METHODS FOR RHIZOREMEDIATION RESEARCH

Approaches to experimental design and microbial analysis

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1. Introduction

Rhizoremediation is an elegant form of bioremediation that seeks to harness light energy via plants to biostimulate pollutant degradation by the indigenous soil microbial community. In pursuit of this goal, rhizoremediation draws upon the fields of rhizosphere ecology and microbial biodegradation, and in doing so confronts many of the same questions that have challenged these fields for decades, including: What mechanisms dictate microbial community structure and activity in the rhizosphere? Which microorganisms and degradative genes are actively involved in biodegradation, and how does their activity respond to different treatments? One major obstacle to answering these fundamental mechanistic questions over the years, and subsequently developing successful rhizoremediation technology, has been our limited methodological capabilities to quantify the degradative potential of microbial communities and to demonstrate and understand their response to the unique and multi-faceted environment of the plant-soil interface.

Microbiological research methods are the windows through we view the hidden world of microorganisms, with each being of different size and dimension, yet none affording a complete view of the diversity, function and abundance of microbial populations within a community. Cultivation methods are notoriously limited in their ability to detect the uncultivable majority of organisms in the environment, yet remain informative and indispensable for studying the metabolic capabilities of particular strains. Molecular biological methods like functional gene detection and stable isotope probing open much larger windows by circumventing culture bias and allowing the direct examination of microbial populations important to bioremediation, although they are limited somewhat by available sequence data. One aim of this chapter is to provide an overview of microbial methodologies, both cultivation-dependent and independent, which have already or are likely to provide new insight into the multitude of mechanistic and applied rhizoremediation questions to be answered.

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Microbial analyses are most valuable to rhizoremediation when conducted within the context of comprehensive, carefully designed experiments to evaluate the effectiveness of different plant species and to uncover the mechanisms that drive rhizodegradation. Accomplishing conclusive, informative experiments can be a challenging task in light of the extraordinary heterogeneity of soil and rhizosphere systems and the long-term nature of rhizostimulation. This chapter considers a variety of different experimental approaches, ranging from controlled greenhouse pot experiments to forensic field studies, and discusses the relationship between the hypothesized mechanism of rhizostimulation and selection of the appropriate experimental system.

While this overview is primarily drawn from rhizoremediation studies of organic aromatic pollutants like polychlorinated biphenyls (PCBs) and polyaromatic hydrocarbons (PAHs), the guidelines are easily adaptable to many other organic contaminants. Together, the experimental design strategies and microbial analytical methods discussed are aimed at assisting researchers in the creation of studies that will successfully answer an array of rhizoremediation questions, both mechanistic and applied.

2. Experimental approaches and design

When planning a study to assess the influence of plants on pollutant degradation, selecting the appropriate experimental approach is critical. Will conclusive results best be achieved by pot studies, field plots or less conventional approaches like forensic field studies of long-term sites? The answer lies in part in the mechanisms and time scale by which detectable rhizostimulation and/or contaminant disappearance is hypothesized to occur. In this section, the benefits and drawbacks of different experimental approaches are discussed and guidelines are provided to assist in their successful implementation.

2.1. FORENSIC FIELD STUDIES

While conventional wisdom may dictate a progression from bench to greenhouse to field-testing of remedial technologies, there are distinct advantages to starting directly in the field to examine the rhizoremediation capabilities of natural vegetation. This approach affords a view of the long-term impacts of a diverse array of plant species, including mature trees, on microbial communities and/or contaminant disappearance otherwise inaccessible due to the practical time limitations of most planted studies. Forensic studies are also invaluable as a means of screening species for their ability to prosper in contaminated soil and under environmental stresses (i.e. drought and nutrient stress) without active cultivation. However, the numerous abiotic heterogeneities and lack of controlled conditions at natural field sites can make determinations of true rhizoremediation effects challenging.

The forensic approach was first applied by Olson *et al.* [1-4] at a naturally-vegetated PAH-contaminated sludge disposal basin in Texas City, Texas. Historical aerial photographs and tree ring analyses revealed that plants colonized the basin over the course of 16 years following the drainage of standing water. Through plant invasion and succession, a diverse plant community (51 species and 22 families) developed that was

dominated by mulberry trees (*Morus* spp.), Bermuda grass (*Cynodon dactylon*) and common sunflowers (*Helianthus annuus*). In the absence of baseline data regarding original contaminant levels, PAH concentrations beneath the root zone were considered representative of the parent sludge which averaged 16,854 mg/kg total PAHs. Coring, excavation and chemical analyses throughout the site indicated that PAH concentrations in the root zones of plants were reduced to 10-50% of that in the original sludge. Microbial analyses indicated that cultivable PAH utilizing bacteria were five-fold more numerous ($p < 0.01$) in the mulberry root zone than in non-rooted samples of the same depth, and these results were confirmed with real-time PCR quantitation of degradative genes [5-8]. The close correspondence between root zone depth, sludge PAH concentration and abundance of PAH-degrading bacteria suggests that plants and associated microflora facilitated pollutant degradation.

Areas with deep and relatively homogeneous contamination like the sludge basin in Texas City are fortuitous opportunities for forensic rhizoremediation studies based on chemical disappearance. However in many cases contaminated field sites are a result of heterogeneous surface spillage of chemicals, making a forensic determination of chemical disappearance nearly impossible. Even in the absence of chemical disappearance data, naturally vegetated contaminated sites afford a good opportunity to screen various plant species for their tolerance to contaminants and their long-term influence on the degradative potential of the associated microbial community.

A forensic field study focusing on rhizosphere microbial populations was performed in a PCB contaminated site near Uherské Hradiste in South Moravia, Czech Republic [9-11]. Accidental spillage of PCBs onto the soil occurred from the mid 1950s to the mid 1980s, resulting in a heterogeneously contaminated area with soil PCB concentrations ranging from 1-500 mg/kg. The site was naturally vegetated with 25 different plant species, including 5 tree species, all rooted directly in contaminated soil of varying concentrations. The abundance and identity of cultivable PCB-metabolising bacteria were determined in soil and rhizosphere samples collected at various depths beneath the trees, grasses and forbs on four different dates during one year. In order to distinguish effects of plants from abiotic factors on microbial populations, PCB concentration and soil moisture content were also analysed but were not found to significantly correlate with numbers of PCB-degrading bacteria. Austrian pine (*Pinus nigra*) and goat willow (*Salix caprea*) trees fostered significantly higher numbers of cultivable PCB-metabolising bacteria in their root zones than other plant species or non-rooted soil of equivalent depth (collected beneath shallow-rooted grasses). The results imply that long term growth of certain plant species can increase numbers of contaminant degraders in the bulk soil.

Forensic field studies, by virtue of their scale and scope, are large interdisciplinary undertakings that incorporate plant taxonomy and ecology, soil biochemistry, soil/rhizosphere microbiology and complex statistical analyses. If planning to embark on a forensic field study, is it recommended that the assistance of collaborators with expertise in these fields be sought early in the process. As much historical information as possible should be collected regarding the time of contamination, previous measurements of concentration, presence of co-contaminants and vegetation history. A comprehensive sampling strategy should be planned (see Sampling Methods section)

and discussed in depth with an ecological statistician. A particular challenge in forensic studies is distinguishing true plant-mediated effects from those created by numerous abiotic heterogeneities. Multiple measurements (i.e. contaminant concentration, soil moisture, pH, nutrients, depth, etc.) should be made of each sample and subjected to statistical analyses along with microbial population data. Multivariate statistical methods are particularly well-suited to this purpose, and a number of useful books on ecological multivariate statistics are available to provide guidance [12-15].

2.2. PLANTED STUDIES

Rhizoremediation experiments conducted by planting and monitoring microbial populations and/or contaminant levels over time are powerful approaches since they can be constructed and operated with replication, randomisation and controlled conditions. Perhaps the major limitation of planted studies is time, since for reasons of practicality planted studies are typically only run for 1-3 years. Because roots are estimated to occupy less than 1% of soil [16], it is reasonable to anticipate that many years of root exploration would be required to thoroughly treat a contaminated area. Rates of contaminant disappearance also indicate that rhizoremediation should be regarded as a long-term process [17]. Nonetheless, losses in soil pollutant concentration and/or increased populations of degraders have been observed within several years or less [17-21]. In the event that pollutant disappearance occurs too slowly to detect, a comprehensive planted study still affords an excellent opportunity to investigate mechanisms of remediation, such as microbial population shifts at the root-soil interface.

When designing planted studies, as with any controlled experiment, careful and statistically conscious design is critical to ensure conclusive results. Replicates of each treatment including unplanted soil as a control should be included. Numbers of replicates should be as high as reasonably achievable without compromising the quality of the data since higher numbers of replicates produce more accurate results and a lower likelihood of producing false positive/negative results. Randomised arrangements of treatments, whether in pots or field plots, should be employed to ensure that differences observed are due to treatments rather than variations in light, soil composition, moisture, etc. associated with different locations in a greenhouse or outdoor area. Additional guidance may be found in books focusing on basic statistics [22] and experimental design [23]. The following sections discuss additional design considerations specific to pot or field plot studies.

2.2.1 Pot studies

Performing studies in containers is an immediately attractive option because it affords greater homogeneity than can be achieved in an intact field site, keeps plant roots contained and therefore maintains discrete species-specific influences on soil. However, pot studies are subject to criticism regarding their ability to realistically replicate natural conditions that may impact rhizoremediation processes, especially when conducted in a greenhouse.

Pot experiments performed in a greenhouse are convenient in that they provide independence from seasonal limitations on outdoor research. Unfortunately, plants are

shielded from environmental cues that influence plant physiological responses important to some rhizoremediation mechanisms. One issue is that greenhouse glass filters out a portion of ultraviolet light from solar radiation. Ultraviolet light is reported to promote the production of plant secondary compounds [24], which are thought to play a major role in biostimulation and/or induction of aromatic pollutant degrading bacteria [25] (detailed in the following chapter by Andrew C. Singer). If studies are conducted in the greenhouse, supplementation with UV light is recommended since it has been demonstrated to increase the concentrations of phenolic compounds produced by plants to levels similar to those obtained under outdoor growth conditions [24].

Greenhouse studies also shield perennial plants from seasonal signals that dictate cycles of senescence and dormancy, including tree root turnover events [26]. Root turnover may be an important mechanism in the delivery of secondary plant metabolites that support the growth of PCB-degrading bacteria [27]. If a concern, this can be remedied by running the pot study outdoors where plants can be exposed to natural environmental conditions. For additional realism, pots may be buried in the soil to help maintain ambient soil temperature. Whether indoors or outdoors, root growth in pots is often very different from bulk soil. Roots tend to grow along the pot edges, coil along the bottom, and may be significantly less branched than field-grown roots, which could deleteriously effect rhizoremediation results (John Fletcher, 2005, personal communication).

Pot studies are advantageous over field studies since they afford the opportunity to minimize heterogeneity of contaminant concentrations. To take advantage of this, care should be taken to homogenize soil thoroughly in a large soil mixer or similar equipment before filling pots, since soil either collected from a contaminated site or manually spiked with pollutants tends to be heterogeneous. At or prior to the initiation of the study, chemical analyses of replicate samples should be performed to assess the variability of pollutant concentrations within and among pots and help determine adequate sample replication. When natural environmental soils are used without additives to provide loose texture (i.e. vermiculite), they can become very compacted after continued watering. If considering the addition of organic material to the potting mix, note that some materials (i.e. bark mulch) contain high concentrations of plant aromatic compounds that can interfere with gas chromatographic analyses of polyaromatic hydrocarbons in soil as well as impact microbial communities.

Another consideration with pot studies is that contaminants may leach out of drainage holes posing not only a chemical hygiene problem but also creating a false positive response when chemical disappearance data are analyzed. Care should be taken to avoid excessive watering, and leachates should be collected for chemical analyses especially if working with water-soluble contaminants. Unplanted controls introduce unique complications with watering regime. If watered uniformly with planted treatments, soil in unplanted pots may remain saturated, generating hypoxic conditions distinctly different from soil conditions in planted pots. This compromises important control data for background levels of microbial activity as well as for volatilization of pollutants through soil pores. A solution to this problem is to maintain soil moisture at similar levels rather than to use equal water volumes, and to monitor outflow for escaping contaminants.

2.2.2 Field plots

Planted field plots afford the opportunity to evaluate the rhizoremediation potential of a variety of plant species under more realistic environmental conditions than pot studies. While heterogeneity of soil contamination is often greater, it can generally be overcome with a randomised layout such as Latin square or randomised block design and careful statistical analyses [22, 23]. Many precautions mentioned in the previous section also apply, including attention to the watering regime and chemical analysis of preliminary samples to determine contaminant variability.

A unified experimental design strategy for field investigations of rhizoremediation has been developed by the U.S. Environmental Protection Agency's Remediation Technologies Development Forum (RTDF) Phytoremediation Action Team. The strategy was designed to evaluate the efficacy of various agricultural and non-crop herbaceous plants for the rhizoremediation of weathered petroleum hydrocarbons. Use of the unified protocol permits direct comparisons of data by researchers in a wide range of geographic locations and climates. Having been carefully developed and debated by numerous researchers, the RTDF experimental design provides a useful general framework for field plot studies, and may be easily adapted to study other plants or contaminants. The experimental method involves three different planted treatments in a randomised block design with each block being a minimum of 6.1 m square in size. The three treatments are 1) a mixture of species optimised for local conditions which may include grasses and trees 2) an unplanted control and 3) a standard seed mixture, including some flexibility in plant species to accommodate local conditions, of 10-15% rye, 20-25% legume and 60-70% fescue. Additional blocks may be added with plants of particular interest to the investigator. Plots are planted and monitored over the course of 3 growing seasons for chemical disappearance and microbial populations. Detailed sampling methods, chemical, microbial and plant analyses are provided in the protocol, which is available at http://www.engg.ksu.edu/HSRC/appa.html.

3. Sampling methods

Strategies for sampling forensic or planted rhizoremediation studies are an integral component of experimental design, and ultimately will impact how accurately the data reflect true conditions. The first step in developing a sampling strategy is to define the compartments of interest within the complex root-soil interface (rhizosphere, rhizoplane, bulk soil, etc.) and select a sampling method that is both appropriate and feasible. Multiple samples must be collected from each replicate treatment in a representative manner, with consideration of temporal and spatial issues. Lastly, samples must be transported and stored for microbial or chemical analyses in ways that will minimize the introduction of artefacts.

3.1. DEFINING AND SAMPLING THE ROOT-SOIL INTERFACE

Before sampling the root-soil interface, one has first to define it. When the term "rhizosphere" was first coined by Lorenz Hiltner in 1904, it was defined simply as the zone of soil in which the microflora is influenced by plant roots. The zone of influence

has since been subdivided into several subgroups to distinguish microbial consortia based on their physical location relative to the root. Although the terminologies and definitions of each subgroup vary somewhat, in general the root-influenced microbial community is divided into those residing in the interior of roots (endophytes or endorhizosphere), on the root surface (rhizoplane), in the soil immediately surrounding the root (rhizosphere), and beyond the root into the root zone or bulk soil. Dead roots are rarely included in rhizosphere studies, however they may harbour distinct microbial populations including bacteria relevant to rhizoremediation [27, 28].

In practice, the rhizosphere is defined operationally. The traditional method for sampling the rhizosphere is to excavate roots carefully and then to shake off loose soil, keeping roots with the small amount of soil that remains adhered for analysis [29]. Although this rhizosphere sampling method may be imprecise since the amount of soil that adheres varies with soil moisture and composition, it remains the most practical, widely used and accepted technique. Following transport to the laboratory, the rhizosphere soil is separated from the root by washing with a diluent, and the resultant suspension is subjected to microbial analyses. The remaining root may then be further processed to recover rhizoplane and endorhizosphere organisms, as detailed later in the microbial analysis section.

The soil beneath a plant that is not defined operationally as the rhizosphere is often referred to as the bulk soil or the root zone. Plant species-specific effects on microbial communities are frequently observed in this soil fraction [9, 30-32], including effects on numbers of pollutant degraders in rhizoremediation studies [9, 30]. These findings indicate that the plant's influence extends beyond the immediate vicinity of roots, which is not surprising considering that fine roots are continually exploring new regions of soil and dying back, impacting the soil both with exudates from living roots and lysates from detritus.

In the field, sampling the root zone of trees and shrubs is achieved by excavation in the densely rooted region of soil, which generally occupies the area beneath the canopy. Root zone soil samples can be accessed by excavation or coring, although the former is often most practical, especially when root and rhizosphere samples are targeted as well. It is recommended that sampling tools (shovels, augers) be disinfected with alcohol between samples to minimize cross contamination.

For pot studies, soil and root sampling may easily be performed by removal of the soil and root complex from the pot followed by dissection. If plants are to be repeatedly sampled, coring may also be used to collect soil samples, although it is generally difficult to obtain sufficient root material for analysis from small cores.

3.2. SPATIAL SAMPLING STRATEGY

Soil is notoriously heterogeneous at both the macro and the micro scale, and plant roots introduce further variation. The main focus of this section is to provide guidelines on sampling schemes that can provide an accurate and statistically valid representation of the treatment in question. As discussed previously, the appropriate sample size can be determined by careful preliminary studies to determine the variability within the experimental system.

When sampling a field plot study with a randomised block design, multiple replicate samples should be collected within each block. The arrangement of the sampling locations within the block may be either random or systematic. There are a number of ways to design systematic sampling schemes, such as along a grid pattern or several transects [33, 34]. Composing of samples from several different locations is not recommended because it reduces sample size and precludes determinations of variability that are important to determining statistical differences among treatments.

In pot studies, multiple samples should be collected from each pot. If the whole plant root system is harvested, different zones of soil may be separated, such as into quadrants and by depth. Analysing multiple separate samples from each pot helps provide the statistical power needed to discern relatively small but significant effects with confidence, as well as to reveal the heterogeneity of the system.

In forensic studies, the distribution of plants will be random and hence rhizosphere sampling will be dictated primarily by the location of the plants. Multiple replicate samples should be collected from each plant, such as in a circumference around a tree. For purposes of site characterization and mapping of contaminant concentration and other factors, it is recommended that a comprehensive set of samples also be collected throughout the entire site on at least one time point using either a randomised or systematic pattern.

3.3. SEASONAL SAMPLING CONSIDERATIONS

Seasonal fluxes in environmental conditions are unavoidable, and not necessarily detrimental, in real-world applications of rhizoremediation technology. Since both plant root physiology (i.e. root turnover) [26] and soil microbial communities [35, 36] respond to seasonal changes, it is anticipated that degradative potential of the microbial community will also fluctuate. For this reason, research efforts should seek whenever possible to understand rhizoremediation process not just in the active summer growing season but throughout the seasonal cycle and under varying moisture conditions. Recognizing these influences on microbial populations important to rhizoremediation may help to formulate site management practices and provide new insights into the mechanisms of rhizoremediation.

At minimum, it is recommended that samples be collected from outdoor rhizoremediation studies at 3-4 times per year to reflect early spring, summer and late autumn conditions. Because major root turnover events typically occur in the autumn in rough synchrony with leaf senescence [26], samples should be collected after occurrence of complete leaf fall for deciduous plants or visible shoot dieback for annuals or biennials. While more frequent sampling events are desirable, care should be taken to ensure that analysing the large number of samples resulting from aggressive sampling is feasible.

At a PCB-contaminated site in the Czech Republic, populations of PCB-degrading bacteria were enumerated in June, August, November and the following May. Significant seasonal differences in population size were detected beneath the Austrian pine tree, in which numbers of degraders increased significantly between August and November [9], coinciding with expected root turnover events. Although the cause of the

increase remains unknown, it exemplifies the seasonal flux in degradative populations that can occur in a rhizoremediation setting.

3.4. SAMPLE STORAGE

Root and soil samples collected in the field can be conveniently stored in self-sealing thin-walled plastic bags, which permit slow gas exchange (O_2 and CO_2) while preventing soil drying [33]. If samples are destined for chemical analyses of contaminants, exposure to common flexible plastics should be avoided because they can introduce phthalate esters into the sample that interfere with analyses of many pollutants including pesticides and PCBs. Instead, inert sample vessels such as glass bottles should be used. For more detailed recommendations regarding sample storage for chemical analyses for particular pollutants, consult U.S. EPA Methods such as Method 8081.

Samples are commonly transported to the laboratory as quickly as possible on ice to reduce microbial activity, and then they are stored at 4°C until analysis. Cultivable or direct microbial analyses should be performed as soon as possible, since sample storage results in changes in microbial properties [33]. For molecular-based analyses of microbial communities, subsamples should be frozen at –20°C or –70°C for later extraction and analyses of DNA or RNA, respectively. Commercially available RNA protectants may be added before freezing to inhibit RNase activity when samples thaw. However, nucleic acid extraction recovery in the presence of the protectant should be tested in advance since these protectants can interfere with some protocols.

If microbial, molecular or pollutant analyses are to be reported per unit of dry weight of soil or root material, then aliquots of each samples should be weighed and dried to obtain ratios of fresh weight to dry weight.

4. Microbial analyses

Once samples have been collected, one turns to the task of investigating the microbial populations contained therein. What is the best approach to identify, quantify or characterize pollutant degraders and to compare them among samples? There are a host of different microbiological methods for these purposes, each with its own set of advantages and limitations. Cultivation-based methods present a notoriously incomplete picture of the microbial community due to the “great plate count anomaly”, in which only 0.1 - 10% of bacteria in the environment are cultivable in the laboratory. However, cultivation methods are widely used for their simplicity and cost-effectiveness, and because they remain the primary way to demonstrate metabolic capabilities of individual strains. Molecular tools including stable isotope probing, quantitative real-time PCR, high-throughput sequencing and microarrays provide unprecedented access to data not subject to issues of culture bias, yet are somewhat constrained by our existing knowledge of target genes and sequence diversity. Because of the unique insights provided by both culture-based and direct molecular approaches, their combined application can provide a complementary view of the microbial community and its function. This section aspires to acquaint investigators embarking on rhizoremediation

studies with an array of both common and innovative approaches to studying the diversity, abundance and activity of microorganisms important to biodegradation.

4.1. CULTIVATION-BASED METHODS

4.1.1 Microbial recovery from the soil-root interface

In preparation for cultivation, it is often necessary to extract microorganisms from the soil, rhizosphere, rhizoplane or endorhizosphere. Typically a bacterial suspension is created that may then be serial diluted or used directly as an inoculum using the following methods.

Soil bacteria are suspended by shaking or vortexing soil in liquid along with sterile glass beads [37], sometimes followed by standing for 30 min or short low-speed centrifugation (500-1000 × g) [33] to separate soil particles from suspended bacteria. Sodium pyrophosphate solution is an effective soil aggregate dispersal agent [38] and so is commonly used as a suspension medium [33, 37]. Alternatively, other buffers, saline solution or simply sterile water may be used. Media containing carbon sources should be avoided since growth may occur that would skew populations during processing. A side-by-side study evaluating cell recovery from soil using water, sodium pyrophosphate and several buffers showed no significant difference (Terence Marsh, 2003, personal communication).

Rhizosphere soil bacteria can be suspended as described for soil, although glass beads may be omitted. Some rhizoplane organisms will invariably be recovered along with the rhizosphere, however gentle washing is thought to leave the rhizoplane largely intact since it reportedly removes only 10% of the bacteria removed by vigorous shaking with glass beads [39]. After shaking a defined quantity (by fresh weight or length) of root with adhering rhizosphere soil in liquid to suspend cells, the roots may be recovered, dried and weighed so that rhizosphere bacterial numbers can be based on root dry weight.

Following removal of the rhizosphere soil, the rhizoplane and endorhizosphere microflora may be recovered separately or together for analyses. Rhizoplane organisms remaining on the surface of the root after rhizosphere washing may be extracted by vigorous treatments such as shaking with glass beads, vortexing, or using a Stomacher blender [40, 41]. For recovery of endorhizosphere organisms, roots may be ground with a small amount of water or buffer in a Warring blender [42] or mortar and pestle [43] to create a suspension. To ensure that truly endophytic organisms are recovered in the absence of rhizoplane contaminants, the roots may be surface disinfected prior to grinding [43].

4.1.2 Liquid enrichment cultures

Because pollutant-degrading bacteria typically comprise a small proportion of the total microbial community, enrichment methods are useful to generate a mixed culture in which degradative bacteria are predominant. Many of the earliest isolates of bacteria capable of degrading pesticides and pollutants were isolated using enrichment methods [44, 45]. Constructing enrichment cultures is a simple matter of inoculating a flask, tube or bottle containing sterile minimal salts medium with an environmental sample, taking

care to minimize introduction of foreign substrates, providing the compound of interest as the sole carbon source, and then incubating with shaking for a period of days to weeks until turbid. Often the enrichment is subjected to several passages to achieve a stable consortium.

Although enrichment cultures are valuable for producing isolates for study, they are not quantitative reflections of the diversity or relative abundance of contaminant-degrading populations within a sample. Studies of environmental samples have demonstrated dramatically reduced diversity of 2,4-D and PAH degrading organisms as well as degradative genes when cultivated using enrichment cultures in comparison to direct agar plates or biofilm culture methods [46, 47]. The difference is explained by competitive interactions in the enrichment culture in which slower-growing organisms are outcompeted by others with higher maximum specific growth rates. Thus, enrichment methods are not recommended for making comparisons of diversity or relative abundance among different samples.

4.1.3 Direct agar plate methods

Direct agar plating methods are a useful means to both enumerate and investigate the diversity of the cultivable fraction of bacteria capable of utilizing pollutants. As opposed to enrichment cultures, direct plates afford the opportunity for organisms of the same functional group but with different growth rates to form colonies and be detected [48]. Direct plates are also relatively simple to perform, require little specialized equipment and can easily be appended to existing protocols for plate counts of total cultivable bacteria. For these reasons, direct plate methods are the most widely used technique for studying abundance and diversity of microbial populations important to pollutant degradation.

Direct plating of soil or rhizosphere samples is performed by suspending bacterial cells from a defined quantity of soil or roots, spread-plating a dilution series onto a minimal medium with the contaminant (or an analogue) provided as a sole carbon source, incubating and colony counting. A secondary screening procedure, such as a clearing-zone test, may be employed to help verify that colonies are truly utilizing the substrate rather than impurities in the medium or carryover from the soil suspension. Details of these procedures are discussed below. For accurate plate counts, 2-3 replicate plates should be inoculated from each dilution generated from a sample and counts averaged. Likewise, multiple subsamples from each soil/root sample should be plated for statistically valid enumeration.

Growth conditions In order to selectively cultivate organisms that utilize a sole carbon source, care should be taken in the design and preparation of agar media to avoid introduction of unwanted growth substrates. A variety of different defined minimal media recipes may be used, taking care to avoid inclusion of any potential energy sources. Addition of yeast extract or vitamins should be avoided unless absolutely necessary for growth since they also function as carbon sources, even in low concentrations. Although expensive, highly purified Noble Agar is preferable over common agars, since most agars contain impurities that act as non-specific carbon sources. However, secondary screening methods discussed later can be used to

distinguish true contaminant utilisers from non-degradative colonies growing on agar plates.

The contaminant or an analogue can be provided as a growth substrate in a number of ways depending on the nature of the compound. Water-soluble compounds may be added directly to the agar medium, preferably when relatively cool following autoclaving to prevent thermal decomposition. Practically insoluble but highly volatile compounds like biphenyl or naphthalene are easily provided in the vapour phase. Biphenyl is commonly provided by sprinkling a few crystals in the lid of the Petri plate [49]. For chemical hygiene, plates are then sealed with Parafilm or enclosing in a plastic sleeve or chamber to minimize volatilization into the ambient air. Naphthalene can be provided to plates all together in a sealed container [37]. Less volatile compounds may be added to the surface of agar plates by dissolving in water, ethanol or acetone and spreading, followed by removal of organic solvents by evaporation from open plates in a laminar flow hood [37]. Alternatively, compounds may be suspended in a small amount of agar or agarose and poured over the basal mineral agar layer [50].

Plates are commonly incubated or below 25°C, which presumably simulates environmental conditions better than the higher temperatures commonly used in microbiological research. The length of incubation required for colony formation of pollutant degraders varies depending on the nature of the population and the compound. It is recommended that when embarking on a new study that a pilot test be performed in which colony forming units are counted repeatedly over a period of up to 1 month in order to identify appropriate incubation times. Brief incubation periods can result in erroneously low counts and bias data toward the faster growing fraction of the population. A biphenyl-utilizing bacterial population comprised predominantly of relatively slow-growing Gram-positive rhodococci required 3 weeks for new colony formation to cease [9].

Colony screening methods Screening colonies pre-grown on agar plates for degradative abilities is often desirable since non-degradative colonies can grow on substrates other than the target compound such as impurities in agar, carryover from the soil suspension, or when cofactors in media are required. Several clearing-zone and colorimetric techniques for secondary screening are described below that that can help confirm that colonies are truly degrading the target substrate. Some of these methods may also be applied to colonies grown intentionally on a rich medium to detect degraders among the total cultivable community.

Following colony formation, clearing zone assays can be performed by depositing a thin cloudy layer of the target compound over the agar plate surface and then incubating until zones of clearing form. A 5 - 10% w/v solution of the target compound prepared in ether or acetone solution may be sprayed onto the agar surface using thin layer chromatography plate spraying apparatus in a fume hood. Alternatively, compounds may be overlayed by sublimation [51]. Spray methods have been used for a variety of aromatic compounds including biphenyl and chlorobiphenyl [52, 53], as well as phenanthrene, fluoranthene, pyrene, naphthalene and anthracene [53, 54]. Agar plates are then sealed in plastic sleeves to minimize volatilization of the compound and

incubated for days to several weeks and checked periodically for the formation of zones of clearing around colonies.

Clearing zone assays may also be performed simultaneously with initial colony formation for compounds such as phenanthrene. The inoculum is mixed in an agarose suspension containing phenanthrene crystals and then spread onto a minimal medium agar plate. As phenanthrene-utilizing colonies form they create clearing zones [50]. This approach is advantageous over spraying methods by reducing the contamination of laboratory fume hoods with chemicals and preventing exposure of organisms to organic solvents, and also provides an effective means for efficient delivery of non-volatile substrates to organisms.

Colonies with aromatic ring dioxygenase activity can be screened using a simple colour indication assay that capitalizes on the ability of naphthalene enzyme to produce indigo from indole [54, 55]. Crystals of indole placed in the lid of the Petri dish will result in rapid (1 day or less) appearance of the blue indigo pigment in colonies in which aromatic dioxygenases are active. However, the assay only detects activity of dioxygenases enzymes that form cis-dihydrodiols from aromatic hydrocarbons [55].

4.1.4 Most probable number (MPN) method

Another useful approach for enumeration of pollutant degraders is the most probable number (MPN) method, which has been adapted for efficient use in 96-well microtiter plates to quantify bacteria that degrade polyaromatic hydrocarbon and alkanes [56] and crude oil [57]. In essence, a suspension of bacterial cells is diluted to extinction in a microtiter plate containing specific substrates as carbon sources. For PAH-degrading organisms, growth is detected in the wells by the presence of a yellow to green-brown colour generated by PAH oxidation products. For substrates without pigmented products such as alkanes, detection using the colour change of an iodonitrotetrazolium violet dye can be used. Empirical testing demonstrated that microtiter MPN produced the same result as enumeration by direct plate count for PAH-degraders [56]. The MPN method may be easily adapted for use with other compounds. When degrader population sizes are low and growth is only detected in wells with very low dilution factors, care should be taken to ensure that the signal is due to utilization of the substrate and not compounds carried over from the soil solution. To minimize carryover, cell suspensions may be washed by repeated centrifugation and resuspension to remove unwanted substrates prior to inoculation.

4.2. MOLECULAR ECOLOGICAL METHODS

Molecular microbial ecology methods bypass culture-bias to provide direct measures of a variety of microbial community parameters. This section highlights some methods that are of particular value to bioremediation studies because of their ability to directly detect the presence, activity, diversity and abundance of degradative organisms.

4.2.1 DNA and RNA extraction methods

Many molecular ecological analyses require bacterial DNA and/or RNA to be extracted from soil. Depending on both the soil itself and the protocol applied, results vary widely

in terms of the quantity, size and purity of nucleic acids yielded. Numerous extraction methods exist in the published literature and in the form of commercial kits. Since the effectiveness of extraction protocols varies among soil types, it is recommended that several methods be tested, compared or sometimes adapted to identify a successful approach for a particular soil.

For the simultaneous extraction of DNA and RNA, a crude nucleic acid extract is first obtained using methods such as the rapid bead-beating method developed by Griffiths *et al.* [58] or a freeze grinding approach by Hurt *et al.* [59]. Bead beating tends to shear nucleic acids, so if high molecular weight extracts are needed (i.e. for metagenomics) then freeze grinding may be preferable. Precipitation of nucleic acids with polyethylene glycol (PEG) [58] is particularly effective in removal of humic materials that interfere with PCR, and may be used with other protocols when needed. RNA and DNA can then be separated from each other by gravity column systems such as the Qiagen DNA/RNA extraction kit (Qiagen Inc., Carlsbad, CA), followed by RNase or DNase treatment to remove residual amounts of undesirable nucleic acids. Alternatively, the crude extract may be split and then each fraction directly subjected to RNase or DNase treatment.

To extract DNA only, simple and effective commercial bead beating kits are commonly used such as those available from QBiogene, Inc. (Carlsbad, CA) or MoBio (Carlsbad, CA) or using a freeze-grinding method [60]. RNA extraction techniques are similar to DNA methods, however since RNA is very labile, extracting it from soil is much more challenging and requires conditions that inhibit RNase activity. Soil RNA extraction protocols are available in the literature [61], and commercial kits have recently arrived on the market (MoBio, Carlsbad, CA and QBiogene Inc., Carlsbad, CA).

To simultaneously extract endophytes, rhizoplane and rhizosphere soil, methods above may be adapted for use with whole roots and adhering soil. For extraction of endophytic root organisms only, other methods in the literature can be consulted [62-64].

4.2.2 Functional gene detection methods

Direct detection of genes that function in degradative processes may be performed on bacterial community DNA extracts either by PCR amplification or using microarrays. The primary limitations of these approaches lie in effective primer or probe design and detection limits. Primers and probes can be designed using bioinformatic methods to either broadly target groups of similar sequences or to be highly sequence specific. Inclusive primers or probes are designed by aligning multiple sequences and identifying conserved regions. Thus, they are limited by current knowledge of sequence diversity in the environment, which may prevent detection of unknown sequences. Detection limits for both PCR and microarray methods can be an obstacle since pollutant degraders often comprise a small proportion of the total community. Nonetheless, direct functional gene detection is a very powerful tool to investigate the presence, diversity and in some cases quantity of certain degradative genes in the microbial community.

PCR and quantitative real-time PCR PCR methods for detecting genes important to aromatic pollutant degradation have frequently targeted initial aromatic dioxygenase genes, which encode enzymes catalyzing the first, rate-limiting hydroxylation step of degradation. The aromatic dioxygenase gene family spans a wide substrate range, including PCBs, mono- and polyaromatic hydrocarbons, and are multimeric enzymes including a reductase subunit, a ferredoxin subunit, a large (alpha) and a small (beta) iron sulphur protein. Sequence variations in the large subunit are associated with substrate specificity [65]. For this reason, as well as the presence of a conserved region encoding the Rieske centre, the large subunit is an attractive target for PCR primer design. PCR primer sets have been successfully developed that can amplify groups of initial dioxygenase genes specific to certain substrates [66], while other primer sets are more organism-specific [66, 67]. Baldwin *et al*., [66] developed multiple primer systems to differentially detect and enumerate subfamilies of aromatic oxygenase genes for biphenyl, naphthalene, toluene dioxygenases as well as monooxygenases involved in toluene/xylene and phenol degradation. Primers have been effectively designed targeting dioxygenases catalysing later steps in aromatic degradation as well [67, 68] For example, Erb and Wagner-Dobler successfully amplified *bphC* encoding the di-oxygenase enzyme catalysing the meta-cleavage step in biphenyl degradation from the environment and achieved a detection limit of five copies per reaction mixture or 100 cells per g wet weight of sediment.

The genetic diversity of catabolic genes can be investigated when PCR products are subjected to sequence analysis. Following PCR amplification of degradative genes, clone libraries may be constructed for sequencing, or alternatively amplicons may be separated by methods such as denaturing gradient gel electrophoresis (DGGE) and excised bands sequenced. The relationship of sequences to previously known genes can be established by searching for similar sequences in public databases such as BLASTn searches of GenBank [69] and conducting phylogenetic analyses [70].

The degradative potential of a microbial community can be quantified using real-time PCR (RTm-PCR) with primers targeting functional genes. In RTm-PCR, a PCR reaction is performed in the presence of either fluorescent probes that hybridize to the specific target sequence (i.e. TaqMan ® probes, Applied Biosciences, Foster City, CA) or alternatively a non-specific fluorescent dye that binds to double stranded DNA (i.e. SYBR green). RTm-PCR thermocyclers are specially designed to measure fluorescence in the PCR reaction at each amplification cycle, permitting quantitation relative to a standard curve containing known copy numbers of the target sequence. When amplifying variable regions of functional genes, use of SYBR green is preferable over highly specific probes, however extra care must be taken to ensure that non-specific products are not being produced and measured. Running a melting curve analysis at the end of the reaction is an important quality control step for this purpose. For additional information regarding RTm-PCR methods, the reader is referred to books dedicated to the subject [71, 72]. RTm-PCR methods to enumerate aromatic dioxygenases have been developed, which used SYBR green and produced detection limits down to 2×10^2 copies per reaction using pure culture DNA [66].

Functional gene microarrays Using microarray techniques, it is now possible to probe community DNA samples for thousands of target sequences simultaneously, opening an enormous window into the functional gene diversity of microbial communities. Microarrays have already been designed that successfully detect a broad range of genes involved in the degradation of pollutants, including monoaromatic and polyaromatic hydrocarbons, PCBs and aliphatics [73-75]. Careful probe design, including the design of several probes per target when possible, followed by comprehensive testing is critical to help ensure the specificity of hybridisation and to prevent false positives or negatives. Unfortunately, as in the design of PCR primers, probe design is limited by current knowledge of the diversity of functional gene sequences so some important genes may not be detected.

Although not yet applied for quantitative purposes, strong linear relationships have been reported between signal intensity of hybridised probes and the number of gene copies in a sample with some functional gene arrays [73-75]. Thus, arrays may eventually be used to evaluate both the diversity and abundance of target genes in a sample.

A major challenge in applying arrays to bioremediation studies is their detection limits. As reported by Denef *et al.* [73], single-copy genes can only be detected from organisms that comprise 1% or more of the community with current technology. This is an issue for many environmental samples where the degradative population often comprises a small proportion of the total community. The detection limits of arrays could likely be overcome, however, if combined with stable isotope probing methods detailed in the following section.

4.2.3 Stable isotope probing

Stable isotope probing (SIP) methods now allow researchers to directly answer the previously evasive question: Which microorganisms are truly active in pollutant degradation? In addition to linking phylogeny with function, SIP also provides the opportunity to thoroughly investigate the genomic content of active degradative organisms. Although it does not provide quantitative measures of degrader abundance, SIP can provide insights into the identity and genetic diversity of organisms that are truly active in rhizoremediation.

SIP methodologies SIP involves provision of a substrate that is labelled with a heavy stable isotope, typically ^{13}C, to a microbial community. Organisms that actively incorporate the substrate are detected by virtue of the presence of ^{13}C in biomarkers such as phospholipid fatty acids (PLFAs), DNA or RNA. SIP based on nucleic acids provides higher resolution phylogenetic data than PLFAs [76], as well as access to functional genes of active species [77]. For DNA or RNA-SIP, nucleic acid extracts are subjected to density gradient centrifugation to separate heavier, ^{13}C-labelled nucleic acids from those of the inactive community. Heavy fractions may then be subject to PCR amplification, community profiling and/or sequence analyses. RNA-SIP is more sensitive than DNA-SIP, and is also advantageous in that growth (and potential community change) during the incubation is not necessary [78-80]. However, if functional genes are to be targeted, DNA-SIP is more attractive because of the difficulty

of extracting mRNA from environmental samples. Recently, SIP methods have been developed to capitalize on the stable isotope, ^{15}N [81], which opens a window into the microorganisms that biodegrade N-containing contaminants.

To perform SIP of soil samples, microcosms are typically constructed in the laboratory, or using an *in situ* method that has also been developed [82] which may prove valuable in studying intact rhizospheres. Closed systems are desirable because they permit the sampling and monitoring of $^{13}CO_2$ evolution in the headspace as an indicator of substrate utilization. Substrates may be provided to soil in aqueous solution or, if volatile, as crystals enclosed in the microcosm. For SIP with ^{13}C-biphenyl, crystals on the interior wall of a sealed serum bottle were effective for enriching bacteria in soil spread loosely on the bottom [83, 84].

Care should be taken to avoid lengthy incubation times to minimize flow of ^{13}C to non-degraders via degradative intermediates or the biomass of primary degraders, and to avoid community change during the incubation. Thus, sampling over a time-course is recommended to ensure that primary utilisers are identified. In a timecourse SIP experiment with ^{13}C-biphenyl, significant $^{13}CO_2$ evolution into the headspace was detected within 24 hours and continued to be produced over the course of the 14-day incubation, indicating that biphenyl was being metabolised [83, 84].

Separation of ^{13}C-labeled DNA from unlabeled DNA can be performed by density gradient centrifugation in caesium chloride (CsCl) [79, 85] or in caesium trifluoroacetate (CsTFA) solutions [83, 84], while RNA is separated in CsTFA with the addition of formamide to reduce secondary structure [86]. Following ultracentrifugation, it is strongly recommended that density gradients be fractionated rather than extracting bands using a syringe needle since fractionation permits the detection of partially labelled nucleic acids. Fractionating samples along with appropriate controls is important to ensuring that organisms detected are truly labelled, since low levels of unlabeled nucleic acids are present throughout the density gradient. Control gradients should be constructed with unlabeled nucleic acids from the same community, such as a sample collected at the initiation of the experiment ($t = 0$). Control fractions should be subjected to the same fractionation and downstream molecular analyses. Then, any sequences detected in the background heavy control fractions can be subtracted from those identified in the ^{13}C-treated sample.

Following fractionation and nucleic acid recovery, PCR amplification of 16S rRNA genes (or RT-PCR of 16S rRNA) and community analyses are helpful to locate the ^{13}C-labeled fractions and to track changes in the labelled populations with increased incubation time. Denaturing gradient gel electrophoresis (DGGE) [78, 86] or terminal restriction fragment length polymorphism (T-RFLP) [79, 80] are common community profiling methods used in SIP.

Following the identification of the fractions containing ^{13}C-labeled nucleic acids, sequence analysis may be performed to phylogenetically identify organisms that derived carbon from the substrate. Clone libraries may be constructed from PCR amplicons, or if DGGE is used, bands of interest may be excised, reamplified and sequenced. Sequence analyses of ^{13}C-labeled DNA and RNA have focused primarily on the SSU rRNA for the purposes of obtaining phylogenetic information. However, a wealth of functional gene information is available in the labelled fraction that can prove informative in

biodegradation studies. Functional gene analyses of SIP-derived ^{13}C-DNA have been reported for methylotrophs [77], but not yet for environmental pollutants.

SIP in bioremediation and the rhizosphere To date, SIP studies targeting aromatic pollutant-degrading bacteria have been achieved in a variety of settings, including phenol in activate sludge [78, 87] naphthalene and phenanthrene in a bioreactor treating soil [88], 2,2'-dichlorobiphenyl in a biofilm growing on PCB droplets [89], naphthalene in sediment [90], toluene in soil [91, 92], phenol, caffeine and naphthalene in soil [82]. Although as yet unexplored, SIP also provides an excellent means for identifying fungi that are significant in bioremediation by simply amplifying 18S rRNA genes in heavy nucleic acid fractions using universal fungal rRNA primers [93].

In the field of rhizosphere ecology, SIP has been applied in efforts to identify microbial communities that derive carbon from root exudates following provision of $^{13}CO_2$ to aboveground plant parts [94, 95]. A major challenge of this approach is the dilution of ^{13}C with the predominantly unlabeled carbon in the plant which can result in isotopic enrichment of the microbial biomarkers below levels needed for SIP [94]. If combined with functional gene detection, SIP of root exudates may prove valuable to demonstrate that pollutant-degrading bacteria are deriving carbon from the plant in the rhizosphere. Unfortunately this approach simultaneously probes for microbes using any of the plethoras of compounds exuded by plant roots, and thus does not permit differentiation of specific plant compound-utilizing bacteria. In order to determine which components of root exudates or lysates specifically feed pollutant degraders, SIP may be performed in a rhizosphere setting, or in root zone samples, with ^{13}C-labelled plant compounds provided individually.

5. Conclusions and research needs

Through the array of windows opened by powerful molecular ecological tools in combination with traditional microbial methods and carefully constructed experiments, new insights can be gained into bioremediation processes in the rhizosphere. What are the mechanisms for rhizostimulation of contaminant degraders? Which bacteria, fungi and degradative genes are most important? Which plant species, communities, or biosystems are most effective at rhizoremediation of various contaminants? Methods now available invite us to answer these questions and assemble the fundamental understanding of rhizoremediation ecology necessary for its successful implementation.

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