 4.95$ cm, $\theta_e = 0.417$, $\eta = 0.437$.

Assuming a saturation level $S_e = 0.2$ then $\Delta\theta = (1 - S_e)\,\theta_e = 0.3336$, if the irrigation rate of water applied is 1 cm/h, then the estimated time where soil becomes saturated is:

Table 5
Infiltration parameters for various soil classes [21]

Type of soil	η	θ_e	Ψ (cm)	K (cm/h)
Sand	0.437	0.417	4.95	11.78
Loam	0.463	0.434	8.89	0.34
Silt loam	0.501	0.486	16.68	0.65
Silty clay	0.471	0.432	27.30	0.10
Clay	0.475	0.382	31.63	0.03

$$t_p = \frac{K\,\Psi\,\Delta\theta}{i\,(i-K)} = \frac{11.78 \times 4.95 \times 0.3336}{1\,(1-11.78)} = -1.8\,\text{h}$$

which means that it does not become saturated before 1.8 h of application. Assuming that the irrigation rate is 12 cm/h then the estimated time where soil becomes saturated is:

$$t_p = \frac{K\,\Psi\,\Delta\theta}{i\,(i-K)} = \frac{11.78 \times 4.95 \times 0.3336}{12\,(12-11.78)} = 7.37\,\text{h}$$

In another case where soil is classified as silt loam and is considered to take up water at slower rate than sandy soil:

$$K = 0.65\ \text{cm/h},\quad \Psi = 16.68\ \text{cm},\quad \theta_e = 0.486,\quad \eta = 0.501$$

Assuming a saturation level $S_e = 0.2$ then $\Delta\theta = (1 - S_e)\,\theta_e = 0.3888$, if the irrigation rate of water applied is 1 cm/h, then the estimated time where soil becomes saturated is:

$$t_p = \frac{K\,\Psi\,\Delta\theta}{i\,(i-K)} = \frac{0.65 \times 16.68 \times 0.3888}{1\,(1-0.65)} = 12.04\,\text{h}$$

which means that it becomes saturated after 12 h of application.

If the irrigation rate is 5 cm/h, then the estimated time where soil becomes saturated is

$$t_p = \frac{K\,\Psi\,\Delta\theta}{i\,(i-K)} = \frac{0.65 \times 16.68 \times 0.3888}{5\,(5-0.65)} = 0.55\,\text{h}$$

which means that it gets saturated after about 30 min of application.

The water that should be applied to reach a water holding capacity of 60% was previously estimated to be 131.1 m^3 and the area to be treated was estimated to be 2,500 m^2. So the estimated level of water that should irrigate this area is calculated to be 5.24 cm. Thus, the application rate should not exceed 5.24 cm/h for a minimum application time of 1 h in order to get the optimum moisture content even though this certain soil gets

saturated after 7 h of constant irrigation (irrigation intensity: 12 cm/h).

3.4 Potential Evaporation

The de facto standard method for estimating potential evaporation, the Penman–Montheith equation, is relatively high data demanding and sensitive to data that are difficult to measure. Therefore, in the absence of adequate raw data empirical methods can be employed. Adopted methods for the estimation of potential evapotranspiration are the Thornthwaite, Blaney–Criddle, Hargreaves, Turc methods, and many others [21, 22].

3.4.1 Calculation of Potential Evaporation with Blaney–Criddle Method

Blaney–Criddle formula $PE = p(0.46\,T + 8.13)$, based on another empirical model, calculates potential evaporation in mm, for the period (day or month) in which p is expressed and requires only mean temperatures T (°C) over the period it is estimated. p is the mean percentage (daily or monthly) of total annual daytime hours [22, 23].

Taking into account meteorological data [24] evaporation can be estimated according to Blaney–Criddle's equation and Table 6 shows the calculated potential evaporation for two extreme cases (seasons).

3.5 Excess Runoff Estimation

To estimate excess runoff due to heavy precipitation meteorological and hydrological data need to be processed. There are a number of methods for excess runoff calculation one of which that is commonly used is SCS method.

3.5.1 The Rational Method Equation

The Rational Method is an empirical relation between rainfall intensity and peak flow that is widely used to estimate the peak surface runoff rate to design the suitable drainage structure. Peak discharge (flow) is the greatest amount of runoff coming out of the watershed at any time. The Rational Method is most suitable for small urban watersheds that don't have storage such as ponds or swamps and it is most applicable to small, uniform, and highly impervious areas. The method is typically used to determine the size of storm sewers, channels, and other drainage structures. This method is not recommended for routing storm water through a basin or for developing a runoff hydrograph. However, for the sake

Table 6
Potential evaporation according to Blaney–Criddle's method

Season	Month daily average percentage (%)	Average temperature (°C)	Evaporation (mm/d)
Winter	0.39	10	4.9
Summer	0.86	29	18.35

of simplicity, we will use the Rational Method in this example to determine the size of the rainfall channel required for the construction site [25–28].

According to this method the potential peak surface runoff is:

$$Q_{ex} = 0.278\ I\ A\ C$$

where A is the area of potential discharge (km^2), I is the precipitation intensity (mm/h), and C is the runoff coefficient which is related to the soil characteristics.

As shown in the schematic representation of the LTU, the treated area can be subdivided into two equal sized areas of 1,250 m^2, 2% slope each oriented at opposite directions though. Estimated runoff from each area will be directed at each channel placed by the two sided berms. Let's assume a precipitation extreme of 200 mm/h and a runoff coefficient for sand soils with slope <2% (0.05–0.1) of 0.1 according to Table 7.

Then excess runoff from each area is $Q_{ex} = 0.278\ I\ A\ C = 0.278 \times 200\,\text{mm/h} \times 0.00125\,\text{km}^2 \times 0.1 = 6.95 \times 10^{-3}\text{m}^3/\text{s}$.

Table 7
Typical runoff coefficients for soils

Type of drainage area	Runoff coefficient
Sand or sandy loam soil, 0–3%	0.15–0.20
Sand or sandy loam soil, 3–5%	0.20–0.25
Black or loessial soil, 0–3%	0.18–0.25
Black or loessial soil, 3–5%	0.25–0.30
Black or loessial soil, >5%	0.70–0.80
Deep sand area	0.05–0.15
Steep grassed slopes	0.70
Sandy soil, flat 2%	0.05–0.10
Sandy soil, average 2–7%	0.10–0.15
Sandy soil, steep 7%	0.15–0.20
Heavy soil, flat 2%	0.13–0.17
Heavy soil, average 2–7%	0.18–0.22
Heavy soil, steep 7%	0.25–0.35
Asphaltic	0.85–0.95
Concrete	0.90–0.95
Brick	0.70–0.85
Drives and walks, roofs	0.75–0.95

Source: http://water.me.vccs.edu/courses/CIV246/table2b.htm

3.6 Infiltration Estimation

Infiltration can be estimated through already mentioned common methods Horton and Green-Ampt, however specific field data (precipitation intensity hydrographs, infiltration coefficients) need to be measured or estimated. It is recommended that f_c, f_0, and k all be obtained through field data, but they are rarely measured locally. Unfortunately the lack of such data forces us to utilize values published in the literature in place of reliable field data.

The following lists include commonly used Horton infiltration parameter values [29] (Tables 8 and 9):

Assuming a Precipitation intensity of 200 mm/h for $t = 1$ h, then Infiltration rate according to Horton's equation is $f(t) = f_c + (f_0 - f_c)e^{-kt} = 9.5$ mm/h where the infiltration

Table 8
Typical initial infiltration rates

Soil type	f_0 (mm/h)
Dry sandy soils with little to no vegetation	127
Dry loam soils with little to no vegetation	76.2
Dry clay soils with little to no vegetation	25.4
Dry sandy soils with dense vegetation	254
Dry loam soils with dense vegetation	152.4
Dry clay soils with dense vegetation	50.8
Moist sandy soils with little to no vegetation	43.18
Moist loam soils with little to no vegetation	25.4
Moist clay soils with little to no vegetation	7.62
Moist sandy soils with dense vegetation	83.82
Moist loam soils with dense vegetation	50.8
Moist clay soils with dense vegetation	17.78

Table 9
Typical constant infiltration rates

Soil type	f_c (mm/h)	k (1/min)
Clay loam, silty clay loams	0–1.3	0.069
Sandy clay loam	1.3–3.8	0.069
Silt loam, loam	3.8–7.6	0.069
Sand, loamy sand, sandy loams	7.6–11.4	0.069

parameters values for sandy soil are: $f_0 = 127$ mm/h, $f_c = 7.6$ mm/h and $k = 0.069\ \text{min}^{-1} = 4.14\ \text{h}^{-1}$

3.7 Leachates Estimation

Leachates can be estimated from water mass balance. Water mass balance in the LTU is:

3.7.1 Mass Balance for LTU

$$\text{Irrigation(Ir)} + \text{Precipitation}(P) + \text{Infiltration}(I) = \text{Evaporation(PE)} + \text{Leachate(LA)} + \text{Excess runoff}\left(Q_{\text{ex}}\right)$$

Where:

LA = leachate from active area

P = precipitation

Ir = Irrigation

PE = evaporation

I = Infiltration

Q_{ex} = Excess runoff from rainfall (all in units of L^3/T)

Let's assume that irrigation during winter where rainfall incidents are often is neglected then according to the above mass balance equation the estimate of leachate generation in active land treatment is:

$$\text{LA} = P + I - \text{PE} - Q_{\text{ex}}$$

Assuming a Precipitation of 200 mm/h, Infiltration rate as calculated from above is 9.5 mm/h and evaporation during winter is 0.204 mm/h (not a significant factor during winter and can be omitted) and given that area A is 1,250 m^2 then the estimated flow rates are:

$P = 0.0694\ \text{m}^3/\text{s}$, $I = 3.3 \times 10^{-3}\ \text{m}^3/\text{s}$, where Q_{ex} was estimated to be $6.95 \times 10^{-3}\ \text{m}^3/\text{s}$.

Then Leachates flow rate is estimated as:

$$\text{LA} = P + IQ_{\text{ex}} = 0.0658\,\text{m}^3/\text{s}$$

3.7.2 Channel Flow

The passage of overland flow or Leachates into a channel can be viewed as a lateral flow and the designing parameters of specific channels can be estimated using Manning's equation [21, 25, 26].

Manning's equation (SI units) for volume flow in open channels:

$$Q = \frac{1}{n} A\, R^{2/3} S^{1/2},$$

where

n = Manning coefficient of roughness

Q = volume flow (m^3/s)

A = cross-sectional area of flow (m^2)

R = hydraulic radius (m)

S = slope of pipe (m/m)

Hydraulic radius can be expressed as: $R = A/P$, where A = cross sectional area of flow (m^2), P = wetted perimeter (m).

Area, wetted perimeter and hydraulic diameter for some common geometric sections which are used in the example are:

- Triangular Channel

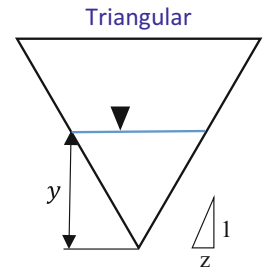
  Flow area of a triangular channel can be expressed as: $A = z \times y^2$

  Wetted perimeter of a triangular channel can be expressed as: $P = 2y\sqrt{1+z^2}$

  Hydraulic radius of a triangular channel can be expressed as: $R = \frac{zy}{2\sqrt{1+z^2}}$
- Circular Channel

  Flow area of a circular channel can be expressed as: $A = \frac{1}{8}(a - \sin a)D^2$

  where D = diameter of channel, $\alpha = 2\cos^{-1}\left(1 - \frac{2y}{D}\right)$

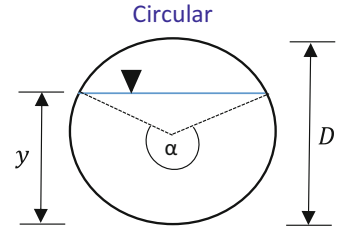


Wetted perimeter of a circular channel can be expressed as: $P = \frac{1}{2}\alpha D$

Hydraulic radius of a circular channel can be expressed as: $R = \frac{1}{4}\left(1 - \frac{\sin a}{a}\right)D$

3.7.3 Overland Flow Channel Design

Typical excess runoff channel's geometric sections can be rectangular, trapezoidal, triangular, or circular. In the current example we are going to design a triangular overflow channel for the estimated



runoff flow $Q_{ex} = 6.95 \times 10^{-3}$ m^3/s. The designed slope is $S = 2\%$ then $z = 0.5$ and the coefficient of roughness $n = 0.02$.

Substituting known and unknown parameters in Manning's equation for flow rate, the only unknown parameter that remains to be solved in the equation is y which is the needed height of the triangular flow channel for construction and is estimated to be $y = 0.25$ m.

3.7.4 Leachates Collection System

Sizing of Leachate Collection Pipes

Perforated pipe size is designed based on Manning's equation taking into account the equations that apply for circular channels, assuming that ratio to full depth (y/D) is 0.7 (1 if flowing full) and $n = 0.012$. Given that $S = 2\%$ and leachates flow is $q = 0.0658$ m^3/s, then pipe diameter is calculated to be 6 cm and the next available HDPE or PVC diameter pipe in the market is 10 cm and such is chosen to be installed.

3.8 Monitoring

In order to effectively monitor the landfarming process a sampling plan of the treatment area should be constructed. Data like samples ID, number and location within the constructed grid of the landfarming zone as well as sampling frequency should be recorded. Fortunately due to regular tilling and relatively thin soil layer, samples can be collected from only one depth [14]. Parameters like pH, moisture content, bacterial population, nutrient content, and contaminant concentrations should be measured for each collected sample. There are several options to do these analyses either in the field with special kits or in the lab with high precision equipment. Analyses' results of the monitoring parameters should be evaluated in order to satisfy maximum degradation rates and thus facilitate the best performance of the landfarming processes [2].

Furthermore it is mandatory to check leachates quality in terms of contaminants concentration and population of possible pathogens by the time they are to be discharged to the environment. It is also useful to measure contaminants concentration at the recirculating leachates to monitor the effectiveness of the bioremediation processes. Due to leachates regular recirculation the groundwater quality should also be monitored in the event of any possible leaking [14]. Nonetheless the monitoring plan that is followed, the parameters that should be checked or any discharging contaminant's levels should comply with the established national action plans and guidelines.

4 Biotreatability/Feasibility Studies of Land Treatment

Feasibility studies serve as a preliminary examination for the potential to implement landfarming as remediation technology to a

particular site. These studies are considered crucial since they determine contaminant's susceptibility to biodegradation and establish the design parameters (indigenous population capability to remediate, optimal nutrients amendments, etc.) for its maximum degradation rate in the field. They also provide information on the time needed to reach the established target concentration levels and whether the contaminants removal is due to biodegradation or due to other abiotic processes like evaporation and photooxidation. Treatability studies can be conducted both in the lab and in the field [10].

4.1 Soil Biotreatability Study

As mentioned above biotreatability studies determine soils capability for landfarming treatment and need to be carried out prior to landfarming application on site. Biodegradation potential of a particular contaminant can be determined through a set of experiments of the polluted soil usually conducted in the lab rather than in the field due to convenience. Biotreatability studies conducted in the lab can be carried out either in flasks or pans. However pan studies are preferred more than flask studies since they are more close to resemble landfarming, whereas flask studies are mostly used to evaluate bioremediation potential in water matrices [2]. Following some useful guidelines on performing laboratory biotreatability studies to evaluate the potential of a petroleum-contaminated soil for landfarming are suggested [2, 30].

Laboratory mesocosms biotreatability studies to evaluate biodegradation of petroleum hydrocarbons in contaminated soil are shown in Fig. 3 and they can be prepared in open pans (glass or metal) as follows:

1. Soil is screened to remove particulates greater than 2 mm in size.
2. Pans depending on the available size are filled with 2–5 kg of contaminated or spiked soil.

Fig. 3 Mesocosm biotreatability study of soil contaminated with petroleum hydrocarbons

3. Initial oil or TPH contamination level is determined and the concentration is adjusted to the desirable range of 0.5–5% w/w after dilution with clean soil.
4. Soil moisture content should be adjusted to the optimal range 40–85% of the field holding capacity to sustain microbial activity. A water content adjusted to 60% of the field-holding capacity is suitable.
5. In order to sustain microbial activities pH should be adjusted to around 7.0 by adding lime, caustic soda, elemental sulfur or ammonium sulfate, etc.
6. Pans should be incubated at the optimum temperature range for microbial processes of 10–35°C depending though on the environmental conditions of the site to be remediated.
7. Determine background concentration levels of essential nutrients (C, N, P) prior to any fertilizers application and detect any possible toxic substances (i.e., metals)
8. Nutrients are added to such amount that results a final concentration equivalent to a C:N:P molar ratio of 100:10:1. The amount of each nutrient can be estimated using the procedure given in Sect. 3.2.4.
9. The expected time span of the biotreatability studies mainly depends on the final goal established for each project and explicitly depends on soil, contaminants characteristics. Traditionally the recommended period for biotreatability studies is between 2 and 6 months.
10. Two basic treatments should be included: (a) control no nutrient additives only aeration by mixing every week and addition of water to maintain 60% of the field holding capacity and (b) sterile control under the same conditions as in control but using sterile soil.
11. Parameters like contaminants concentration (oil or TPH), moisture, and pH should be monitored regularly.
12. The soil should be mixed at least twice a week to ensure that the desired aeration, mixing, and moisture requirements are satisfied for the best performance of the landfarming processes.
13. The water content should be weekly monitored and the soil should be irrigated to the optimum moisture level.
14. Measuring and monitoring the remaining contaminants level (oil or TPH) with chromatographic techniques (GC-FID, GC-MS), as well as moisture, pH, and nitrogen regularly is necessary during the treatability studies to evaluate treatment's performance. Another important aspect to evaluate biodegradation processes is to monitor microbial activity of either total heterotrophs or hydrocarbon-degraders through

microbiological or molecular techniques. The same sampling protocol and methods must be followed throughout the treatment period.

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Anaerobic Digestion of Lipid-Rich Waste

A.J. Cavaleiro*, M.A. Picavet*, D.Z. Sousa, A.J.M. Stams, M.A. Pereira, and M.M. Alves

Abstract

Lipids present in waste and wastewater, also referred as fat, oil, and grease (FOG), can be efficiently converted to methane. This fact constitutes an opportunity for conserving the high energy content of waste lipids, thus facilitating its storage and future use as fuel, electricity, and heat. In anaerobic bioreactors, long-chain fatty acids (LCFAs) are released during hydrolysis of FOG. LCFAs tend to form stable emulsions, adhere to all available surfaces, and adsorb on the microbial cell walls leading to foam formation, sludge flotation, and washout, as well as temporary inhibition of microbes. These problems can be prevented if a correct balance between LCFA accumulation and biodegradation is assured, by sequential feeding and degradation steps. Appropriate reactor operation is the key strategy to prevent the excessive accumulation of LCFA and to stimulate microbial acclimation, especially during the start-up phase. After successful acclimation, a continuously feeding operation is possible, provided that there is proper process control through an adequate monitoring protocol. In addition to adequate operation, a suitable reactor design is recommended. Among other technologies, the inverted anaerobic sludge blanket (IASB) was recently developed for the direct treatment of FOG-containing wastewater. This chapter reports a protocol with a detailed operation and monitoring strategy for achieving effective methane production from FOG-containing waste and wastewater and presents a brief description of the basic concepts behind the development of the reactor.

Keywords: Cycles, IASB reactor, Lipids, Methane, SLS technology, Wastes, Wastewater

1 Introduction

1.1 Concept and Framework

Aligned with recent policies toward an increased use of energy from renewable sources and directives that restrict landfill treatment of biodegradable wastes, methane production from waste lipids has captured the interest of industries, stakeholders, and practitioners, besides the scientific community. Lipids hold high energy content within their long carbonaceous chains, which can be conserved in the form of methane during anaerobic treatment processes. At standard temperature and pressure (STP) conditions (i.e.,

*Both authors contributed equally to this chapter.

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temperature of 273 K and absolute pressure of 10^5 Pa), approximately 1 L methane can be expected per gram of biodegraded lipids, corresponding in mass to about 0.7 $g \cdot g^{-1}$ [1]. Fat, oil, and grease (FOG) are readily available high-strength organic wastes originated from diverse locations such as households, restaurants, wastewater treatment plants, or slaughterhouses, among others. If amended in certain amounts to biogas plants treating waste or wastewater, a significant increase can be observed in the biogas yield and productivity, thus enabling a better economic performance of these systems. This fact is confirmed empirically by biogas plant operators, but in general, there is a lack of adequate monitoring strategies that assure the correct balance between feeding and degradation of FOG. For example, co-digestion of FOG with municipal biosolids, at a rate of 10–30% (v/v) of FOG in the feed, increased the biogas production by 30–80% in two full-scale wastewater biosolid anaerobic digesters [2, 3]. Several other examples of higher methane production resulting from lipid addition to biogas plants were reported by operators in Austria, Denmark, and Italy. However, in some cases, a severe inhibition is observed resulting in process interruption associated to important economic losses. Thus, inhibition by lipids in anaerobic digesters is still poorly understood.

Due to the large lipid-water interface, consequence of the small particle size of lipid emulsions or micelles, hydrolysis of FOG or lipids in general is not the limiting step in anaerobic bioreactors [4] and proceeds fast toward glycerol and LCFA. Once released from the triacylglycerol molecules, LCFAs are not easily biodegraded and accumulate in the bulk. Thus, overall degradation of lipids to methane is limited by LCFA conversion [5–8].

LCFA tend to adsorb onto the surface of microbial cells, wrapping the microbial aggregates with a hydrophobic layer that may hamper the transfer of substrates and products [9]. Moreover, lipids and LCFA are less dense than water, thus decreasing sludge density, resulting in flotation [10–12]. A thick layer of sludge completely enclosed by LCFA is formed on the top of the water surface, where biogas bubbles are frequently retained leading to foam formation (Fig. 1). This foam causes problems in the biogas

Fig. 1 Floating layer of sludge enclosed by LCFA in anaerobic bioreactors

line and inefficient biogas recovery. Washout follows consequently, since high upflow velocities are usually imposed in currently applied anaerobic technologies, which are based on settling as sludge retention mechanism. Granule disintegration has also been reported to occur in the presence of LCFA and is related with the surfactant behavior of these compounds at neutral pH, lowering the surface tension and inhibiting aggregation of hydrophobic bacteria [13]. Microbial toxicity of LCFA toward acetogenic bacteria and methanogenic archaea was also described. Recently, Sousa et al. [14] showed that LCFAs have an effect on methanogenic activity and membrane integrity of methanogens, being more critical for unsaturated than saturated LCFA.

In industrial and municipal wastewater treatment plants, lipid removal prior to the biological treatment, using energy-intensive processes, is frequently applied. At laboratory and pilot scale, several other strategies were tentatively studied. Calcium ions or inert materials, e.g., activated carbon, bentonite, or other clays, were added to anaerobic bioreactors, aiming to reduce the bioavailability of LCFA through mechanisms of precipitation or adsorption, and thus decrease potential inhibitory or toxic effects [15–17]. Possible solutions to overcome flotation problems were also searched, as, e.g., sieve drums, biomass recirculation, biomass adhesion, or partial phase separation [18–20]. The need for periodic reseeding of anaerobic reactors treating oily wastewater, due to sludge washout, was suggested by Hwu et al. [21] and Jeganathan et al. [12].

1.2 The Primary Strategy: Operating Conditions

Although LCFA adsorption on the sludge is a nuisance for anaerobic processes treating waste lipids, LCFA adsorption is a prerequisite for further biodegradation of these compounds [22]. Therefore, besides the reactor's design, the key for effective conversion of lipids/LCFA to methane is a controlled equilibrium between adsorption and biodegradation. Specific LCFA content ($g \cdot g^{-1}$) can be calculated by dividing the LCFA concentrations (expressed as chemical oxygen demand, COD) by the volatile solid (VS) content. LCFA accumulation onto the sludge should be favored, but it must not exceed a specific LCFA content of around $1\ g \cdot g^{-1}$ [23]. When this critical value is surpassed, the maximum degradation rate of the accumulated LCFA drops sharply, due to metabolic or physical inhibitory effects. Degradation of the accumulated substrate may then be promoted by stopping the feed to the reactor, allowing a full recovery of methane production from lipids/LCFA. An operation mode based on repeated cycles of continuous feeding alternated with batch periods was shown to induce adaptation of the mixed microbial community in the start-up of a lab-scale upflow anaerobic column reactor [24]. A subsequent continuous operation was possible at a COD-organic loading rate (OLR) of $20\ kg \cdot m^{-3} \cdot day^{-1}$ with 80% conversion of LCFA to methane, using a synthetic wastewater composed of 50%

COD as oleic acid. This approach was also successfully applied to the anaerobic treatment of olive mill wastewater [25]. Similar approaches with discontinuous addition of FOG have been used successfully in the co-digestion of solid wastes and fat [26–28].

1.3 The Secondary Strategy: Reactor Technology

In the last 30 years, high-rate anaerobic wastewater treatment technology (HR-AnWT) has been developed, giving rise to reliable reactor configurations such as the upflow anaerobic sludge blanket (UASB) reactor and the derived designs – expanded granular sludge bed (EGSB) and internal circulation (IC) reactors – that promoted the confidence on anaerobic digestion technology. These technologies became standard for a certain range of industrial wastewaters. Thousands of full-scale installations are in operation worldwide, treating mainly wastewater containing readily degradable organic pollutants such as volatile fatty acids and carbohydrates. However, HR-AnWT applications are still centered essentially in biodegradable effluents from distillery, pulp and paper, brewery, and beverage industries.

The coverage of HR-AnWT in the waste and wastewater treatment fields can be further enhanced if the range of suitable substrates is expanded. It is however clear that wastewaters with high lipid content are not effectively treated yet by granular sludge-based anaerobic reactors, such as the UASB, EGSB, and IC configurations. Different reactor technologies have been tested and recently implemented at full scale for the treatment of wastewater containing FOG, such as the inverse fluidized bed reactor and the inverse turbulent reactor [29]. Haridas et al. [30] described a novel technology, the buoyant filter bioreactor (BFBR), for the treatment of a fat-rich wastewater. In this reactor, biomass and insoluble COD retention time is decoupled from the hydraulic retention time, by means of a granular filter bed made of buoyant polystyrene beads. Filter clogging is prevented by an automatic backwash driven by biogas release, which fluidizes the granular filter bed in a downward direction. Three reactor technologies are presently in the market, claiming to be able to treat complex wastewater containing FOG. The BIOPAQ®AFR reactor was designed to treat wastewater with FOG and/or solids such as proteins and starch. This anaerobic flotation reactor (AFR) with an effective sludge retention system is an all-in-one technology that converts organic compounds into valuable biogas. According to the manufacturer, this compactly designed bioreactor treats wastewater with a COD content from 5 to 70 $g \cdot L^{-1}$ with vegetable or animal fats, at hydraulically short retention times from 1 to 8 days. The intensive contact between the open bacteria flocks and the organic compounds, in combination with the biomass retention in the integrated flotation unit, is the success factor for this technology (http://en.paques.nl/products/featured/biopaq/biopaqafr).

Another technology for high-strength wastewater containing lipids is the Biothane's Memthane® anaerobic MBR (AnMBR) reactor. According to the manufacturer, this reactor is suitable for effluents from dairy whey, ethanol facilities, FOG-containing streams, and ice cream and biodiesel production, among others (http://technomaps.veoliawatertechnologies.com/memthane/en/?bu=biothane.en).

The third technology especially designed for the treatment of effluents with high content of lipids is the inverted anaerobic sludge blanket (IASB) reactor [31, 32]. Contrary to conventional anaerobic technologies, it does not depend upon sludge with good settling properties for sludge retention in the system, since this is accomplished by sludge flotation resulting from lipid/LCFA adsorption. In addition, the specific contact surface between sludge and lipids/LCFA is maximized to prevent mass transfer limitations. The IASB is fed from the top and is equipped with a separation step at the bottom. Reactor contents are thoroughly mixed through the combined action of a gas lift loop and a liquid recycle over the reactor. The IASB can be operated in continuous or sequential mode and, although specifically designed for lipid degradation, it can be used for many other applications (http://www.biofatrecovery.eu/). A schematic representation of the reactor concept is shown in Fig. 2.

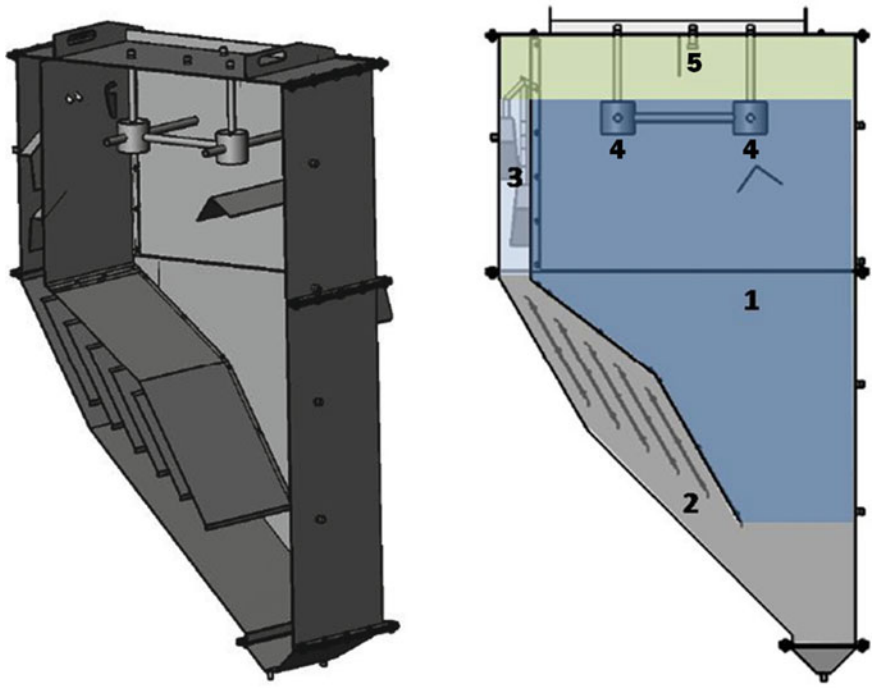


Fig. 2 Schematic representation of the IASB reactor concept. *1*, reactor vessel; *2*, tilted plate separator; *3*, sludge lift system; *4*, cylindrical downers; *5*, spray heads

The IASB concept was tested at lab scale and at pilot scale (1.2 m^3) for 1 year on a real slaughterhouse wastewater. Its robustness and capacity for treating wastewater strongly fluctuating in quality, while maintaining treatment efficiency, were demonstrated [33]. An organic loading rate of 16 $kg_{COD} \cdot m^{-3} \cdot day^{-1}$ was applied, of which 63% consisted of fat. Volatile suspended solids in the effluent were around 500 $mg \cdot L^{-1}$. COD removal efficiency was consistently above 80% and excessive LCFA accumulation was prevented (specific LCFA content $<1\ g \cdot g^{-1}$). Then, it was scaled up for industrial applications, using a modular approach to reduce investment costs associated with the technology. A sludge lift system (SLS) was also designed and patented [34]. This technology uses the gas lift effect and flotation to stimulate reactor mixing and sludge retention. The IASB concept and assemblage details are described in this chapter.

In this chapter, we describe two complementary approaches for lipid biodegradation and effective conversion to methane in anaerobic bioreactors, including:

Bioreactor assemblage: the inverted anaerobic sludge blanket reactor

Bioreactor start-up through repeated continuous/batch cycles and further continuous operation

2 Materials

For full-scale operation with liquid effluents, use one of the available reactor technologies: Memthane®, BIOPAQ®AFR, or IASB. For pilot-scale treatability tests, use prototypes of the available technologies of at least 1 m^3 volume. For lab-scale treatability tests, use a glass or Plexiglas upflow or downflow column of at least 10 L volume with an external jacket (see Fig. 3 for examples). Remove the effluent from the middle height of the column. If necessary, use an external settler and recirculate the settled biomass to the reactor.

For treating lipids in co-digestion with slurries or solid waste, use a continuous stirred tank reactor either at lab scale or at full scale.

3 Methods

Example of assemblage and operation is detailed for the IASB reactor.

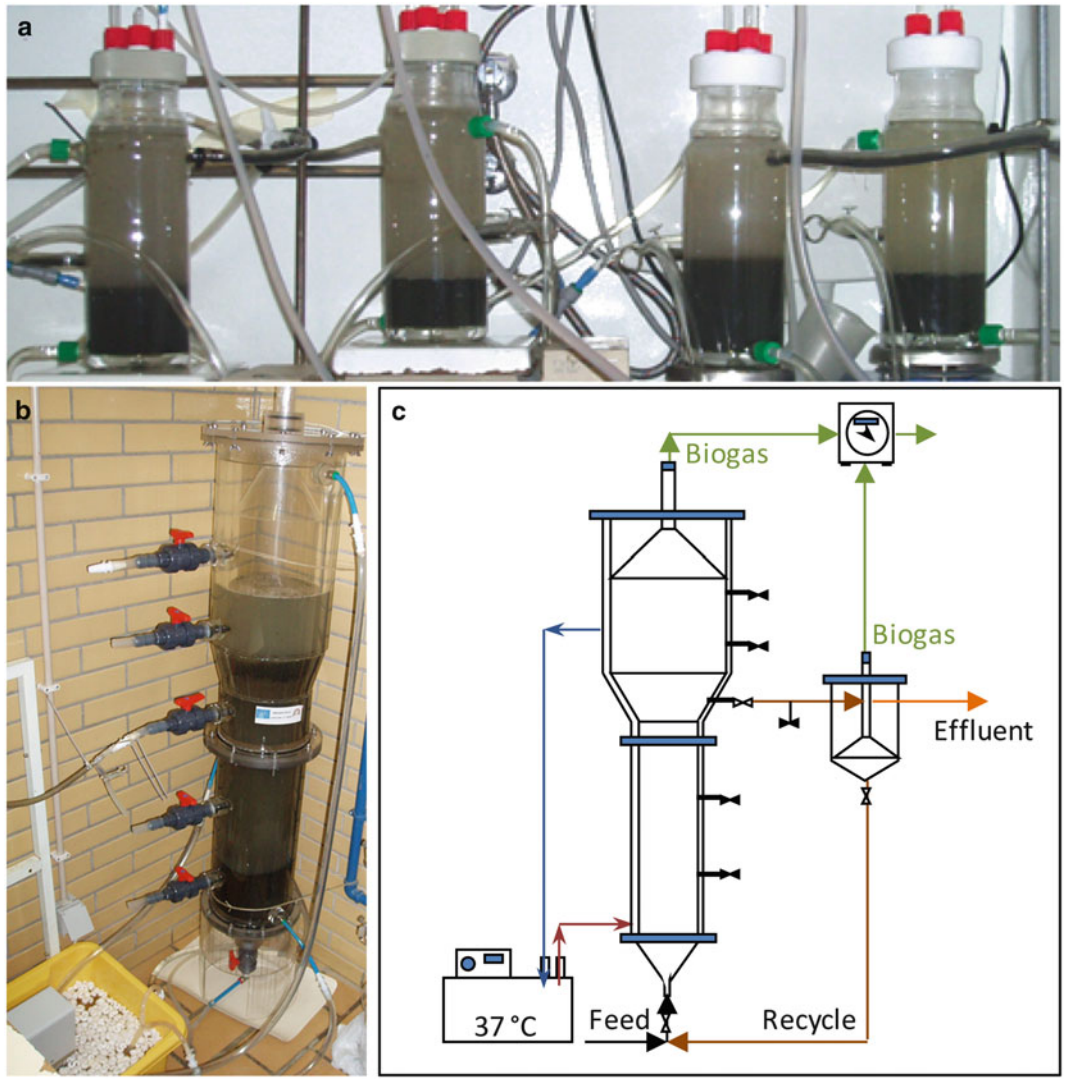


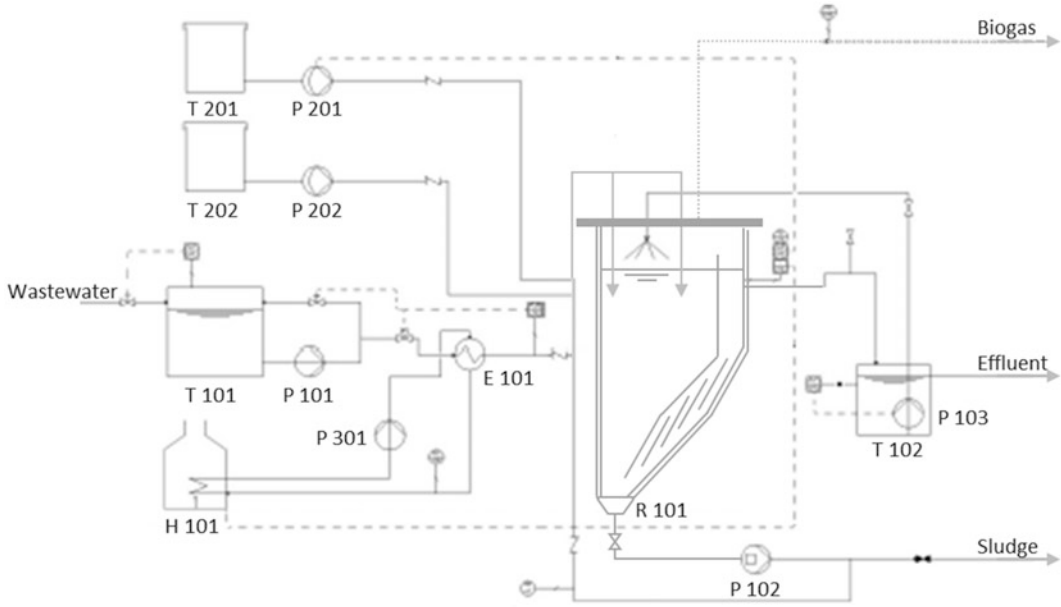
Fig. 3 Examples of glass (**a**) or Plexiglas (**b**) laboratory-scale reactors and a schematic representation of an experimental setup (**c**)

3.1 Assemblage and Operation of the IASB Reactor to Treat a Liquid Effluent Containing FOG

1. Begin by collecting quantitative and qualitative data on the wastewater to treat, e.g., volume, flow rate, COD concentration, FOG, and total and free LCFA content (see **Note** 1). If very heterogeneous, samples should be freeze-dried before analyzing COD or LCFA, and several replicates should be performed.

2. Construct or buy a cylindrical vessel of appropriate dimensions, considering the volume and physical-chemical characterization of the wastewater to treat. Example of possible dimensions may be a total volume of 1.2 m^3 subdivided into four main compartments: 0.700 m^3 reaction compartment, 0.280 m^3 separation compartment, 0.035 m^3 effluent compartment, and 0.165 m^3 headspace. Plexiglas, stainless steel, and concrete are some materials than can be used. Include sampling ports for liquid and gas analysis.
3. Operate the reactor under downflow mode, i.e., feed should be applied from the top (4 in Fig. 2) and the effluent discharged from the bottom. Floating sludge will be easily retained in the system with these conditions.
4. Assemble and install a settling sludge retention system at the bottom of the reactor. Settling sludge is released from the top floating layer due to LCFA biodegradation. A commonly used technology is a tilted plate separator (TPS) (2 in Fig. 2). The treated water leaves the reactor through this TPS and is directed to a narrow and high chamber (effluent compartment). Here any remaining solids have the opportunity to settle before the effluent leaves the system at the top of this chamber.
5. A sludge lift system (SLS) may also be installed in the effluent compartment (3 in Fig. 2), to transport back to the reaction section the sludge and biogas that eventually still entered this compartment.
6. Recycle the settled sludge to the top of the reactor and mix this stream with incoming raw wastewater, to stimulate lipid adsorption and biodegradation.
7. Inject this sludge/raw wastewater mixture into downer sections (4 in Fig. 2), for sludge entrainment and production of mild shear stress that disrupts encapsulated sludge and liberates the biogas. This procedure contributes for maximizing the contact surface between sludge and lipids/LCFA, preventing mass transfer limitations. The mixing regime inside the reactor can be controlled with the sludge recycle and the wastewater flow rate.
8. To counteract foam formation, install sprays on the top of the reactor that will distribute treated effluent above the floating layer (5 in Fig. 2).

As an example, a flow sheet of a pilot plant treatment facility is shown in Fig. 4, including the different devices used and the flow directions.



Displayed text	Description	Pump type	Model
E 101	Heat exchange		Not determined
H 101	Heater		Not determined
P 101	Feeding pump	Centrifugal	500 L/h
P 102	Sludge pump	Positive displacement	500 L/h
P 103	Spray pump	Centrifugal	50 L/h
P 201	NaOH dosing pump	Diaphragm	25 L/h
P 202	Nutrients dosing pump	Diaphragm	25 L/h
P 301	Heating pump	Centrifugal	Not determined
R 101	Anaerobic reactor		1000 L
T 101	Influent tank		250 L
T 102	Effluent tank		100 L
T 201	5 % NaOH tank		50 L
T 202	Nutrients tank		50 L

Fig. 4 Example of a process flow diagram of a wastewater treatment facility

3.2 Recommendations for the Start-Up of the IASB Reactor

1. After assembling the reactor as described in Sect. 3.1, test its tightness and absence of gas or liquid leaks.
2. Install and test the heating system, if the reactor is to be operated at mesophilic or thermophilic conditions.
3. Guarantee the availability of granular or suspended sludge with good methanogenic activity, as defined by Angelidaki et al. [35] (*see* **Note** 2).
4. Plan the operational conditions for bioreactor start-up: based on the working volume and influent COD concentration, define the initial hydraulic retention time and thus the applied organic loading rate. Make all the necessary calculations:

organic loading rate (OLR) applied and desired flow rates. Consider the possibility of applying directly a relatively high organic loading rate (e.g., 2–3 $kg_{COD} \cdot m^{-3} \cdot day^{-1}$), because it is the alternation of sludge shock and recovery that induces biomass acclimation to LCFA degradation.

5. Consider the possibility of adding macro- and micronutrients to the feed (*see* **Note** 3).
6. Calibrate all the necessary pumps and gather feed and storage tanks.
7. Fill the reactor with a mixture of anaerobic sludge and feeding (the waste/wastewater to treat), and close the system until biogas production can be recorded, showing that microbial activity is thriving inside the reactor. The amount of sludge can be defined based on the preferred amount of volatile solids in the reactor, typically around 10 $g \cdot L^{-1}$.
8. Start feeding the reactor in continuous mode applying the previously defined OLR. Measure biogas flow rate production continuously with a biogas counter, and analyze the methane content in the biogas regularly (e.g., two times a week). Other monitoring parameters include pH, temperature, total and soluble + colloidal COD in the feed and in the effluent, volatile fatty acids, and total and volatile suspended solids in the effluent.
9. Calculate the COD removal efficiency (%) and the organic loading removed ($kg_{COD} \cdot m^{-3} \cdot day^{-1}$). Calculate also the methane production rate, i.e., the amount of methane produced per day. Calculate the methane yield that provides information on how much of the removed COD is being recovered as methane (*see* **Note** 4).
10. In a first operating period, a low methane yield is expected, indicating that lipids and LCFA are being accumulated inside the reactor but are not converted to methane. This period depends on the inoculum and applied OLR and may vary between 15 days and 1–2 months. When the methane yield reaches a value as low as 20%, turn off the feeding pump. At industrial scale, it is mandatory to have a second IASB reactor operating in parallel for alternating the feeding. Then impose a batch period to the reactor, where the accumulated LCFA will be converted to methane. Monitor the cumulative methane production and estimate the amount of substrate COD that was converted to methane in the batch period.
11. Collect samples for LCFA analysis at least at the beginning and end of the batch period, and perform the analysis as described by Neves et al. [36] – *see* **Note** 1.
12. Once the cumulative methane production stabilizes, achieving a plateau, restart the feeding and repeat the cycle of operation by sequencing feeding and batch operating modes.

13. After repeating several times steps 8–12, i.e., cycles of continuous/batch phases, biomass will adapt to the applied feeding and will convert LCFA to methane practically without LCFA accumulation in the system. In this case, after restarting the fed, the methane production rate will increase and then stabilize around an average value. At this point, the system is prepared to work in continuous.
14. Operate the reactor in a continuous fashion and step-increase the OLR applied, as long as performance stability and good methane recoveries can still be guaranteed. Cavaleiro et al. [24] reported a maximum OLR around 20 $kg_{COD} \cdot m^{-3} \cdot day^{-1}$ during the treatment of wastewater containing 50% COD as LCFA.

3.3 Recommendations for the Addition of FOG to a Continuous Stirred Tank Reactor Treating Slurry or Solid Waste

When FOG is available, it can be used as a co-substrate in anaerobic digestion plants treating, for instance, manure, sewage sludge, or municipal solid waste. The addition of FOG should be intermittent. The monitoring plan should include analysis of LCFA.

Neves et al. [36] refer a maximum oil concentration (in COD) of 12 $kg \cdot m^{-3}$ in the co-digestion process of manure and food waste supplemented with discontinuous pulses of oil. A pulse feeding of oil at a COD concentration of 18 $kg \cdot m^{-3}$ induced a persistent inhibition of the process, detected by the decrease in pH to a minimum of 6.5 and an increase in effluent soluble COD and VFA. This experiment also demonstrated that threshold values for LCFA accumulation onto the solid matrix, expressed in COD-LCFA per total solids, of about 180–220 $g \cdot kg^{-1}$, should not be surpassed in order to prevent persistent reactor failure in co-digestion plants.

4 Notes

1. Brief description of LCFA analysis method

   Total LCFA

   Total LCFA is quantified by capillary gas chromatography, after performing hydrolysis of the samples for LCFA release and further esterification. Pentadecanoic (C15:0) acid in chloroform ($1 g \cdot L^{-1}$) is used as internal standard (IS) solution.

   Transfer a defined amount of freeze-dried sample to a glass vial and add 1.5 mL chloroform, 1.5 mL IS solution, and 3 mL methanol-H_2SO_4 (85:15% v/v). The mixture is vortex-mixed, to promote good contact between the two phases, and is digested at 100°C for 3.5 h [37]. Phase separation and GC analysis is performed as described for free LCFA analysis.

Free LCFA

A method for free LCFA extraction from liquid and solid samples and further quantification by capillary gas chromatography (GC) is briefly described. Lauric (C12:0), myristic (C14:0), pentadecanoic (C15:0), palmitic (C16:0), palmitoleic (C16:1), stearic (C18:0), oleic (C18:1), and linoleic (C18:2) acids (puriss. p.a. standard for GC analysis), oleic acid sodium salt (puriss p.a. for GC analysis $\geq$99%), dichloromethane (DMC) (puriss p.a. stabilized with amylene), hydrochloric acid solution (37%), and 1-propanol (p.a. ACS) should be used.

Calibration curves are made from a series of standard solutions (25, 50, 100, 250, 500, and 1,000 mg L^{-1}) prepared with the following acids in a DCM solution (represented by their chemical notation): C12:0, C14:0, C16:0, C16:1, C18:0, C18:1, and C18:2. Pentadecanoic acid (C15:0) is used as the LCFA internal standard (IS).

The standard (in DCM solution), liquid (aqueous solution), and solid (anaerobic biomass) samples are submitted to a similar procedure, ensuring that the organic phase and the aqueous phase always comprised equal amount (3.5 mL), in a total volume of 7 mL. For the standard and liquid samples, once homogenized, 2 mL is transferred into glass vials. Afterward, 1.5 mL of the IS solution (1,000 mg $\cdot$ L^{-1}) and 1.5 mL of HCl:1-propanol (25% v/v) are added. For the liquid samples, 2 mL of DCM is subsequently added, whereas, for the standard solutions, 2 mL of ultrapure water is added instead. For solid samples, a defined amount is transferred to the glass vials and dried for 12 h at 85°C. The content of the vial is weighed, and the solutions of IS (1.5 mL), HCl:1-propanol (1.5 mL), DCM (2 mL), and ultrapure water (2 mL) are further added.

The mixture is vortex-mixed, to promote good contact between the two phases, and is digested at 100°C for 3.5 h. After digestion, the content of the vial is transferred with 2 mL of ultrapure water to a different vial, rubber covered, and the contact between the two phases is further promoted. These new vials are kept in inverted position for 30 min, after which 1 mL of the organic phase is collected. 1 µL of this subsample is analyzed by GC.

This analysis is carried out in a GC system (CP-9001 Chrompack) equipped with a flame ionization detector (FID). LCFAs are separated using an eq.CP-Sil 52 CB 30 m $\times$ 0.32 mm $\times$ 0.25 µm column (Teknokroma, Tr-wax), with helium as the carrier gas at 1.0 mL $\cdot$ min^{-1}. Temperatures of the injection port and detector are 220 and 250°C, respectively. Initial oven temperature is 50°C for 2 min, with a 10°C min^{-1} ramp to 225°C, and a final isothermal for 10 min.

2. Specific methanogenic activity of the inoculum
   Activity tests should be performed using acetate ($1\ g \cdot L^{-1}$) or H_2/CO_2 (80:20% v/v, 10^5 Pa overpressure) as specific substrates for the acetoclastic and hydrogenotrophic methanogens. The assays are performed in closed bottles that should be flushed continuously with a mixture of N_2/CO_2 (80/20% v/v) before transferring the substrate and inoculum. Generally, N_2/CO_2 (80/20% v/v) is also used to flush the headspace of the inoculum storage vessel. Further details on the experimental procedure can be found in Colleran et al. [38]. Methane production (mL) is measured during the assays and plotted as a function of time (hours or days). The slope of the initial methane production is calculated and represents the methanogenic activity of the sludge ($mL \cdot h^{-1}$ or $mL \cdot day^{-1}$). Dividing this value by the VS (g) of the sludge gives the specific methanogenic activity ($mL \cdot g^{-1} \cdot day^{-1}$). The specific acetoclastic activity of the inoculum should be higher than $105\ mL\ g^{-1} \cdot day^{-1}$ for granular sludge and $35\ mL\ g^{-1} \cdot day^{-1}$ for flocculent sludge [35].
3. Macronutrient and micronutrient solutions
   Macro- and micronutrients, as well as vitamins, are necessary for the optimum growth and metabolism of anaerobic microorganisms. Supplementation of the anaerobic processes with these components is particularly important for certain substrates (e.g., solid wastes and energy crops) that can lack or be deficient in some nutrients. Macro- and micronutrient solutions specifically defined considering the requirements of methanogenic archaea, the most sensitive among the different trophic groups in anaerobic digesters, are described [39].

Macronutrient solution

Compound	Concentration ($g\ L^{-1}$)
$MgSO_4\ 7H_2O$	30.2
KH_2PO_4	28.3
NH_4Cl	99.0
KCl	45.0

Supplement the feed to the reactor with this solution at $0.6\ mL\ g^{-1}$ COD fed. When the substrate is rich in nitrogen (e.g., dairy wastewater), do not add NH_4Cl to this solution

Micronutrient solution

Compound	Concentration (g L^{-1})
$FeCl_2$ $6H_2O$	2.0
H_3BO_3	0.05
$ZnCl_2$	0.05
$CuCl_2$ $2H_2O$	0.038
$MnCl_2$ $4H_2O$	0.5
$(NH_4)_6Mo_7O_{24}$ $4H_2O$	0.05
$AlCl_3$ $6H_2O$	0.09
$CoCl_2$ $6H_2O$	2.0
$NiCl_2$ $6H_2O$	0.092
Na_2SeO_3 $5H_2O$	0.164
EDTA	1.0
Resazurin	0.2
HCl 37%	1 mL L^{-1}

Add 1 mL L^{-1} of micronutrient solution to the feed

4. Calculation details
   During the operation in cycles, calculate COD removal efficiency and methane yield in each operating period as follows:

   4.1. COD removal efficiency (%) is

$$\frac{\mathrm{COD}_{\text{removed continuous}} + \mathrm{COD}_{\text{removed batch}}}{\mathrm{COD}_{\text{fed}}} \times 100 \qquad (1)$$

   where:

   COD removed continuous (kg COD) = average OLR removed (kg COD removed m^{-3} day^{-1}) × reactor working volume (m^3) × phase time (days)

   COD removed batch (kg COD) = [COD at the start of the reaction phase (kg COD m^{-3}) – COD at the end of the reaction phase (kg COD m^{-3})] × reactor working volume (m^3)

   COD fed (kg COD) = average OLR applied (kg COD fed m^{-3} day^{-1}) × reactor working volume (L) × phase time (days)

   4.2. Methane produced in each phase (batch or continuous) during a cycle (%) is

$$\frac{CH_4 \text{ (kg COD) produced during the phase (batch or continuous)}}{CH_4 \text{ (kg COD) produced during the entire cycle}} \times 100 \quad (2)$$

4.3. Methane yield (%) is

$$\frac{CH_4 \text{ produced during the cycle or phase (Kg COD)}}{(COD_{\text{removed continuous}} + COD_{\text{removed batch}})(\text{Kg COD})} \times 100 \quad (3)$$

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Oil Droplet Generation and Incubation for Biodegradation Studies of Dispersed Oil

Odd Gunnar Brakstad, Mimmi Throne-Holst, and Trond Nordtug

Abstract

Biodegradation of oil in the marine water column is mainly associated with oil dispersions. The operational use of chemical dispersants may therefore enhance oil biodegradation in seawater. The dispersant treatment results in oil droplet size reductions, increasing the surface-to-oil ratio of the oil. Several laboratory systems have been developed for testing of oil dispersions. The protocol presented here describes a system for generating oil dispersions with small droplets (10–30 μm) and maintaining the dispersions during biodegradation experiments. The dispersions are generated in a system consisting of a series of nozzles. The system is designed to generate accurately controlled oil concentrations and droplet sizes. The dispersions are transferred to a carousel system for incubation with slow continuous rotation around the carousel axis. This carousel system has been developed for keeping the oil droplets in suspension due to the combined effect of turbulence created in the flasks and the fact that the rising velocity of the oil droplets will make them constantly change direction in relation to any fixed point at the flasks inner surface. Biodegradation is determined by quantification of selected oil compounds and by microbiological analyses during the biodegradation period. Results from these studies have been used as input data in dynamic environmental models as part of fate predictions after oil spill and for describing successions of microbial communities related to the biodegradation of different groups of oil compounds.

Keywords: Biodegradation, Dispersions, Droplet size, Oil, Seawater

1 Introduction

When oil is spilled to the marine environment a number of weathering processes occur. In surface spills, these processes include evaporation of volatile compounds, dissolution of water-soluble compounds, the formation of water-in-oil emulsions, oil-in-water dispersions and generation of surface oil films. In addition, processes like photo-oxidation and sedimentation may contribute to the weathering processes [1]. In subsurface spills, volatiles and gases may be dissolved to the water column before the oil reaches the surface. In deep water spills, parts of the oil may be entrained in the water masses as a plume of dispersion, as exemplified during the *Deepwater Horizon* (DWH) spill in the Gulf of Mexico [2].

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Biodegradation of oil in seawater is mainly related to oil dispersions. Chemical dispersants have been introduced as an oil spill response (OSR) tool to avoid large surface slicks drifting onshore. Dispersant treatment has been used both on surface and subsurface oil spills. Treatment of the oil with dispersants changes the characteristics of the oil, creating a more hydrophilic oil surface, and results in the generation of small droplet dispersions in the water column with low positive buoyancy. For subsurface oil spills, dispersants may be used in order to reduce the amount of oil reaching the sea surface, thereby lowering the human hazard in OSR operations and reducing the amount of oil reaching shorelines and other vulnerable environments.

An important effect of dispersant treatment of oil discharges is the enhancement of oil biodegradation. Generation of oil dispersions, and reduction of the droplet sizes of the dispersions, will increase the oil attachment areas for the indigenous seawater microbes, an important prerequisite for the biodegradation process. Several studies that aimed at investigating the effect of dispersants on biodegradation efficiency have shown increased oil compound biodegradation, as compared to physical dispersion alone (e.g. [3–6]). Other studies have shown slow or negligible effects of the use of dispersants on biodegradation, often using high concentrations of oil and dispersants (e.g. [7, 8]), which may result in initial inhibition of oil biodegradation [9]. However, during biodegradation studies of dispersed oil it is important to account for the rapid dilution of the oil dispersions in the water column, using low concentrations of oil and dispersants [9].

Results from laboratory studies of oil biodegradation can be used to determine degradation rates that are used in environmental oil spill models. One such model is the three-dimensional and dynamic OSCAR model, in which biodegradation rates of a number of oil compound groups are determined as rate coefficients [10]. The compound groups are separated between saturate and aromatic compounds, and by different boiling-point ranges, covering approximately 70–80% of light crude oils, according to true boiling-point curves [10, 11].

Several laboratory methods have been developed for preparing oil dispersions. In the MNS (Mackay–Nadeau–Szeto) system, energy is applied from blowing air along the walls of a chamber, thereby creating a breaking wave [12]. In the IFP system (developed by Institute Française du Pétrole), dispersions are generated in a test chamber where the oil/emulsion is applied on the seawater surface within a test ring and dispersant is added. Energy is applied to the system by a metal ring [13]. The dispersant is usually applied to the oil in the system with a dispersant-to-oil ratio (DOR) of 1:25 to 1:100. The oil is often applied as a water-in-oil emulsion, which can be generated in rotating cylinders [14]. Another test is the baffled flask incubation system with orbital rotation [15]. In

addition, mesoscale systems like flume tanks may be used to simulate weathering processes [16]. The systems described above are mostly relevant for testing of dispersant treatment of surface spills, and evaporation will be an important depletion process for low boiling-point oil compounds, like small alkanes and monoaromatic hydrocarbons.

During the DWH spill, subsurface dispersant injection (SSDI) was introduced to reduce the quantity of surfaced oil. A major part of the oil was trapped as a deepwater plume, consisting of small droplets with estimated sizes between 10 and 50 μm [17]. As a tool to study ecotoxicity and biodegradation of dispersed oil we have developed a system for generation of small oil droplet dispersions in seawater. The oil droplet generator consists of a series of nozzles creating repeated turbulence inside a tube when pumping water through the system (Fig. 1A) [18]. The generated oil concentration in the water can be accurately controlled by the rate of oil added, and the oil droplet size distribution can be adjusted by varying the amount of shear in the turbulent zones by adjusting the water flow rate through the system. For studies of oil dispersant biodegradation, we have developed a slowly rotating carousel incubation system. Dispersions are transferred to flasks of 1–2 L that are completely filled (no headspace or air bubbles) and then mounted on the carousels (Fig. 2). Biodegradation experiments are performed with the wheels of the carousels slowly rotating around the carousel axis (0.75 r.p.m) by a gear motor [19].

To simulate conditions found during a potential deepwater oil spill, and with the use of dispersants as OSR method, we here describe a system with small oil droplet dispersions (median droplet size close to 10 μm), low oil concentrations (2–3 mg/l nominal

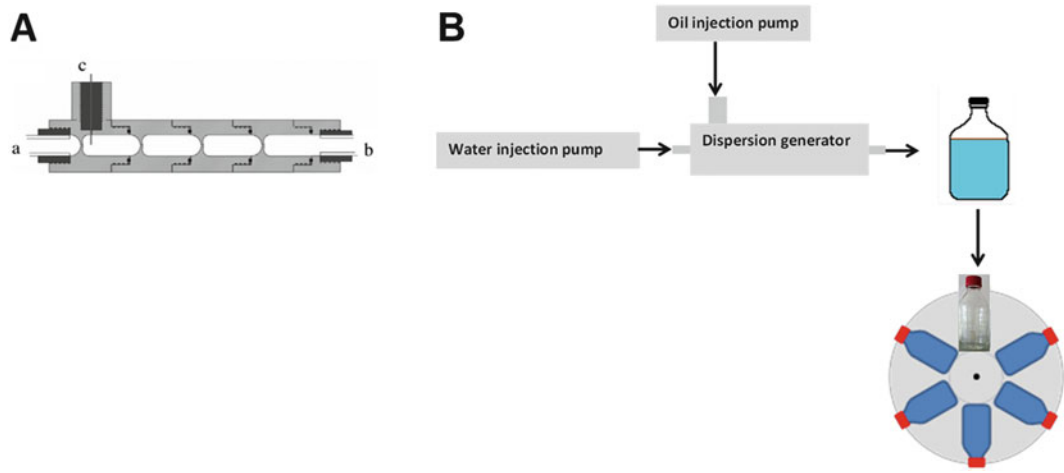

Fig. 1 Transect of the dispersion generator consisting of a series of chambers with connecting nozzles (**A**). Seawater inlet (*a*), dispersion outlet (*b*) and inlet capillary for oil (*c*) are shown [18]. The carousel incubation system (**B**) shows wheels with 8 × 2-L flasks mounted on each wheel [19]

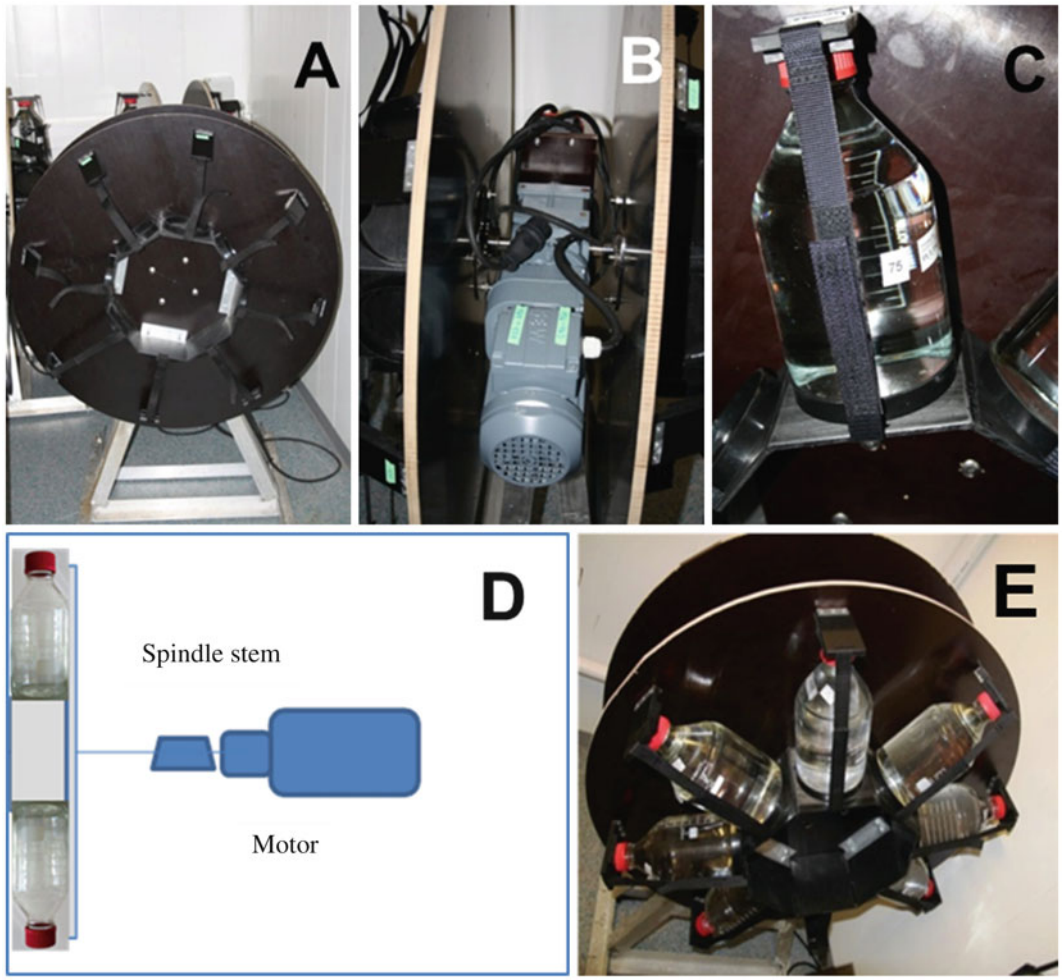

Fig. 2 Carousel incubation system. Wheel with holders for eight 2-L flasks (**A**), gear motor placed between the two wheels of a carousel unit (**B**), system for mounting of flasks consisting of a base socket, a head lid and a band for tightening the flask with a hook and loop fastener (**C**). In (**D**), the system is described with a gear motor connected with the wheel system with a spindle stem driving the system. In (**E**), the carousel incubation system shows wheels with 8 × 2-L flasks mounted on each wheel [19]

concentrations), pre-mixing of the oil with a dispersant and an incubation temperature of 5°C. However, the system can be operated at any relevant seawater temperature above freezing point. The experiment is performed in 2-L glass flasks completely filled with the oil dispersions.

2 Materials

2.1 Oil Dispersion Generator System

The capillary system and its connections to seawater and oil injection pump systems are shown in Fig. 1A, B. The system consists of the following units:

1. Dispersion generator with a system of multiple ($n = 4$) nozzles (ID = 0.5 mm) and inner chamber diameter of 8 mm (*see* Fig. 1A). The seawater inlet consists of polyethylene feed tubes (OD = 6 mm, ID = 4 mm) for inlet water ((a) in Fig. 1A) and outlet dispersion is fed through a Teflon tube (PFTE or PFA, OD = 6 mm, ID = 4 mm) ((b) in Fig. 1A). An inlet capillary ((c) in Fig. 1A) is used for introduction of oil (*see* **Note 1**). Both inlet and outlet of the generator are supplied with a simple pulse damper (*see* **Note 1**).
2. Laboratory pump for seawater flow-through (Fluid Metering Inc., Model QV (http://fluidmetering.com)).
3. Stroke rate controller (Fluid Metering Inc., Model V200 (http://fluidmetering.com)).
4. Programmable syringe pump (World Precision Instruments, Model AL-2000 (http://www.wpiinc.com)).
5. Gas-tight glass syringe (e.g. 5 ml) for the syringe pump (http://www.sge.com)
6. Teflon tubings for connections of the various parts.
7. Flasks or barrels for collecting excess oil dispersions to be discarded (*see* **Note 13**).

2.2 Carousel Incubation System

The carousel incubation system is shown in Fig. 2. The system consists of the following parts:

1. Gear motor with Movitrac LT (SEW Eurodrive, helical worm gear motor S-series (http://www.seweurodrive.com)).
2. Frequency converter (SEW Eurodrive, Movitrac LT (http://www.seweurodrive.com)).
3. Wheels ($D \sim 100$ cm) with system for mounting of up to 8 flasks with a volume of 2 L (Fig. 2; *see* 7 in Subheading 2.4). Flasks may be mounted on the carousel as shown in Fig. 2C. The wheels are used for biodegradation experiments (incubations).
4. Wheels ($D \sim 65$ cm) with system for mounting up to 6 flasks with a volume of 1 L (*see* 6 in Subheading 2.4). This wheel is used for preparation of stock dispersions (*see* 2 to 6 in Subheading 3.3 and **Note 2**).

2.3 Temperature-Controlled Room

1. Temperature-controlled room set at 5°C.

2.4 Materials for Preparation of Seawater and Oil

1. Filter units, nominal exclusion limits 25 and 1 μm in standard filter cartridge systems (e.g. Aqua-Pure Whole House Filtration System, AP055T (http://solutions.3m.com)).
2. Sterile-filtering unit, 0.2 μm exclusion limit (Nalgene Rapid Flow (http://www.thermoscientific.com)).

3. Gas-tight glass syringe (e.g. 1,000 µl) (http://www.hamiltoncompany.com).
4. Pipette for mixing of oil and dispersant, e.g. volume 10 µl.
5. 10-L plastic containers with faucet at bottom.
6. One-litre flasks (SCHOTT or similar quality) with PBT screw caps, pouring rings for PBT screws caps and PTFE protected seal for preparation of stock oil dispersions. The flasks should be washed and autoclaved prior to use (*see* **Note 3**).
7. Two-litre flasks (SCHOTT or similar quality) with PBT screw caps, pouring rings for PBT screws caps and PTFE protected seal for preparation of final oil dispersions to be mounted on the carousels. The flasks should be washed as described in **Note 3** prior to use.
8. Volatile organic compound (VOC) tubes (40 ml volume) for storage of samples for analyses of VOC, screw-capped "EPA" vials with Teflon inserts cleaned by baking, 450°C in min. 4 h.
9. Two-litre flasks (SCHOTT or similar quality) with PBT screw caps, pouring rings for PBT screws caps and PTFE protected seal for storage of samples for analyses of semi-volatile organic compounds (SVOC). The flasks should be baked prior to use.

2.5 Substances and Chemicals

1. Natural seawater from a suitable source (*see* **Note 4**).
2. Filtered seawater. Natural seawater is filtered through 25 µm filter, then through 1 µm filter (*see* **Note 5**).
3. Sterile-filtered seawater. Filtered seawater is sterile-filtered through 0.2 µm filters. For further explanation, *see* **Note 5**.
4. Dispersant (*see* **Note 6**).
5. Oil, fresh or evaporated to simulate time on sea (*see* **Note 7**). The oil should be heated at 50°C for 30 min to melt wax and used for generation of dispersions (*see* **Note 7**) at room temperature.
6. Inorganic nutrients (optional; *see* **Note 8**).
7. Chemical for sterilizing seawater, e.g. $HgCl_2$, stock solution 50 g/L (*see* **Note 5**).
8. HCl 15 % (vol/vol), p.a. quality.

2.6 Analytical Instruments

1. Coulter counter, e.g. Multisizer 4 fitted with 280 µm aperture with Multisizer 4 software, Coulter counter size L30 quality control standard bead suspension (https://www.beckmancoulter.com), and sterile-filter (0.22 µm filter) seawater to be used as electrolyte (*see* **Note 9**).

3 Methods

3.1 Preparations Before Dispersion Generation

1. Place natural, filtered and sterile-filtered seawater (*see* 1–3 in Subheading 2.5) overnight in flasks or containers in the temperature-controlled room at 5°C at least for 24 h. Measure temperature before start of experiment. Optionally, the natural seawater may be pre-incubated with oil for seawater adaption to the oil (*see* **Note 10**).
2. Place all 2-L flasks to be used in the experiment at 5°C.
3. When seawater has reached 5°C, fill all 2-L flasks to be run with natural seawater (*see* 1 in Subheading 2.5) almost completely. Leave approximately 50 ml headspace.
4. Fill the 2-L flasks to be used with sterile-filtered seawater (*see* 3 in Subheading 2.5) almost completely, leaving approximately 50 ml headspace. Add $HgCl_2$ to these flasks to achieve final concentrations of 100 mg/L of $HgCl_2$ in sterilized seawater.
5. Prepare additional volumes of sterile-filtered seawater supplemented with $HgCl_2$ to fill up flasks during the experiment with sterilized seawater.
6. Prepare seawater filtered through 25 μm filter and 1 μm filter (*see* 2 in Subheading 2.5) to be used for generation of stock oil dispersions (*see* also **Note 5**).

3.2 Determination of Dispersion Volumes To Be Used

1. Mix oil and dispersant (*see* 4 and 5 in Subheading 2.5) at a DOR of 1:100 (*see* **Note 11**).
2. Transfer the oil–dispersant mixture to the syringe (*see* 5 in Subheading 2.1) of the oil injection pump (*see* 4 in Subheading 2.1).
3. Remove enough volume of filtered seawater (*see* 2 in Subheading 2.5) from the temperature-controlled room for preparation of stock oil dispersions.
4. Adjust the dispersant generator for preparation of 200 mg/l dispersed oil with 10 μm median oil droplet size (stock oil dispersion). The seawater and oil flow rates must be calibrated for each dispersant generator system and oil (*see* **Note 12**). Prepare stock oil dispersion in filtered seawater. Stabilize the dispersion generation for about 10 min. This dispersion generated during the stabilization period is discarded (*see* **Note 13**)
5. Prepare approximately 500 ml dispersion in a 1-L flask and measure with Coulter counter (*see* Subheading 2.6). Before measurement dilute the stock dispersion 100 times in filtered seawater. Determine the oil concentration (mg/l) in the original stock dispersions based on the measured particle concentration (μm^3/ml) corrected for the oil density.

6. Based on the determined oil droplet concentration, calculate the dilution of the stock dispersion that will give a certain oil concentration (e.g. 3 mg/l) by filling the 2-L flasks completely:

$$\frac{(\mathbf{X} \times 100 \times \mathbf{Y}) \times \text{volume for dilution}}{2,300} = 3 \qquad (1)$$

where **X** is the Coulter counter results (X × 10^6 μm^3), dilution factor is 100, **Y** is the oil density (**Y** mg/ml), the total volume of the 2-L SCHOTT flasks is 2,300 ml and final oil concentration 3 mg/l. The volume for dilution is therefore:

$$\text{volume for dilution} = 6,900/(\mathbf{X} \times 100 \times \mathbf{Y}) \text{ ml} \qquad (2)$$

If, for example, a concentration of 2.52 × 10^6 μm^3 is measured in the Coulter counter, a volume of 32.84 ml stock dispersion shall be applied to 2,300 ml seawater to achieve a final concentration of approximately 3 mg/l oil dispersion and an oil density of 0,8337 mg/ml.

3.3 Generation of Dispersions and Mounting on Carousels

Prior to the experiment the selected oil should be tested in the dispersion generator to determine the flow rates of seawater and oil that result in planned oil concentrations and oil droplet sizes (*see* Subheading 3.2 and **Note 12**). The oil dispersions are prepared at room temperature, with oil and dispersant at room temperature, and filtered seawater at 5°C (*see* 1 in Subheading 3.1). During the oil dispersion preparation and mounting of flasks with dispersions on the carousel system, it is important to be well organized and to work fast. The procedures described here are recommended for oil dispersions with an average oil droplet size of 10 μm to be incubated at a temperature of 5°C. For larger oil droplet dispersions, *see* **Note 14**.

1. The carousels should be placed in the temperature-controlled room at 5°C. Set the carousel system at 0.75 r.p.m. (*see* **Note 15**).
2. Prepare flasks with natural seawater in a 1-L SCHOTT flask. These flasks are to be used for preparation of stock dispersions in a carousel holding 1-L SCHOTT flasks (*see* **Note 16**). Prepare stock dispersions of oil (200 mg/l oil droplets) in the 1-L flask and mount the flask on a carousel for 1-L flasks (*see* 4 in Subsection 2.2) at the speed to be used in the incubations (*see* **Note 15**). Several flasks with stock dispersions can be mounted in the carousel at the same time.
3. For transfer from stock dispersions to the flasks used for incubation, remove a 1-L flask with stock dispersion from the carousel. Apply the volume of stock dispersion as calculated from Eq. (2) (*see* 6 in Subheading 3.2) in 2-L SCHOTT flasks nearly filled with natural seawater and fill immediately with natural seawater. Check that no air bubbles are left in the flasks.

4. After removal of flasks with stock dispersion from the carousel, each stock dispersion can be used for dilution for a period of not more than 30 min when 10 μm droplet size is used. After a period of 30 min, remove a new flask from the carousel with stock dispersions, or prepare a new flask (1 L) with stock dispersion (*see* **Note 16**).
5. Mount the flasks prepared with dispersions at final concentrations immediately on the incubation carousel system (*see* 3 in Subheading 2.2) running at 0.75 r.p.m.
6. During these operations, it is recommended that at least two persons are working in an organized way, one filling the flasks and the other mounting the flasks on the carousels.
7. Apply stock dispersions of oil (200 mg/l oil droplets; *see* Subheading 3.2) in the volume as calculated from Eq. (2) (*see* 6 in Subheading 3.2) in 2-L flasks nearly filled with sterilized seawater and fill immediately with sterilized seawater. Check that no air bubbles are left in the flasks.
8. Mount the flasks in the carousel (*see* 3 in Subheading 2.2) running at 0.75 r.p.m. Use a new flask with stock dispersions if time exceeds 30 min (*see* 4 and **Note 16**).
9. Seawater without oil dispersions should also be included. Fill 2-L flasks completely with natural seawater and mount on carousels running at 0.75 r.p.m. Alternatively, these flasks may be incubated on bench in the temperature-controlled room at 5°C.

3.4 Incubation, Sampling and Analyses

At the conditions described here (5°C), the experiment should be run for at least 2 months. We have experienced that more frequent sampling is required for the first period, for instance, with at least three samplings for the first week (e.g. days 0, 3 and 7), two the next week (e.g. days 10 and 14), weekly samplings the next two weeks (days 21 and 28) and then monthly or bi-weekly sampling for the last period (e.g. days 42 and 64). The 0-day samples should be placed on the carousel for at least 15 min before sampling.

Samples collected with dispersions in natural seawater should be analyzed for oil droplet concentrations and size distributions, oil chemistry and microbiology, while samples in sterilized seawater should be analyzed for droplet concentrations/size and chemistry. Microbiology analyses may also be performed with some sterilized samples to determine inhibitory effects of the biocide used (*see* **Note 17**).

1. For sampling of flasks containing dispersions, each flask should be removed and sampled individually before proceeding to the next flask to be sampled. It is important to work quickly during sampling to prevent droplets from rising and thereby creating inhomogeneous dispersions. To collect samples for oil droplet and chemical analysis, each flask should be sampled as follows:

Remove the flask with dispersion to be sampled from the carousel and collect 20 ml for Coulter counter measurements.

Measure the dissolved oxygen (DO) in the flasks, for instance, with a DO probe (e.g. YSI dissolved oxygen meter with BOD probe (https://www.ysi.com)).

For chemical analyses of the low oil concentrations recommended for these studies, 2-L flasks should be sacrificed for analyses. Remove 40 ml sample for analyses of VOC, transfer to a VOC tube (*see* 8 in Subheading 2.4) and add 4 drops of HCl (*see* 8 in Subheading 2.5). Fill the VOC tubes completely and store at 4–5°C until analyses (*see* **Note 18**). Acidify the rest of the sample volume in the flask (appr. 2.2 L) by adding 17 ml of HCl (*see* 8 in Subheading 2.5) for analyses of SVOC (*see* **Note 18**).

2. To collect samples for microbiological analyses each flask should be sampled as follows:

   Remove 10 ml sample for epifluorescence microscopy and MPN analyses. For microscopy analyses, dilute 1 ml samples in a recommended diluent, depending on the choice of method (*see* **Note 19**). MPN analyses should be performed for quantification of viable heterotrophic and oil-degrading microbes on 3 or 5 replicates of dilutions in the growth medium (*see* **Note 20**).

3. For DNA extraction for microbial community analyses (e.g. 16S rRNA gene amplification), we usually filter volumes of dispersions or seawater without oil through membrane filters (exclusion limit 0.22 μm). Filtered volumes will depend on the analyses, and for 16S rRNA gene amplification analyses we have used from 600 ml to 2,000 ml volumes. Filters are stored (−20°C) until analyses (*see* **Note 21**).

4 Notes

1. The principle of the oil droplet generator is to create repeated zones of turbulence by pumping water through a number of small chambers separated by small ($Ø = 0.5$ mm) nozzles (Fig. 1A). The flow rate of water through the system determines the degree of turbulence. Oil is added into the water stream behind the first nozzle through a thin capillary (syringe needle Gauge 21), and oil droplets are formed by the shear forces created by the turbulence behind each nozzle. Repeated steps of turbulence increase the uniformity of the oil droplet sizes and thus reduce the width of the droplet size distribution. The parameters with the biggest impact on the resulting oil droplet sizes are the flow rate of water through the system and the properties of the oil used. The water flow rate through the generator is limited upwards by the pressure generated by the resistance in the nozzles and should be kept below

200 ml/min. At low flow rates, the turbulence eventually becomes too low to generate oil droplets and carry the oil through the system. Water flow rates below 70 ml/min are thus not recommended. When using a valveless piston pump as described above, a pulse damper must be placed between the pump and the inlet of the droplet generator. Optionally an additional pulse damper may be placed at the outlet of the generator. Simple pulse dampers can be made using a T-shaped tube connector with a short piece of air filled and closed tubing directed upwards from the inlet tube. Further details, as well as the theory behind the construction, are found as supplementary material in Nordtug et al. [18]. The generator is custom made by SINTEF, and further details can be supplied by the authors.

2. The carousel incubation system consists of wheels with flasks mounted as shown in Fig. 2. The wheels are slowly rotated around the carousel axis by a gear motor connected to a spindle stem (Fig. 2D). The carousel system has been developed for maintaining small oil droplet dispersions while incubating the dispersions over time. According to Stoke's law, small oil droplets of 10 μm have a rising velocity of 1–2 cm/h, depending on the oil density, while larger droplets have higher rising velocities (e.g. 30 μm droplets may rise by velocities of 15–20 cm/h). We believe that the ability of keeping the droplets in suspension is due to the combined effect of turbulence created in the flasks and the fact that the oil droplets rising velocity will make them constantly change direction in relation to any fixed point at the flasks inner surface.
3. Prior to use all flasks to be used for oil dispersions should be washed to reduce attachment of oil to glass walls (wall effects). We compared various washing procedures and recommend the following one: Flasks are baked in an oven (450°C; 3 h), placed overnight in Deconex bath (4% vol/vol) and washed in a laboratory dishwasher with detergent (e.g. Neodisher N). The flasks are then washed in a laboratory dishwasher without detergent and autoclaved (120°C; 20 min). The lids of the flasks should be closed as soon as the flasks have cooled after autoclaving to prevent the inner surface from drying.
4. Seawater should be collected from a non-polluted source. Concentrations of inorganic nutrients (e.g. NO_2 + NO_3-N, NH_4-N, PO_4-P and Fe), organic content (TOC/DOC), pH, temperature and dissolved O_2 should be measured in the natural seawater.
5. To correct for non-biotic degradation, we recommend to include sterilized controls in the experiments. The seawater is filtered through 25 μm filters and 1 μm filters to remove coarse and fine particles and to facilitate sterile-filtration through

0.22 μm filters. Risk of filter clogging is thereby reduced during filtering of large volumes of seawater. To avoid growth of microbes possibly escaping the filtering process, we also include the use of a biocide ($HgCl_2$) in the sterilized controls. Seawater is also filtered through 25 μm filters and then 1 μm filter for preparation of stock dispersions to avoid interference of potential particles in seawater when dispersion volumes to be used as final concentrations are determined.

6. The dispersant is used for at least two reasons: (1) the oil droplets generated in the oil dispersion generator (*see* **Note 1**) are of a droplet size distribution relevant for chemically dispersed oil, and (2) the use of dispersant delays surface attachment of the oil [6] (*see also* **Note 3**). Typical dispersants that have been used in these experiments are Corexit 9500A (supplier Nalco Environmental Solutions LLC (http://www.nalcoesllc.com)), Slickgone NS (supplier Dasic International (http://www.dasicinter.com)) and Finasol OSR-52 (supplier Total Special Fluids (http://www.totalspecialfluids.com)).
7. Oils that we have used include both paraffinic oils (e.g. Macondo 252 and Statfjord), naphthenic oils (Troll), and asphalthenic (Grane). Both fresh and weathered/evaporated oils as well as chemically dispersed emulsions have been used in the system. Crude oils stored for some time may contain wax precipitates. In our laboratory, it is therefore a standard procedure to heat the oils at 50°C for 60 min to melt this precipitated wax and to shake well for 1 min before use.
8. Inorganic nutrients are used if there is a risk of limitations of these during the biodegradation period, due to low natural background concentrations (*see* **Note 4**), and if experiments are to be performed with initial oil concentrations expected to be able to consume essential nutrients like nitrate and phosphate. A "marine" Bushnell-Haas solution (*see* **Note 20**) of inorganic nutrients may be used if amendments are required [20]. Some precipitation may be observed for this solution. We preferably recommend the use of low oil concentration and unamended natural seawater.
9. For seawater-based dispersions, sterile-filtered seawater should be used as electrolyte in the Coulter counter instrument. For more information, see the User's Manual of the instrument. Alternatively to a 280 μm aperture, a 100 μm aperture for measuring smaller oil droplets. Quality control standard beads L10 are then used for quality control.
10. In some experiments, the seawater may be pre-incubated with low oil concentrations to induce growth of oil-degrading microbes before starting the experiment. This approach may be used to simulate oil discharges to already oil-impacted seawater. If the experiment is to be performed with initial oil

Table 1
An example of median oil droplet size and measured oil concentrations measured by Coulter counter analyses for different settings of the oil droplet generator system for an oil with low viscosity (Statfjord fresh; viscosity 12 mPas, 13°C)

% of flow (seawater pump)	ml/min (flow as measured)	Oil added (μl/min)	Median of droplets generated (μm)	Oil concentration (ppm)
63.1	152	2.00	12.30	11.14
51.9	129	1.64	15.35	13.78
41.5	112	1.32	18.43	11.86
31.1	83	1.00	25.39	10.10
28.4	54	0.66	42.37	4.365

The % of flow is set on the stroke rate controller of the seawater pump (see 3 in Subheading 2.1), while oil added is set on the oil pump (*see* 4 in Subheading 2.1). This system must be calibrated in each droplet generator for individual oils (*see* **Note 12**)

concentrations of the 2–3 mg/L, a pre-adaption of the seawater may be run for, e.g. one week ahead of the experiment with an oil concentration of 0.2–0.3 mg/L (1/10 of the concentration used in the experiment).

11. With well-known dispersants (e.g. Corexit 9500 and Slickgone NS) we have experienced that a DOR of 1:100 efficiently disperses oils that are dispersible, although higher DORs (up to 1:25) are used in many instances.
12. The oil dispersion generator has been proven to generate reproducible results for oil concentrations and droplet size distributions for crude oils with low viscosity (e.g. <100 mPas, 13°C) within a range of 10–30 μm median oil droplet size. An example of oil dispersion generator settings is shown for a fresh Statfjord oil in Table 1. However, flow rates of seawater and oil must be calibrated for each generator and oil/oil weathering degree. In our system, with a fresh Statfjord oil, we have used a seawater flow of 178 ml/min, seawater pump settings of 73.2 % flow and an oil flow of 42.16 μl/min for the generation of oil dispersions with 10 μm median oil droplet size. The relation between seawater flow rates and average oil droplet distribution is shown in Table 1 for the fresh Statfjord oil. However, with more viscous oils the median droplet size was larger at the same conditions (e.g. oils with viscosity > 100 mPas, 13°C).
13. Oil-containing water should be handled as hazardous waste and discarded according to approved procedures for this type of waste.
14. For dispersions with large oil droplets, with low seawater and oil flows, a vertical system may be used. Large oil droplets will

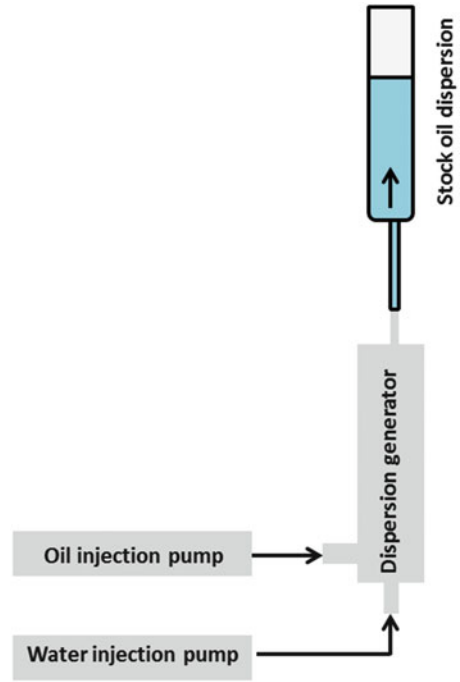

Fig. 3 Illustration of a vertical oil dispersion generator system for "large" droplets (e.g. 30 μm median oil droplet size). Seawater and oil are pumped into the oil dispersion generator which is placed in a vertical position with the outlet in the top. The dispersion is collected from the bottom of a tube (e.g. 100 ml volume)

rise faster than small oil droplets, resulting in inhomogeneous dispersions with most of the droplets in the upper parts of the water. This effect may be reduced by a vertical position of the dispersion generator and an upward collection of the stock dispersion (*see* Subsection 3.3). Such a system is illustrated in Fig. 3.

15. We have experienced that a slow speed of 0.75 r.p.m works fine with small oil droplet dispersions. If dispersions with larger oil droplets are used, higher speeds may be considered, but should be tested prior to each experiment.
16. Stock dispersions (200 mg/L oil droplets) should be prepared in "small" 1-L SCHOTT flasks (rather than in larger 2-L flasks) to reduce unnecessary waste of dispersions. Small volumes of stock dispersions are recommended, since transfer of stock dispersions (200 mg/L oil droplets) to low oil droplet concentrations (2–3 mg/L oil droplets) has to be performed fast to secure homogenous dispersions. By experience, we have observed that 10 μm oil droplet dispersions are homogenous for at least 30 min, while dispersions with larger oil droplets (e.g. 30 μm) should be distributed within 10–15 min. We have therefore designed a

carousel holding 1-L SCHOTT flasks for preparation of the stock dispersions.

17. We recommend separation between samples used for chemical and microbiological analyses, while oil droplet analyses (Coulter counter measurements) should be performed for all samples. If chemical and microbiological analyses are to be performed from the same sample, the dispersion or seawater (blank) for microbiological analyses should be transferred to new autoclaved flasks. Microbiological analyses may also be performed on some samples of sterilized dispersions, primarily late in the biodegradation experiment. The purpose of these analyses will be to control that sterilized controls are not contaminated. Microbial analyses of sterilized controls should then be performed as Most probable number (MPN) counts of heterotrophic prokaryotes (*see* **Note 20**).
18. Acidified samples for analyses of volatile organic compounds (VOC) and semi-volatile organic compounds (SVOC) should be stored at 4–5°C for not more than 14 days before extraction (SVOC) or analyses (VOC). We perform VOC analyses by direct injection into a Purge and Trap GC-MS. For SVOC analyses, the samples are extracted by liquid–liquid extraction, using, e.g. dichloromethane, before measurements of total extractable organic material (TEOM), n-alkanes and isoprenoids (pristane and phytane) by GC-FID, while decalines, naphthalenes, phenols, 3- to 5-ring polyaromatic hydrocarbons (PAH) and hopanes are quantified by GC-MS analyses.
19. Epifluorescence microscopy is performed with fluorescent dyes like 4′,6-diamidino-2-phenylindole [21], acridine orange [22], or by live/dead staining [23].
20. MPN counts can be performed in sterile tissue culture plates with lids, e.g. 24-well plates, using Difco™ Marine Broth for MPN counts of heterotrophic prokaryotes and Difco™ Bushnell-Haas Broth (http://www.bd.com) for oil-degrading prokaryotes. For obtaining "marine" conditions in the Bushnell-Haas Broth, we add 30 g/L NaCl, while the crude oil (0.1 % [vol/vol]) is used as carbon source in the medium. The Sheen screen method is used for detection of positive growth in the Bushnell-Haas medium [24]. MPN concentrations are then determined from MPN tables [25].
21. For 16S rRNA gene amplicon analyses, we have filtered dispersions and seawater through Durapore membrane filters (0.22 μm exclusion limit and diameter 45 mm (http://www.merckmillipore.com)). The filters are stored dry (−20°C). For DNA analyses, the filters are cut in small pieces (sterile scalpels) and transferred to tubes for extraction of DNA, performed by FastDNA Spin Kit for Soil (http://www.mpbio.com).

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Protocol for Inferring Compound Biodegradation at Low Concentrations from Biomass Measurements

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Abstract

Biodegradation tests for organic compounds are usually performed at relatively high carbon concentrations (2–100 mg L^{-1}), which can be problematic for toxic compounds. Here we describe a protocol to test compound biodegradation through the concomitant formation of bacterial biomass at relatively low carbon concentrations (0.5–5 mg C/L). The protocol is based on accurate cell counting of dilute cell suspensions by flow cytometry coupled to cell staining with fluorescent dyes.

Keywords: Bacterial population growth, Cell staining, Flow cytometry

1 Introduction

Bacterial degradation (metabolism) of organic compounds is usually inferred from two different lines of evidence: (1) increase of biomass or cell numbers over time at the expense of a single uniquely present carbon source and (2) disappearance of the carbon source over time, inversely proportional to the increase of the amount of cells in culture. Appropriate negative controls, consisting of cultures without added carbon source and media with the added carbon source but without inoculated bacteria under sterile conditions, serve to correctly interpret the results on the compound's biodegradability.

The strength of this experimental concept is its basic simplicity, yet in its current and most applied form, it demands relatively "high" amounts of available carbon (of the tester compound) in order to detect sufficient bacterial biomass formation (typically quantified from culture turbidity or similar) [1–3]. For example, as a rule of thumb, one can assume 0.2–0.5 pg C per bacterial cell [4, 5], which with a carbon yield coefficient of 20% would dictate that 1–2.5 pg C is needed per cell division. Culture turbidity is reliably detectable at between 10^7 and 10^8 cells per mL, meaning

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that in order to detect population growth one would need to add the equivalent of some 1–2.5 × 10^8 pg C mL^{-1} = 100–250 μg C mL^{-1} (or mg C L^{-1}). This concentration is much higher than what is typically found in the environment. Furthermore, mg L^{-1} concentrations of organic pollutants can easily become toxic and inhibit bacterial growth, thereby leading to an underestimation of the potential for biodegradation [6, 7].

In order to overcome this toxicity issue and to approach lower more environmentally relevant carbon concentrations (0.1–1 mg C L^{-1}), we and others recently developed adaptations to the classical experimental biodegradation concept [8, 9]. The most important adaptation to the experimental protocol is a more sophisticated way of counting cells in a population with higher accuracy and at much lower cell densities by using flow cytometry coupled to cell staining [10–12]. These adaptations not only permit to study biodegradation by pure cultures at lower carbon concentrations [9] but also to study biodegradation by native microbial communities [8]. The second important adaptation of the experimental protocol involves working in a low C environment and avoiding as much as possible the use of non-inert (plastic) material, which can leak carbon. Theoretically, it might be interesting to push for the ultralow limit, for example, the amount of carbon needed to observe a single cell division, but it will be very difficult to create experimental conditions which exclude all other available carbon for microbial growth. Kinetics and yields of biodegradation at very low C-concentrations are still problematic to assess, but recent data suggest that extrapolations can be made from kinetic data obtained at slightly higher (10–50 mg C L^{-1}) concentrations [9]. Ideally and if possible, growth assays at low C-concentrations should be complemented with measurements of compound disappearance or metabolite appearance, in order to make appropriate kinetic calculations [8, 9].

The protocol presented here describes the materials and methods to detect biomass growth from pure culture or communities at the expense of added organic compound in the range of 0.5–5 mg C L^{-1}, by using flow cytometry cell counting. The protocol can be adapted for water-soluble as well as poorly water-soluble organic substrates.

2 Materials

2.1 Glassware and Materials

1. Sterile glass Erlenmeyer (100 mL) for regular culturing of bacteria
2. HCl-treated glass Erlenmeyer (100 mL) for examining bacterial growth at low substrate concentrations

3. Glass filtration system and cellulose acetate filters (0.2 μm pore size, sartorius) to prepare filter-sterile medium
4. Regular aluminum foil, glass pipettes (10 and 25 mL), and glass cylinders (500 mL, 1 L)
5. 96-well plate (e.g., flat bottom, plastic, NuncTM, Denmark) for the preparation of culture dilutions for the flow cytometer autosampling device

2.2 Culture Medium Preparation

1. All assays were performed in glass Erlenmeyer closed with aluminum foil caps. Avoid using plastic tools to avoid unintended carbon release as much as possible.
2. Rinse all glassware twice with 6 M HCl followed by four rinses with Milli-Q water. Empty bottles and let them dry to the air at room temperature, cover them with aluminum foil, and autoclave before adding the medium. Alternatively, dry heat flasks at 180°C to remove any contaminating carbon.
3. Prepare mineral salts medium for general cultivation, such as *Pseudomonas* minimal medium [13], but without any carbon source (*see* **Note 1**). Filter the medium over a 0.2 μm membrane to remove cells and particles. Fill in flasks cleaned under point 2 (*see* **Note 2**).
4. *Pseudomonas* minimal medium (PMM) is, per liter, 1.0 g NH_4Cl, 3.5 g $Na_2HPO_4{\cdot}2H_2O$, 2.8 g KH_2PO_4, pH 6.8. After filter-sterilization (point 3), add 20 mL Hutner's mineral base solution (50×) and 2 mL vitamin solution (500×).
5. Hutner's vitamin-free mineral base (50-fold concentrated) is, per liter, 10 g nitrilotriacetic acid (NTA), 14.45 g $MgSO_4 \cdot 7H_2O$, 3.33 g $CaCl_2 \cdot 2H_2O$, 9.74 mg $(NH_4)_6Mo_7O_{24} \cdot 4H_2O$, 99 mg $Fe_2SO_4 \cdot 7H_2O$, and 50 mL Metals 44. Dissolve metals one by one, ensuring that everything has dissolved before adding the next metal salt. Sterilize by filtration.
6. Metals 44 is, per 100 mL, 387 mg $Na_4EDTA \cdot 4H_2O$, 1.095 g $ZnSO_4 \cdot$ mg $Fe_2SO_4 \cdot 7H_2O$, 154 mg $MnSO_4 \cdot H_2O$, 39.2 mg $CuSO_4$, 24.8 mg $Co(NO_3)_2 \cdot 6H_2O$, and 17.7 mg $Na_2B_4O_7 \cdot 10H_2O$). Sterilize by filtration.
7. Vitamin solution (500-fold concentrated) is, per 100 mL, 0.5 mg biotin, 50 mg nicotinic acid, and 25 mg thiamin hydrochloride (*see* **Note 3**). Sterilize by filtration.
8. Prepare series of the concentrations and carbon sources to be tested. Include a positive (e.g., 1 mg L^{-1} of a compound which is known to be used by the tester strain) and a negative control (i.e., no C added).
9. Prepare a regular "high" C-growth medium, for example, with 5 mM succinate in *Pseudomonas* minimal medium, to be used for preculturing the tester strains.

2.3 Flow Cytometry

1. The instrument we use is a Becton Dickinson LSRFortessa (BD Biosciences, Erembodegem, Belgium) equipped with three lasers (blue 488 nm, 50 mW; red 640 nm, 40 mW; UV 355, 20 mW). However, any flow cytometer capable of volumetric counting can be deployed.
2. Sterile-filtered phosphate buffered saline (PBS) solution (PBS contains, per L, 8 g NaCl, 1.44 g Na_2HPO_4, 0.24 g KH_2PO_4, pH 7.4).
3. BD FACSDiva software (v. 6.2, Becton Dickinson Biosciences, Erembodegem, Belgium), for data acquisition and processing.
4. BD Cell Quest, FlowJo, or a similar program, in order to analyze and interpret the flow diagrams.

2.4 Cell Staining

1. 37% formaldehyde solution for fixation of cells (*see* **Note 4**).
2. Sodium azide stock solution for fixation of cells (NaN_3, 400 mg mL^{-1} in dimethyl sulfoxide [DMSO], Sigma-Aldrich, Switzerland),
3. NaN_3 working solution (400-fold diluted NaN_3 stock solution in DMSO, *see* **Note 4**). Freshly made every day.
4. SYBR Green I stock solution (Invitrogen).
5. SYBR Green I working solution (100-fold diluted SYBR Green I stock solution in DMSO), used for staining cells in suspension. The working solution can be stored for several weeks at −20°C. Protect from light.
6. Milli-Q water (LaboStar, Siemens; 0.5 μS cm^{-1}).

3 Methods

3.1 Initial Considerations

The method can be carried out with pure bacterial cultures or with native bacterial communities [8]. For the accuracy of quantifying population or community growth at the expense of low amounts of carbon in the medium, it is important to start at low cell densities (10^4 cells per mL). Else, the expected cell density increase is too small to become detected. It is advised to calibrate the accuracy of flow cytometric cell counting by staining and analyzing serial dilutions of a culture with known density.

3.2 Preculturing Procedure for Pure Culture

1. Recover the tester strain from −80°C and grow on a regular agar-solidified medium to have single colonies. Incubate the plates at temperature and duration optimal for the chosen pure culture, until the appearance of bacterial colonies of reasonable size (1–2 mm).
2. Inoculate 20 mL sterile *Pseudomonas* minimal medium with 5 mM sodium succinate in a glass Erlenmeyer (100 mL) with a single colony from a freshly grown agar plate.

3. Cultivate the cells until late stationary phase (e.g., 48 h at 30°C, with shaking at 180 rpm). For the procedure, it is important that the bacteria have used **all** the available carbon and that there is no carryover of carbon from the preculture in the "low-C" test cultures. Further washing steps (i.e., centrifuging cells and resuspending in carbon-free medium) can be included to remove traces of carbon.
4. Dilute the cell suspension 1,000-fold in (filter-sterilized) PBS or PMM.
5. Stain the cells in a 1 mL subsample of the suspension by SYBR Green using the procedure in point 3.5.
6. Count the number of cells in the suspension using flow cytometry according to procedure 3.6.
7. Dilute the suspension, if necessary, to be able to achieve a starting inoculum density of 10^4 cells per mL.

3.3 Sample Preparation of a Natural Community for Testing

1. Sample 500 mL of lake water (we used Lake Geneva water) and filter through a 30 μm pore-size nylon net filter (Millipore) to remove bigger particles.
2. Take a subsample of 1 mL and stain the cells in the filtrate subsample by SYBR Green using the procedure outlined under point 3.5.
3. Count the number of cells in the filtrate using flow cytometry as under point 3.6.
4. Dilute the filtrate, if necessary, in order to be able to achieve a starting inoculum density of 10^4 cells per mL.
5. Use the adjusted filtrate as inoculum in the growth exposure assays.

3.4 Growth Assays at Low C-Concentrations

1. Prepare quadruplicate flasks of 25 mL of *Pseudomonas* minimal medium (without carbon) in 100 mL aluminum-capped treated glass Erlenmeyer, for every C-concentration to be tested.
2. Add carbon substrate to achieve 0.5, 1.0, and 5 mg C L^{-1} (*see* **Note 5**).
3. Prepare a quadruplicate positive control, e.g., 1 and 5 mg C L^{-1} of a C-source which the community or pure culture is known to utilize (e.g., benzoate or succinate).
4. Prepare a quadruplicate negative control of *Pseudomonas* minimal medium only (no added C). This important control serves to measure background growth on the leftover assimilable carbon in the water used to prepare the media, on any carryover C from the precultures, or autotrophic growth as a result of dissolved CO_2 from the air.

5. Inoculate the pure culture or the tester community at a starting cell density of around 10^4 cells per mL.
6. Incubate flasks on a rotary shaker (180 rpm) at a temperature of 21°C, or any temperature adapted to the primary objectives of the experiment.
7. Sample cultures (1 mL) directly after inoculation and addition of carbon substrate (T_0) and after appropriate time intervals to capture the expected growth (*see* **Note 6**).

3.5 Cell Staining Procedure

1. To test the accuracy of the procedure, prepare serial dilutions of the stationary phase preculture in sterile-filtered PBS or PMM, with (ideally) 10^1, 10^2, 10^3, 10^4, 10^5, and 10^6 cells per mL.
2. Add 10 μL formaldehyde solution (37%) per mL cell suspension or samples from the growth cultures (final concentration: 0.37% formaldehyde) to fix the cells.
3. Incubate for 1–2 h at 4°C (*see* **Note 4**).
4. Stain (fixed or not) cell suspensions with one-hundredth volume of SYBR Green I working solution (e.g., pipet 2.5 μL SYBR Green I working solution in the wells of a 96-well plate and add 250 μL culture sample).
5. Incubate for 15 min in the dark at room temperature, and continue immediately afterward with flow cytometric analysis.

3.6 Volumetric Cell Counting by Flow Cytometry

1. We use volumetric counting on a BD LSRFortessa flow cytometer. Set side scatter (SSC) and forward scatter (FSC) threshold levels such that background noise from remaining particles in solution is reduced.
2. Acquire FSC, SSC, and green fluorescence intensity (FITC-A, 530 ± 20 nm) for each event (cell). Calibrate the FITC-A gate settings using the diluted and stained bacterial culture of 3.5.1.
3. Aspire a constant sample volume (200 μL) onto the flow cytometer from 96-well microtiter plates by using the high-throughput screening device (HTS system, BD Biosciences) at a flow rate of 1 μL s^{-1}.
4. Count all events in the aspired volume (200 μL).
5. Wash flow lines four times in between each sample with 600 μL of sterile-filtered PBS solution.

3.7 Data Analysis

1. Define the bacterial population from the appropriate gating in the flow diagram.
2. Average the mean population or community size at each time point from the quadruplicates.
3. Compare specific population growth in incubations with added carbon source to that in the no-added carbon controls using,

e.g., pair-wise one-tailed T-testing (null hypothesis being that the population size in the carbon-amended samples is significantly higher than in the control).

4. Plot the net population growth as a result from the amended carbon as a function of time compared to the no-added carbon control, or by subtracting the values from the no-added carbon control at each time point.
5. Calculate biomass yield (in mg C biomass per mg C compound) by converting the net population growth (total number of cells per flask) into biomass by assuming 0.1–0.5 pg C/cell (*see* **Note** 7).

4 Notes

1. General mineral salts media will have an "overdose" of N-, P-, and S-source compared to the amount of C to be tested. If necessary, this can be optimized and balanced further. When using water-based medium only without any added N-, P-, or S-source, one risks underestimating potential C-degradation because of limiting other nutrients and trace elements.
2. Sterile filtration can release assimilable carbon into the medium from the filter matrix (e.g., cellulose acetate or nitrocellulose). Since filtration is necessary to remove particles that can disturb flow cytometry analysis, we wash filters with Milli-Q water before filtering the growth medium itself. For example, to prepare 1 L of filter-sterilized PMM on a new filter, we first pass 1 L of Milli-Q water, discard this, and then only filter the PMM solution.
3. The use of vitamins may have to be reconsidered because this introduces traces of metabolizable carbon, as do NTA and EDTA (used for solubilizing metals). On the other hand, the absence of vitamins in the medium may slow down bacterial growth and result in an underestimation of compound degradation.
4. Test whether fixation is necessary and test which fixation procedure is optimal. Cell fixation prior to cell staining is not absolutely required to obtain optimal staining. This should be tested on a dilution series of decreasing cell numbers. As an alternative to formaldehyde fixation, cells can be fixed with a solution of 1 g L^{-1} sodium azide for 1 h at 4°C.
5. Water-soluble carbon substrates can be added from a concentrated stock (100- to 1,000-fold). Poorly soluble substrates should ideally be dosed at 1 mg L^{-1}, without using additional solvents. In this case one can increase the volume of the used flasks and media for more accurate dosing of small quantities of

compound. In case of volatile substrates, the aluminum caps for the Erlenmeyer should be replaced by screw caps with Teflon lining, to minimize adsorption and volatilization losses.

6. For pure cultures and easily degradable carbon substrates, one expects the exponential phase to be very short due to the low amount of carbon. In that case, sample at 1, 2, 3, 4, 6, and 8 h after inoculation and after 1 and 2 days. In case of community growth and poorly degradable carbon substrates, sample every day during a period of a week.
7. A value of 0.1 pg C per cell should be used for oligotrophic bacteria. A value of 0.5 pg C per cell corresponds to a typical laboratory strain of *Escherichia coli* [4, 5]. For a further discussion on cell number to biomass conversion factors and on other confounding factors in kinetic parameter estimation, see recent work such as [9].

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Bioremediation of Sludge Obtained from Oil/Biofuel Storage Tanks

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Abstract

Crude oil refinery leads to the production of a considerable amount of oil tank bottom sludge which can contaminate the environment and is toxic to human and environmental health. Among the methods available for cleaning up sludge-contaminated environments is bioremediation, which is a widely acceptable method for the remediation of different hydrocarbon-contaminated soils. This method is based on using microbes, mainly bacteria and fungi to degrade or remove the contaminants. Bioremediation technology can be applied to soil contaminated with oil tank bottom sludge using natural attenuation, biostimulation (addition of nutrients to enhance indigenous microbial activities), and bioaugmentation (addition of hydrocarbon-degrading microorganisms to contaminated soils) or a combination of both biostimulation and bioaugmentation strategies. In addition to the type of the bioremediation methods applied, isolation and identification of microorganisms involved in the biodegradation process and the monitoring of their activities are important steps for any successful bioremediation project. In this chapter, simple and effective protocols are provided on how to isolate, screen, and identify hydrocarbon degrading-bacteria from oil sludge or sludge–soil complexes. In addition, two laboratory-scale methods for bioremediation of oil sludge and sludge-contaminated soil (microcosms and slurry phase) together with protocols for determining the concentration of hydrocarbon contaminants in soils are presented.

Keywords: Biodiesel, Biolog MT2 plates, Crude oil tank bottom sludge, Hydrocarbon degradation, Microcosms

1 Introduction

Crude oil, petroleum, and related products such as fuel, diesel, and gas are important in the modern world. They not only play an important role in providing energy for industrial, commercial, and residential use and are indispensable to transportation but are raw materials for many products such as plastics, cosmetics, and paints [1, 2].

However, crude oil refining is a complex process, and oil is often kept in storage tanks prior to downstream processing and transportation to other destinations. During this time, a substantial amount of heavy fractions such as paraffins, olefins, and aromatic

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compounds settles at the bottom of the storage tanks [3] and is called crude oil tank bottom sludge (COTBS). These storage tanks are cleaned periodically, usually once in every 5–10 years; this process is time-consuming, labor-intensive, and expensive. The cleaning process results in a huge amount of waste sludge or COTBS being generated which is toxic to the environment.

The crude oil sludge (oil tank bottom sludge) contains different hydrocarbon fractions, some of which (e.g., PAHs) are recognized as mutagenic and carcinogenic compounds [4]. In addition, alkanes and other fractions in the hydrocarbon sludge can be adsorbed to soil particles, with their adverse effects on the soil structure preventing such soils from being used for agriculture and other productive purposes. Consequently, detoxification of oil sludge or sludge–soil complex is very important.

Different physical, chemical, and biological approaches can be used to treat oil sludge and sludge-contaminated environments [4]. However, physicochemical treatments are expensive and labor-intensive and can often generate secondary pollutants. Biological methods, for example, bioremediation, are therefore widely accepted approaches for cleaning up oil sludge or soils contaminated with sludge because they are efficient, cost-effective, and environmentally friendly. Bioremediation is defined as the use of microorganisms (mostly bacteria and fungi) to degrade or remove the contaminants from environments. Different types of bioremediation strategies are available, ranging from natural attenuation (contaminant reduction via natural processes), biostimulation (addition of nutrients to contaminated soil), to bioaugmentation (addition of hydrocarbon-degrading microorganisms to contaminated soil) and a combined approach of biostimulation and bioaugmentation. For details of bioremediation technology, *see* references [5–7].

The quest for renewable alternatives to fossil fuels has led to the development of biofuels such as biodiesel. Biofuels are not only renewable, being produced from crops and microalgae, but are also less polluting and easier to degrade than fossil fuels. For example, biodiesel contains fatty acid methyl esters derived from triglycerides which can be used as a source of energy [8] but are easily degraded by microorganisms. Given its mild environmental footprint compared to petroleum hydrocarbons, the storage and treatment of biofuel is less problematic than crude oil. However, the methods used for oil (petroleum) sludge can also readily be applied to biofuel and associated sludge [9, 10]. Therefore, in this book chapter, the bioremediation methods described for treating hydrocarbon sludge can also be used to treat biofuel and biofuel–sludge-contaminated environments.

The aim of this book chapter is to present protocols for the treatment of oil sludge or sludge–soil complexes. The protocols presented here include those for the isolation and identification of hydrocarbon (oil)-degrading bacteria, the use of Biolog plates for

screening hydrocarbon degrading microorganisms, the extraction of petroleum hydrocarbon from contaminated soil (in order to monitor the rate of contaminant removal), and laboratory-scale study of different bioremediation technologies (ex situ microcosms and slurry phase).

2 Materials

2.1 Isolation of Bacteria

1. Crude oil sludge or oil sludge–soil complex.
2. A hydrocarbon source such as crude oil or diesel or biofuel (*see* **Note** 1).
3. Full range of pipettes and sterile tips.
4. Bushnell Haas (BH) mineral salts medium.

   BH medium contains 0.2 g L^{-1} $MgSO_4 \cdot 7H_2O$, 0.02 g L^{-1} $CaCl_2 \cdot 2H_2O$, 1 g L^{-1} KH_2PO_4, 1 g L^{-1} $(NH_4)_2HPO_4$, 1 g L^{-1} $(NH_4)_2SO_4$, and 0.05 g L^{-1} $FeCl_3 \cdot 6H_2O$ (pH 7.0) and can be solidified with 1.5% agar (Oxoid Ltd, UK). The reagents can be obtained from Sigma-Aldrich. BH medium broth can also be purchased directly from Sigma-Aldrich. In this case, 3.75 g of BH medium powder should be dissolved in 1,000 mL distilled water and agar (1.5%, Oxoid Ltd, UK) should be added before autoclaving.
5. Sterile distilled water
6. Phosphate-buffered saline (PBS) (Sigma-Aldrich, Australia)

   Phosphate-buffered saline solution also can be prepared using 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4, and 0.24 g KH_2PO_4 per 1 L, pH 7.4 (the reagents can be purchased individually from Sigma-Aldrich).
7. Agar (Oxoid Ltd, UK).
8. Sterile 10 mL tubes.
9. Disposable plastic spreaders.
10. Glycerol (Sigma-Aldrich, Australia).
11. Nutrient Agar (Oxoid Ltd, UK).
12. Nutrient Broth (Oxoid Ltd, UK).
13. Parafilm tape.
14. Shaker.
15. 25°C incubator for incubating inoculated nutrient media plates.

2.2 Bacterial Identification

1. Glass beads (212–300 μm, Sigma-Aldrich, Australia).

   Beads (0.5 g) should be weighed into Eppendorf tubes (2 mL) and sterilized at 121°C for 15 min.

2. FastPrep®-24 Instrument (MP Biomedicals, Australia) for homogenizing the samples.
3. Disposable plastic loops.
4. Agarose for gel electrophoresis in 1× TAE buffer (final agarose concentration of 1.5%).
5. NanoDrop 1000 spectrophotometer (Thermo Scientific, USA)
6. Sodium acetate (3M, pH 5.2).
7. Ice cold 100% ethanol.
8. 70% ethanol.
9. Nuclease-free water (Sigma-Aldrich, Australia).
10. dNTPs (Promega, Australia).
11. Taq polymerase (5 U μL^{-1}) (Promega, Australia).
12. 10× buffer and $MgCl_2$ (50 mM) provided with Taq polymerase (Sigma-Aldrich, Australia).
13. PCR primer (GeneWorks, Australia) [11]

    Primer sequences: 63F Bacteria CAGGCCTAACACATGCAAGTC and 1389R ACGGGCGGTGTGTACAAG (*see* **Note** 2).
14. DNA template (bacterial isolates).
15. PCR machine (e.g., T100, Bio-Rad, USA).
16. Wizard®SV Gel and a PCR Clean-Up System (Promega, Australia).
17. Sequencher software (version 5) (Gene Codes Corporation, USA).

    Sequencher software is commercially available; however, BioEdit freeware software (http://www.mbio.ncsu.edu/bioedit/bioedit.html) can also be used to edit and assemble the sequence.
18. Mega 6 software [12] or TreeDyn software in PhyML [13]. Mega 6 software can be freely downloaded from http://www.megasoftware.net, while TreeDyn is an online platform (http://www.phylogeny.fr/version2_cgi/index.cgi).

2.3 Biolog MT2 Plates

1. Centrifuge (Heraeus Multifuge, Thermo Scientific, USA).
2. Spectrophotometer (Pharmacia LKB Biochrom, UK).
3. Hydrocarbon degraders isolated from contaminated soil and oil sludge.
4. Sterile distilled water.
5. Biolog MT2 plates (Biolog, USA).
6. Shaker.
7. 25°C incubator for incubating environmental bacteria.

8. Biolog substrates.

   Different hydrocarbon fractions such as naphthalene, phenanthrene, eicosane, octadecane and hexadecane, crude oil, and diesel can be used for Biolog MT2 plate. These substrates can be purchased from Sigma-Aldrich. The solid substrates can be dissolved in hexane at a final concentration of 2% (w/v).

9. Microplate reader (Bio-Rad iMark Microplate Reader, USA)

2.4 Petroleum Hydrocarbon Extraction from Sludge–Soil Complex

1. Teflon-coated centrifuge tubes (25 mL) (Thermo Scientific, USA)
2. Shaker
3. Centrifuge (Heraeus Multifuge, Thermo Scientific, USA)
4. Glass bottles
5. Fume hood
6. Hexane (Sigma-Aldrich, Australia)
7. Dichloromethane (DCM) (Sigma-Aldrich, Australia)
8. Chromatographic vials (PerkinElmer, USA)
9. GC–MS (Agilent 6890 GC and LECO Pegasus II TOF-MS or equivalent/others)
10. Hydrocarbon standards (e.g., TPH standard, Sigma-Aldrich)

2.5 Microcosms

1. Contaminated soil (waste soil) or oily sludge/slurry with the desired concentration of oil tank bottom sludge (e.g., 5% diesel, up to 20%).
2. 300 g contaminated soil with oil tank bottom sludge or sludge–soil complex.
3. 1 L glass flask.
4. Bushnell Haas (BH) mineral salts (Sigma-Aldrich, Australia).
5. Sterile distilled water.
6. Hydrocarbon-degrading microorganisms.

   For studying the bioremediation of contaminated soils, hydrocarbonoclastic isolates such as *Bacillus* sp. and *Pseudomonas* sp., isolated from hydrocarbon-contaminated soil can be used as part of a biostimulation–bioaugmentation strategy. A fungus, *Scedosporium angiospermum*, has also been used in some studies [14, 15]. Commonly employed strategies include bioaugmentation (aeration and the addition of hydrocarbonoclastic isolates), biostimulation (aeration and addition of nutrients), natural attenuation (aeration), and biostimulation–bioaugmentation (aeration, addition of hydrocarbonoclastic isolates and nutrients). Bioremediation is usually carried out at suitable soil water content (40–70% of soil water holding capacity).

The selected microbial strains should be kept at −80°C in 50% glycerol and individually streaked on nutrient agar plates before being used in bioremediation. The streaked plates can be incubated at 25–35°C for up to 4 days. A loopful of bacteria can also be inoculated into nutrient broth and incubated at 30°C for 48 h at 120 rpm. Cultures of OD_{600} 0.6–1 are harvested by centrifugation at 4°C at 5,000 rpm for 5 min and washed twice with sterile distilled water. The pellet is then resuspended in 10 mL sterile distilled water. Bacterial cultures can be pooled together in 50 mL tubes before the start of the bioremediation experiments.

7. MX6 iBrid portable gas analyzer (Industrial Scientific Corporation, USA).

   The equipment can analyze CO_2 and volatile organic compounds (VOCs).

8. Incubator.

2.6 Slurry Phase

1. 200 g oil tank bottom sludge-contaminated soil.
2. 2 L glass flask.
3. Bushnell Haas (BH) mineral salts (Sigma-Aldrich, Australia).
4. Sterile distilled water.
5. Hydrocarbon degraders.

   For studying the bioremediation of sludge-contaminated soils, *Bacillus* sp. and *Pseudomonas* sp. or other hydrocarbon-degrading bacteria isolated from hydrocarbon-contaminated soil can be used. A fungus, *Scedosporium angiospermum*, has also been used [12, 13].

6. MX6 iBrid portable gas analyzer (Industrial Scientific Corporation, USA).
7. Incubator.

3 Method

3.1 Isolation of Hydrocarbonoclastic Bacterial Species

Isolation of hydrocarbon-degrading bacteria from oil tank bottom sludge or sludge–soil complex can be carried out with selective enrichment method involving the use of Bushnell Haas (BH) mineral salts medium [16].

1. Prepare Bushnell Haas (BH) mineral salts medium amended with 0.2–1.0% of hydrocarbons (e.g., crude oil) as source of carbon.

   For heavy oils such as some types of crude oil, use the lower concentrations (e.g., 0.2%), while for lighter oils such as diesel, use higher concentrations of hydrocarbons (1%). It is difficult to observe the bacterial colony when oil at higher concentrations are used.

2. Prepare serial dilution tubes, up to 10^{-3} in sterilized phosphate-buffered saline (PBS).
3. Spread 10^{-2} and 10^{-4} dilutions of hydrocarbon sludge or sludge–soil complex onto BH hydrocarbons agar plates supplemented with 0.2–1.0% (w/v) crude oil with sterile spreader to spread the dilution on the plate.
4. Seal the edges of plates with Parafilm tape.
5. Incubate for up to 14 days at room temperature (in the dark) or 25°C in the incubator again up to 14 days.
6. Select bacteria colonies based on their colonial morphologies.
7. Subculture selected colonies onto new sterile nutrient agar plates and incubate for up to 4 days (or until good colonial growth has been attained).
8. Aseptically inoculate selected purified bacteria (isolates) into nutrient broth and incubate for 48 h at 120 rpm at 30°C for the generation of cells for storage.
9. Maintain stocks of pure cultures at −80°C in 50% glycerol for further analyses.

3.2 Identification of Hydrocarbonoclastic Bacterial Species

Apart from traditional identification methods based on colonial morphology and biochemical tests, identification of bacteria using polymerase chain reaction (PCR) can be carried out based on the 16S rRNA gene [17].

3.2.1 DNA Extraction (see Note 3)

1. Take 2–4 loopfuls of bacterial culture from pure bacterial culture plates (nutrient agar) for DNA isolation.
2. Dislodge the colonies on the loop into the bead solution tube containing 500 μL sodium phosphate buffer (pH 8) and 500 μL phenol–chloroform–isoamyl alcohol (25:24:1).
3. Lyse the solution by bead beating for 2 × 20 s using FastPrep®-24 Instrument.
4. Centrifuge at 12,000 rpm for 5 min at 4°C.
5. Transfer (up to 500 μL) the supernatant to a sterile 1.5 mL clean tube.
6. Add an equal amount of phenol–chloroform–isoamyl alcohol (25:24:1).
7. Centrifuge at 12,000 rpm for 5 min at 4°C.
8. Transfer (up to 350–450 μL) the supernatant to 1.5 mL clean tube.
9. Add 1/10 volume sodium acetate and two volumes of ice cold 100% ethanol.
10. Incubate the tubes on ice or −20°C for at least 20 min.
11. Centrifuge at 12,000 rpm for 10 min at 4°C.

12. Carefully discard the supernatant without disturbing the pellet.
13. Wash the pellet with 500 µL of 70% ethanol twice.
14. Centrifuge at 12,000 rpm for 2 min at 4°C.
15. Dry the pellet by keeping the tube lids open for 10 min at room temperature.
16. Dissolve the DNA pellet in 100 µL nuclease-free water.
17. Use 1.5% agarose gel in 1 X TAE and NanoDrop 1000 spectrophotometer to check the quality and quantity of the extracted DNA.

3.2.2 Polymerase Chain Reaction (PCR)

1. Prepare a PCR reaction mix (48 µL) according to Table 1.
2. Add 2 µL of DNA sample to each PCR tube, 2 µL of amplifiable DNA to the positive control tube, and 2 µL nuclease-free water to the negative control tube.
3. Run the PCR program as shown in Table 2.
4. Check the size of PCR product (bp) with 1.5% agarose gel in 1× TAE.
5. Clean up the PCR amplicons using Wizard® SV Gel and PCR Clean-Up System according to the manufacturer's protocol.
6. Quantify the cleaned-up PCR products with a NanoDrop 1000 spectrophotometer or any other suitable spectrophotometer prior to sequencing.
7. Send purified DNA samples to any appropriate DNA sequencing laboratory.

Table 1
Master mix preparation for PCR

Reagent	Volume for 1 reaction (µL)	Final concentration
10 mM dNTP	1	0.2 mM
GoTaq Flexi Buffer (10×)	10	1X
25 mM $MgCl_2$	3	2.5 mM
GoTaq polymerase, 10 units μL^{-1}	0.25	1.25 units μL^{-1}
Forward primer, 63F (10 pmol μL^{-1})	2	0.4 pmol μL^{-1}
Reverse primer, 1389R (10 pmol μL^{-1})	2	0.4 pmol μL^{-1}
Sterile purified H_2O	29.75	–
Template DNA	2	–
Total volume (µL)	50	

Table 2
Thermocycling program for primer 63F and 1389R

Stage		Time	Temperature (°C)	Cycles	Touchdown
1	Initial denaturation	5 min	94	X1	
2	Denaturation	1 min	94	X10	
2	Annealing	1 min	65		−1 per cycle
2	Extension	1 min	72		
3	Denaturation		55	X20	
3	Annealing	1 min			
3	Extension	1 min	72		
4	Final extension	10 min	72	X1	
5	Incubation	–	12 (*see* **Note** 4)		

8. Assemble and edit obtained sequence data using Sequencher software (version 5).
9. Search GenBank (http://www.ncbi.nlm.nih.gov) with BLASTN searches for sequence homology and similarity [18] in order to identify the isolate.
10. Draw a phylogenetic tree with the relevant software such as MEGA 6 [12] or TreeDyn in PhyML [13]. This is optional.

3.3 Biolog MT2 Plates

"Biolog MT2 plate is a 96-well microplate designed to test the ability of the inoculated microorganism suspension to utilize (oxidize) a panel of different carbon sources (e.g., different petroleum hydrocarbons). Each well of the panel contains a tetrazolium redox dye and a buffered nutrient medium that has been developed and optimized for a wide variety of bacteria". Unlike other Biolog MicroPlates however, the carbon sources have been omitted so the wells in this regard are "empty" (MT2). For biodegradation studies, this provides the user with flexibility in selecting an array of appropriate carbon sources. At the user's discretion, carbon sources may be added either before or after inoculating with a cell suspension. Volatile and hydrophobic carbon sources such as naphthalene can also be used. However, visual confirmation of the formation of the naphthalene crystals should be carried out prior to any inoculation.

Ideally, about 0.3 mg of carbon source (e.g., 15 μL of a 2% stock solution) should be added to each well; however utilization of much lower levels (e.g., 20–200 ppm) can often be detected (http://www.biolog.com/products-static/microbial_identification_literature.php):

1. Activate pure bacterial isolates from −80°C by inoculation into nutrient broth (15 mL) in 50 mL tubes and incubate at 30°C and 120 rpm for up to 2 days.
2. Transfer 5 mL of the broth culture into 10 mL tubes.
3. Determine the optical density of the culture with 1 mL of broth culture at 600 nm in a spectrophotometer.

   The OD_{600} should be around 1. If the OD of some samples are below 1 (e.g., 0.7), all other samples should be diluted to the minimum value using sterile distilled water.
   In the case of OD_{600} values above 1 OD, samples also should be diluted to 1.
4. Centrifuge the remaining 4 mL of culture for 3 min at 4,700 rpm and discard the supernatant.
5. Add 4 mL of sterile distilled water, shake and centrifuge for 3 min at 4,700 rpm, and discard the supernatant.
6. Repeat **steps 4** and **5** twice.
7. Resuspend pellet in 4 mL of sterile distilled water.
8. Inoculate the wells on the Biolog plates first with 15 μL of desired hydrocarbon fractions (in triplicate) (*see* **Notes** 5–9).
9. Incubate the plate on ice for 30 min to allow for the evaporation of hexane.

   This step only applies to solid substrates which need to be dissolved in hexane.
10. Inoculate the wells on the Biolog plates with 150 μL of optimized culture (in triplicate).
11. Keep a control lane without hydrocarbon fraction (i.e., inoculated with only culture).
12. Take zero time reading in the Biolog plate using a multiscan microplate reader equipped with an automated shaker-loader cassette at 595 nm and record the values.
13. Incubate the plates at 30°C for 7 days.
14. Readings should be taken every 2 h over the first 12 h, then every 2 h from 12 to 36 h, and every 12 h thereafter for up to day 7 (*see* **Notes** 10 and 11).

3.4 Petroleum Hydrocarbon Extraction from Sludge–Soil Complex

1. Weigh 5 g sludge–soil complex or soil contaminated with crude oil sludge into Teflon-coated centrifuge tubes.
2. Add 15 mL of hexane/DCM (50:50) into the tubes.
3. Incubate the tubes in an ultrasonic bath for 20 min.
4. Centrifuge the tubes at 5,000 rpm for 5 min at room temperature.
5. Transfer the supernatant to clean pre-weighed glass bottles.

6. Concentrate the supernatant by using a rotary evaporator or allow for solvent evaporation overnight at room temperature in a fume hood.
7. Add 1 mL of hexane to the concentrated extract and transfer into a 2 mL chromatographic vial.
8. Carry out gas chromatography mass spectrometry (MS) equipped with an autosampler following established protocols (*see* **Note** 12).

3.5 Laboratory-Scale Study of Different Bioremediation Technologies

As soil properties are important factors that affect any bioremediation project, soil properties should be characterized prior to carrying out any bioremediation. Soil characterization can be carried out in the soil laboratory or outsourced to commercial companies. However, standard protocols [19–21] are available for researchers who need to carry out soil analysis in their own laboratory.

3.5.1 Set Up the Microcosms

For the application of bioremediation technology for the treatment of oil tank bottom sludge, 200–500 g of sludge–soil complex is usually needed for the experimental setup. The samples can be soils already contaminated with hydrocarbons or clean soil spiked with the desired concentration of oil tank bottom sludge or hydrocarbon contaminant (e.g., 5% diesel). However, clean soils have been replaced with previously bioremediated soils (waste soil) as an alternative to using pristine soil in our laboratory [14].

Our experience showed that soil contaminated up to 20% oil tank bottom sludge can be successfully bioremediated in a microcosms study. Soil/sludge properties such as pH, water holding capacity, texture, status of nutrients, and heavy metals are the key parameters to be considered prior to starting the experiment. Here, we are presenting a general approach, and the methods should be optimized for different areas and conditions as required.

Microcosms represent a small simplified version of a real environment which mimics the real conditions. The use of microcosms offers a number of advantages. For example, a vast number of samples can be tested, microcosms are cheap to process, and parameters such as temperature, pH, nutrition, level of oxygen and CO_2, etc., can be controlled individually [22]. Therefore, optimization of microcosms can be achieved in a short time period.

The degradation rate of hydrocarbons can be used as a good indicator of successful biostimulation and bioaugmentation. The treatments will be suitable for use in the field when the degradation rate of hydrocarbons is significantly higher than the control. Field conditions differ from laboratory conditions as the control of all environmental factors in the real environment is impossible. There are only relatively few reports on the implementation of laboratory bioremediation studies in the field. However our unpublished results showed that laboratory studies can represent a very accurate,

useful, and cost-effective approach for the optimization of bioremediation prior to field implementation. In addition, Lors et al. [23] conducted a study with PAH-contaminated soil at field and laboratory scales, and they found that results obtained from the laboratory experiments were similar to those obtained in field experiments.

Two common methods, ex situ microcosms and slurry phase microcosms, are presented:

1. In this section, we will apply four different bioremediation strategies: bioaugmentation (BA), biostimulation (BS), biostimulation–bioaugmentation (BSBA), and natural attenuation (NA).
2. Weigh 300 g oil tank bottom sludge-contaminated soil into a clean 1 L flask (for each treatment or strategy employed, at least three replicates are needed).
3. Prepare and sterilize Bushnell Haas (BH) mineral salts in 1 L distilled water without any agar and hydrocarbon source (*see* **Note** 13).
4. Add Bushnell Haas (BH) mineral salts in the labeled flasks as (8% w/w) (BS treatment).
5. Add hydrocarbon degrader/degraders into the flasks (BA treatment).

   50 mL of inoculant suspension (approximately 10^4 CFU $mL^{-1)}$ is enough for bioaugmentation studies.
6. Add Bushnell Haas (BH) mineral salts and hydrocarbon degraders in right combination into the flasks (BSBA treatment).
7. Incubate the flask at suitable temperature (e.g., 25°C) up to the end of experiments (e.g., 12 weeks).
8. Maintain moisture content with sterile water at 40–70% of the maximum water holding capacity of the soil.
9. Monitor the CO_2 and volatile organic compounds (VOCs) production by using MX6 iBrid.
10. Carry out sampling on 2–4 weekly basis for downstream analyses.

    Sampling can be performed using a clean spatula from soil in the middle of the microcosm at each point time. In most experiments, 10–20 g soil (depending on analysis methods) will be enough for each soil sample.

3.5.2 Slurry Phase Set-Up

Bioavailability of hydrocarbons in soil could be limited due to the nature of hydrocarbon contaminant, time frame of pollution (whether fresh or old), and soil type. Some bioremediation projects have stalled or not worked as a result of the low or reduced bioavailability of hydrocarbon contaminant even though the microbial catabolic capacity to degrade contaminant was available [24].

One approach for increasing of hydrocarbon bioavailability would be to use slurry phase technology. In this method, laboratory-based slurry phase bioremediation projects are prepared with water or other treatments (e.g., active sludge from wastewater treatment plant) in different ratios such as 1:3 and 1:5 ratios of water to contaminated sample. The concept is that the slurry phase approach will enhance interaction between microorganisms, contaminants, nutrients, and oxygen and leads to an increase in hydrocarbon removal from contaminated soil [25]. Here, a useful laboratory-scale protocol for slurry method is presented:

1. Weigh 200 g of oil tank bottom sludge-contaminated soil and keep in a 2 L flask (for each treatment with at least three replicates needed for each treatment) (*see* **Note** 14).
2. Add 1,000 mL of distilled water to achieve ~20% (w/v) slurry (control treatment, natural attenuation treatment).
3. Add 1,000 mL of sterile water to achieve ~20% (w/v) slurry and $(NH_4)_2SO_4$, KH_2PO_4, and K_2HPO_4 during the slurry preparation phase to achieve a soil C:N:P molar ratio (C determined from hydrocarbon concentration) of approximately 100:10:1 (biostimulation treatment).
4. Add 1,000 mL of sterile water to achieve ~20% (w/v) slurry plus hydrocarbon degraders as outlined in previous sections (bioaugmentation treatment). 50 mL of inoculant suspension (approximately 10^4 CFU mL^{-1}) is enough for a bioaugmentation study for each flask.
5. Add 1,000 mL of distilled water to achieve ~20% (w/v) slurry plus hydrocarbon degraders and nutrients as outlined in previous sections (biostimulation–bioaugmentation treatments).
6. Incubate flasks on shaker incubators with continuous rotation of 150 rpm for 45 days at 30°C in dark conditions.
7. Monitor the CO_2 and volatile organic compounds (VOCs) production by using MX6 iBrid.
8. Take 25–50 mL of sample from each flask weekly over the incubation for further analyses.

4 Notes

1. Petroleum hydrocarbons and biofuel sources should be filter-sterilized using solvent stable filter (0.22 μm) before pouring into plate.
2. Apart from 63F and 1389R primers, any other suitable eubacterial primers can be used with the appropriate thermocycling conditions.

3. DNA extraction from bacterial cultures can be performed by using commercial kits such as UltraClean® Microbial DNA Isolation Kit or PowerSoil™ DNA extraction kit (MO BIO Laboratories, Inc, USA) by following the manufacturers' protocol.
4. After completion of PCR, samples can be kept in the thermocycler at 12°C rather than at 4°C as keeping samples at the lower temperature can damage the thermocycler over time.
5. At least three replicates are needed for each hydrocarbon substrate.
6. A fume hood should be used for the inoculation of different hydrocarbons.
7. As some hydrocarbons are known as hazardous, special care has to be taken into account when using these substrates.
8. The microplates should be kept on ice for 30 min before loading the substrates to prevent the microplates from cracking.
9. For nonvolatile fractions, different compounds can be used in one plate (e.g., phenanthrene and pyrene). However, for volatile substrate (e.g., naphthalene), it is recommended to use only one substrate per plate to avoid cross contamination.
10. The plates' lids should be closed except when taking readings.
11. For Biolog analysis, control absorbance should always be subtracted, irrespective of the time of observation (initial and during incubation).
12. For hydrocarbon determination, hydrocarbons should be separated on a nonpolar capillary column, Supelco SPB (30 m by 0.25 mm with 0.25-μm film thickness). The injection temperature is 350°C and injection volume is 1 μL. Helium gas (2 mL min^{-1}) is used as a carrier gas at a constant flow rate. The % weight of each hydrocarbon fraction can be analyzed and compared to the weight of each fraction using standards (e.g., TPH standard).
13. Bushnell Haas (BH) mineral salts can be replaced with other treatments such as manure, $(NH_4)_2SO_4$, KH_2PO_4, and K_2HPO_4.
14. Soil (contaminated soil) for slurry phase needs to be sieved before use, and the soil particles need to be less that 2 mm.

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