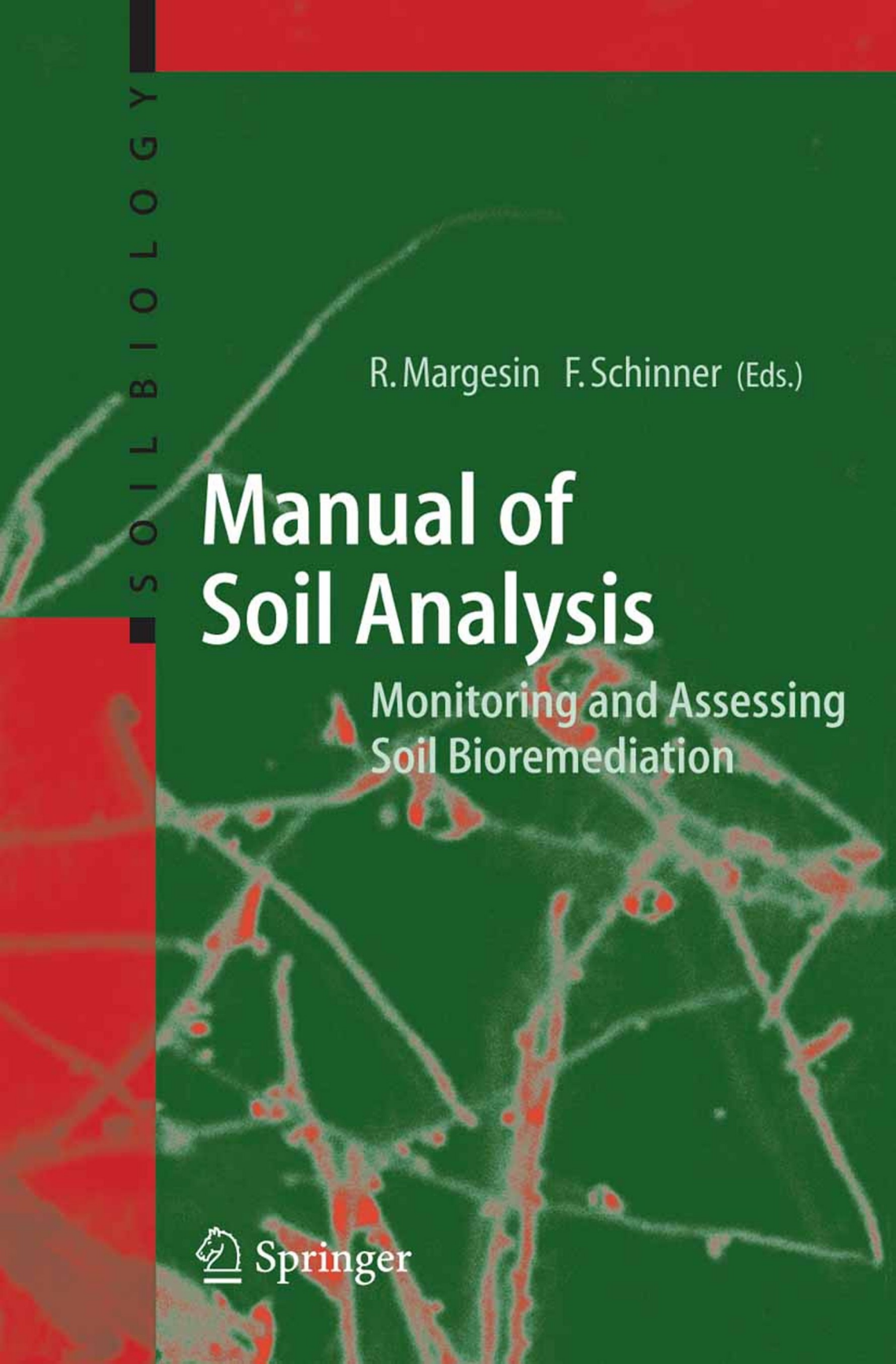
SOIL BIOLOGY

R. Margesin F. Schinner (Eds.)

Manual of Soil Analysis

Monitoring and Assessing Soil Bioremediation



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Manual for Soil Analysis – Monitoring and Assessing Soil Bioremediation

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Preface

The increasing use of soil bioremediation technologies requires new concepts and methods to assess the feasibility of a remediation technology and to monitor the success of the treatment. The knowledge of the reaction of the soil microflora to contamination facilitates the optimization of biodegradation. *Manual of Soil Analysis – Monitoring and Assessing Soil Bioremediation* differs from other books on soil analysis in that the monitoring and assessing of soil bioremediation are the central themes.

In this comprehensive laboratory manual, sampling, pretreatment and storage of soil, feasibility studies for soil bioremediation, and the most important methods to analyze physical, chemical, and biological soil parameters are presented. Chapters written by experts for those involved in research, teaching, and routine analyses outline molecular and immunological techniques, the use of conserved internal markers, radiorespirometry, bioreporter technology, the interpretation of fatty acid profiles, soil microbial and enzymatic methods, and the assessment of ecotoxicity using bioassays. Particular emphasis has been placed on the comprehensible and complete description of the experimental procedures. The broad spectrum of modern soil biological methods provides an excellent complementation of traditional soil investigation and characterization. Our book, however, does not claim to present all modern methods available, it rather contains a selection of the most suitable methods for investigating contaminated soil. More biological methods can be found in our volume *Methods in Soil Biology* (Schinner, Öhlinger, Kandeler and Margesin 1996, Springer).

We are most grateful to the authors for their excellent contributions and to Springer, especially to Dr. Jutta Lindenborn and Dr. Dieter Czeschlik, for continuous support and cooperation. We also thank Dr. Ajit Varma for the possibility to publish this book in the Soil Biology Series.

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1 Soil Sampling and Storage

Andreas Paetz, Berndt-Michael Wilke

1.1 Objective of Soil Sampling

1.1.1 Principal Objectives

General

Samples are collected and examined primarily to determine their physical, chemical, biological, and radiological properties. This section outlines the more important factors which should be considered when devising a sampling program for soil and related material. More detailed information is given in subsequent sections.

Whenever a volume of soil is to be characterized, it is generally not possible to examine the whole and it is therefore necessary to take samples. The samples collected should be as fully representative as possible, and all precautions should be taken to ensure that, as far as possible, the samples do not undergo any changes in the interval between sampling and examination. The sampling of multiphase systems, such as soils containing water or other liquids, gases, biological material, radionuclides, or other solids not naturally belonging to soil (e.g., waste materials), can present special problems. In addition, the determination of some physical soil parameters may require so-called undisturbed soil samples for correct execution of the relevant measurement.

Before any sampling program is devised, it is important that the objectives be first established since they are the major determining factors, e.g., the position and density of sampling points, time of sampling, sampling procedures, subsequent treatment of samples and analytical requirements. The details of a sampling program depend on whether the information needed is the average value, the distribution, or the variability of given soil parameters.

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Some consideration should be given to the degree of detail and precision that will be required, and also to the manner in which the results are to be expressed and presented, for example, concentrations of chemical substances, maximum and minimum values, arithmetic means, median values, etc. Additionally, a list of parameters of interest should be compiled and the relevant analytical procedures consulted; these will usually give guidance on precautions to be observed during sampling and subsequent handling of soil samples.

It may often be necessary to carry out an exploratory sampling-and-analysis program before the final objectives can be defined. It is important to take into account all relevant data from previous programs at the same or similar locations and other information on local conditions. Previous personal experience can also be very valuable. Time and money allocated to the design of a proper sampling program are usually well justified because they ensure that the required information is obtained efficiently and economically.

It is emphasized that complete achievement of objectives of soil investigations depends mainly on the design and execution of an appropriate sampling program. The four principal objectives of soil sampling may be distinguished as follows and are discussed below:

- Sampling for determination of general soil quality
- Sampling for characterization purposes in preparation of soil maps
- Sampling to support legal or regulatory action
- Sampling as part of a hazard or risk assessmenthack

The utilization of the soil and site is of varying importance depending on the primary objective of an investigation. For example, while consideration of past, present, and future site use is particularly relevant to sampling for risk assessment, it is less important for soil mapping where the focus is on description rather than the evaluation of a soil. Objectives such as soil quality assessment, land appraisal, and soil monitoring take utilization into account to varying degrees.

The results obtained from sampling campaigns to assess soil quality for mapping may indicate a need for further investigation. For example, if contamination is detected, a need arises for identification and assessment of potential hazards and risks.

Sampling for Determination of General Soil Quality

This is typically carried out at irregular time intervals to determine the quality of the soil for a particular purpose, e.g., agriculture. As such, it will tend to concentrate on factors such as nutrient status, pH, organic matter content, trace element concentrations, and physical factors, which provide

a measure of current quality and which are amenable to manipulation. Sampling is usually carried out within the main rooting zone and also at greater depths but sometimes without exact distinction of horizons or layers. The guidance given in ISO 10381-4 (2003) will be particularly relevant.

Sampling for Preparation of Soil Maps

Soil maps may be used in soil description, land appraisal (taxation), and for soil monitoring sites to establish the basic information on the genesis and distribution of naturally occurring or man-made soils, their chemical, mineralogical, biological composition, and their physical properties at selected positions. The preparation of soil maps involves installation of trial pits or core sampling with detailed consideration of soil layers and horizons. Special strategies are required to preserve samples in their original physical and chemical condition. Sampling is nearly always a once-off procedure. The guidance given in ISO 10381-4 (2003) is particularly relevant.

Sampling to Support Legal or Regulatory Action

Sampling may be required to establish base-line conditions prior to an activity that might affect the composition or quality of soil, or it may be required following an anthropogenic effect such as the input of an undesirable material that may be from a point or a diffuse source. Sampling strategies need to be developed on a site-specific basis. To adequately support legal or regulatory action particular attention should be paid to all aspects of quality assurance including, for example, "chain-of-custody procedures." The guidance given in ISO 10381-5 (1995) is particularly relevant; that in ISO 10381-4 (2003) may also be relevant.

Sampling for Hazard and Risk Assessment

When land is contaminated with chemicals and other substances potentially harmful to human health and safety or to the environment, it may be necessary to carry out an investigation as a part of a hazard and/or risk assessment i.e., to determine the nature and extent of contamination, to identify hazards associated with the contamination, to identify potential targets and routes of exposure, and to evaluate the risks relating to current and future use of the site and neighboring land. A sampling program for risk assessment (in this context: phase I, phase II, phase III, and phase IV investigations) may have to comply with legal or regulatory requirements, and careful attention to sample integrity is recommended. Sampling strategies should be developed on a site-specific basis. The guidance given in ISO 10381-5 (1995) is particularly relevant, and that in ISO 10381-4 (2003) may also be relevant.

1.1.2 Specific Objectives

General

Depending on the principal objective(s) it will usually be necessary to determine for the body of soil or part thereof:

- The nature, concentrations, and distribution of naturally occurring substances
- The nature, concentrations, and distribution of contaminants (extraneous substances)
- The physical properties and variations
- The presence and distribution of biological species of interest

It will often be necessary to take into account changes in the above parameters with time, caused by migration, atmospheric conditions, and land/soil use. Some detailed objectives are suggested in the clauses below. The list is not exhaustive.

Sampling for the Determination of Chemical Soil Parameters

There are many reasons for chemical investigation of soil and related material and only a few are mentioned here. It is important that each sampling routine is tailored to fit the soil and the situation. Chemical investigations are carried out

1. To identify immediate hazards to human health and safety and to the environment
2. To determine the suitability of a soil for an intended use, e.g., agricultural production, residential development
3. To study the effects of atmospheric pollutants including radioactive fallout on the quality of soil (which may also provide information on water quality and indicate if problems are likely to arise in near-surface aquifers)
4. To assess the effects of direct inputs to soil; there may be contributions from:
   - naturally occurring substances that exceed local background values, e.g., certain mineral phases in metal deposits
   - (un)expected contamination by application of agrochemicals
   - (un)expected contamination due to industrial processes

5. To assess the effect of the accumulation and release of substances by soils on other soil horizons or on other environmental compartments, e.g., the transfer of substances from a soil into a plant
6. To study the effect of waste disposal, including the disposal of sewage sludge on a soil (which, apart from contributing to the pollution load, may produce other chemical reactions such as the formation of persistent compounds, metabolites, or the evolution of gases, such as methane)
7. To identify and quantify products released by industrial processes and by accident (usually done by investigation of suspect sites or contaminated sites)
8. To evaluate soil derived from construction works with view to possible or further utilization of such soils or disposal as waste

Commonly, sampling strategies are employed that require samples to be taken either from identifiable soil horizons or from specified depths (below ground surface). It is best to avoid mixing the two approaches, particularly when sampling natural strata, as this can make it difficult to compare results. However, a coherent combination of the two approaches can sometimes be useful on old industrial sites where there is variation in both the nature of fill and the depth of penetration of mobile contaminants into the ground. i.e., where there are two independent reasons for changes in soil/fill properties.

Knowledge of the way in which particular chemical substances tend to be distributed between the different compartments (air, soil, water, sediment, and living organisms) is advantageous for the design of some sampling programs. Similarly, knowledge of the behavior of living organisms affected by chemical substances, or that influence the availability of substances due to microbiological procedures, is also advantageous.

Sampling for the Determination of Physical Soil Parameters

The sampling of soil for the determination of some physical properties requires special consideration since the accuracy and extrapolation of measured data rely on obtaining a sample that retains its in situ structural characteristics. In many circumstances, it may be preferable to conduct measurements in the field since the removal of even an undisturbed sample can change the continuity and characteristics of soil physical properties and lead to erroneous results. However, certain measurements are not possible in the field. Others require specific field conditions, but the field situation can only be controlled to a very limited extent; e.g., it may be possible to modify the hydrological situation temporarily for measurement purposes by irrigation. The time and expense necessary for field measurements may

not be affordable. Laboratory measurements of physical properties are therefore frequently necessary.

Differences and changes in soil structure affect the choice of size of sample. Hence, a representative volume or minimum number of replicates must be determined for each soil type to be studied. The moisture status of the soil at sampling can influence physical measurements, e.g., hysteresis on rewetting can occur. Many physical properties have vertical and lateral components, and this should be considered prior to sampling. Where small undisturbed soil samples are required, manual excavation of cores, clods, or soil aggregates can be applied. Sampling equipment should be designed such that minimal physical disturbance to the soil occurs. For larger samples, the use of hydraulic sampling equipment and cutting devices is preferable in order to obtain a sample with minimal disturbance. Care should be taken in both equipment design and manufacture to ensure that internal compression or compaction of the sample does not occur. Where it is difficult to obtain an undisturbed sample for laboratory measurements, e.g., in stony or iron pan soils, then in situ measurements may be the most appropriate method.

Sampling for the Assessment of Biological Soil Parameters

Biological soil investigations address a number of different questions related to what is happening to or caused by life forms in and on the soil, including both fauna and flora in the micro and macro range. Ecotoxicological questions are usually given first priority. For example, tests should be made to verify the effects of chemicals added to the soil on life-forms and also the possible effects of life-forms in the soil on plants (e.g., high-value crops) and on the environment, especially on human health. In some cases, biological soil test procedures operate with fully artificial soils, but normally the major task of sampling is to choose a reliable soil or site to carry out the tests. Sampling for the assessment of aerobic microbial processes is covered in ISO 10381-6 (1993). The sampling for the assessment of anaerobic processes is described in ISO 15473 (2002).

1.2 Selection of Sampling Technique

The selection of appropriate sampling equipment depends on the objective of sampling and should be done after consideration by the analyst or scientist responsible for subsequent determination. ISO 10381-2 (2002) gives guidance on commonly used equipment for sampling soil and related material. Parts 4, 5, and 6 of ISO 10381 describe needs for specific purposes within their scopes.

1.3 Sampling Strategy

1.3.1 General

The strategy for the site investigation (whether preliminary, exploratory, or main) will be determined by the objectives. For example, the different requirements of site investigations for the purpose of selling, determining whether contamination is present as suspected, or redevelopment will influence the spacing of sample locations and the number of samples analyzed, and hence the cost of the investigation.

Before embarking on any phase or stage of investigation it is important to set data quality objectives in terms of the type, quantity, and quality (e.g., analytical quality) of the data and other information to be collected. These data quality objectives will depend in part on the nature of the decisions to be made on the basis of the investigation and the confidence required in those decisions. Failure to set data quality objectives at the outset can lead to considerable waste of money if, for example, the data collected are not suitable or sufficient for a reliable hazard assessment, or leave too many uncertainties about the "conceptual model" developed for the site.

1.3.2 Preliminary Investigation

General

This is an investigation comprising a desk study (see below) and site reconnaissance (walk-over survey, site inspection). It is carried out using historical records and other sources to obtain information on the past and present usage of the site together with information about local soil properties, geology, hydrogeology, and environmental setting. From this investigation, the possibility of contamination can be deduced, and hypotheses can be formulated on the nature, location, and distribution of the contamination.

These hypotheses form part of the overall conceptual model of the site that should be developed, encompassing not only the contamination aspects but also the geology, hydrogeology, geotechnical properties, and environmental setting. The current and planned site uses are also important aspects of the conceptual model. The preliminary investigation should provide sufficient information:

- For initial conclusions about potential hazards and hazards to actual or potential human and other receptors, and
- For determination as to need for further action.

The amount and type of information required will depend on the objectives of the investigation and the ease with which the information can be obtained, i.e., the amount of work required will vary with the age of the site, the complexity of its historic usage, the complexity of the underlying geology, etc.

It shall be remembered that the contamination on a site may be more complex than initially indicated (for example by current usage) and adequate information on site history should always be obtained in the preliminary investigation.

Desk Study

This includes collection of relevant information on the site, e.g., location, infrastructure, utilization, history. Possible sources of this information are publications, maps (check accuracy of map used), aerial photographs, and satellite imagery from, e.g., land surveyor's offices, geological surveys, water management boards, industrial inspection boards, mining boards, mining companies, geotechnical institutions, regional and local (city) archives, agricultural and forestry authorities, and building supervisory boards. Particularly important is information on the physical and chemical properties and the possible spatial distribution of the soil parameter under investigation; special attention must be paid to geological features such as stratigraphy and hydrogeology.

Site Reconnaissance

A visit of the site should be part of the preliminary investigation, preferably in conjunction with the desk study, although it may be independent. Depending on the local variability of the site and the technical difficulty of the planned investigation, an experienced person should be chosen for this task. Such a visit gives a first impression about the correlation of existing maps with reality, and it will provide much additional information in a comparatively short time. In some cases, it may be necessary to draw a first or additional map at this stage.

Samples are not often taken during preliminary investigations; if they are, they are usually needed to obtain an overview of the kind of soil in order to chose the right equipment for later activities. Parts 4, 5, and 6 of ISO 10381 specify the range of preliminary investigations used within their scopes.

Output from Preliminary Investigation

A report should be prepared summarizing the findings of the preliminary investigations and stating the conclusions (or hypotheses) drawn concern-

ing the anticipated site conditions (e.g., geology, hydrology, possible contamination) relevant to the design of the sampling program. This should enable the appropriateness of the sampling strategy adopted to be assessed at a later date.

1.3.3 Exploratory Investigation

This involves on-site investigation including collecting samples of soil or fill, surface water, groundwater, and soil gas, where appropriate, to be analyzed or tested. The data and information produced are then assessed to determine if the hypotheses from the preliminary investigation are correct and, where appropriate, to test other aspects of the conceptual model. It is therefore mainly a qualitative investigation rather than quantitative.

In some cases, where the hypotheses are found to be correct, no further investigation may need to be carried out. However, it may become apparent as a result of the exploratory site investigation, for example, that the contamination pattern is more complex or concentrations of contamination are higher than anticipated and may have already caused or in the future may cause a hazard. In this situation the information obtained may be inadequate to make decisions with a satisfactory degree of confidence. It will be necessary to carry out a main site investigation to produce sufficient information to make a full hazard assessment, to determine the need for protective or remedial measures, and in due course (and possibly following further stages of investigation), to select, design, and apply protective or remedial measures.

1.3.4 Main Site Investigation

The main site investigation quantitatively determines the amount and spatial distribution of contaminants, their mobile and mobilizable fractions, and the possibilities of spread into the environment. Also included is the possible future development of the contamination situation. This will involve the collection and analysis of soil or fill, surface water, ground water, and soil gas samples in order to obtain the information necessary to enable a full assessment of the hazards presented by the contamination to humans and other potential receptors and also to enable appropriate containment or remediation actions to be identified (sometimes) together with an initial estimate of costs. The analysis of samples can be supported by model calculations and investigation techniques that do not make use of sampling.

Detailed design of protective or remedial works may require further stages of investigation.

The amount and nature of the information required from the main site investigation (or any particular stage of it) will vary depending on the nature of the site and the objectives of the investigation. The implications of the decisions on what actions should be implemented on a site will vary from site to site. Additionally, the amount and quality of the information required will also vary according to the requirements of the decision-making processes (e.g., the risk assessment, decisions regarding the need for and type of remedial actions). All parties involved in the decision-making process should be kept fully informed as information is produced to ensure that the information is sufficient for the purpose intended.

After completion of the interpretation of the information generated, including any risk assessment, it should be possible to determine whether protective or remedial measures are required and to make generalizations about the type of measures that might be appropriate.

1.3.5 Samples and Sampling Points

General

The selection, location and preparation of the sampling points depend on:

- The objectives of the investigation
- The preliminary information available
- The on-site conditions

The nature of samples to be obtained shall be appropriate to the aim of the investigation and shall be specified in the program before fieldwork begins.

Sampling Patterns

Sampling patterns are based on the estimation of the distribution of soil constituents (in most cases chemical substances) in an area or, when appropriate, on the type of substance input. Four major fixed sampling patterns can be identified as being based on:

- No specific estimate of substance distribution
- Local substance distribution and known as a “hot spot”
- Distributions along a line
- Strip-like distributions

Along with these, several other patterns exist (e.g., based on deposition of substances from the air, input due to flooding). All fixed patterns have to be adjusted to local conditions and are subject to modification.

In agricultural sampling a small number of convenient sampling patterns are established in order to obtain information on, e.g., nutrient demand or pesticide residues of rather large areas. For additional information refer to ISO 10381-4 (2003). However, it must be emphasized that most grid sampling patterns are not very efficient during the growing season, and are rarely applicable. The investigation of contaminated sites which may have profound health and economic consequences usually requires a much more detailed selection and application of sampling patterns, to give calculated, estimated, or randomly chosen sampling points on a one-, two- or three-dimensional figure. The choice of pattern should be the result of preliminary investigation of a site rather than of an ad hoc decision taken in the field.

Some investigations are carried out without predetermined pattern plans. This should not be confused with the application of random distribution of sampling points, because a person usually cannot distribute sampling points randomly without preparation, i.e., without ensuring that at every point in the area, despite the position of the other sampling points, a sample will be obtained with equal probability. Where sampling is to be carried out without a predetermined pattern (ad hoc sampling) care shall be taken that sampling is carried out by an appropriately experienced investigator. It also should not be confused with the application of sampling plans to verify special hypotheses which, with regard to the problem, will be developed and justified by the investigator (judgmental sampling).

In the following are examples of a number of commonly applied sampling patterns which meet different statistical requirements (Figs. 1.1–1.5). Experience (and theoretical considerations) show that in many cases systematic sampling on a regular grid is both practical and sufficiently productive to allow the creation of a detailed picture of variations in soil properties. The number of sampling points can be easily increased (e.g., in areas meriting more detailed investigation), the grid is easy to mark out on site, and sampling points are usually easily relocated. Systematic sampling can be supplemented by judgmental sampling when appropriate. ISO 10381-5 (1995) provides examples of pattern application for sampling contaminated sites. For selection of sampling patterns see Fig. 1.5.

Most natural properties of the soil vary continuously in space and, as a consequence, the values at sites that are close together are more similar than those further apart. They depend upon one another in a statistical sense. This property is known as spatial dependence and its implications for sampling are covered by methods of geostatistics, i.e., spatial statistics. Viewed mathematically, the value of a soil property at any place is a function of its position. The only practicable approach is to regard such

a property as a random variable and to treat its variation in space statistically. Such properties are known as regionalized variables. The application of regionalized variable theory by developing variograms is a common tool in geostatistics.

Another geostatistical approach is multi-stage or nested sampling and analysis which also can be linked with a regionalized variable theory.

The applicability of geostatistical methods does not depend on the observed values at those sites, but on the configuration of the sampling points in relation to the area (or block if three dimensions are considered) to be estimated. A general criterion for the usefulness of a sampling pattern lies in the smallness of the largest parcel not being sampled. In terms of statistically efficient sampling, a regular equilateral triangular grid provides the best selection of sampling points. For a grid with one node per unit area, the sampling points are 1.0746 units of distance apart, and no other point is more then 0.6204 units of distance away from a sampling point. For practical purposes, sampling patterns are based on rectangular grids. For such a grid with one node per unit area, no point is more than 0.7071 units of distance from a sampling point, i.e., the greater ease of use of the square grid is offset by the slightly greater area of unsampled site.

Example 1: Non-Systematic Patterns (Irregular Sampling)

Widely used in agricultural/horticultural land investigations are the "N", "S", "W", and "X" patterns of sampling (Fig. 1.1). The general premise is that the distribution of soil constituents is relatively homogeneous. The patterns used are simplifications of the stratified random sampling method. Along the outline of such a pattern, a number of samples are taken and then may be bulked and mixed to provide one sample for analysis. The distribution of sampling points is likely to be inadequate to provide the location of point pollution, and in any event high contaminant levels will be lost in mixing of these samples. Thus, in most contaminated-land investigations, these patterns are unlikely to be useful because they obscure high levels of point contamination. Wherever there are likely to be differences in soil type or conditions, crop growth, plant species, previous cultivation, etc., the site should be subdivided according to these differences and a separate sample taken from each area.

Sampling along a single diagonal of a field or a unit is only recommended in case of strip-like distribution of contaminants on agricultural areas due to application of fertilizers. Applying a diagonal for sampling avoids systematic bias by simple and effective means, which would arise with strip-parallel sampling. However, the more diagonals, the better. Two diagonals (X-shape) introduce a serious bias to the central area of the field (Fig. 1.1). This should be considered for the evaluation of the results of the

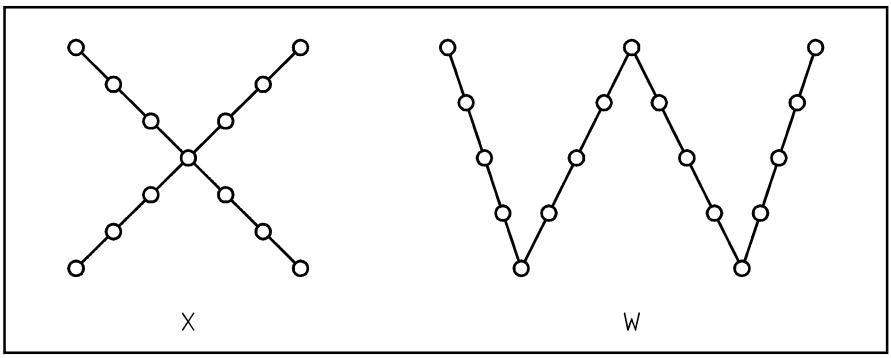

Fig. 1.1. Non-systematic patterns

determinations. Application of diagonal patterns should be based on the following:

- Estimation that substances are distributed uniformly
- Recognition of usefulness only for uniformly developed areas, and of the need to sample deviating parts separately
- Application of more than one diagonal if possible (e.g., parallel or X-shape)
- Equidistant placing of sampling points for all diagonals, i.e. shorter diagonals have fewer sampling points
- Selection of sampling point independent of local characteristics, points being fixed (preferably by pacing)

Traversing the area in a zigzag manner similar to that shown in Fig. 1.2 is another way of applying a non-systematic pattern.

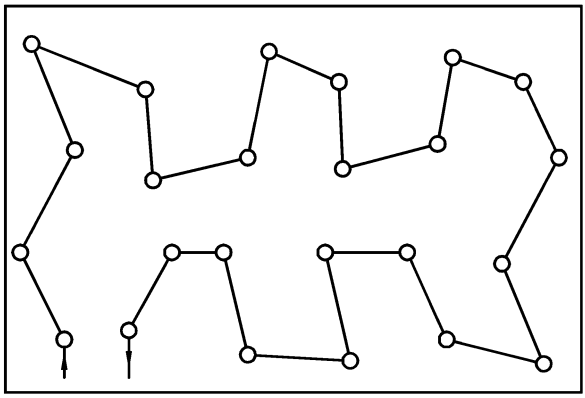

Fig. 1.2. Zigzag traverse sampling pattern

A general exception to the "biased diagonals pattern" was developed for permanently monitored areas within selected sites to achieve information about long-term changes due to human influence. The aim is to make samples available from an area representative of the surrounding environment for a defined number of examinations to be carried out over a period of some years. The following procedure is recommended (Fig. 1.3):

1. Select a representative area of approx. 1,000 m^2.
2. Divide this area into four squares, each of 250 m^2.
3. Within each square draw two diagonals, along each of which nine samples are obtained (Fig. 1.3).
4. Take samples according to the specified requirements.
5. Prepare composite samples 1, 2, and 3 by:
   - Mixing single samples of positions 1, 4, 7, 10, 13, and 16 to give composite sample 1
   - Mixing single samples of positions 2, 5, 8, 11, 14, and 17 to give composite sample 2
   - Mixing single samples of positions 3, 6, 9, 12, 15, and 18 to give composite sample 3

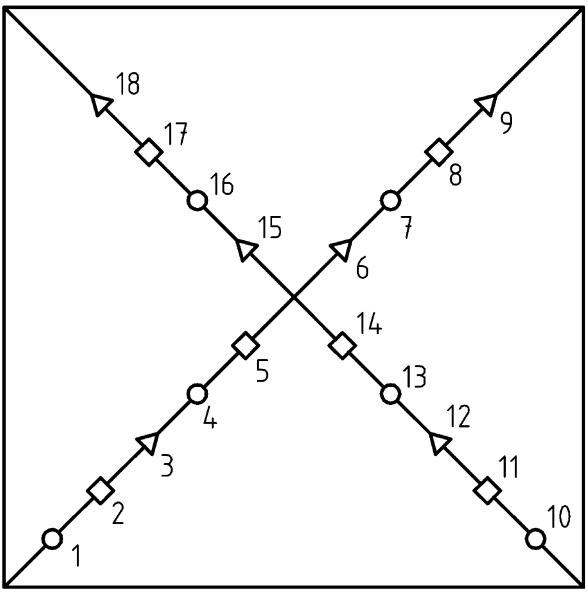

Fig. 1.3. Rotating diagonals pattern for permanently monitored areas

6. Rotational sampling of the area may be conducted by:
   - Taking samples in the intersections of the sampling points (positions 1–18 in Fig. 1.3)
   - Rotating the diagonals clockwise around the center of the square in steps of 22. 5° so that, all in all, four series of samplings can be carried out at undisturbed positions

An area selected and sampled according to the above-mentioned scheme serves for eight sampling series. After the final series, the area may be considered unsuitable for further sampling. Extensions or reductions in the dimensions of the test area may impose changes in the total number of samples, and thus also affect composite samples.

Example 2: Circular Grids

Circular grids are useful for delineating local contaminations, such as from storage tanks, but also for indicating influence around a regional emitting source, e.g., precipitation from an industrial plant. Sampling is carried out at the intersection of concentric circles (the radii of which will depend on the suspected area of contamination) and the radial lines of the main eight points of the compass (Fig. 1.4). Sampling based on circular grids may lead to information on:

- Substance concentrations at the grid center (maximum values)
- Distribution of contamination (size of particular area with increased contamination)
- Shape of distribution of contamination

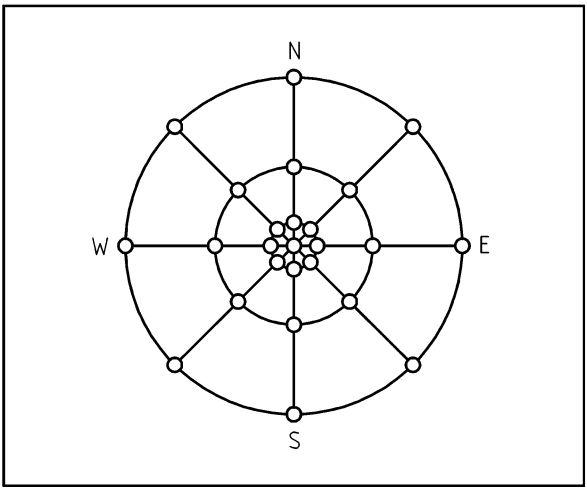

Fig. 1.4. Circular grid

Disadvantages of circular grids are:

- Star-shaped (radial) location of sampling points is practicable but not optimal. Rotation of concentric circles by 22. 5° leads to a higher quality pattern (Fig. 1.3).
- Relationship of sampling-point densities of the (usually) eight samples close to the center and those (usually) eight samples at greater distance might not to be optimal in every case. If, for example, borders of distribution of a contaminated area are looked for, fewer central points should be sampled and more toward the margins of the grid.
- Circular grids might imply a uniform extension of contamination in all directions. This is usually not the case. Preferred directions, e.g., due to main wind direction in case of airborne contaminants, should be considered in modifying of the circular grid, e.g., an increased number of sampling points in critical directions, an extended distance of sampling in critical directions.
- Circular grids generally do not serve for taking composite samples because the values thus measured give information neither on the average nor on the maximum concentration in the area sampled.

Example 3: Systematic Sampling (Regular Grids)

In many cases a regular grid is selected (Fig. 1.5). Because there is a direct relationship between optimal sampling point distance and the (estimated) dimension of the contamination, spacing between sampling points should not exceed the greatest (estimated) extent of the contamination. Grid dimensions will depend on how much detail is required. The assigned spacing will differ according to the objective of sampling, e.g., to collect samples of average degree of contamination, to locate isolated sources of contamination, or to establish the extent of contaminated zones (horizontal and vertical). The latter is of particular importance in cases where a contamination is already located and a follow-up sampling program becomes necessary. Although more frequently used for the investigation of soil contamination, regular grids are also suitable for soil fertility investigations, general soil monitoring programs, etc. An advantage of a regular grid is that it may be set up easily and grid dimensions may be readily varied. Interpolation between sampling points and return to the grid to carry out a more intensive sampling in localized areas to further delineate point sources of contamination is easy. It is also possible to fix the sampling points at the intersections of the grid lines.

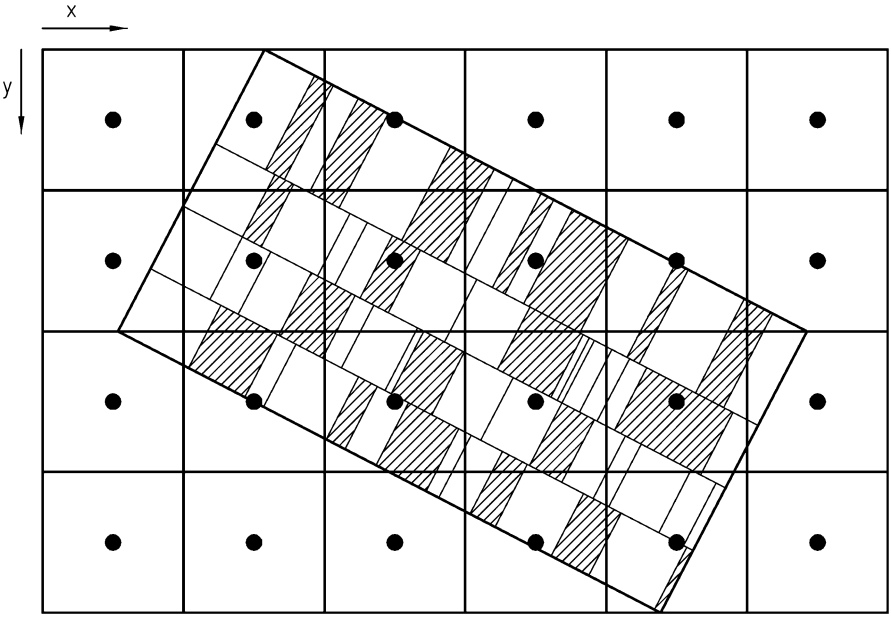

Fig. 1.5. Regular distribution of sampling points on a regular grid *hatched areas* indicate contamination

Example 4: Random Sampling

In cases of presumed irregular occurrences of contaminated zones, random sampling may be applied. Sampling points within the area are selected by using random numbers, which can be found in tables included in manuals on statistics or which may be generated by computer programs. This technique has the disadvantage of irregular coverage and makes interpolation between sampling points difficult (Fig. 1.6). In general, random sampling can also be applied for soil fertility investigations, etc. In practice, random sampling (in its purest form) is rarely used in soil surveys.

Example 5: Stratified Random Sampling

This method avoids some of the disadvantages of strictly random sampling. The site is divided into a number of grid cells, and a given number of randomly distributed sampling points is chosen in each cell (Fig. 1.7). In general, stratified random sampling can also be applied for soil fertility investigations, etc. The method has disadvantages in terms of interpolation between the sampling points. Further sampling of the site to identify local areas of contamination based on the original sampling locations is difficult.

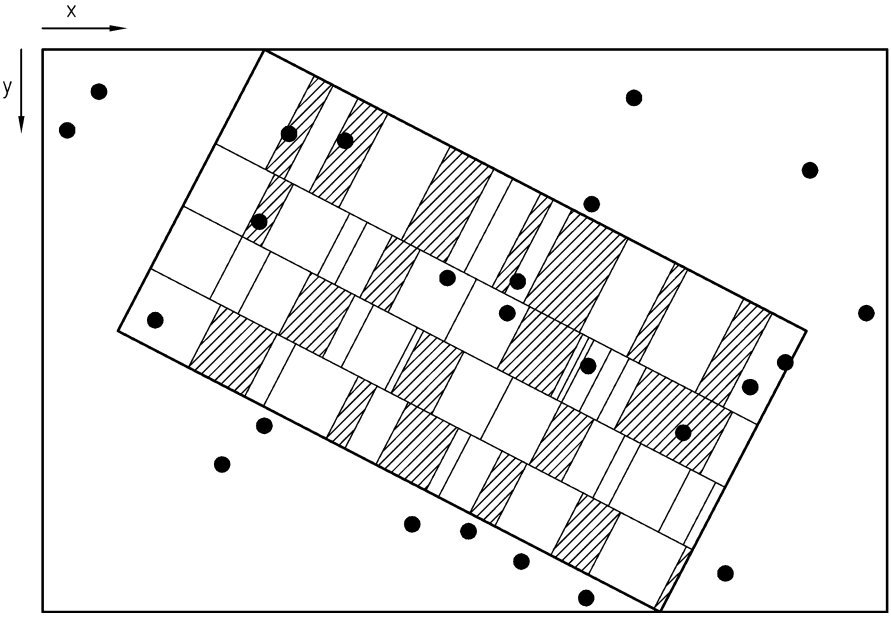

Fig. 1.6. Random sampling without grid

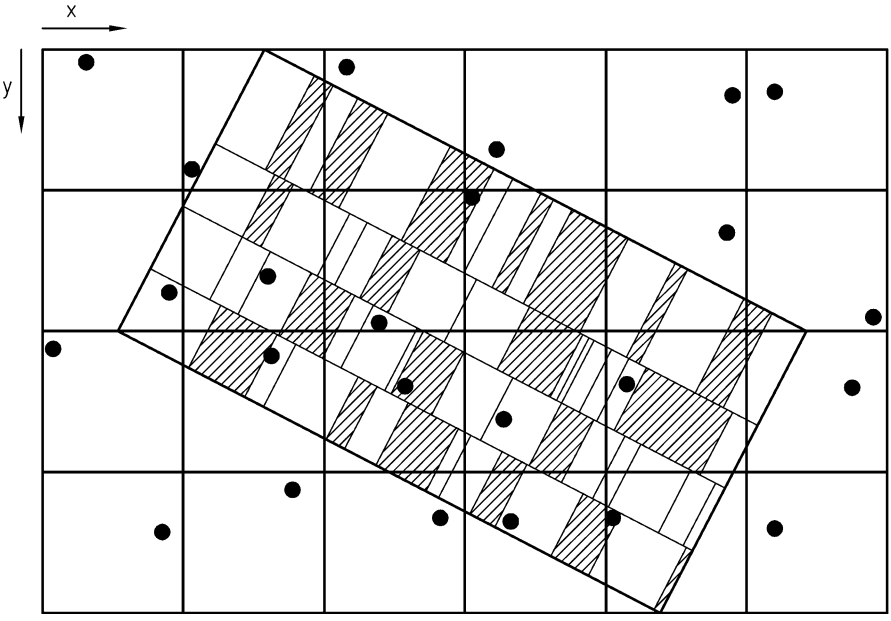

Fig. 1.7. Stratified random sampling

Example 6: Unaligned Random Sampling

The term "unaligned" means "irregular" in the sense of "not-in-a-line." The method is similar to stratified random sampling but in this case only one of two coordinates is chosen at random. The procedure is as follows. For example: given a grid with 24 cells (squares), arranged in 4 lines and 6 columns (Fig. 1.8):

1. For the first cell (line 1, column 1), x- and y-coordinates are chosen at random.
2. For cells 2, 3, 4, 5, and 6 only the y-coordinates are chosen at random.
3. For cells 7, 13, and 19, only the x-coordinates are chosen at random.
4. All sampling points are now located on the grid: For all sampling points in the columns, the y-coordinates of cells 2, 3, 4, 5, and 6 are valid, and for all sampling points in the lines the x-coordinates of cells 7, 13, and 19 are valid.

The method has disadvantages in terms of interpolation between the sampling points. Further sampling of the site to identify local areas of contamination based on the original sampling location is difficult.

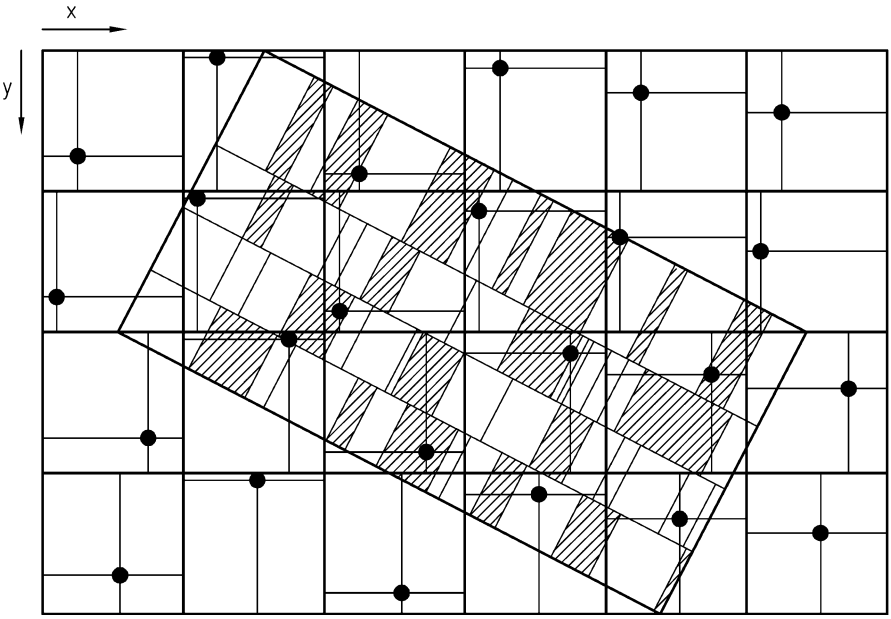

Fig. 1.8. Unaligned random sampling on a regular grid

Example 7: Systematic Sampling on a Non-Rectangular Grid

In case of an equilateral triangular grid (Fig. 1.9), each grid point is neighbored by three grid points at the unique distance d_x. No other adjacent points exist. The free, unsampled distance between the related adjacent points has a radius of

$$r = \frac{d_x}{3} \cdot \sqrt{3} \tag{1.1}$$

The circular area (A) not being sampled therefore is

$$A = \pi \cdot r^2 = \pi \cdot \frac{d_x^2}{3} \tag{1.2}$$

Example: given an area of 10×10 m and using 99 sampling points arranged in 11 rows with 9 sampling points each (distance between rows = 1.11 m) the area not being sampled is 1.29 m^2. This unsampled area is thus smaller than for example a rectangular grid of the same size and using 100 sampling points arranged at a distance of 1 m one from another, where the area not being sampled is 1.57 m^2. Any circular contamination with $r > 0.64$ m is certain to be detected. Thus, just by changing the pattern (and with one sample less) the size of the unsampled area decreases to approx. 18%.

Sampling points at the site are fixed at a distance of d_x in parallel rows spaced at a distance

$$d_y = \frac{d_x}{2} \cdot \sqrt{3} \tag{1.3}$$

That is, approx. $0.87 \times d_x$. The sampling points on the parallel rows are staggered by

$$\frac{d_x}{2} \tag{1.4}$$

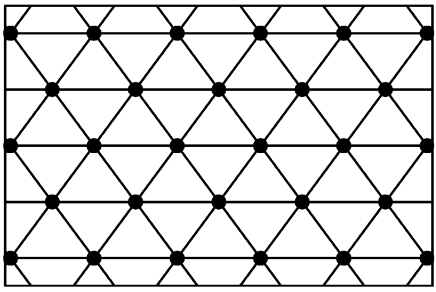

Fig. 1.9. Triangular grid

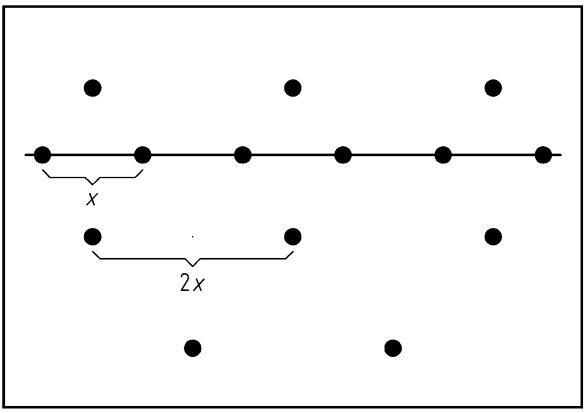

Fig. 1.10. Sampling along a linear source

Example 8: Sampling Along a Linear Source

In case of contamination following a line, e.g., caused by leaking pipelines, sampling points can be arranged in the covering soil directly above the pipeline or, if not practicable for certain reasons, close to the pipeline. If the distribution of contaminants caused by a line-like structure is also of interest, it is recommended to take samples at a distance x one from another above the line and further samples at increasing distances (e.g., 2x) parallel to the line (Fig. 1.10).

Identifying the Sampling Location

Identification of sampling points is not usually necessary when taking composite samples for agricultural purposes. Where samples are taken at pre-defined points, their accurate location and identification is important for three principal reasons:

1. To enable actual sampling locations to be revisited if necessary
2. To enable accurate plotting of data in relation to site features so that any needed treatment (e.g., additions of nutrients or removal of contamination) can be properly planned
3. To enable the data to be stored and processed by computers (e.g., for modeling studies, preparation of maps, input into geographic information systems)

Moreover, it is recommended that a sketch map be prepared presenting all relevant information on the sampling location. Both maps and photographs should include a scale and a direction marker. It is important for the interpretation of data, particularly on abandoned industrial sites to have detailed information on surface levels at sampling locations.

Sampling locations should be determined with an appropriate degree of accuracy. Because it may be necessary to vary the actual location away from the predetermined location because of the presence of obstructions, it may be preferable to do the accurate surveying of sampling locations once the sampling exercise is completed or as it progresses. Surface levels can be determined at the same time.

When investigating abandoned industrial, waste disposal, or other potentially contaminated sites, the horizontal and vertical location of sampling points or probing points should be recorded. The location of sampling points should be marked before sampling begins using poles/markers with color sprays. Color sprays should not be used if soil air has to be sampled.

Preparation of the Sampling Site

Depending on the objective of the investigation, a sampling pattern is chosen at the design stage and is then applied in the field. Within the range of patterns are some very complex ones developed with the help of computer-aided statistics. Preparation for sampling with the use of such patterns, e.g., location of desired sampling points on the ground, can be very time-consuming, especially when samples are to be obtained by boring/drilling techniques or from trial pits. Preparation of the site includes, for example, removal of superficial deposits (e.g., uncontrolled deposition of urban wastes), establishment of safety measures, installation of measurement devices (if field tests are carried out together with sampling), as well as exactly locating the sampling points. In many cases, preparation of the site takes longer than the actual sampling procedures. Both during and on completion of sampling all necessary measures must be taken to avoid hazards to the health and safety of anyone entering the site, and to the environment.

Barriers to Sampling

It may not be possible to sample at a planned location due to a variety of reasons (e.g., trees, large rocks, buildings, buried foundations or public utility services, difficulties of access) and contingency plans for dealing with such situations should be made in advance. The action to take will depend on the circumstances. The investigator may ignore the unavailable point or follow predetermined rules for choosing a nearby substitute location (e.g., alternative position within 10% of grid spacing or paired sampling along grid lines on either side the obstruction). Ad hoc decisions made in the field can lead to bias. An attempt should be made when mapping out the site to identify such obstructions in advance of actual field work. In all cases when a sampling point has to be relocated, this fact, and the reasons for relocation, should be clearly indicated in the report.

Preliminary investigations as described in Sect. 1.3.2 should provide as much detail as possible about conditions expected to exist on the site and should therefore guide the design and execution of the sampling program. However, such investigations cannot totally prevent the danger of misinterpretation of the results of borings, and the selection of sampling points should take this into account.

Depth of Sampling

No general recommendation can be given on the depths at which samples should be taken or on the final depths to which trial pits or boring/drilling should extend. This depends on the objectives and might be subject to change during a running program. Investigation of soil for chemical characteristics can be divided into two general types:

1. The investigation of agricultural and similar near-natural sites, where information is required mostly on the topsoil or plowed horizon or arable zone but often over an extended area.
2. The investigation of sites which are known or suspected to be contaminated, where information is required from deeper layers, sometimes to a depth of several tens of meters, the extent of the area usually being rather small compared to agricultural sites.

A mixture of both cases is realized in so-called "soil-monitoring sites," which represent larger areas of homogeneous soil development and in most cases are established to monitor environmental effects to the complete profile over a long-term scale. A precise description should be made of all soil horizons or layers encountered during the sampling exercise and included in the report.

If a profile is to be sampled, care should be taken that every horizon/layer of interest is sampled and that different horizons/layers are not mixed. In general, contaminated sites should be sampled horizon by horizon unless stated otherwise by the client. Care should be taken in a site investigation to ensure that pathways for migration of contamination are not created, particularly where impermeable strata may be penetrated.

When trial pits are used it may be appropriate to sample from more than one site. A depth-related sampling program is based on a number of conventions, depending on the project. It is not as representative with regard to the soil as a horizon-related sampling program can be. The mode of sampling from each depth should be carefully specified; e.g., the maximum depth range (usually not more than 0.1 m) and how horizontal variations are to be dealt with.

The total depth reached, the thickness of the horizons/layers penetrated, and the depth from which the samples are obtained should be recorded. All

data should be recorded in meters below surface. The soil depth should be measured from the ground surface with the thickness of the humus litter layer recorded separately.

Mountain regions or hilly areas with pronounced slopes require special consideration. For slopes of 10° and greater, vertical drilling lengths should be extended according to the cosine rule in order to maintain constant slope-parallel thicknesses of soil layers. The extension factor is $1/\cos$ of slope. Without correction, for example, the error will be 2% at a slope of 11.5°.

Timing of Investigation

In some circumstances, it may be necessary to restrict sampling to specific periods of the year. For example, if the characteristic or substance to be determined is likely to be affected by seasonal factors or human activities (weather, soil conditioning/fertilization, use of plant protecting agents), this should be taken into account in the design of the sampling program. This is particularly important where monitoring continues for several months or years or is repeated periodically, and therefore requires similar conditions every time sampling is carried out.

Sample Quantity

At least 1,000 g of fine soil should be obtained for chemical analysis. This figure applies both to single samples and composite samples, in the latter case after sufficient homogenization. Samples obtained to serve as reference material or to be stored in a soil specimen bank should be of larger size, usually larger than 2,000 g.

Where the sampling of soil involves the separation of oversized material (i.e., mineral grains, sand, pebbles, and all other materials) due to very coarse-grained or heterogeneous soil conditions, the material removed shall be weighed or estimated and recorded and described to enable the analytical results to be given with reference to the composition of the original sample. These procedures should be carried out in accordance with ISO 11464 (1994).

Details on the amount of sample materials needed for determination of specific physical soil parameters are given in the respective methods (Chap. 2). In particular, the determination of the particle size distribution may need a very large mass of soil material. The actual mass required will usually depend on the largest grain size to be determined (see ISO 11277 1998). The quantity of soil sample needed for biological or ecotoxicological investigations is highly dependent on the aim of the investigation and the related soil organisms.

Single Samples vs. Composite Samples

Composite samples are usually required in cases where the average concentration of a substance in a defined horizon/layer is to be determined. Single samples are required in cases in which the distribution of a substance over a defined area and/or depth is sought. In most guidelines on sampling for agricultural or similar investigations, it is recommended that composite samples be collected by taking a number of increments (according to ISO 10381-4 (2003) at least 25 increments should be obtained) and combining them to form a composite sample. When preparing composite samples regard should be paid to analytical requirements. For example, composite samples should never be used if volatile compounds are to be determined.

1.4 Sampling Methods

1.4.1 General

The most commonly used methods of sampling and forming holes in the ground to collect samples are covered in this text. This does not preclude the use of other techniques that are suited to the problems of a particular location, e.g., areas of permafrost, nor does it preclude the use of other methods that have been developed. Whatever technique is used, the principles of sample collection and the approach to sampling to obtain an appropriately representative sample should be adhered to. This will include the minimization of contamination of the sample and the protection of the samplers and other personnel involved. The choice of sampling method will be determined by taking into account all the needs of the investigation, including distribution of sampling locations, size and type of sample, and the nature of the site, including any problems the site poses in carrying out the investigation.

1.4.2 Type of Sample

There are three basic approaches to taking samples from the ground for the purpose of investigating soil and ground conditions. A sample may be:

Type 1 Material collected from a single point (disturbed or undisturbed sample).

Type 2 A composite of small incremental point samples taken close together [disturbed sample; perhaps not suitable for certain tests, e.g., the determination of volatile organic compounds; (VOCs)].

Type 3 A composite of small incremental point samples taken over an area (such as a field; disturbed sample).

Samples taken to identify the distribution and concentration of particular elements or compounds will normally be samples of type 1 or perhaps type 2 within the area being examined. Such samples would be appropriate for geological or contamination investigations and any other investigation involving disturbed samples. Samples taken to assess the overall quality or nature of the ground in an area would be type 3. Such samples would be taken for agricultural purposes.

Disturbed samples may be taken by any of the three basic methods since these samples do not require the maintenance of the original ground structure. Undisturbed samples will always require type 1 sampling because the original ground structure needs to be retained in the sample. Undisturbed samples can be taken using a coring tool or cylinder or with a sampling frame. Whichever of these sampling devices is used, the mode of operation is the same. The sampling device is pushed into the ground to be sampled and then subsequently removed complete with the sample so that the ground is collected in its original physical form.

Type 1 samples can be readily collected using hand augers and other similar sampling techniques. Any of the following tools (as well as others) may be used as appropriate:

- Cutting cylinders of different size, cutting frame
- Special hand augers [gauge auger (shallow-profile sampler), bucket auger to bring down borings for cutting cylinder application];
- Protective cap, hydraulic or handpowered supporting ring

Special bags should be used for storage and transport of "sample rings" (actually sample cylinders of limited height) to prevent disturbance and drying out. Where undisturbed samples are required, special equipment (see above) will be necessary in order to collect the sample while maintaining the original ground structure.

Type 2 samples will be appropriate when using machines for excavating ground to obtain samples. In these circumstances the samples should be formed by taking portions from locations within the bucket of excavated material (e.g., nine-point sample, according to Fig. 1.4).

Type 3 samples can be collected using hand or powered augers, but care needs to be taken to ensure the auger repetitively collects the same amount of sample.

Disturbed samples are suitable for most purposes except for some physical measurements, profiles, and microbiological examinations when undisturbed samples may be required. Undisturbed samples should be collected where it is intended to determine the presence and concentration of VOCs, since disturbance will result in loss of these compounds to the atmosphere.

Choices of sampling method include the use of machinery or manual methods. The sampling may be carried out near the ground surface, at some depth below ground level, or from locations deep below the ground surface. Methods of achieving the desired depth for sampling are either by excavating (e.g., trial pits), driving probes, or drilling (e.g., boreholes).

Sampling during borehole creation allows the required integrity for the chemical, physical, and biological investigation of selected soil horizons. Gas and water sampling may also be undertaken for specific purposes relating to the need to acquire information rapidly, for example monitoring a borehole for methane and carbon dioxide or VOCs on occasions when the rapid identification of chemical constituents in groundwater is required. It is recommended that monitoring groundwater horizons over time for hydrogeological and chemical parameters, as well as ground composition, be undertaken from cased wells or standpipes installed in boreholes. The requirements of the sampling strategy should identify the nature of borehole construction so that the appropriate monitoring design can be specified.

1.4.3 Undisturbed Samples

If undisturbed samples are required for soil sampling, these can easily be taken, for example, using a Kubiena box, a coring tool, or cylinder. In each case the sampling device is pushed into the soil and subsequently removed with the sample so that the soil is collected in its original physical form. Beside these simple techniques, many others exist, some of which are described later.

Hand-Operated Auger Techniques

There are many designs of hand auger samplers available. The designs have been developed over many years to deal with different soil types and conditions. Ease of use depends upon the nature of the ground to be sampled. In general, handaugers are easier to use in a sandy soil than in other soils, particularly where obstructions such as stones are encountered. In sandy soils, hand augers can be used to sample to a depth of about 5 m. Hand augers are usually used for sampling homogeneous soils, e.g., agricultural soils. When using hand augers, care should be taken to ensure that the soil is not contaminated by material dropping into the sample from

higher up the bore either during augering or during withdrawal of the samples. Lining the borehole carefully with a plastic tube can prevent this cross contamination.

Preferred forms of hand augers to be used for collection of soil samples are those which take a core sample. Other types of auger may be used to facilitate drilling to the requisite depth for sampling providing it is possible to clean the bore to prevent cross contamination.

Sampling by hand augers allows observation of the ground profile and the collection of samples at preselected depths. Particular care should be taken to obtain representative samples if localized contamination is penetrated. When a hand auger is to be used to take samples for testing soil for agricultural purposes, and the samples are to be composited, it is essential that the auger should be capable of consistently collecting the same sample volume. Such sampling of the near-surface soil is normally done at approx. 150–250 mm depth.

Power-Operated Auger Techniques

It is possible to obtain augers powered by small motors to reduce the labor required to carry out the sampling. The need to avoid cross contamination within the bore applies equally to augering with power-operated augers as with hand augers. Powered augers mounted on rough-terrain vehicles are available for repetitive sampling for agricultural purposes. Care should be exercised when using fuel-driven motors to avoid contamination of the sample by the fuel, the motor lubricant, and the exhaust fumes. Augers powered by electric motors that minimize the risk of such contamination are available.

Light Cable Percussion Boring

Light cable percussion boring general uses a mobile rig with winch of 1–2 t capacity driven by a diesel engine and a tripod derrick of about 6 m height. With many types the derrick folds down so that the rig can be towed by a small vehicle (frequently four-wheel drive). The light cable percussion technique is commonly used for geotechnical purposes, and boreholes over 20 m deep can be created. This technique can be of particular use in investigating deep sites such as refuse tips and other unstable ground. The ground is penetrated using different tools, depending on the strata. A clay cutter is used for cohesive soils and a shell (or bailer) for cohesionless soils. Chisels may be used to penetrate very hard ground and obstructions. The borehole formed by these tools is supported by a steel casing that is advanced as the borehole proceeds.

Depending upon the nature of the ground, the tool may form the borehole in advance of the steel casing being pushed down the hole, e.g., in clay

strata. This often results in material from the side of the borehole being dislodged as the casing is pushed down the borehole, and can result in cross-contamination. If the borehole is being formed in sands or gravels, particularly in the saturated zone, the steel casing may be pushed into place to support the borehole sides before the material is removed with the shell. This can disturb the ground and make sampling difficult.

In some strata it may be necessary to add water to the borehole to provide lubrication. In this situation tap water may be used, if available, and care should be taken with respect to the effects on both soil and water samples. The addition of water should be recorded on the borehole log and, if appropriate, on the sample details. The clay cutter and the shell bring up disturbed material from the borehole which is generally sufficiently representative to permit recording of the strata, but care has to be taken to avoid misinterpretation due to ground being pushed down within the borehole – for example, when the casing is moved. The casing avoids most of the problems of cross contamination, but the borehole should be cleaned out each time the supporting casing is driven further into the borehole, before taking a sample. Samples may be collected from both the clay cutter and the shell. The resultant sample size, although larger than obtained by hand-augering techniques, is still restricted. Undisturbed samples may be collected in cohesive strata and in weak rock (e.g., chalk) by driving a hollow tube (100 mm open-tube sampler) into the ground and withdrawing the resultant core for examination and analysis. Use of such undisturbed sampling equipment may be preferred in order to minimize cross-contamination of samples collected for testing purposes.

Water samples may be obtained as drilling proceeds and, because the casing of the borehole seals the borehole from the surrounding ground as the borehole advances, it is possible to sample water horizons at different depths with minimal risk of cross contamination. However water samples that are truly representative of the ground water necessitate the installation of an appropriately designed monitoring well. The borehole atmosphere can be monitored for gas concentrations as the borehole proceeds, or gas samples may be taken so that the profile of the ground gas composition can be determined.

Rotary Drilling

Powered rotary cutting tools use a shaft fitted with a cutter head that is driven into the ground as it rotates. The system requires some form of lubrication (air, water, or drilling mud) to keep the cutting head cool and remove the soil and other material that has been cut through. The lubricant lifts the debris from the cutting head up the borehole formed and ejects the material at ground level. This results in the potential for

cross contamination due to contact with the ground forming the sides of the hole. This technique is particularly useful for digging a hole quickly in order to form a deep observation well or for obtaining samples using a technique appropriate at greater depths only. The uncontrolled ejection of material that can occur with this technique (for instance where air or water is used for lubrication) can lead to extensive surface contamination when drilling through contaminated ground. This may be hazardous, both to the investigation team and the environment.

There are two basic types of rotary drilling, (1) open hole (or full hole) drilling in which the drill cuts all the material within the diameter of the borehole, and (2) core drilling where an annular bit fixed to the bottom of the outer rotating tube of the core barrel assembly cuts a core that is recovered within the inner most tube of the core barrel assembly and is brought to the surface for examination and testing. Rotary drilling requires well-maintained equipment operated by a specialist driller with adequate training and considerable experience.

Driven Auger

The driven auger is powered by machine, so that great force can be exerted downwards. The cutter head consists of one or more 360° spirals, usually with a shallow pitch to prevent ground falling off when withdrawn from the borehole. The method of forming the borehole is to advance the cutter head approx. 1 m into the ground, withdraw the head from the hole and spin off the spoil. This process is repeated until the required depth is reached. This method is not very satisfactory for sampling, because of the potential for cross contamination, nor is it suitable for strata logging. The method does enable the formation of a large diameter hole (up to 25 cm) into the ground relatively quickly. Lubrication of the auger is not required, but some dispersal of contaminated material may occur as the spoil is spun from the cutter head.

Continuous Flight Auger

A similar system is the continuous flight auger, which consists of a continuous helix welded to the center shaft. Downward force is again provided by the machine and continuous rotation lifts the ground to the surface from the base of the hole. This technique is only of use in site investigations in forming a hole rapidly to give depth in the ground and cannot be used for sampling or strata logging. Lubrication of the auger is not required.

Hollow Stem Auger

Hollow stem augers are a form of continuous flight auger in which the continuous helix is attached to a hollow central shaft. The drill head is

formed of two pieces, a circular outer head and an inner pilot or center bit that is fixed on a plug on the hollow shaft that can be withdrawn through the center of the auger up to the surface. This ability to withdraw the center bit and plug whilst leaving the auger in place is the principal advantage of the hollow stem auger. Withdrawing the plug provides an open cored hole into which samplers, undisturbed samplers, instruments, borehole casing, and numerous other items can be inserted to the depth achieved.

Removal of any such equipment and replacing the center plug and bit enables the continuation of the borehole. The technique provides a fully cased hole and can avoid some of the potential cross-contamination problems of percussion boring. Ground samples are collected by open drive samplers or core barrels inserted down the hollow stem. The method has been successful on some landfill sites and can be used for the installation of groundwater monitoring wells and gas standpipes. Some versions of the hollow stem auger allow continuous access to the bottom of the borehole and will permit percussion drilling or driven sampling through the center, while the hollow stem auger is actually forming the hole. The technique will allow collection of samples, particularly undisturbed samples, in addition to other down-hole testing, and also enables strata logs to be produced. Lubrication of the auger is not required.

Percussive window sampling involves driving cylindrical steel tubes into the ground using a high frequency percussive hammer. Usually, the hammer is driven by a hydraulic power pack, but electric and pneumatic hammers are also available to suit particular site conditions. Sample tubes are 1 or 2 m long and have a broad slot or window cut down one side. The soil material passes into the sample tube, through a cutting shoe at the end, as it is driven into the ground. Drill rods are used to drive the sample tubes to greater depths. On reaching the required depth for sampling, the sample tube and any drill rods are withdrawn using a mechanical jack. After removal from the probe hole, the soil material can then be inspected and the strata logged and sampled from the window.

Soil samples may also be obtained using split tubes or split spoon samplers. These are effectively tubes linearly split in half but held together by securing rings during sampling. Such devices are often used in conjunction with driven bar probes, and they allow ready retrieval of the core. Soil samples may also be obtained using a tube combined with an inert liner to enable ease of removal of the core from the sampler. The system can be used to collect samples at different depths, to rapidly penetrate to the depth at which the sample is to be taken, or to provide a continuous core.

Sample tubes of various diameters are available (35–80 mm) and selected according to the ground conditions. Tubes are normally selected in a sequence of reducing diameters to penetrate to depth. The depth that can be achieved depends on the soil type and particularly on the presence

(or absence) of obstructions. Depths of 10–12 m can be achieved where the probe hole remains open without support. Piezometers and ground gas monitoring pipes can be installed in the resultant probe holes where the ground is sufficiently stable. Systems are available to allow a probe head, with a sampling device, to be inserted into the previously formed hole to the desired sampling depth. The probe head is then unscrewed and withdrawn up the inside of the shaft, and the exposed sampling device is pushed into the ground to collect the sample. The sampling head is then withdrawn and removed for analysis. This system also enables undisturbed samples to be collected.

Continuous Samplers

Continuous soil samplers can produce core samples up to 30 m length in ground such as fine alluvial deposits. This may be of particular value and is considered to yield superior samples to those obtained by consecutive drive-in sampling. The samplers normally are made in sizes between 30 and 70 mm diameter and consist of an outer driven tube with an internal system providing a sheath to the core as the sampler is driven into the ground. Extension tubes of 1 m length are added to the sampler as the ground is penetrated. On removal from the ground, the continuous core is cut to suitable lengths, frequently 1 m, and placed in purpose-made sample cases for storage. Samples may be removed from the core for testing and the core itself observed and recorded.

Driven Probes

Driven probes may be used to make continuous geophysical measurements, for example, resistance to penetration, or may be fitted with instruments for gathering other data. Care should be taken to avoid cross contamination from the sides of the probe hole and from the base of the probe hole. This system can be used to either monitor ground water parameters (such pH, electrical conductivity, temperature, etc.) using monitors in the probe, or to access groundwater so that a representative sample can be taken without the need for purging as associated with conventional monitoring wells. Ground gases can be similarly accessed and sampled. Driven probes have the usual disadvantage of difficulty in penetrating ground with obstructions, and cannot be used for logging the ground strata unless continuous soil samples are taken. Driven probes are, however, considerably faster than traditional boreholing techniques.

Excavations (Trial Pits)

This is a widely used technique for collecting samples for site investigations related to contamination. The advantages of the method are the applicability

over a wide range of ground conditions, the opportunity for close visual examination of the strata, and the speed with which the work can be carried out. Trial pits can be dug where the ground will stand temporarily unsupported and permit the observation of the in-situ condition of the ground both vertically and laterally. Where there is water present in the excavation, problems are presented due to instability of the sides and the difficulty of obtaining representative samples of the ground (finer material tends to wash out with the water as the sample is collected). In this situation the trial pit may be dewatered by pumping, providing there is a safe and suitable means of disposal of the water – or an alternative technique of sampling should be used. In deeper trial pits formed by machines, samples of the ground can be collected by careful use of the machine bucket, thereby avoiding any need to enter the pit. In carrying out excavations, whatever technique is used to form a trial pit, the excavated material should be placed on the adjacent ground (this should be protected as necessary from contamination) in a way that ensures it will not fall back into the excavation causing cross contamination.

The surface soil layer should be kept separate so that it can be replaced on the surface after the trial pit is backfilled. It may be necessary to separate other material as it is excavated so that any deep lying contamination is replaced at the same depth when back filling and not mixed with other material or replaced near the surface. For environmental reasons and due to legislation, it may be necessary to dispose of excavated material off-site and to complete the backfilling of the trial pit and restoration of the site using clean imported material.

Entry of the excavation by personnel should be avoided where possible since the unsupported sides of a trial pit may readily collapse. If it is essential that an excavation is to be entered for sampling purposes, e.g., the collection of undisturbed samples, then shoring should be used and reference should be made to the guidance given in ISO 10381-3 (2001). In unstable ground the trial pit may collapse and extra care should be taken when observing the excavation and collecting samples. If necessary, the sides should be supported or made to slope to improve stability. For all ground conditions, if the depth of excavation is greater than 1–1.2 m and the excavation is to be entered by personnel, the sides should be adequately shored to prevent collapse.

Manual

Shovel, pick, and fork may be used to excavate trial pits down to about 2 m and, if only a small number of such excavations are required, this may be the easiest technique for collecting soil samples. The trial pit should have a plan area of approx. 1×1 m to enable easy collection of samples

and recording of the soil profile. Hand excavation is necessary particularly in urban areas if services (water, gas, electricity, etc.) are known to exist in the vicinity, and particularly if their location is uncertain. Once the base of the excavation is below the depth at which any services may exist, then the excavation or boreholing may be continued using the appropriate machinery.

1.4.4 Cross-Contamination

Whatever method is used, it is important that nothing connected with the sampling system itself contaminates the sample. This includes avoiding contamination by contact with the sampling equipment or containers and also avoiding the loss of contaminants from the sample by adsorption or volatilization. The sampling equipment should be kept clean so that parts of a previous sample are not transmitted to a subsequent sample causing cross contamination. For agricultural purposes, even with repetitive sampling across a field to form a composite sample, the sampling device should at least be brushed clean between each location. For geological and contamination investigations, all sampling equipment should be thoroughly cleaned between each sample. Contamination of samples due to lubrication used to ease sample collection, or contamination due to equipment lubricants, oils, greases, or fuels should be avoided. Where it is necessary to use lubrication, e.g., water, to ease forming a borehole to enable sample collection, only lubrication that will not conflict with nor confound the analysis of the samples (in the sense of matrix effects or contribution to the contamination) should be used.

A hand trowel of stainless steel should be used to place samples into sample containers. The quality of the stainless steel should, however, first be verified to ensure that cross contamination of the samples will not occur or interfere with the quality of the analytical data. The most commonly used methods of drilling, excavating, and sampling of the ground produce disturbed samples. If undisturbed samples are required, special sampling equipment is required and extra care should be taken in collection.

1.4.5 Sampling Containers

General Considerations

Samples of soils and related materials are liable to change to differing extents as a result of physical, chemical or biological reactions that may take place between the time of sampling and the analysis. This is especially true

of soils contaminated with volatile constituents. The causes of variations are numerous and may include:

- Changes of certain constituents due to the activities of living organisms in the soil
- Oxidation of certain compounds by atmospheric oxygen
- Changes in the chemical nature of certain substances due to changes of temperature, pressure, and hygroscopicity (e.g., loss to the vapor phase);
- Modification of pH, conductivity, carbon dioxide content, etc., by the absorption of carbon dioxide from the air
- Irreversible adsorption on the surface of containers by metals in solution or in a colloidal state, or by certain organic compounds
- Polymerization or depolymerization

The extent of these reactions is a function of the chemical and biological nature of the sample, its temperature, its exposure to light, the nature of the container in which it is placed, the time between sampling and analysis, conditions such as rest or agitation during transport, seasonal conditions, etc. It must be emphasized, moreover, that these variations are often sufficiently rapid so as to modify the sample considerably within several hours. It is therefore essential in all cases to take the necessary precautions to minimize these reactions, and in the case of many parameters to analyze the sample with a minimum of delay. Any of the procedures should be mentioned in the sampling report if applied during sampling.

Preservation

The addition of chemical preservatives or stabilizing agents is not a common practice for soil sampling. This is because a single soil sample is usually used for a large number of different determinations, and moreover has to undergo preparation (drying, milling, etc.) during which unwanted and unquantifiable reactions of the preservatives may occur. If, in special cases, it is necessary to preserve samples a method that does not introduce unacceptable contamination should be chosen. Generally, stability of samples can be considered in three classes:

1. Samples in which the contaminant(s) is/are stable
2. Samples in which the contaminant(s) is/are unstable but stability can be achieved by a preservation method
3. Samples in which the contaminant(s) is/are unstable and cannot be readily stabilized

For those contaminants that are unstable, loss or change (chemical or biological) of the contaminant should be minimized by either preserving the contaminant (e.g., freezing or adding a stabilizing agent) or by arranging for analysis to be undertaken immediately or soon after sampling. The use of liquid nitrogen for immediate deep freezing of soil samples in vapor phase is effective, and containers made of stainless steel (not chromium or nickel plated) are recommended. Some contaminants are not easily stabilized in a manner compatible with subsequent analysis. Volatile solvents fall into this category and some of them may begin to volatilize as soon as the soil is exposed by sampling. A special sampling procedure is needed to minimize such loss. In spite of numerous investigations carried out in search of methods that will enable soil samples to be stored without modification of their composition, it is impossible to give absolute rules that cover all cases and all situations and that do not have exceptions. In every case, the method of storage must be compatible with the analytical techniques to be used and should be discussed with the analytical laboratory.

Use of Appropriate Containers

The choice and the preparation of containers can be of major importance. The most frequently encountered problems are:

- Adsorption onto the walls of the containers
- Improper cleaning resulting in contamination of the container prior to sampling
- Contamination of the sample by the material of which the container is made
- Reaction between constituents of the sample and the container

The purpose of the container is to protect the sample from losses due to adsorption or volatilization, or from contamination by foreign substances. Other factors to be considered in selection of the sample container used to collect and store the sample include:

- Resistance to temperature extremes
- Resistance to breakage
- Water and gas tightness
- Ease of reopening
- Size, shape, and mass
- Availability
- Potential for cleaning and re-use

Cleaning of the sample container is a very important part of any sampling/analysis program. Two basic situations can be distinguished: (1) cleaning of new containers to remove dust and packing material; (2) cleaning of used containers prior to re-use. The type of cleaners used depends on the kind of container material and on the material to be analyzed. The selection of acids or other cleaning agents should ensure that no contamination of the containers results with regard to the constituents to be analyzed and, moreover, that there is no harm to the environment or human health.

Containers already used for investigations of contaminated sites should not be used again because cleaning containers of soils containing unknown substances may cause risks to health. The determination of organic constituents may require drying or cooling procedures under carefully controlled conditions to avoid microbial contamination. Sterilization is required whenever biological or microbiological determinations are to be carried out.

1.5 Pretreatment

1.5.1 Chemical Analysis

Inorganic Parameters and Soil Characteristics

Soil samples are dried in the air or in an oven at temperature not exceeding 40 °C, or are freeze-dried. If necessary, the soil sample is crushed while still damp and friable and again after drying. The soil is sieved and the fraction smaller than 2 mm is divided into portions mechanically or by hand, to enable representative subsampling for analysis. If small subsamples (< 2 g) are required for analysis, the size of the particles of the fraction smaller than 2 mm is further decreased.

- A drying temperature of 40 °C in an oven is preferable to air drying at room temperature because the increased speed of the drying limits changes due to microbial activity.
- It should be noted that every type of pretreatment will have an influence on several soil properties.
- The sieve aperture size of 2 mm is generally used. However, before the pretreatment is started, check should be made to see if any of the analytical methods to be applied require other sieve sizes.
- Storing soil samples, including samples that are air dried, refrigerated or stored in the absence of light, for a long time may have an influence

on a number of soil parameters, especially solubilities of both inorganic and organic fractions.

- Special measures should usually be taken for samples from contaminated soils. It is important to avoid contact with the skin, and special measures should be taken when drying such samples (ventilation, air removal, etc.). Samples may be hazardous because of the presence of chemical contaminants, fungal spores, or pathogens such as leptospirosis, and appropriate safety precautions should be taken.
- According to the international standard, it is generally assumed that at least 500 g of fresh soil shall be available.
- Keeping an archive sample is optional and should be clearly stated in the overall description of the investigation program.

Organic Contaminants

The properties of organic micro-pollutants may differ greatly according to chemical species:

- They can range from non volatile to very volatile compounds (low to high vapor pressure).
- They may be labile or reactive at ambient or elevated temperatures.
- They may be biodegradable or UV degradable.
- They may have considerably different solubilities in water.
- They require different analytical procedures.

Because of these differences a general pretreatment procedure cannot be proposed. The goal of a pretreatment procedure is to prepare a test sample in which the concentration of the contaminant is equal to the concentration in the original soil, provided, however, that this procedure does not alter the chemical species to be analyzed. If the sample contains only small particles and the contaminant is homogeneously distributed it is, for instance, not necessary to grind the sample. According to the international standard the size 2 mm is used to distinguish between small and large soil particles. Care should be taken to ensure consistency among the following aspects:

- Soil diversity
- The aim and accuracy of the analysis
- The nature of the chemical species to be analyzed

Important to pretreatment is the particle size distribution of the sample in relation to the mass of sample taken for analysis. For the analysis of

organic contaminants, the mass taken in most cases is about 20 g. With such a sample mass, and provided that the contaminant is homogeneously distributed and the particles in the sample are smaller than about 2 mm, further grinding of the sample is not necessary. If the sample contains large particles or if the contaminant is heterogeneously distributed (for instance, tar particles), it is not possible to take a representative test sample of about 20 g without grinding the sample. To improve the homogeneity, samples are grinded to a size smaller than 1 mm. Prior to analysis very often no information about the distribution of the contaminant in the soil is known. Some analytical procedures start with a field-moist sample. Drying of the sample will give lower extraction results, But because the sample is not dry, grinding is not possible. In a situation in which accurate results are needed, the best available pretreatment procedure should be used. If it is necessary to establish whether the concentration is above a certain limit, and it is already known that the soil is heavily polluted, the simplest pretreatment procedure will perhaps meet the needs despite drawbacks. In that case, however, the result may have to be presented as not representative of the whole sample.

Three methods for the pretreatment of soil samples in the laboratory prior to the determination of organic contaminants are applied in routine analysis:

1. A method for pretreatment if VOCs are to be measured. Core test samples are taken from the sample and extracted according to the specific analytical procedure. If composite samples are required, extracts of individual samples are mixed. It is usually not possible to obtain composite samples without severe losses of volatiles.

2. A method for pretreatment of moderately volatile to non-volatile organic compounds where the result of analysis must be accurate and reproducible. The sample contains particles larger than 2 mm and/or the contaminant is heterogeneously distributed: Samples are chemically dried at a low temperature (−196 °C, liquid nitrogen). The freeze-dried samples are ground with a cross beater mill with a sieve of 1 mm (cryogenic crushing). After grinding suitable test portions are processed according to the specific analytical procedures. Composite samples can be prepared by mixing of the ground samples. If the extraction procedure prescribes a field-moist sample, drying and grinding is not possible. If the original samples only contain a small fraction of particles greater than 2 mm and the distribution of contaminants is likely to be homogeneous, grinding may be omitted. In these two cases suitable test portions are directly taken after mixing of the sample. To distinguish more volatile from less volatile organic compounds, boiling points are used instead of vapor pressure at ambient temperature. For some specific components in the

group of moderately volatile compounds, freeze drying may give good results. (In the International Standard freeze drying is not described.)

3. A method for pretreatment if non volatile organic compounds are to be measured and the extraction procedure prescribes a field-moist sample, or if the largest particles of the sample are smaller than 2 mm and the contaminant is homogeneously distributed, mixing by hand is the only pretreatment that need be applied. This procedure may also be used if reduced accuracy and repeatability are acceptable.

The choice depends above all on the volatility of the organic compounds under analysis. It also depends on the soil particle size distribution, the heterogeneity of the sample, and the analytical procedure that is to follow.

1.5.2 Physical Analysis

Usually, the determination of soil physical parameters requires undisturbed soil samples. Thus, pretreatment plays only a minor role. Exceptions are:

- Determination of the water content, which can be carried out to support calculation of the analytical result, i.e., to standardize the result on dry soil mass. In this case, the analysis can be performed in the laboratory based on disturbed soil sample material. On the other hand, if the soil water content needs to be determined on a volume basis, an undisturbed sample must be taken and no further treatment applied before testing.
- The determination of the particle size distribution. Depending on the range of particle sizes, the nature (chemistry) of the soil material, and the objective of the investigation, a suite of different pretreatment procedures may be applied, including drying, slightly breaking aggregates, removing specific kinds of materials, chemically breaking aggregates down, etc. The matter is very complex and needs specialists' advice in most cases.

1.5.3 Biological Analysis

As a rule, soil for microbiological analyses under laboratory conditions should be sampled in the field with a water content that facilitates sieving. In the laboratory the soil should be processed (sieving) as soon as possible after sampling. Soil fauna and plant tests can be also carried out on air-dried samples. In this case the samples must be pretreated in order to achieve optimum conditions for the species present.

1.6 Storage of Samples

1.6.1 General

- Soils samples for laboratory determinations are collected in many studies. In general the samples are taken at the site, mixed, or otherwise treated at the site, packed in containers, and then transported to the laboratory. Upon arrival at the laboratory the samples may again be treated before being sent for analysis. Some samples may be stored directly for later analysis. After analysis the samples may be discarded or stored. The samples are stored when there is a need for further analysis, either because parameters already determined require rechecking or a need exists for making additional determinations in the future.
- The conditions for storage should be selected carefully at all stages from the point of taking the sample. Examples of storage conditions are light, temperature, humidity, accessibility, duration of storage, type of containers, and amount of storage. The documentation is also important. Risk and security problems should be considered. Well-designed storage conditions, such as provisions for monitoring, are particularly important in large-scale studies where the number of samples may become quite large over the years. Incorrectly chosen storage conditions may lead to high costs and may render the samples unfit for future use.
- The effect of storage on biodiversity is not discussed because of the difficulty to define this parameter.
- Radioactivity decay is generally not affected by storage and is not treated in this standard. Radioactive change caused by loss or gain of matter should be considered in connection with the appropriate compounds.
- Containers holding samples should be protected and sealed in such a way that the samples do not deteriorate or lose any part of their content during transport. Packaging should protect the containers from possible external contamination, particularly near the opening, and should not itself be a source of contamination. Most of the analytical procedures used in chemical soil analysis recommend that soil samples be taken to the laboratory immediately after sampling, but in some cases a range of time is given during which the sample should arrive in the laboratory.
- Soil samples should be kept cool and dark during transportation and storage.

- Cooling or freezing procedures can be applied to increase the period available for transport and storage. A cooling temperature of $4 \pm 2\,^{\circ}C$ has been found suitable for many applications. But cooling and freezing procedures should only be used in consultation with the analytical laboratory. Freezing especially requires detailed control of the freezing and thawing process in order to return the sample to its initial equilibrium after thawing.
- Light-sensitive soil constituents require storage in darkness or, at least, in light-absorbent containers.
- Undisturbed samples should be transported in the absence vibration or other physical disorder in order to maintain the original structure.
- Disturbed samples, and especially non-cohesive, very dry soils, tend to separate into different particle fractions during transportation. In such cases the soil material should be re-homogenized before pretreatment and analysis.
- Any national regulations regarding the packaging and transport of hazardous materials should be observed.

1.6.2 Specific Considerations for Biological Parameters

Biological tests can be separated into soil microbiological, fauna, plant, and biodegradation tests, and tests for the ecotoxicological characterization of soils and soil materials. Storage conditions for soils used for these tests vary over a wide range and depend on the organism or parameter to be tested.

Microbiological Tests

Samples should be stored in the dark at $4 \pm 2\,^{\circ}C$ with free access of air. It is preferable to use soils as soon as possible after sampling. If storage is unavoidable, this should not exceed 3 months unless evidence showing continued microbial activity is provided. The active soil microflora decreases with storage time, even at low temperatures, and the rate of decrease depends on the composition of the soil and the microflora involved (see also ISO 10381–6 1993).

If soil samples have to be stored for longer periods than 3 months, freezing of samples at −20, −80, or $-150\,^{\circ}C$ may be appropriate, although not generally recommended. It has been shown for a number of soils from temperate climates that storage at $-20\,^{\circ}C$ for up to 12 months does not inhibit microbial activity (e.g., ammonium oxidation). Soil samples for phospholipid fatty acid (PLFA) and DNA analyses can be stored at $-20\,^{\circ}C$

for 1–2 years. Samples for rRNA analyses can be stored at –80 °C for the same period. In the latter case the samples should be frozen immediately at −180 °C (shock freezing with liquid nitrogen).

Longer storage periods are mainly needed if the influence of added pollutants on soil microbes and microbial processes has to be tested with the same soil material, or if the community structure (structural diversity; PLFA, DNA, RNA) of soils has to be evaluated at a distinct point of time during the year. In these cases the time needed for analyses can easily exceed 3 months (chemical, pollutant testing). For structural analyses of the microflora, storage at −4 °C is not suitable.

If longer storage of samples at temperatures below −20 °C is used, special attention has to be given to the thawing of samples. Freeze–thaw cycles can increase the availability of organic matter to micro-organisms (Haynes and Beare 1996). For analyses of microbial activity (e.g., soil respiration) a thawing period of 1 day at 4 °C and another 3 days at 20 °C is recommended. Generally drying of soils is not recommended although air drying and rewetting is a common physiological stress for the microbial communities in surface soils. It has been shown that drying–rewetting events can induce significant changes in microbial carbon and nitrogen dynamics that can last for more than a month after the last stress (Fierer and Schimel 2002). Rewetting after drying causes bursts of respiration and growth of distinct populations of bacteria (Lund and Goksør 1980).

Investigations indicate that fast thawing (1 day at 20 °C in an incubator) results in smaller variations in microbiological parameters (e.g., microbial activity) than the recommended slow thawing when compared to a frozen control (Weinfurtner et al. 2002).

Biodegradation Tests

For testing the biodegradation of organic chemicals in soils (ISO 11266 1994; ISO 15473 2002), storage of soils should be avoided if possible because activity of soil microorganisms will decrease in the course of time. Storage at 4 °C up to 3 months is permissible. For the assessment of degradation of chemicals in anaerobic soils under anaerobic conditions, the access of oxygen should be avoided during storage.

Tests Involving Soil Fauna and Higher Plants

There are no specific recommendations for soil storage with respect to soil fauna and higher plant tests in ISO standards. It is recommended to store the soil samples under the same conditions as for testing of microbes and microbial processes. The reason for this is that the availability and effectiveness of pollutants is essentially governed by microbial activity. The same is also true for plant testing. Additionally, the nutrient supply of

test soils should be considered, especially if unknown contaminated soils are tested, to avoid false negative results.

Ecotoxicological Testing

Generally, sieved samples should be stored in darkness. For microbial analyses, soils and soil materials should be handled as described above. For terrestrial analyses (e.g., plant tests, earthworm tests) samples can be stored at $4 \pm 2\,^{\circ}C$ for 3 months. For testing the leaching potential/retention function of soils and soil materials, water extracts for aquatic tests should be prepared immediately after sieving. If the tests cannot be performed within 7 days (storage of the extracts at $4 \pm 2\,^{\circ}C$ in the dark), extracts should be stored at $-20\,^{\circ}C$.

1.6.3 Preparing the Samples After Storage

The procedures for preparing the samples after storage will depend on the storage conditions and the analyses. It is not possible to give a general specification. Existing standards (e.g., ISO 11464 1994) have to be considered. When a soil sample is stored for a long period of time, a vertical redistribution may occur. A new mixing in a suitable mixer is advisable. For large samples, this may not be sufficient. It is recommended that the sample be spread in a thin layer on a plastic foil, and then the layer repeatedly folded and spread it out again. Especially, the conditions of thawing have to be defined because this can influence the determination of biological, microbiological, and organic parameters. The soil samples stored below $0\,^{\circ}C$ must be unfrozen in original bags or containers.

References

Fierer N, Schimel P (2002) Effects of drying-rewetting frequency on soil carbon and nitrogen transformations. Soil Biol Biochem 34:777–787

Haynes RJ, Beare MH (1996) Aggregation and organic matter storage in meso-thermal, humid soils. In: Carter MR, Steward BA (eds) Soil structure and organic matter storage. CRC/Lewis, Boca Raton, pp 213–262

ISO 10381–1 (2002) Soil quality – Sampling – Part 1: Guidance on the design of sampling programmes

ISO 10381–2 (2002) Soil quality – Sampling – Part 2: Guidance on sampling techniques

ISO 10381–3 (2001) Soil quality – Sampling – Part 3: Guidance on safety

ISO 10381–4 (2003) Soil quality – Sampling - Part 4: Guidance on the procedure for investigation of natural, near-natural and cultivated sites

ISO 10381–5 (1995) Soil quality – Sampling

ISO 10381–6 (1993) Soil quality – Sampling – Part 6: Guidance on the collection, handling and storage of soil for the assessment of aerobic microbial processes in the laboratory

ISO 11266 (1994) Soil quality – Guidance on laboratory testing for biodegradation of organic chemicals in soil under aerobic conditions
ISO 11277 (1998) Soil quality – Determination of particle size distribution in mineral soil material – Method by sieving and sedimentation
ISO 11464 (1994) Soil quality – Pretreatment of samples for physico-chemical analysis
ISO 15473 (2002) Soil quality – Guidance on laboratory testing for biodegradation of organic chemicals in soil under anaerobic conditions
ISO 15799 (2003) Soil quality – Guidance on the ecotoxicological characterization of soils and soil materials
Lund V, Goksør J (1980) Effects of water fluctuations on microbial mass and activity in soil. Microbial Ecol 6:115–123
Weinfurtner K, Koerdel W, Schlueter C (2002) Probenahmerichtlinie für eine Kryolagerung von Bodenproben. Jahrestagungen GDCh-Fachgruppe Umweltchemie und Ökotoxikologie / SETAC-GLB, Braunschweig 2002

2 Determination of Chemical and Physical Soil Properties

Berndt-Michael Wilke

2.1 Soil Dry Mass and Water Content

Introduction

Objectives. Measures of soil water content and dry mass are needed in practically all types of soil studies, e.g., determination of water holding capacity, plant available water, infiltration, pore size distribution, permeability. With respect to soil microbial processes and biological soil remediation, determination of optimum water content for measurement of microbial parameters and activity, as well as determination of soil permeability for estimation of the success of in situ remediation, is of essential importance.

Principle. Soil samples are dried at $105 \pm 5\,^{\circ}C$ until mass constancy is reached. 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 ($cm^3{}_{water}/cm^3{}_{soil}$). The method described below can be used for disturbed and undisturbed (sampling of soil using coring sieves) soil samples. It is a direct laboratory measurement. The procedure described can be used for the determination of dry mass on a mass basis (ISO 11465 1993).

Theory. Under natural conditions all soils contain water. The amount of water can be very low in air-dried soils. As a convention the total water content and dry mass of soils are measured after drying at $105\,^{\circ}C$ (ISO 11465 1993). Thus, the water content of a soil is given as percent by weight or volume of oven-dried soil. Water which is removed at higher temperatures is not included in the definition of soil water. The soil water content can be determined with direct and indirect methods. Direct methods are more precise but time consuming. Indirect methods are mainly used for continuous determination of water contents in the field. The most appropriate indirect method is the time domain reflectometry (TRD) method (Topp et al. 2000). The optimum water content for microbial processes is in

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the range of 40–60% of maximum water-holding capacity (WHC, Sect. 2.2), or corresponds to the water content that is held in soil at suction pressures of −0. 01 to −0.031 MPa.

Equipment

- Drying oven, thermostatically controlled with forced air ventilation and capable of maintaining a temperature of 105 ± 5 °C
- Desiccator with an active drying agent
- Analytical balance, accuracy 1 mg
- Container (moisture box, 25−100 mL) with lid, made of waterproof material that does not adsorb moisture, capacity 25−100 mL for air-dried soil samples and at least 100 mL for field-moist soil samples
- Spoon

Procedure

Air-Dried Soil Samples

1. Dry container with lid at 105 ± 5 °C and then cool it, with the lid closed, in a desiccator for at least 45 min. Determine the mass (m_o) of the closed container with an accuracy of ±1 mg.
2. Transfer 10−15 g of air-dried soil to this container using a spoon.
3. Determine the mass (m_1) of the closed container and soil with an accuracy of ±1 mg.
4. Dry the container and soil in an oven at 105 °C until constant mass is achieved. Dry the lid at the same time.
5. Cool the container with the lid closed in a desiccator for at least 45 min.
6. Remove the container from the desiccator and immediately determine the mass (m_2) of the closed container containing the oven-dried soil with an accuracy of ±10 mg.

Field-Moist Soil Samples

1. Place the soil on a clean surface that does not absorb moisture (e.g., a glass plate) and mix well. Remove particles with a diameter > 2 mm.
2. Dry container with lid at 105 ± 5 °C and then cool it, with the lid closed, in desiccator for at least 45 min. Determine the mass (m_o) of the closed container with an accuracy of ±1 mg.
3. Transfer 30−40 g of soil to this container using a spoon.

4. Determine the mass (m_1) of the closed container and soil with an accuracy of ±10 mg.
5. Dry the container and soil in an oven at 105 °C until constant mass is achieved. Dry the lid at the same time.
6. Cool the container with the lid closed in a desiccator for at least 45 min.
7. Remove the container from the desiccator and immediately determine the mass (m_2) of the closed container containing the oven-dried soil with an accuracy of ±10 mg.

Calculation

Calculate 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% (*m/m*) using the following equations:

$$w_{dm} = \frac{m_2 - m_0}{m_1 - m_0} \times 100 \quad (2.1)$$

$$w_{H_2O} = \frac{m_1 - m_2}{m_2 - m_0} \times 100 \quad (2.2)$$

m_0 mass of the empty container with lid (g)

m_1 mass of the container with air-dried soil or field-moist soil (g)

m_2 mass of the container plus oven-dried soil (g)

Notes and Points to Watch

- With contaminated soil samples, special measures must be taken. Avoid any contact with the skin. Special measures must be taken during the drying process in order to prevent contamination of the laboratory atmosphere. The procedures must be performed as quickly as possible to prevent evaporation.
- In general decomposition of organic material can be neglected at temperatures up to 105 °C. However, for soil samples with a high organic matter content (> 10% *m/m*) the method of drying should be adapted by drying to a constant mass at 50 °C.
- Some minerals similar to gypsum lose chemically combined water at a temperature of 105 °C.
- If volatile organic substances are present, the method will not give a reliable determination of the water content.

2.2 Water-Holding Capacity

Introduction

Objectives. Microbiological laboratory tests (e.g., respiration measurements ISO 16072 2002; nitrogen mineralization ISO 14238 1997; biodegradation ISO 11266 1994) are carried out under optimum water conditions. These range from 40–60% of soil WHC. In order to adjust the optimum water content of a given soil the maximum WHC has to be determined.

Principle. A cylinder with a perforated base is filled with soil, capped and immersed in water and drained. The quantity of water taken up by the soil is determined by weighing, drying to constant mass at 105 °C, and reweighing.

Theory. Microbial transformations in soils are moisture dependent. Moisture must be adequate for decomposition to proceed. High moisture levels reduce activities of aerobic microorganisms due to a deficiency of oxygen. Therefore, the soil moisture content is adjusted to optimum conditions in microbial laboratory experiments. For most aerobic processes it ranges from 40 to 60% of WHC. Alternatively, the water content can be also adjusted by means of pore water pressure. Water contents of 40–60% of WHC equal 0.01−0.03 MPa.

Equipment

- Cylinder (glass, plastic, metal; with cap) of known volume, of about 50−150 mm length and 50−100 mm in diameter with a perforated base
- Water bath at room temperature
- Tray with a drainage hole, containing wet, fine, quartz sand (20−50 mm)
- Oven, capable of maintaining a temperature of 105 ± 2 °C
- Beaker, 250 mL
- Desiccator
- Balance, accurate to ±0.01 g.

Sample Preparation

As rule fresh soil samples screened through a 2 mm sieve are used.

Procedure

Determination of Maximum WHC

1. Cover the perforated base of the cylinder with a filter paper and fill it with field-moist soil (three parallels per sample). Fill the soil in small portions and provide homogeneous spreading by gentle tapping of the cylinder.
2. Submerge the cylinder in the water bath at room temperature with the water level lower than the soil surface. When the soil is moistened to the surface, lower the cylinder to the soil surface and leave it in this position overnight.
3. Remove the cylinder from the water and place the capped cylinder on the tray of sand and allow to drain. Capping of the cylinder is crucial to avoid evaporation of water.
4. Weigh the cylinder hourly beginning after 3 h until constant weight is achieved. Remove the soil from the cylinder into a 250 mL beaker and dry it at 105 °C in an oven for 24 h (minimum). Cool the samples in a desiccator and weigh again.

Adjustment of a Defined Water Content

If the actual water content is lower than wanted, the soil is spread as a thin layer and the needed amount of water is evenly sprayed in small portions on the surface. The soil should be mixed thoroughly after each water addition. Reduction of soil volume (formation of aggregates) should be avoided during addition of water.

In case of a higher actual water content the soil is dried at room temperature until the wanted moisture is reached. Drying of the soil surface has to be avoided by periodic mixing.

The water content can also be adjusted by using a device (e.g., porous funnel apparatus) whereby the saturated soil can be drained stepwise to a known soil water (matric) potential. First, the field-moist soil is saturated on a ceramic plate. Subsequently the surplus of water is drained until the wanted water content is reached using a vacuum pump.

Calculation

Calculate the WHC using the following equation:

$$\mathrm{WHC_{max}}(\%\ \text{dry mass}) = \frac{m_s - m_t}{m_t - m_b} \times 100 \tag{2.3}$$

m_s mass of beaker containing water saturated soil (g)

m_t mass of beaker containing oven-dried soil (g)

m_b mass of beaker (g)

2.3 Bulk Density – Total Porosity

Introduction

Objectives. Determination of bulk density is a widely used soil parameter. Bulk density is needed for converting water percentage by weight to content by volume, calculating the porosity and void ratio when the particle density is known (Blake and Hartge 1986). It can by used to estimate the weight of a volume of soil too large to weigh and to calculate the total mass of a pollutant in a given soil volume.

The bulk density gives a rough estimation of the aeration and permeability of a soil. The lower the bulk density, the higher is the permeability. Bulk density varies with structural conditions of the soil. Therefore, it is related to packing and often used as a measure for soil structure. In swelling soils (e.g., clay soils) it varies with soil water content (Hartge 1968). In these soils the bulk density obtained should be compared with the soil water content at the sampling time. There are three methods available for the determination of soil bulk density: core method, excavation method, and clod method. All methods are standardized (ISO DIS 11272 1998).

Theory. Soil is a porous three-phase system composed of air, water, and solids. The relative distribution of these three components is important to understand the hydraulic properties of the soil. The dry bulk density is the ratio of oven-dried solids to volume of soil. It is expressed in SI units, e.g., g/cm^3, kg/m^3, or Mg/m^3. It reflects the structural condition of the soil at given depth. Bulk densities of mineral soils may range from < 0.8 to > 1.75 g/cm^3 (Schlichting et al. 1995). The total porosity (S_t) can be calculated if the particle density (ρ_p) and the bulk density (ρ_b) are known, according to the following equation:

$$S_t = 1 - \left(\rho_b/\rho_p\right) \tag{2.4}$$

(Danielson and Sutherland 1986). As a rule of thumb the density of quartz ($\rho_p = 2.65$ g/cm^3) is used as particle density (ρ_p) of mineral soils.

2.3.1 Core Method

Principle. This method is only applicable to stoneless and slightly stony soils. A cylindrical metal sampler is pressed or driven into the soil to the desired depth. It is carefully removed to preserve a known volume of sample as it existed in situ. The sample is dried in an oven at 105 °C and weighed.

Equipment

- Core sampler holder, thin walled metal cylinders with a volume of 100–400 cm^3, a steel cap for driving into the soil, and a driver
- Oven, heated and ventilated, capable of maintaining a temperature of 105 °C
- Desiccator
- Laboratory balance, capable of weighing to an accuracy of 1/1,000 of the measured value

Procedure

1. Drive or press the core sampler into either a vertical or a horizontal soil surface enough to fill the sampler but not so firmly as to compress the soil in the confined space of the sampler.
2. Carefully remove the sampler and its contents to preserve the natural structure, and trim the soil extending beyond each end of the sample holder with a straight-edged knife or a sharp spatula. The soil sample volume thus established is the same as the volume of the sample holder.
3. Take at least six core samples from each soil horizon.
4. Place the holders containing the sample in an oven at 105 °C until constant mass is achieved (minimum 48 h).
5. Remove the samples from the oven and allow them to cool in the desiccator.
6. Weigh the samples immediately after removal from the desiccator (m_t). Control mass is reached when the differences in successive weighing of the cooled sample, at intervals of 4 h, do not exceed 0.01% of the original mass of the sample.

Calculation

The dry bulk density is calculated using the following equations:

$$\rho_b = \frac{m_d}{V} \tag{2.5}$$

$$m_d = m_t - m_s \tag{2.6}$$

ρ_b bulk density (g/cm^3)

m_d mass of the core sample dried at 105 °C minus mass of the core sample holder (g)

V volume of the core sample holder (cm^3)

m_t mass of the sample holder plus soil sample dried at 105 °C (g)

m_s mass of the empty core sample holder (g)

Notes and Points to Watch

- Swell/shrink soils (e.g., clays, muds, peats) change their bulk density with changing water content. Such soils should be sampled in a moist state (e.g., field capacity). In addition they should be sampled in a wet state (water saturation) and a dry state.
- If bulk density and water content (Sect. 2.1) are the only parameters of interest, it is not necessary to keep the samples in their holders. A single core sample holder can be reused if each sample is transferred to another container.
- The undisturbed samples in the core sample holder can be also used for other measurements such as pore-size distribution (Sect. 2.5), conductivity, or water retention.
- It is normally worthwhile to combine a measurement of the water content with a measurement of bulk density. In this case, it is necessary to transport the samples without allowing loss of water by evaporation and to begin the laboratory operations by weighing the fresh sample.

2.3.2 Excavation Method

Principle. Dry bulk density is determined by excavating a quantity of soil, drying and weighing it, and determining the volume of the excavation by filling it with sand. The method is applicable to soils containing gravel and/or stones.

Equipment

- Earth-digging equipment, e.g., spade with sharp-edged blade
- Sampling equipment (flat blade spade, knife, pick, spade chisel, hammer)
- Equipment for collecting and cleaning (plastic sheet, brush, heat-resistant plastic bags or containers)
- Plastic film, thin, flexible but stable

- Equipment for spreading sand, including funnel with a gauging rod (falling height beneath the funnel mouth should be 5 cm), graduated cylinder of 1 dm^3 capacity
- Dry, graded sand of known volume, particle diameter 500–700 µm
- Balance capable of weighing 1 g
- Oven, heated and ventilated, capable of maintaining a temperature of 105 °C
- Vacuum desiccator with self indicating desiccant
- Sieve with 2-mm mesh size

Procedure

Field

1. Level off the soil surface with the straight metal blade (Fig. 2.1a).
2. Dig a hole in the leveled soil having a representative content of larger gravel and stones (volume 20 dm^3 containing 30% stones) avoiding compaction of sides (Fig. 2.1b).
3. Put the excavated soil in bags or containers for laboratory analysis. (Large nonporous stones such as granite can be separated in the field, cleaned with a stiff brush, and weighed on a field balance).
4. Line the hole with a plastic film.
5. Fill the hole to excess with a known volume of sand from a height of 5 cm using the funnel (Fig. 2.1c). Level the surface with the blade without packing down.
6. Pour the excess sand into the graduated measuring cylinder and read the volume (Fig. 2.1d). The difference from the initial volume of sand is the volume V in the hole.

Laboratory

1. Determine the mass of the moist excavated soil (in g) with a balance (m_{pw}).
2. Separate the stones and gravel from the fine soil with a 2-mm sieve and weigh them on a balance (m_{xw}).
3. Dry the stones and the gravel in the oven at 105 °C and weigh them after cooling on the laboratory balance (m_x).
4. Determine the water content of the fine soil (< 2 mm) by drying a representative sample (5–10 g) of known mass in the oven (105 °C) until

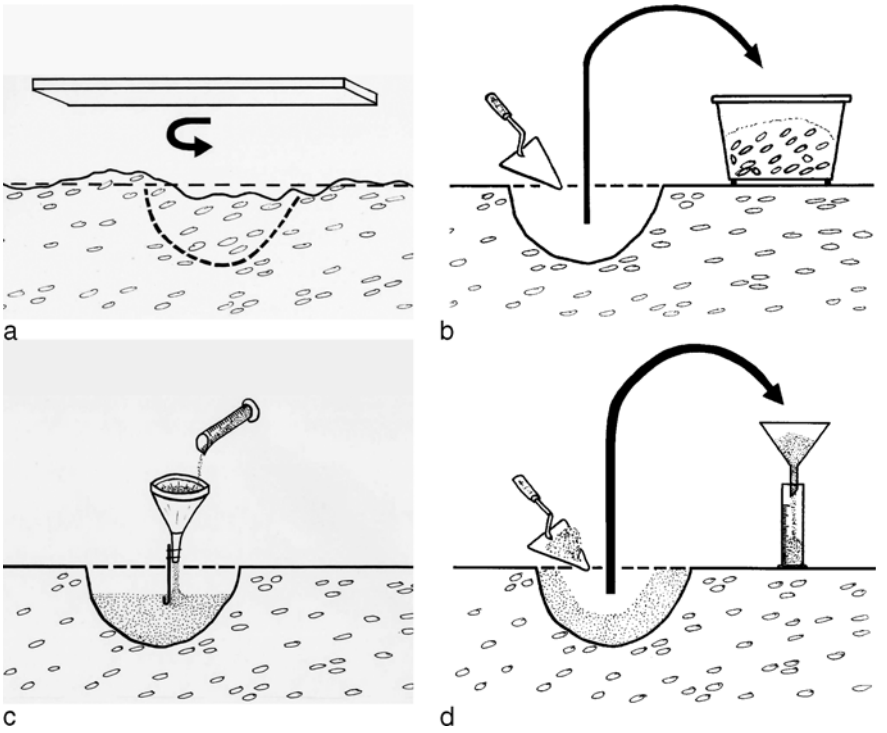

Fig. 2.1. Excavation method, field procedure (adapted from ISO 11272 1998). **a** Level off soil surface; **b** dig a hole; **c** fill with sand; **d** remove excess sand and measure its volume

constant mass is reached. Remove the sample from the oven and cool it in the desiccator. Weigh the sample on a laboratory balance. Calculate the water content (w) as a mass ratio of the moist sample.

Calculation

The bulk density of the soil layer is calculated using the following equations:

$$\rho_{\mathrm{b}} = \frac{m_x - m_{tp}}{V} \tag{2.7}$$

$$m_{tp} = m_{pw} - m_{xw} - m_{\mathrm{w}} \tag{2.8}$$

$$m_{\mathrm{w}} = m_{pw} \times m_{tw} \tag{2.9}$$

$$m_{tw} = m_{pw} - m_{xw} \tag{2.10}$$

ρ_{b} bulk density (g/cm^3)
m_x mass of stones and dry gravel (g)
m_{tp} mass of the dry fine soil (g)

V volume of the hole (cm^3)

m_{pw} mass of the excavated moist soil (g)

m_w mass of the water from excavated fine soil (g)

w water content of the excavated moist fine soil (g/g oven-dried soil)

m_{tw} mass of the moist fine soil (g)

m_{xw} mass of moist gravel and stones (g)

Notes and Points to Watch

- Holes should have smooth, rounded walls.
- Protruding stones should be included in the sample
- A heavy pair of scissors can be used to cut roots at the wall surface.

2.3.3 Clod Method

Principle. The bulk density of clods, or coarse peds, is calculated from their mass and volume. The volume is determined by coating the clod with a water-repellent substance and by weighing it first in air, then again while immersed in a liquid of known density, making use of Archimedes' principle.

Equipment

- Earth digging equipment (flat shovel, spade, pick)
- Sampling equipment (small flat-bladed spade, knife, chisel, hammer)
- Container of molybdenum sulfide (MoS_2) in heavy oil
- Laboratory balance, capable of weighing suspended samples (Fig. 2.2)
- Thermometer

Procedure

1. Separate clods or peds of about 50–200 cm^3, trim off protrusions and cut off roots with scissors.
2. Weigh soil clods or peds with a laboratory balance and coat them in oil.

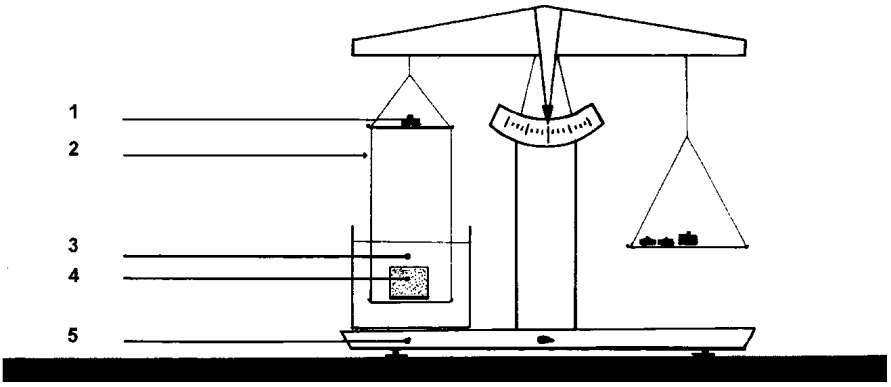

Fig. 2.2. Laboratory balance to determine the volume of clods by weighing in air and water. *1* Compensating weights; *2* thin wire; *3* small container; *4* large container filled with water; *5* balance

3. Weigh the coated clod again once in air and once immersed in water.
4. Measure the temperature of the water and determine its density
5. To obtain a correction for the water content of the soil, break the clod, remove an aliquot of soil, and weigh it before and after drying in an oven at 105 ± 2 °C.

Calculation

1. The oven dry mass of the soil clods is calculated using the equation

$$m_d = m/(1 + w) \tag{2.11}$$

m_d net mass of the oven-dried clod (g)
m net mass of the moist clod in air (g)
w water content of the subsample (g of water/g of oven-dried soil)

2. Calculate the bulk density of the dried clod using the equation

$$\rho_b = \frac{\text{mass}}{\text{volume}} = \frac{p_w \times m_d}{m - m_w + m_0\left(\rho_0 - \rho_w\right)} \tag{2.12}$$

ρ_b bulk density of oven-dried sample (g/cm^3)
ρ_o density of the coating oil (g/cm^3)
ρ_w density of water at temperature of determination (g/cm^3)
m_d oven-dried mass of soil sample, i.e., clod or ped (g)

m mass of soil sample in air (g)
m_w mass of soil sample plus coating in water (g)
m_o mass of coating in air (g)

Notes and Points to Watch

- The clod method gives usually higher bulk density values than the other methods as it does not take interclod space into account.
- Clods on or near the soil surface are likely to be unrepresentative as these are often formed by packing or plowing.
- Several other substances have been used to seal the clods against water including Saran solution (Dow Chemical, Rolling Meadows, IL, USA), paraffin, and wax mixtures.
- See also Blake and Hartge (1986).

2.4 Water Retention Characteristics – Pore Size Distribution

Introduction

Objectives. The spaces between soil particles are known as the soil pores. They are filled either with soil-air or water (soil solution) depending on the pore size and the water saturation of the soil. With respect to their equivalent diameter, soil pores can be divided into wide coarse (> 50 µm), tight coarse (10–50 µm), medium (0.2–10 µm) and fine (< 0.2 µm). Pore sizes were assigned in accordance with adaptation to the water content at characteristic matric pressures. Equivalent diameters of 50 and 10 µm comply with the water content of soils at field capacity (6 and 30 kPa), 0.2 µm with the water content at the permanent wilting point (1,500 kPa). The range of water available to plants and microorganisms is between field capacity and the permanent wilting point. Water stored at matric pressures > 1,500 kPa is neither accessible to fine plant roots nor to microorganisms. The pore size distribution of a given soil depends on its density and texture. Thus, it influences its aeration, permeability, transport of chemicals dissolved in soil water, and the water-retention characteristics of a soil. Direct evaluation of the size, configuration, and distribution of soil pores is impossible due to their extremely complicated nature. However, the size distribution can be measured by determination of water content at different matric pressures. Besides providing an assessment of the equivalent pore size distribution (e.g., identification of coarse, medium, and fine pores), the results using the methods to be described can be used for other purposes, for example:

- For assessment of the water retention characteristics
- To determine water content at specific matric pressures (e.g., for microbial degradation studies)
- To ascertain the relationship between the negative matric pressures and other soil physical properties (e.g., hydraulic conductivity, thermal conductivity)
- To determine the drainable pore space (e.g., pollution risk assessment)
- To determine indices for plant-available water in the soil (e.g., for irrigation purposes)

Principle. Undisturbed soil samples (soil cores) are used for the measurement at the high matric pressure range 0−100 kPa. The samples are saturated with de-aerated water or calcium sulfate solution (0.005 mol/L) and subsequently drained using sand, kaolin, or ceramic suction tables (for pressures from 0 to 20 kPa) and pressure plate extractors (for determination of pressures from −5 to −1,500 kPa). At equilibrium status, soil samples are weighed, oven dried and reweighed to determine the water content. The results are given either as volume fraction or mass ratio. The differences in volume fractions at different suction pressures give the pore volume (e.g., medium pores in vol%), the differences in mass fractions give the water content retained in these pores. Two standardized (ISO 11274 1998) methods are described, namely use of sand, kaolin, or ceramic suction tables for determination of water contents at pressures of 0 to −50 kPa, and use of pressure plates for determination of pressures from −5 to −1,500 kPa.

Theory. Soil water content and matric pressure are related to each other. At zero matric pressure the soil is saturated and all pores are filled with water. As the soil dries matric pressure decreases and pores will empty according to their equivalent diameter. Large coarse pores (> 50 µm) will drain at a matric pressure of > -6 kPa, tight coarse pores (10−50 µm) at −6 to 30 kPa, medium pores at −30 to −1,500 kPa, and fine pores at $< -1{,}500$ kPa.

Sampling

1. It is essential that undisturbed soil samples be used for measurement at the matric pressure range 0 to −100 kPa, since soil structure has a strong influence on water-retention properties. Use either undisturbed cores or, if appropriate, individual peds for low matric pressure methods (< -100 kPa). Soil cores shall be taken in a metal or plastic cylinder of a height and diameter such that they are representative of the natural soil variability and structure. The dimensions of samples taken in the field are dependent on the texture and structure of the soil and the test

Table 2.1. Recommended sample sizes (height × diameter) for the different test methods

Test method	Structure		
	Coarse	Medium	Fine
Suction table	50 × 100 mm	40 × 76 mm	24 × 50 mm
Pressure plate		10 × 76 mm	10 × 50 mm

method which is to be used. Table 2.1 gives guidance on suitable sample sizes for the different methods and soil structure.

2. To ensure minimal compaction and disturbance to structure, take soil cores carefully, either by hand pressure in suitable material or by using a suitable soil corer. Take a minimum of three representative replicates for each freshly exposed soil horizon or layer; more replicates are required in stony soils. Dig out the cylinder carefully with a trowel, roughly trim the two faces of the cylinder with a knife. If necessary adjust the sample within the cylinder before fitting lids to each end, and label the top clearly with the sample grid reference, the direction of the sampling (horizontal or vertical), the horizon number, and the sample depth.

3. Wrap the samples (e.g., in plastic bags) to prevent drying. Wrap aggregates (e.g., in aluminum foil or plastic film) to retain structure and prevent drying. Alternatively, excavate undisturbed soil blocks measuring approx. 30 cm^3 in the field, wrap in metal foil, wax (to retain structure and prevent drying), and take to the laboratory for subdivision. Store the samples at 1–2 °C to reduce water loss and suppress biological activity until they can be analyzed. Treat samples having obvious macrofaunal activity with a suitable biocide, e.g., 0.05% copper sulfate solution.

Sample Preparation

1. To prepare samples for water-retention measurements at pressures greater than −50 kPa, trim undisturbed cores flush with the ends of the container and replace one lid with a circle of polyamide (nylon) mesh (or similar close-weave material or paper if the water-retention characteristic is known) secured with an elastic band. The mesh will retain the soil sample in the cylinder and enable direct contact with the soil and the porous contact medium. Avoid smearing the surface of clayey soils. Remove any small projecting stones to ensure maximum contact and correct the soil volume if necessary. Replace the other lid to prevent drying of the sample by evaporation. Prepare soil aggregates for high matric pressure measurements by leveling one face and wrapping other

faces in aluminum foil to minimize water loss. Disturbed soils should be packed into a cylinder with a mesh attached. Firm the soil by tapping and gentle pressure to obtain a specified bulk density.

2. Weigh the prepared samples. Ensure that the samples are brought to a pressure of less than the first equilibration point by wetting them, if necessary, by capillary rise, mesh side or leveled face down on a sheet of foam rubber saturated with de-aerated tap water or 0.005 mol/L calcium sulfate solution. Weigh the wet sample when a thin film of water is seen on the surface. The time required for wetting varies with initial soil water content and texture. Soils are ideally field moist when the wetting is commenced. General guidelines for wetting times are:

   sand: 1–5 days

   loam: 5–10 days

   clay: 5–14 days or longer

   peat: 5–20 days.

   Very coarse pores are not water filled when the soil sample is saturated by capillary rise.

2.4.1 Determination of Soil Water Characteristics Using Sand, Kaolin, and Ceramic Suction Tables

Principle. Suction tables are suitable for measurement of water contents at matric pressure from 0 to −50 kPa. A negative matric pressure is applied to coarse silt or very fine sand held in a rigid watertight non-rusting container (a ceramic sink is particularly suitable). Soil samples placed in contact with the surface of the table lose pore water until their matric pressure is equivalent to that of the suction table. Equilibrium status is determined by weighing samples on a regular basis, and soil water content by weighing, oven drying, and reweighing.

Equipment

- Large ceramic sink or other watertight, rigid, non-rusting container with outlet in base (dimensions about (50 × 70 × 25 cm) and close-fitting cover
- Tubing and connecting pieces to construct a draining system for the suction table
- Sand, silt, or kaolin, as packing material for the suction table (Commercially available graded and washed industrial sands with a narrow

particle size distribution are most suitable. The particle size distributions of some suitable sand grades and the approximate suctions they can attain are given in Table 2.2. It is permissible to use other packing materials, such as fine glass beards or aluminum oxide powder, if they can achieve the required air entry values. Alternatively to sand, silt, or kaolin suction tables, ceramic plates can be used.

- Leveling bottle, stopcock, and 5-L aspirator bottle
- Tensiometer system (optional)
- Drying oven, capable of maintaining a temperature of 105 ± 2 °C
- Balance capable of weighing with an accuracy of 0.1% of the measured value

Procedure

1. Prepare suction tables using packing material that can attain the required air entry values (Table 2.2).
2. Prepare soil cores as described (see above).
3. Weigh the cores and then place them on a suction table at the desired matric pressure.
4. Leave the cores for 7 days. The sample is than weighed, and thereafter weighed as frequently as needed to verify that the daily change in mass of the core is less than 0.02%. The sample is than regarded as equilibrated.

Table 2.2. Examples of sands and silica flour suitable for suction tables

Type	Coarse sand	Medium	Fine sand	Silica flour
Use	Base of suction tables	Surface of suction tables (5 kPa matric pressure)	Surface of suction tables (11 kPa matric pressure)	Surface of suction tables (21 kPa matric pressure)
Typical particle size distribution	Percent content			
> 600 µm	1	1	1	0
200–600 µm	61	8	1	0
100–200 µm	36	68	11	1
63–100 µm	1	20	30	9
20–63 µm	1	3	52	43
< 20 µm	0	0	5	47

5. Move the equilibrated sample to a suction table of a lower pressure or dry it in an oven at 105 ± 5 C.
6. Samples which have not attained equilibrium should be replaced firmly onto the suction table and the table cover replaced to minimize evaporation from the table.

Calculation

Soils Containing < 20% Stones (> 2 mm)

1. Calculate the water content mass ratio at a matric pressure p_m using the formula:

$$w(p_m) = \frac{m(p_m) - m_d}{m_d} \tag{2.13}$$

$w(p_m)$ water content mass ratio at a matric pressure p_m (g)
$m(p_m)$ mass of the soil sample at a matric pressure p_m (g)
m_d mass of the oven-dried soil sample (g)

2. Calculate the water content on a volume basis at matric pressure p_m using the formula:

$$\theta\left(p_m\right) = \frac{m\left(p_m\right) - m_d}{V \times p_w} \tag{2.14}$$

$\theta(p_m)$ water content mass ratio at a matric pressure p_m (cm^3 water/cm^3 soil)
$m(p_m)$ mass of the soil sample at a matric pressure p_m (g)
m_d mass of the oven-dried soil sample (g)
V volume of the soil sample (cm^3)
p_w density of water (g/cm^3)

Conversion of Results to a Fine Earth Basis

The stone content of a laboratory sample may not accurately represent the field situation. Therefore, conversion of data to a fine earth basis may be required. Conversion of results derived from suction methods to a fine earth basis (f) is required for soils containing stones (> 2 mm) according to the following equation:

$$\theta_f = \frac{\theta_t}{\left(1 - \theta_s\right)} \tag{2.15}$$

θ_f water content of the fine earth expressed as a volume fraction

θ_s volume of stones, expressed as a fraction of total core volume

θ_t water content of the total soil, expressed as a volume fraction

2.4.2 Determination of Soil Water Characteristics by Pressure Plate Extractor

Principle. Pressure plate extractors are suitable for measurement of water contents at matric pressure −5 to −1,500 kPa. Several small soil cores are placed in contact with a porous ceramic plate contained within a pressure chamber. A gas pressure is applied to the air space above the samples and soil water moves through the plate to be collected in a burette/measuring cylinder or similar collecting device. At equilibrium status, soil samples are weighed, oven-dried, and reweighed to determine the water content at the predetermined pressures.

Equipment

- Pressure chamber with porous ceramic plate
- Sample retaining rings/soil cores with plastic discs or lids
- Graduated burette
- Air compressor (1.700 kPa), nitrogen cylinder, or other suitable pressurized gas
- Pressure regulator and test gauge
- Drying oven capable of maintaining a temperature of 105 ± 2.0 °C
- Balance capable of weighing to ±0.01 g

Procedure

1. Take small soil cores of approx. 5 cm diameter and 5–10 mm in height in situ or from larger undisturbed cores.
2. Place at least three replicates on a pre-saturated plate of appropriate bubbling pressure.
3. Wet the samples by immersing the plate and the samples to a level just above the base of the core and waiting until a thin film of water can be seen on the surface of the sample.

4. Cover the bottom of the extractor with water to create a saturated atmosphere.
5. Place a plastic disc lightly on top of each sample to prevent evaporation.
6. To apply the desired pressure, remove excess water from the porous plate and connect the outflow tube to the burette via the connector in the chamber wall. The pressure is supplied via regulators and gauges from a nitrogen cylinder or by a mechanical air compressor.
7. The pressure (from whatever source) should slightly exceed the lowest matric pressure required.
8. Apply the desired gas pressure p, check for any gas leaks, and allow the samples to come to equilibrium by recording on a daily basis the volume increase in the burette. When this remains static, the samples have come to equilibrium; the matric pressure p_m of the samples equals $-p$.
9. To remove the samples, clamp the outflow tube to prevent a backflow of water, and release the air pressure.
10. Weigh the samples plus sleeve immediately.
11. Carry out sequential equilibration of the core at different pressures by removing and weighing the core at equilibrium, reinserting it, and resetting the pressure.
12. Moisten the ceramic plate with a fine spray of water to re-establish hydraulic contact.
13. When the last equilibrium has taken place, dry at 105 °C and determine the oven-dried mass of the soil plus sleeve.

Calculation

Stoneless Soils

Calculate the water content volume fraction (θ) using the formula:

$$\theta\left(p_m\right) = \frac{m\left(p_m\right) - m_d}{V \times p_w} \tag{2.16}$$

$\theta(p_m)$ water content mass ratio at a matric pressure p_m (cm^3 water/cm^3 soil)

$m(p_m)$ mass of the soil sample at a matric pressure p_m (g)

m_d mass of the oven-dried soil sample (g)

V volume of the soil sample (cm^3)

p_w density of water (g/cm^3)

Stony Soils

Samples containing any stones (> 2 mm) shall not form part of the pressure chamber or membrane sample since the sample volume is very small. After oven-drying, determine the volume of stones in the original soil core from a field measurement and make a correction to convert θ_f values to total soil (θ_t).

$$\theta_t = \theta_f(1 - \theta_s) \tag{2.17}$$

θ_f water content of the fine earth in the pressure vessel at equilibrium expressed as volume fraction

θ_s volume of stones, expressed as a fraction of total core volume

θ_t water content of the total soil, expressed as a volume fraction

For a soil containing a volume fraction of non porous stones of 0.05 the water content is:

$$\theta_t = \theta_f \times 0.95 \tag{2.18}$$

Evaluation of Results: Pore Size Distribution

Pore volumes of coarse, medium, and tight pores in vol% of total soil volume can be calculated as follows:

Large Coarse Pores (Equivalent Diameter > 50 µm)

$$V_{lcp} = \left(\theta_{pm0} - \theta_{pm-6}\right) \times 100 \tag{2.19}$$

V_{lcp} volume of large coarse pores (% of total soil volume)

θ_{pm0} volumetric water content at water saturation ($p_m = 0$ kPa)

θ_{pm-6} volumetric water content at a matric pressure of $p_m = -6$ kPa

Tight Coarse Pores (Equivalent Diameter 10–50 µm)

$$V_{tcp} = \left(\theta_{pm-6} - \theta_{pm-30}\right) \times 100 \tag{2.20}$$

V_{tcp} volume of large coarse pores (% of total soil volume)

θ_{pm-6} volumetric water content at water saturation ($p_m = -6$ kPa)

θ_{pm-30} volumetric water content at a matric pressure of $p_m = -30$ kPa

Medium Pores (Equivalent Diameter 0.2–10 µm)

$$V_{\mathrm{mp}} = \left(\theta_{-30} - \theta_{\mathrm{pm}-1500}\right) \times 100 \tag{2.21}$$

V_{mp} volume of large coarse pores (percent of total soil volume)

$\theta_{\mathrm{pm}-30}$ volumetric water content at water saturation ($p_{\mathrm{m}} = -30\,\mathrm{kPa}$)

$\theta_{\mathrm{pm}-1500}$ volumetric water content at a matric pressure of $p_{\mathrm{m}} = -1{,}500\,\mathrm{kPa}$

Fine Pores (Equivalent Diameter < 0.2 µm)

$$V_{\mathrm{fp}} = \theta_{\mathrm{pm}-1500} \times 100 \tag{2.22}$$

V_{fp} volume of fine pores (% of total soil volume)

$\theta_{\mathrm{pm}-1500}$ volumetric water content at a matric pressure of $p_{\mathrm{m}} = -1{,}500\,\mathrm{kPa}$

Notes and Points to Watch

- If a containing sleeve is used, it should be weighed and the mass deducted from the total mass of the soil core to give $m(p_{\mathrm{m}})$.
- If stones are porous, carry out separate water retention measurements and correct fine earth values according to their volume.

2.5 Soil pH

Introduction

Objectives. Soil pH is one of the most indicative measurements of the soil chemical properties. All (bio)chemical reactions in soils are influenced by proton (H^+) activity, which is measured by soil pH. Values of pH of most natural soils (measured in 0.01 M $CaCl_2$) vary between < 3. 00 (extremely acid) and 8.00 (weakly alkaline). Solubility of various compounds in soils is influenced by soil pH (e.g., heavy metals) as well as by microbial activity and microbial degradation of pollutants. The optimum pH values for pollutant-degrading microorganisms range from 6.5 to 7.5 (Kästner 2001). Determination of soil pH is standardized in ISO DIS 10390 (2002).

Principle. A pH measurement is normally made by either a colorimetric or an electrometric method. The former involves suitable dyes or acid-base indicators. Indicator strips can be used for rough estimation of soil pH. Normally, pH values of soils are measured by means of a glass electrode in a soil solution slurry that contains a fivefold volume of water containing 1 M KCl or 0.01 M $CaCl_2$.

Theory Soil pH is a measure of the activity of ionized H (H^+, H_3O^+) and defined as the negative logarithm of the H^+/H_3O^+ ion activity in mol/L. Soil acidity results from soluble acids in the soil solution, e.g., organic acids and carbonic acid. Further acidic cations in the soil solution are Al^{3+} and Fe^{3+}. Al^{3+} ions exists in water as an $[Al(H_2O)_6]^{3+}$ complex which dissociates into H_3O^+ ions according to $[Al(H_2O)_6]^{3+} + H_2O \Leftrightarrow [Al(H_2O)_5]^{2+} + H_3O^+$ ($pK_a = 5.0$). A stronger cationic acid producer is Fe^{3+} ($pK_a = 2.2$), which due to the low solubility of iron oxides only exists below pH 3.

Soil pH is influenced by various factors, namely, the nature and type of inorganic and organic constituents (that contribute to soil acidity), the soil/solution ratio, the salt or electrolyte content, and the CO_2 partial pressure. A pH measurement in water includes easily dissociated protons while 0.01 M $CaCl_2$ and 1 M KCl solutions also mobilize exchangeable H^+. They are used to simulate soil solutions of arable soils ($CaCl_2$) and forest soils (KCl) in temperate humid climates. Values of pH measured at constant salt concentrations reflect seasonal variations to a lower degree (Page et al. 1982); and those measured in 0.01 M $CaCl_2$ are 0.6 ± 0.2 units lower than pH_{H_2O} values, because H^+ and Al^{3+} ions are partly exchanged by Ca^{2+}.

Equipment

- Shaking or mixing machine
- pH meter with slope adjustment and temperature control (in case of pH values > 10, an electrode specifically designed for that range is to be used)
- Glass electrode and a reference electrode or a combined electrode of equivalent performance
- Thermometer capable of measuring to the nearest 1 °C
- Sample bottle (50 mL) made of borosilicate glass or polyethylene with a tightly fitting cap
- Spoon of known capacity (at least 5.0 mL)

Reagents

- Water with a specific conductivity not higher than 0.2 mS/m at 25 °C and a pH > 5. 6
- Potassium chloride solution (KCl 1 mol/L)
- Calcium chloride solution ($CaCl_2$ 0.01 mol/L)
- Solution for the calibration of the pH meter
- Buffer solution, pH 4.00 at 20 °C: dissolve 10.21 g of potassium hydrogen phthalate ($C_6H_5O_4K$, dried at 110–120 °C for 2 h before use) in water and dilute to 1,000 mL at 20 °C.
- Buffer solution, pH 6.88 at 20 °C: dissolve 3.39 g of KH_2PO_4 and 3.53 g of Na_2HPO_4 in water and dilute to 1,000 mL at 20 °C.
- Buffer solution, pH 9.22 at 20 °C: dissolve 3.80 g of $Na_2B_4O_7 \times 10H_2O$ in water and dilute to 1,000 mL at 20 °C. The buffer solutions are stable for 1 month when stored in polyethylene bottles. Alternatively, commercially available buffer solutions may be used.

Sample Preparation

Use the fraction of particles of air-dried soil or soil dried at temperatures $\leq$ 40 °C and passed through a square-hole sieve with 2-mm mesh size. Alternatively, field-moist soil passed through a 2-mm sieve can be used.

Procedure

1. Take a representative test portion of at least 5 mL from the soil sample using the spoon.
2. Place the test portion in the sample bottle and add five volumes of water, potassium chloride solution, or calcium chloride solution.
3. Shake or mix the suspension for 60 ± 10 min using a mechanical shaker (never longer than 3 h). The stirring should be at a rate that achieves a homogenous soil suspension. Entrainment of air should be avoided.
4. Calibrate the pH meter as prescribed in the manufacturer's manual using the buffer solutions.
5. Adjust the pH meter as indicated in the manufacturer's manual. Measure the temperature of the suspension and take care that the temperature of the buffer and the soil solution does differ more than 1 °C. Measure the pH in the suspension while or immediately after being stirred. Read the pH after stabilization is reached. Record the pH values to two decimals.

Notes and Points to Watch

- Drying may influence the pH of soils, especially those containing sulfides. In such soils drying will lower the pH substantially.
- In calcareous soil samples the pH depends on calcium ion activity and CO_2 partial pressure (pCO_2), and also on the quality of the laboratory air (Schlichting et al. 1995).
- If a swinging-needle pH meter is used, the second decimal place should be estimated (ISO DIS 10390 2002).
- In samples with a high content of organic material (e.g., peat soils, pot soils) the suspension effect can play a role. In calcareous soils it is possible for carbon dioxide to be adsorbed by the suspension. Under these circumstances it is difficult to reach equilibrium pH values (ISO DIS 10390 2002).
- Magnetic stirring of the suspension is not suitable since this can affect the reading of pH.
- pH indicator strips may be used for rough estimations.

2.6 Soil Organic Matter – Soil Organic Carbon

Introduction

Objectives. Soil organic matter (SOM) is one of the most important indicators of soil quality. It influences many soil properties including nutrient supply (mainly N, P, S), cation exchange capacity, adsorption of pollutants, infiltration and retention of water, soil structure, and soil color, most of which in turn affect soil temperature. SOM consists of microbial cells, plant and animal residues at various stages of decomposition, stable humus (humic acids, humins) synthesized from residues by microorganisms, and highly carbonized compounds (e.g., charcoal, graphite, coal; Nelson and Sommers 1996). The term humus is used synonymously with SOM; that is, it denotes all organic material in the soil. Organic material is essential as a nutrient source for all heterotrophic soil organisms, which in turn hold a key position in the processes of humification and mineralization of humic substrates that lead to the production of stable humus, degradable organic compounds, and carbon dioxide (Forster 1995a). There is often a direct relationship between the organic carbon contents of soils and microbial biomass and activity. Several methods are available for the determination of SOM in soils. Most often SOM content of soils is determined by carbon

analysis. Two methods are described in this Section, namely dry combustion and loss on ignition (LOI).

Theory. Carbon is the chief element (48–58%) in SOM. Therefore, organic C determination is used as a basis of SOM estimates in soils. Based on the assumption that SOM contains 58% organic C, a conversion factor of 1.724 has been proposed for the conversion of organic C content to SOM (humus content) of soils (Nelson and Sommers 1996). C content of soil can be determined by wet and dry combustion techniques. If inorganic C is also extracted, corrections have to be made for the inorganic portion. This can be done either by destruction of inorganic C prior to C analysis or by separate measurement and subtraction of inorganic C from total C content. Wet digestion procedures are based on oxidation of organic C compounds by $Cr_2O_7^{2-}$. Because of the high toxicity of Cr(VI) compounds, this method should not be used. Dry combustion techniques are based on heating the soil gradually up to $\geq 900\,^{\circ}C$ and subsequent measurement of evolved CO_2 trapped in a suitable reagent and determined titrimetrically or gravimetrically. There are also other measuring devices in use (see below and ISO 10694 1995). A simple technique for the estimation of SOM is the LOI method that was standardized in Germany under DIN 19684-3 (1977).

2.6.1 Dry Combustion Method

Principle. The soil sample is gradually heated in a stream of purified oxygen to $\geq 900\,^{\circ}C$. Organic and inorganic soil carbon is converted to CO_2. The CO_2 evolved is measured by titrimetry, gas chromatography, infrared spectrometry, or gravimetry. In the presence of carbonates, the samples are pretreated with HCl. If the carbonate content is known (determination according to ISO 10693 1995), the organic carbon can be calculated. Soils with $pH(CaCl_2) < 6.5$ are unlikely to contain carbonates!

Equipment

- Analytical balance, accuracy 0.1 mg, or microbalance, accuracy 0.01 mg.
- Apparatus for determination of total organic carbon by dry combustion at a temperature of $\geq 900\,^{\circ}C$ equipped with an appropriate CO_2 detector. The following detection devices are currently available: titrimetry, gravimetry, gas chromatography, conductometry, and infrared spectroscopy. Some of the devices are able to measure separately inorganic and organic carbon, others also measure total C and N contents (CN analyzer)

- Crucibles made of porcelain, quartz, silver, tin, or nickel of different size; crucibles made of tin and nickel are not acid resistant.

Reagents

- Distilled or demineralized water with an electric conductivity of $\leq$ 0.2 mS/m at 25 °C
- Reagents for calibration, e.g., acetanilide (C_8H_9NO); atropine ($C_{17}H_{23}NO_3$); calcium carbonate ($CaCO_3$); graphite powder for spectroscopy (C); sodium hydrogen phthalate ($C_8H_5KO_4$)
- HCl (4 mol/L)

Sample Preparation

Use air-dried, sieved (< 2 mm) soil.

Procedure

1. Weigh out m_1 g of the air-dried sample or subsample into a crucible. The amount for analysis depends on carbon content and on the apparatus used!
2. Carry out the analysis according to the manufacturer's manual.
3. Soils containing carbonates should be pretreated as follows: add an excess of HCl to the crucible containing a weighed quantity of air-dried soil and mix. Wait 4 h and dry the crucible for 16 h at a temperature of 60–70 °C. Then carry out the analysis in accordance to the manufacturer's manual. The quantity of HCl depends on the weight of the subsample and its carbonate content. In all cases an excess of acid should be added!

Calculation

Organic Carbon Content

The total carbon content is calculated according to the following equation:

$$w_{Ct} = 1000 \times \frac{m_2}{m_1} \times 0.2727 \times \frac{100 + w_{H_2O}}{100} \tag{2.23}$$

w_{Ct} total carbon content on the basis of oven-dried soil (g/kg)

m_1 mass of the test portion (g)

m_2 mass of carbon dioxide released by the soil sample (g)

0.2727 conversion factor for CO_2 to C

w_{H_2O} water content expressed as a percentage by mass on a dry mass basis (Sect. 2.1)

Organic Matter Content

The organic matter content of the soil sample can be calculated using the following equation:

$$w_{om} = f \times w_{Corg} \tag{2.24}$$

w_{om} organic matter content of the soil on the basis of oven-dried soil (g/kg)

w_{Corg} organic carbon content of the soil on the basis of oven-dried soil (g/kg)

f conversion factor

2.6.2 Loss On Ignition Method (LOI)

Principle. The LOI method is based on ignition (550 ± 25 °C) of a dried (105 °C) soil sample until mass constancy is achieved. The SOM content is calculated from the mass difference before and after heating.

Equipment

- Sieves, 2- or 5-mm mesh size
- Drying oven, capable of maintaining a temperature of 105 ± 2 °C
- Muffle furnace, capable of maintaining a temperature of 550 ± 25 °C installed under a fume hood
- Analytical balance, accuracy 0.01 g
- Porcelain crucibles or bowls
- Desiccator with an active drying agent

Sample Preparation

Use field-moist, sieved (< 5 mm) soil or air-dried, sieved (< 2 mm) soil. Dry the soil to 105 °C prior to organic matter determination.

Procedure

1. Determine the dry mass (m_s) of the soil according to Sect. 2.1.
2. Heat crucibles or bowls in the muffle furnace at 550 ± 25 °C for 20 min, cool in a desiccator and determine tare mass (m_t) to 0.1 g.
3. Weigh 5–20 g (accuracy 0.01 g) of oven-dried (105 °C) soil (see step 1) depending on its organic matter content in crucibles or bowls, and place them in the cold muffle furnace.
4. Heat the muffle furnace gradually to 550 ± 25 °C for 2–4 h until mass constancy is achieved.
5. Open the door and cool the muffle furnace down to 100 °C.
6. Place the crucibles/bowls in the desiccator and cool them to room temperature (approx. 1 h).
7. Measure the mass of the filled crucibles/bowls ($m_c + m_t$) twice. The difference of each individual measurements from the mean should not exceed 5% of the mean.

Calculation

1. Calculate the loss of mass (Δm; g) after ignition at 550 °C using the following equation:

$$\Delta m = \left(m_s + m_t\right) - \left(m_c + m_t\right) = m_s - m_c \tag{2.25}$$

2. The LOI corresponds to the SOM content and can be calculated using the following equation:

$$\mathrm{LOI}\left(\%\right) = \frac{\Delta m}{m_s} \times 100 \tag{2.26}$$

Δm loss of mass of the soil after ignition at 550 °C (g)
m_s mass of the soil dried at 105 °C (g)
m_t mass of the crucibles/bowls ignited to 550 °C (g)
m_c mass of the soil ignited to 550 °C (g)

Notes and Points to Watch

- Humus-rich samples should be weighed in the crucibles/bowls in a field-moist state and dried and heated in the same crucible. In order to avoid dusting the organic samples, the crucibles/bowls should be covered with a porcelain lid or a metal mesh.

- The incineration of the samples should be controlled. The process is complete if black particles cannot be found in the sample or if it has a light gray to reddish color.
- Samples which do not show complete incineration should be treated with a few drops of saturated ammonium nitrate solution or hydrogen peroxide and heated again to 550 °C for 1 h.
- The LOI is assumed to be equal in most surface soils. Losses of crystalline water of clay minerals and gypsum may result in an overestimation of SOM contents. The same is true for carbonate-rich soils, because decomposition of $CaCO_3$, which starts at temperatures of approx. 500 °C. Therefore, the method is mainly recommended for sandy and carbonate-free soils and peats. Nevertheless, results for clayey soils and soils rich in gypsum can be corrected by subtraction of 0.1% SOM per 1% of clayey soil and 0.26% SOM per 1% of gypsum-rich soil.
- The error caused by the destruction of clay minerals may be avoided by pre-heating at 430 °C in an N_2 atmosphere.
- For peat soils the LOI method is advantageous over the carbon determination procedures because the carbon content of these materials varies between 40 and 100 mass%.

2.7 Soil Nutrients: Total Nitrogen

Introduction

Objectives. Analysis of total N, the C/N ratio, and inorganic N (ammonium, nitrate) provides an insight into the nitrogen supply to soil microflora and plants. The total N content ranges from < 0.02% (subsoils) to > 2.5% (peats). A-horizons of mineral soils contain 0.06–0.5% N. Nitrogen, phosphorous, and/or potassium deficiency may limit the microbial decomposition (mainly cometabolic) of pollutants in soil. Optimum conditions are achieved at C:N:P ratios of 100:10:2 (Kästner 2001). Therefore, the concentrations of these nutrients have to be analyzed and adjusted if necessary. Two methods have gained general acceptance for the determination of total N in soils, namely the Kjeldahl and the Dumas methods (Bremner 1996). The Kjeldahl method is a wet oxidation procedure, the Dumas method a dry oxidation (combustion) method. Both methods have been standardized (ISO 11261 1995; ISO 13878 1998).

2.7.1 Dry Combustion Method ("Elemental Analysis")

Principle and Theory. The soil is heated in a purified oxygen stream to a temperature of $\geq$ 900 °C. Mineral and organic N species are oxidized and/or volatilized. Products are oxides of N (NO_x) and molecular N (N_2) mainly. After transforming into N_2 by reduction on surfaces of metallic copper, the N content is measured by means of thermal conductivity detection (method adapted from ISO 13878 1998).

Equipment

- Balance, capable of weighing accurately to 0.1 mg, or microbalance, capable of weighing accurately to 0.01 mg
- Combustion apparatus to determine total N at a temperature $\geq$ 900 °C, including a detector for measuring the nitrogen gas formed
- Crucibles of various sizes, e.g. 10 or 20 mL, special requirements being given in the manual of the apparatus used

Reagents

- Combustion gas (oxygen), special requirements being given in the instruction manual of the apparatus used
- Chemicals and/or catalysts for reduction, oxidation, and/or fixing of combustion gases that interfere with the analysis
- Calibration substances, for example acetanilide (C_8H_9NO), amino acids of known composition, or soil samples with certified N contents, the N content of the calibration substance being as similar to the suspected soil N content as possible

Sample Preparation

Soil samples dried in the air, dried in an oven at a temperature not exceeding 40 °C, or freeze dried (see Chapt. 1) are sieved (2 mm); if a soil mass < 2 g is required for the analysis, mill a representative subsample to 0.1–0.15 mm.

Procedure

1. Calibrate the apparatus as described in the manufacturer's manual.
2. Weigh out m_1 g of the air-dried sample or subsample into a crucible. The amount for analysis depends on N contents and on the apparatus used.

3. Carry out the analysis according to the manufacturer's manual.
4. Determine the percentage of water content (mass fraction) according to the method described in Sect. 2.1.

Calculation

1. Normally, the primary results (from the apparatus) are given in mg N (X_1) or a mass fraction of N (X_2), expressed as a percentage of the air-dried soil used (m_1). Calculate total N content (w_{Nt}), in mg/g, on the basis the oven-dried soil according to following equations:
   For primary results given in mg of N:

$$w_{Nt} = \frac{X_1}{m_1} \times \frac{(100 + w)}{100} \tag{2.27}$$

   For primary results, given as percent mass fraction of N:

$$w_{Nt} = X_2 \times 10 \times \frac{(100 + w)}{100} \tag{2.28}$$

   w_{Nt} content of N (mg/g oven-dried soil)
   X_1 primary result in N (mg)
   X_2 primary result in percentage N (mass fraction)
   m_1 mass of air-dried soil for analysis (g)
   w percentage of water content (mass fraction) on the basis of oven-dried soil (Sect. 2.1)

2. If oven-dried samples are used, the N content is calculated as follows:
   For primary results given in mg of N:

$$w_{Nt} = \frac{X_1}{m_1} \tag{2.29}$$

   For primary results given as percent of mass fraction of N:

$$w_{Nt} = X_2 \times 10 \tag{2.30}$$

Notes and Points to Watch

- Today, several automated elemental analyzers for the determination of total N are in use (see Bremner 1996). Most of them can be used for determination of total N and total C, others also for hydrogen or sulfur.

- One of the problems linked with the use of elemental analyzers is sample-size limitation. This makes it essential to grind soil samples very finely in order to get representative subsamples.
- Soil pores are filled with air, which contains up to 80% N_2. Nitrogen gas can also enter the combustion cell when it is opened for sample exchange. Both facts may lead to overestimation of the soil N content. Therefore, sufficient purging should be carried out by oxygen gas flow before the combustion step.

2.7.2 Modified Kjeldahl Method

Principle. The method (as in ISO 11261 1995) is based on the Kjeldahl digestion. Additional reagents are salicylic acid to avoid loss of nitrates, and thiosulfate to detect azo-, nitroso- and, nitrocompounds. Instead of selenium, titanium dioxide is used as catalyst.

Theory. The Kjeldahl method generally employed for determination of total N involve two steps: (1) digestion of the sample to convert organic N into NH_4^+-N and (2) determination of NH_4^+-N in the digest. The digestion is usually performed by heating the sample with H_2SO_4 containing substances that promote the oxidation of organic matter and conversion of organic N into NH_4^+-N. For increasing the temperature K_2SO_4 or Na_2SO_4 are used. Catalysts such as Hg, Cu, Se, or TiO_2 increase the rate of oxidation of organic matter by H_2SO_4. A general equation for the digestion process is given below:

$$\text{Organic-N} + H_2SO_4 \rightarrow (NH_4)_2SO_4 + H_2O + CO_2 + \text{other matrix by-products.}$$

For the determination of NH_4^+-N several methods are possible, namely ion-sensitive electrodes, colorimetric analyses, or analyses by steam distillation and titration (Forster 1995b). In the distillation method employed by most workers, NH_4^+-N in digests is converted under excess alkali into NH_3, which is collected in boric acid. By this procedure ammonium borate is formed, which is titrated back to boric acid with hydrochloric acid according to the following equations:

$$2NH_3 + H_3BO_3 \rightarrow (NH_4)_2HBO_3$$

$$(NH_4)_2HBO_3 + 2HCl \rightarrow 2NH_4Cl + H_3BO_3$$

Equipment

- Digestion flasks or tubes, of nominal volume 50 mL, suitable for the digestion stand
- Digestion stand, glass tubes placed in holes drilled into an aluminum block also being suitable
- Distillation apparatus, preferably of the Parnass-Wagner type
- Burette, graduated in intervals of 0.05 mL or smaller

Reagents

- Salicylic acid/sulfuric acid: dissolve 25 g of salicylic acid in 1 L of conc. sulfuric acid (ϱ = 1.84 g/cm^3).
- Potassium sulfate catalyst mixture: grind and thoroughly mix 200 g of potassium sulfate, 6 g of copper(II) sulfate pentahydrate, and 6 g of titanium dioxide with the crystal structure of anatase.
- Sodium thiosulfate pentahydrate: crush the crystals to form a powder that passes through a sieve with 0.25-mm mesh size.
- NaOH solution (10 mol/L).
- Boric acid solution (H_3BO_3, 20 g/L).
- Mixed indicator: dissolve 0.1 g of bromocresol green and 0.02 g of methyl red in 100 mL of ethanol.
- Sulfuric acid (0.01 mol/L).

Sample Preparation

Soil samples dried in the air, dried in an oven at temperature not exceeding 40 °C, or freeze-dried (see Chapt. 1) are sieved (2 mm) and ground to 0.1–0.15 mm.

Procedure

1. Place a test portion of the air-dried soil sample of about 0.2 g (expected N content ca. 0.5%) to 1 g (expected N content ca. 0.1%) in the digestion flask.

2. Add 4 mL of salicylic/sulfuric acid and swirl the flask until the acid is thoroughly mixed with the soil.
3. Allow the mixture to stand for at least several hours (or overnight).
4. Add 0.5 g of sodium thiosulfate through a dry funnel with a long stem that reaches down into the bulb of the digestion flask, and heat the mixture cautiously on the digestion stand until frothing has ceased.
5. Cool the flask.
6. Add 1.1 g of the catalyst mixture and heat until the digestion mixture becomes clear.
7. Boil the mixture gently for up to 5 h so that the sulfuric acid condenses about 1/3 of the way up to the neck of the flask. Ensure that the temperature of the solution does not exceed 400 °C. In most cases a boiling period of 2 h is sufficient.
8. After completion of the digestion step, allow the flask to cool, and add about 20 mL of water slowly while shaking.
9. Swirl the flask to bring any insoluble material into suspension and transfer the contents to the distillation apparatus.
10. Rinse three times with water to complete the transfer.
11. Add 5 mL of boric acid solution to a 100 mL conical flask and place the flask under the condenser of the distillation apparatus in such a way that the end of the condenser dips into the solution.
12. Add 20 mL of sodium hydroxide solution to the funnel of the apparatus and run the alkali slowly into the distillation chamber.
13. Distill about 40 mL of condensate (the amount depends on the dimensions of the conical flask).
14. Rinse the end of the condenser.
15. Add a few drops of indicator to the distillate.
16. Titrate with sulfuric acid to a violet endpoint.

Calculation

Calculate total N content (w_{Nt}) using the following equation and round the result to two significant figures:

$$w_{Nt}(\text{mg N/g soil}) = \frac{(V_1 - V_0) \times c(H^+) \times M_N}{m} \times \frac{100 + w_{H_2O}}{100} \qquad (2.31)$$

V_1 volume of the sulfuric acid (mL)

V_0 volume of the sulfuric acid used in the blank test (mL)

$c(H^+)$ concentration of H^+ in the sulfuric acid (mol/L; e.g., if 0.01 mol/L sulfuric acid is used, $c(H^+)$ is 0.02 mol/L

M_N molar mass of nitrogen (g/mol; 14)

m mass of the air-dried soil subsample used (g)

w_{H_2O} water content expressed as a percentage by mass on the basis of oven-dried soil (Sect. 2.1)

Notes and Points to Watch

- The modified Kjeldahl procedure is satisfying for the analyses of most N compounds in soils, but it detects compounds containing N–N and N–O linkages and some heterocyclics (e.g., pyridine) only partially (Bremner 1996; ISO 11261 1995).
- Losses of nitrogen can occur with samples of high NH_4-N and NO_3-N content. Therefore, excessive drying prior to analysis should be avoided.
- A potentiometric titration is also possible. The endpoint of the titration is pH 5.0.
- If a steam distillation is used, a distillation rate up to 25 mL/min is applicable. Stop the distillation when about 100 mL of distillate have been collected.

2.8 Soil Nutrients: Inorganic Nitrogen

Introduction

Objectives. Nitrogen is a main nutrient for plants and soil organisms. Ammonium and nitrate in the soil are the N sources immediately available to plants. They are produced by mineralization of organic compounds or fertilized to soil. Besides ammonium and nitrate, nitrite (NO_2) may also be present, although its content is usually negligible except in neutral and alkaline soils recently treated with NH_4^+ salts or NH_4^+-forming fertilizers (Mulvaney 1996). Quantification of nitrate and ammonium N in soil extracts and soil solution can be performed using colorimetric, microdiffusion, and ion electrode methods (Mulvaney 1996).

Principle. Fresh soil is extracted with a calcium chloride solution (0.01 mol/L) in a 1:10 ratio (m/v) at 20 ± 1 °C. After reaching equilibrium (2 h), the

solution is centrifuged for the determination of ammonium and nitrate. A segmented flow analysis (SFA) system equipped with a colorimetric detector is used to quantify nitrate and ammonium N.

Theory. Up to 90% of total N in upper soil layers exists in organic forms. Organic N is mineralized by soil microorganisms to ammonium, nitrite, and nitrate, which are easily available to plants. In loamy and clayey soils 20–25% of total N are present as fixed ammonium in clay minerals (Li et al. 1990; Sahrawat 1995). Fixed ammonium occurs between the layers of 2:1-type clay minerals. It cannot be replaced by neutral potassium solution (Mulvaney 1996). In contrast to exchangeable ammonium, it is not available to plants. The method described here is adapted from ISO 14255 (1998). It is valid for the determination of soluble inorganic N and weakly adsorbed ammonium. In acid clayey forest soils, ammonium specifically bound at the edges of clay mineral interlayers may be the main N source of plants. For the determination of available N in these soils the use of KCl (1 mol/L) is recommended as extractant (VDLUFA 2000).

2.8.1 Extraction

Equipment

- Balance, accuracy 10 mg
- Polyethylene bottles, nominal volume 250 mL, with screwcaps
- Shaking machine or reciprocating shaker, 150–250 rpm
- Centrifuge, capable of holding the tubes used
- Polyethylene centrifuge tubes, nominal volume 100 mL or other sufficient volume

Reagents

- Water, with a specific conductivity not higher than 0.2 mS/m at 25 °C (according to grade 2 of ISO 3696 1987)
- Extraction solution: $CaCl_2$ (0.01 mol/L)

Sample Preparation

Field-moist soil samples shall be stored in a cooling box immediately after sampling. In the laboratory they are homogenized, sieved (< 2 or < 5 mm), and analyzed immediately. If the analysis cannot be carried out the same day, storing at 4 °C (up to 5 days) or freezing at −25 °C is necessary. In

order to avoid microbial transformation of soil N, slow thawing of frozen samples should be avoided. Homogenized and frozen samples stored in plastic bags can be reduced to small pieces by slamming the frozen plastic bags on a hard pad. A separate part of the homogenized sample is used for the determination of the water content (see Sect. 2.1).

Procedure

1. Weigh 10.00 g of the laboratory sample in a polyethylene bottle.
2. Add 100 mL of extraction solution at a temperature of 20 °C and shake mechanically for 2 h. Perform a blank test by adding only the extraction solution to the polyethylene bottle.
3. Decant the required quantity of the extract suspension into centrifuge tubes and centrifuge for 10 min at about 3,000 *g*.
4. Decant the supernatant solution in measuring cups and measure the contents of nitrate, nitrite (if necessary), and ammonium as described below (see Sects. 2.8.2, 2.8.3).

Notes and Points to Watch

- In order to avoid microbial transformation of N, sampling and storage conditions described above have to be observed strictly. For the handling of large sample series, quick drying at 105 °C or air drying is used. In both cases microbial transformations cannot be avoided totally. Furthermore, drying at high temperatures increases the ammonium content of soil samples significantly (VDLUFA 2000).
- Sampling of arable soils should be carried out in early spring. At this time inorganic N mainly exists as nitrate.
- If the homogenization of samples is difficult, larger sample weights (up to 150 g) are recommended (VDLUFA 2000). In any case the soil solution ratio shall be kept at 1:10 (m/v).
- The soluble N fractions should be measured immediately if possible, but not later than 1 day after the extraction. If this is not possible, the extracts should be stored in a refrigerator at a temperature not exceeding 4 °C for a maximum of 1 week.

2.8.2 Quantification of Nitrate Nitrogen

Principle. In an SFA system, the sample is first subjected to dialysis. Nitrate and nitrite ions of the samples pass through the membrane. The nitrate

is then reduced to nitrite by means of cadmium. Next a-naphthylethylenediamine dihydrochloride and sulfanilamide are added, so that a red-colored diazo compound is formed in the acidic medium. Its absorbance is measured at a wavelength of 543 nm.

Equipment

- SFA system consisting of a sampler, pump, dialysis unit, reduction column, nitrate unit, photometer, and recorder
- Cd/Cu reduction tube consisting of U-shaped glass tubing about 15 cm long, with internal diameter of 2 mm, and provided with ferrule for connection to the SFA tubing (may be purchased from the SFA system manufacturer)

Reagents

- Wetting agent, polyoxyethylene lauryl ether (30%).
- Buffer solution: dissolve 25 g of NH_4Cl in water, add 12.5 mL of NH_4OH solution (3%) and 1 mL of wetting agent. Make up to 1 L with water and mix.
- Cd/Cu reducing agent: swirl approx. 5 g of cadmium powder (particle size 0.3–0.8 mm) for 1 min with about 30 mL of HCl (1 mol/L). Wash with water until acid free. Then add about 50 mL of a $CuSO_4$ solution (20 g/L) and swirl for 3 min. Wash at least ten times with water to remove any flocculated copper. Store the Cd/Cu reducing agent in a dark place.
- Cd/Cu reduction tube: fill the U-shaped column with buffer solution, taking care not to introduce air bubbles. Introduce the activated cadmium powder with the aid of a funnel on both sides of the column. Apply vibration now and then to pack the powder. Fill the column up to 5 mm from the top and seal the ends with small plugs of glass wool. The column is now ready for use and can be placed in the SFA system.
- Color reagent: in a 1 L volumetric flask, add 150 mL of conc. H_3PO_4 (85%) to 0.5 L of water. Add 0.5 g of α-naphthylethylenediamine dihydrochloride ($C_{12}H_{16}N_2Cl_2$) and swirl until dissolved. Then dissolve 10 g of sulfanilamide ($C_6H_8N_2S$) in this mixture and fill up to the mark with water.

Procedure

The analysis of nitrate nitrogen in calcium chloride soil extracts is carried out with the SFA system. Details are given in the manufacturer's manual.

2.8.3 Quantification of Ammonium Nitrogen

Principle. In an SFA system, the sample is first subjected to dialysis. The determination of ammonium is based on the Berthelot reaction, in which a phenol derivative (here salicylate) forms an indophenol in the presence of ammonia and hypochlorite under the catalytic action of sodium nitroferricyanide (nitroprusside). In alkaline medium, the indophenol thus formed has a green-blue color, the absorbance of which is measured at a wavelength of 660 nm.

Reagents

- Buffer solution, pH 5.2: dissolve 24 g of sodium citrate ($Na_3C_6H_5O_7$) and 33 g of sodium potassium tartrate ($KNaC_4H_4O_6$) and make up to 1 L with water. Add 1 mL of wetting agent (30% polyoxyethylene lauryl ether.
- Color reagent: in a 1 L volumetric flask containing about 800 mL of water, dissolve 80 g of sodium salicylate ($C_7H_5O_3Na$) and 25 g of NaOH. Fill up to the mark with water.
- Nitroferricyanide (nitroprusside) solution: dissolve 1 g of sodium nitroferricyanide dihydrate ($Na_2[Fe(CN)_5NO] \times 2H_2O$) in 1 L of water.
- Isocyanurate solution: Dissolve 2 g of sodium dichloroisocyanurate ($Cl_2C_3N_3NaO_3 \times 2H_2O$) and 25 g of NaOH in 1 L of water.

Procedure

The analysis of ammonium nitrogen in calcium chloride soil extracts is carried out with the SFA system. Details are given in the manufacturer's manual.

Calculation

The content of the different N fractions in the soil material (w_N), expressed in mg/kg, is calculated using the following equation:

$$w_N = \frac{(a - b) \times 10 \times (100 + w)}{100} \tag{2.32}$$

a content of NO_3-N and NH_4-N in the soil extract (mg/L)

b content of NO_3-N and NH_4-N in the blank extract (mg/L)

w percentage of water content (m/m) on the basis of the air-dried soil (Sect. 2.1)

2.9
Soil Nutrients: Phosphorus

Introduction

Objectives. Soil phosphorus is, besides nitrogen, potassium, calcium, and magnesium, a main nutrient for soil organisms and plants. It exists in inorganic and organic fractions with varying percentages between 5 and 95%. The soil organic P fraction may be derived from plant residues, soil flora, and soil fauna tissues and residues that resist rapid hydrolysis (Kuo 1996). Inorganic fractions consist of Ca-, Al-, and Fe-phosphates. The most prominent phosphate mineral in soils is apatite ($Ca_5(PO_4)_3OH$). The total concentration in soil is generally in the range from 200 to 800 mg/kg. A considerable amount of P is also bound in the amorphous mineral fraction. Part of this is specifically adsorbed on surfaces of iron and aluminum oxides. Only a small part appears in a soil solution (< 0.1 mg/L in unfertilized soils and subsoils, 0.1–5 mg/L in Ap-horizons of arable soils (Scheffer 2002).

Soil microbes are involved in the mineralization of P from organic debris (Forster 1995c). Extracellular phosphatases are produced by microorganisms and roots and contribute to the mineralization of organic P. Deficiency of P may limit the growth of plants and the microbial decomposition of pollutants in soil. P is likely to be deficient in hydrocarbon-impacted soils and subsoils. Therefore, its concentration has to be analyzed and, if necessary, adjusted. Regarding available fractions of nutrients, optimum conditions are achieved at C:N:P ratios of 100:10:2 (Kästner 2001).

There are several methods available for the determination of total, inorganic, organic, and labile (plant available) P (VDLUFA 1991; Kuo 1996). Only a minor amount of soil P is available to plants. The estimation of plant available P is essential for optimum nutrient supply and can be applied within the scope of phytoremediation. Numerous tests have been developed to extract varying amounts of P, depending on the types of extractants used (e.g., $CaCl_2$, lactate, acetate, EDTA, ammonium bicarbonate; see also Kuo 1996). The test described here uses a sodium hydrogen carbonate solution. It is useful for both acid and calcareous soils and has been standardized (ISO 11263 1994). There are several methods for the quantification of P in soil extracts and soil solution, namely, spectophotometry (most common), ion chromatography, and inductively coupled plasma spectrometry (for details see Kuo 1996). The spectrophotometric method described below was developed by Murphy and Riley (1962).

Principle. Total phosphorus is extracted from finely ground soil with conc. sulfuric acid, hydrogen peroxide, and hydrofluoric acid (Bowman 1988). Labile P is extracted with a sodium hydrogen carbonate solution at pH 8.50.

Clear extracts are quantitatively analyzed for P by a spectrophotometric method involving the formation of an antimony–phosphate–molybdate complex reduced with ascorbic acid to form a deep-blue-colored complex.

Theory. Organic and non-silicate inorganic forms of P are dissolved in sulfuric acid and hydrogen peroxide. P in silicate lattice is released by the hydrofluoric acid treatment. Labile P is extracted using a $NaHCO_3$ solution. The OH^- and CO_3^{2-} ions in the $NaHCO_3$ solution decrease the concentration or activity of Ca^{2+} and Al^{3+} in the soil solution, resulting in an increased solubility of P. Quantification of P is based on the reaction of phosphoric acid with molybdate ions, which forms a heteropoly molybdophosphate complex:

$$H_3PO_4 + 12H_2MoO_4 \rightarrow H_3P(Mo_3O_{10})_4 + 12H_2O$$

The complex has a yellow color. In the presence of reducing agent such as ascorbic acid, the Mo in the complex is partially reduced from 6+ to 3+ and/or 5+, which results in a characteristic blue color.

2.9.1 Extraction of Total Phosphorus

Equipment

- Pebble mill for grinding sieved soil
- Analytical balance, accuracy 0.01 mg
- Hot plates or heated sand bath
- Teflon beakers, 100 mL

Reagents

- Conc. sulfuric acid (95–97%)
- Hydrogen peroxide (30%)
- Conc. hydrofluoric acid (40%)

Sample Preparation

Air-dried soil samples are sieved (2 mm) and ground to 0.1–0.15 mm using a pebble mill. The dry mass portion (percentage) is determined as described in Sect. 2.1.

Procedure

1. Weigh 0.5 g finely ground, well mixed soil into a 100 mL Teflon beaker. For high organic matter soils use 0.25 g.
2. Add 5 mL of conc. sulfuric acid and swirl gently.
3. Add 3 mL of 30% hydrogen peroxide in 0.5 mL portions.
4. Swirl vigorously (caution: foaming may lead to an overflow of samples high in organic matter).
5. When the reaction with hydrogen peroxide has subsided, add 1 mL of conc. hydrofluoric acid in 0.5 mL portions and swirl gently.
6. Place the beaker on a hot plate at 150 °C for 10–20 min to eliminate excess hydrogen peroxide.
7. After slight cooling, wash down the sides of the beaker with approx. 15 mL distilled water.
8. Mix and cool to room temperature.
9. Transfer the beakers content quantitatively to a 50 mL volumetric flask, passing it through a filter paper.
10. Make two additional washings of the beaker with 10 mL of distilled water, filter, and make up to volume.
11. Measure the P concentration of the extract using the molybdenum blue method described in Sect. 2.9.3.

Calculation

The total content of P (w_{PT}; mg/kg oven-dried soil) is calculated using the following equation:

$$w_{PT} = \frac{\varrho_P \times 50}{m} \times \frac{(100 + w_W)}{100} \tag{2.33}$$

ϱ_P concentration of P (mg/L) measured according to the method described in Sect. 2.9.3

m mass of air-dried soil (g)

w_W percentage of water content (mass fraction) on the basis of oven-dried soil (Sect. 2.1)

Notes and Points to Watch

- The extraction procedure described may be also used for determination of total contents of other elements (e.g., K, Ca, Mg, Al, Fe).

2.9.2 Extraction of Labile Phosphorus

Equipment

- Analytical balance, with a readability of ±0.01 g
- Shaker, end over end (30–35 rpm) or eccentric horizontal (140 thrusts per min)
- pH meter, with a readability of ±0. 01 pH units
- Fluted filters, free of P, fine to medium porosity
- Apparatus to filter extracts simultaneously

Reagents

- Sodium hydroxide solution (NaOH, 1 mol/L); store in an inert hermetically sealed bottle.
- Extracting solution: dissolve 42 ± 0.1 g of sodium hydrogen carbonate ($NaHCO_3$) in 800 mL of water. Adjust the pH to 8. 50 ± 0. 02 with sodium hydroxide solution. Make up the solution to 1 L with water.
- Carbon, activated, allowing the absorbance of the blank to be less than 0.015.

Sample Preparation

Soils samples are dried in the air, or in an oven at temperature not exceeding 40 °C, or freeze-dried (see Chapt. 1). Then samples are sieved (2 mm). The dry mass portion (percentage) is determined according to Sect. 2.1

Procedure

1. Weigh 5.00 ± 0.01 g of pretreated soil into a 250-mL flask.
2. Add 1.0 g of activated carbon and 100 ± 0.5 mL of extracting solution.
3. Stopper the flask and place it immediately on the shaker.

4. Shake for exactly 30 min at 20 ± 1 °C.
5. Filter immediately (within 1 min) into a dry vessel using a P free-fluted filter paper.
6. Prepare a blank by following the above procedure without soil.
7. Measure the P concentration of filtrates as given below.

Calculation

The content of phosphorous soluble in sodium hydrogen carbonate (w_P; mg/kg of oven-dried soil) is calculated using the following equation:

$$w_P = \varrho_P \times 20 \times \frac{100 + w_W}{100} \tag{2.34}$$

ϱ_P concentration of P (mg/L) as measured in the extract according to the method described in Sect. 2.9.3

20 quotient of the volume of extracting solution (100 mL) and the mass of air-dried soil (5 g)

w_W percentage of water content (mass fraction) on the basis of oven-dried soil (Sect. 2.1)

Notes and Points to Watch

- The extracting solution must be used within 4 h after preparation.
- A $NaHCO_3$ test level of 10 mg P/kg soil is considered to be in the "high" category (see Kuo 1996).

2.9.3 Quantification of Phosphorus

Equipment

- Analytical balance, with a readability of ±0.001 g
- Spectrophotometer, capable of measuring the absorbance in wavelength up to 900 nm and accepting cells of path 10 mm (readability 0.001 units of absorbance)
- Optical cells, of path length 10 mm
- Volumetric flasks, 50 mL

Reagents

- Sulfuric acid, (H_2SO_4 2.5 mol/L)
- Ammonium molybdate solution: dissolve 20 g of ammonium heptamolybdate tetrahydrate ($[(NH_4)_6Mo_7O_{24}\ 4H_2O]$) in 500 mL of deionized water. Store the solution in a glass-stoppered bottle.
- Antimony potassium tartrate solution (1 mg Sb/mL): dissolve 0.2728 g of $K(SbO)\ C_4H_4O_6 \times 1/2H_2O$ in 100 mL of deionized water.
- Ascorbic acid solution (0.1 mol/L): dissolve 1.76 g of $C_6H_8O_6$ in 100 mL of deionized water. Prepare the solution fresh daily.
- Mixed reagent: thoroughly mix 50 mL of H_2SO_4 (2.5 mol/L), 15 mL of ammonium molybdate solution, 30 mL of ascorbic acid solution, and 5 mL of antimony potassium tartrate solution. Prepare fresh daily.
- Phosphate stock solution (50 mg P/L): dissolve 0.2197 g of oven-dried (40 °C) KH_2PO_4 in deionized water. Add 25 mL of H_2SO_4 (2.5 mol/L) and dilute to 1 L with deionized water.
- Working phosphate standard solution (5 mg P/L): dilute 10 mL stock solution to 100 mL with deionized water.

Sample Preparation

Soil samples are extracted as described in Sects. 2.9.1 and 2.9.2.

Procedure

1. Transfer into 50 mL volumetric flasks:
   - A standard series, ranging from 0.5 mL up to 8 mL of the working phosphate standard solution, corresponding to P concentrations in the measuring solution between 0.05 and 0.8 mg/L.
   - An aliquot of 10 mL of the soil extract containing total phosphorus if the expected concentration of P in the soil is less than 400 mg/kg; otherwise use 5 mL or less.
   - An aliquot of 25 mL of the soil extract containing labile phosphorus.
2. Dilute the aliquots with deionized water to about 25 mL (if necessary), and add 8 mL of mixed reagent.
3. Dilute the solution to volume and mix well.
4. Measure the absorbance at 880 nm after 10 min in a spectrophotometer.
5. Prepare a blank that contains all reagents except the P solution.

Calculation

For the evaluation of the spectrophotometric measurements prepare a calibration graph by plotting absorbance units versus the P concentrations of standard solutions (mg P/L). The correlation between both parameters is linear in the relevant range up to 0.8 mg P/L.

The phosphorous concentration of the measuring solution (ϱ_P; mg/L) can be calculated using the following equation:

$$\varrho_P = \frac{\left(A_{ES} - A_B\right) \times 50}{f \times V_s} \tag{2.35}$$

A_{ES} absorbance of the soil extract

A_B absorbance of the blank

f slope of the regression line (absorbance per mg P/L)

V_s volume of the aliquot (mL)

50 volume of the volumetric flasks (mL)

References

Blake JR, Hartge KH (1986) Bulk density. In: Klute A (ed) Methods in soil analysis, part 1. Physical and mineralogical methods, 2nd ed, Am Soc Agron, Madison, WI, pp 363–376

Bowman RA (1988) A rapid method to determine phosphorus in soils. Soil Sci Soc Am J 52:1301–1304

Bremner JM (1996) Nitrogen – total. In: Bigham JM (ed) Methods of soil analysis, part 3, chemical methods. Soil Sci Soc Am Am Soc Agron, SSSA Book, Series no 5, Madison, WI, pp 1085–1121

Danielson RE, Sutherland PL (1986) Porosity. In: Klute A (ed) Methods in soil analysis, part 1, physical and mineralogical methods, 2nd edn. Soil Sci Soc Am Am Soc Agron, Madison WI, pp 443–461

DIN 19684–3 (1977) Bodenuntersuchungsverfahren für den Landwirschaftlichen Wasserbau – Chemische Laboruntersuchungen – Teil 3: Bestimmung des Glühverlustes und des Glührückstandes

Forster JC (1995a) Soil sampling, handling, storage and analysis – organic carbon – In: Alef K, Nannipieri P (eds) Methods in applied soil microbiology and biochemistry. Academic Press, pp 59–65

Forster JC (1995b) Soil sampling, handling, storage and analysis – soil nitrogen. In: Alef K, Nannipieri P (eds) Methods in applied soil microbiology and biochemistry. Academic Press, pp 79–87

Forster JC (1995c) Soil sampling, handling, storage and analysis – soil phosphorus. In: Alef K, Nannipieri P (eds) Methods in applied soil microbiology and biochemistry. Academic Press, pp 88–93

Hartge KH (1968) Heterogenität des Bodens oder Quellung? Trans Int Congr Soil Sci 3:591–597

ISO 10693 (1995) Soil Quality – Determination of carbonate content – volumetric method
ISO 10694 (1995) Soil Quality – Determination of organic and total carbon after dry combustion (elementary analysis)
ISO 11261 (1995) Soil Quality – Determination of total nitrogen – Modified Kjeldahl method
ISO 11263 (1994) Soil Quality – Determination of phosphorous – spectrometric determination of phosphorous soluble in sodium hydrogen carbonate solution
ISO 11266 (1994) Soil Quality – Guidance on laboratory testing for biodegradation of organic chemicals in soil under aerobic conditions
ISO 11272 (1998) Soil Quality – Determination of dry bulk density
ISO 11274 (1998) Soil Quality – Determination of the water-retention characteristic – Laboratory methods
ISO 11465 (1993) Soil Quality – Determination of dry matter and water content on a mass basis – Gravimetric method
ISO 13878 (1998) Soil Quality – Determination of total nitrogen content by dry combustion ("elemental analysis")
ISO 14238 (1997) Soil quality – Biological methods – Determination of nitrogen mineralization and nitrification in soils and the influence of chemicals on these processes
ISO 14255 (1998) Soil Quality – Determination of nitrate nitrogen, ammonium nitrogen and total soluble nitrogen in air-dry soils using calcium chloride solution as extractant
ISO 16072 (2002) Soil quality – Laboratory methods for determination of microbial soil respiration
ISO 3696 (1987) Water for analytical laboratory use – Specifications and test methods
ISO DIS 10390 (2002) Soil Quality – Determination of pH
ISO/TS 14256–1 (2003) Soil Quality – Determination of nitrate, nitrite and ammonium in field moist soils by extraction with potassium chloride solution – Part 1: Manual method
Kästner M (2001) Parameter und Methoden zur Beurteilung der biologischen Sanierbarkeit von Böden. In: Michels J, Track Th, Gehrke U, Sell D (eds) Leitfaden, Biologische Verfahren zur Bodensanierung. DECHEMA Gesellschaft für Chemische Technik und Biotechnologie eV, Bericht im Auftrag des BMBF Projektträger Abfallwirtschaft und Altlastensanierung im Umweltbundesamt Berlin. Förderkennzeichen 1491064, pp 191–234
Kuo S (1996) Phosphorous. In: Bigham JM (ed) Methods of soil analysis, part 3, chemical methods. Soil Sci Soc Am Am Soc Agron, SSSA Book, Series no 5, Madison WI, pp 869–919
In: Bigham JM (ed) Methods of soil analysis, part 3, chemical methods. Soil Sci Soc Am Am Soc Agron, SSSA Book, Series no 5, Madison WI, pp 1123–1184
Li C, Fan X, Mengel K (1990) Turnover of interlayer ammonium in loess-derived soil grown with winter wheat in the Shaanxi Province of China. Biol Fert Soils 9:211–214
Mulvaney RL (1996) Nitrogen – inorganic forms. In: Bigham JM (ed) Methods of soil analysis, part 3, chemical methods. Soil Sci Soc Am Am Soc Agron, SSSA Book, Series no 5, Madison WI, pp 1123–1184
Murphy J, Riley HP (1962) A modified single solution method for the determination of phosphate in natural waters. Anal Chim Acta 27:31–36
Nelson, DW, Sommer LE (1996) Total carbon, organic carbon, and organic matter. In: Bigham JM (ed) Methods of soil analysis, part 3, chemical methods. Soil Sci Soc Am Am Soc Agron, SSSA Book, Series no 5, Madison WI, pp 961–1010
Page AL, Miller RH, Keeney DR (eds, 1982) Methods in soil analysis, part 2, chemical and microbiological properties. Soil Sci Soc Am Am Soc Agron, SSSA Book, Madison WI
Sahrawat KL (1995) Fixed ammonium and carbon-nitrogen ratios of some semi-arid tropical Indian soils. Geoderma 68:219–224

Scheffer F (2002) Lehrbuch der Bodenkunde, Scheffer/Schachtschabel. 15. Auflage, neubearbeitet und erweitert von Blume HP et al. Akademischer Verlag GmbH, Heidelberg
Schlichting E, Blume H-P, Stahr K (1995) Bodenkundliches Praktikum, 2nd ed, Blackwell, Berlin Wien
Topp GC, Dow B, Edwards M, Gregorich EG, Curnoe WE, Cook EJ (2000) Oxygen measurements in the root zone facilitated by TRD. Can J Soil Sci 80:33–41
VDLUFA (2000) Methodenbuch I 3. Teilliefg. 2000, Nmin-Methode Labor A6.1.4.1, VDLUFA-Verlag Darmstadt

3 Quantification of Soil Contamination

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3.1 General Introduction

Even though better toxicity tests have become available and their use is increasing in risk assessment of contaminated sites and of the reuse of bioremediated soil, chemical data is still today the main information used in decision-making. The awareness of soil contamination increased in the 1980s and large-scale bioremediation became frequent during the 1990s. However, the development and standardization of reproducible chemical methods for the determination of specific organic contaminants in soils have been very slow (Karstensen et al. 1998). The methods for the determination of heavy metals have been in use longer, metal examination of soil samples having been performed for geological purposes, e.g., in the search for ore by the mining industry. The older methods for determination of oil and polyaromatic hydrocarbons (PAHs) were often based on extraction of dried and sieved samples (resembling the procedures for heavy metal pretreatment) followed by an extraction with non-polar solvents. The use of halogenated solvents such as CCl_4 and Freon (ISO/TR 11046 1994) were common, but due to occupational health and environmental aspects these are being phased out. Methods that are based on the extraction of field-moist samples with a mixture of polar (e.g., acetone, methanol) and non-polar (hexane, pentane, heptane) solvents have been proven to give sufficient yield and to be reproducible. Methods that are feasible and reproducible in the laboratory are currently being standardized by the International Standardization Organization (ISO).

The objectives of determining contaminant concentrations in soil may be to assess the appearance of particular contaminants at a site or to monitor the progress of a bioremediation action either in the field or in laboratory feasibility studies. The monitoring of bioremediation involves repeated measurements of contaminant concentrations over time. Based on a time series with, e.g., five points, a biodegradation rate can be obtained.

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The biodegradation rate can be linear or represent first order decay dependent on, e.g., the contaminant concentration and bioavailability. Field-scale bioremediation can be classified as either in situ methods, where the treatment takes place without excavating the soil, and ex situ methods, where excavated soil is treated typically in piles. When monitoring a site undergoing in situ treatment by drilling for subsurface samples, it is essential to remember that true replicate samples cannot be obtained and a large variation is to be expected. When sampling stock piles or biopiles, a combination sample consisting of subsamples from different places in the piles typically will be assembled, and parallel combination samples can be made (Jørgensen et al. 2000). When monitoring biodegradation by laboratory microcosms, it is of great importance that all the sample material representing a certain depth, treatment, etc., is homogenous. This is best ensured by homogenizing and sieving a larger batch from the field and by distributing this into separate parallel bottles or other containers for laboratory incubations. A mesh size of 8 mm has proven to be a good size for sieving field-moist soil (Laine and Jørgensen 1997; Salminen et al. 2004). However, the measurement of contaminant disappearance only shows that the parent compound has been transformed; it does not reveal whether the degradation is complete to CO_2 or CH_4 or if other degradation products are produced.

Contamination with petroleum hydrocarbon products is one of the most frequent types of soil contamination. Refineries, surface and underground storage tanks, petrol service stations, etc., are the most common sites for such contamination. Most petroleum products also contain minor amounts of PAHs. No single method is reliable for the determination of all petroleum hydrocarbons, and we therefore describe three methods for the determination of different fractions of hydrocarbons in soil samples.

Volatile hydrocarbons (Sect. 3.2) should be determined at sites where gasoline and jet fuel are the sources of contamination. The pertinent method here quantitatively determines these separate compounds: benzene, toluene, ethylbenzene and xylenes (BTEX compounds), naphthalene, and gasoline additives such as MTBE (methyl *tert*-butyl ether) and TAME (*tert*-amyl methyl ether). This method can also be used to determine halogenated volatiles, which may often be found together with fuel products because such solvents often are used, e.g., for cleaning engines.

Contamination with oil products such as heating oil, diesel or lubricating oil is best determined using the method (Sect. 3.3) for hydrocarbons in the range C_{10} to C_{40}. The result is a sum parameter, which does not give concentrations of specific compounds. But still the sum of the hundreds of compounds in this range is very useful for quantifying contamination with them and for monitoring bioremediation. Based on the chromatogram, a qualitative estimation of the type of contamination can be obtained. This $C_{10–40}$ parameter is often referred to as mineral oil or total petroleum

hydrocarbons (TPH), but these terms are somewhat unspecific. Crude oil is often determined with this method, but it also includes volatiles and PAHs that should be determined separately with the methods for volatiles and PAHs, respectively.

Contamination with PAHs is commonly found at gas works and at sites where coal tar and oil shale are handled. Oil containing heavy fractions or waste oil may also contain significant amounts of PAHs. The method described here (Sect. 3.4) allows for a single determination of 16 different PAH compounds. In the literature the sum of PAHs is often reported, but the fact that different countries and different laboratories analyze different number of compounds has made this term very unspecific. Guideline values for clean-up needs also differ between countries, so it is important to check which compounds require reports. Since the toxicities of the PAH compounds differ, there may not be any guideline value set out for all compounds.

Contamination with heavy metals is difficult to assess because clean soil itself may contain many heavy metals, depending on the geological structure. Furthermore, many metals are not necessarily bioavailable in soil, and for that reason different types of less exhaustive extractions are being developed to determine the bioavailable fractions. The background contents of metals in soil are in many countries known and they are taken into account when guideline values for clean-up are determined. Still today most guideline values are based on the total or near-total content of metals. The method described here (Sect. 3.5) reveals the near-total content and is aiming at determining the anthropogenic contamination.

3.2 Volatile Hydrocarbons

Introduction

Objectives. The volatile organic compounds (VOCs) in soils primarily originate from petroleum products and solvents. The spectra of the VOCs depend on their source. The analysis of benzene, toluene, and ethylbenzene and xylenes (BTEX) is widely used as an indicator of contamination with light petroleum products, e.g., petrol and kerosene. Furthermore, the gasoline additives MTBE and TAME as well as halogenated volatile compounds can be analyzed with this method.

Principle. A soil sample is extracted with methanol. A defined volume of the methanol extract is transferred into water and the water sample is heated to 80 °C in a headspace vial. When equilibrium is established between the gaseous and liquid phases, an aliquot of the gaseous phase is injected on

a column of a gas chromatograph and the VOCs are determined with a mass selective detector.

Theory. VOCs are a group of compounds that have a boiling point from 20 to 220 °C and usually they have two to ten C atoms. They are mainly unsubstituted or substituted monoaromatics and short-chain aliphatic compounds that differ in solubility and in toxicity. The individual compounds are quantitatively determined using this method, as can also be the diaromatic compound naphthalene. We do not recommend measuring the sum of VOCs because such a sum is unspecific and depends on the compounds included.

The sampling (ISO 10381–1 1994; ISO 10381–2 1994; Owen and Whittle 2003) is a crucial step in the analysis of VOCs. In order to prevent their loss during preparative steps, field-moist samples are used (ISO 14507 2003). The sample is added into a pre-weighed glass container containing a known amount of methanol. To control the quality of the determination, field duplicates, a procedural blank, and a control sample are analyzed. The two main methods of analysis of VOCs are static headspace/gas chromatography (e.g., ISO/PRF 22155 in prep.) and purge and trap/gas chromatography (e.g., ISO 15009 2002). In the analysis of volatile aliphatic and aromatic hydrocarbons a mass selective detector (MSD) is used. VOCs can also be detected with a photo ionization detector (PID), a flame ionization detector (FID), and an electron capture detector (ECD; Owen and Whittle 2003). The identification of target compounds (ISO/DIS 22892 in prep.) is easy with a MSD, and a possible matrix effect can be eliminated. The method described here is that using static headspace/gas chromatography (MSD) and is based on the proof of a new international standard ISO/PRF 22155 and has earlier been described by Salminen et al. (2004).

Equipment

- Usual laboratory glassware, free of interfering compounds
- Shaking machine
- Headspace analyzer and gas chromatograph with a mass selective detector (MSD)
  - Oven temperature program: maintain 35 °C for 2 min, then steadily raise by 14 °C/min up to 90 °C. Maintain 90 °C for 5 min, then raise by 12 °C/min up to 190 °C. Maintain 190 °C for 1 min, then raise by 40 °C/min up to 225 °C, and maintain at 225 °C for 1 min.
  - Carrier gas: helium.
  - Gas flow: 10 mL/min.
  - Split ratio (gas flow rate through split exit: column flow rate): 5.7:1.

- Column: stationary phase non-polar or low polar fused silica capillary column; film thickness 1.4 µm; column length 30 m; internal diameter 0.25 mm

Reagents

- Methanol
- Internal standards, e.g., toluene-d_8, α,α,α-trifluorotoluene
- Helium
- Synthetic air
- Volatile aromatic and halogenated hydrocarbons for standard solutions: MTBE, TAME, benzene, ethylbenzene, toluene, m-xylene, p-xylene, o-xylene, styrene, naphthalene, dichloromethane, chloroform, carbon tetrachloride, 1,2-dichloroethane, 1,1,1-trichloroethane, cis-1,2-dichloroethene, trichloroethene, tetrachloroethene, chlorobenzene, 1,2-dichlorobenzene, 1,4-dichlorobenzene, 1,2,3-trichlorobenzene, 1,2,4-trichlorobenzene, 1,3,5-trichlorobenzene
- Standard stock solutions
  - Standard solutions: for each analyte, 10 mg/mL of methanol
  - Internal standard (see above) solution, 10 mg/mL of methanol
- Working standard solutions
  - Standard solutions: 1 mg mixed analyte solution/mL of methanol
  - Internal standard (see above) solution, 10 µg/mL of methanol
- Calibration solutions: at least five different concentrations by suitable dilutions of the working standard solutions within the range of 0.05–10 µg/L

Sample Preparation

In the field, approximately 20 g of field-moist soil sample is taken directly into a pre-weighed headspace vial containing 20 mL of methanol. No sieving of the samples is recommended. A separate sample is taken for dry mass determination in a glass jar leaving no headspace.

Procedure

1. Weigh the vial containing the soil sample and methanol.
2. Shake the vial containing sample and methanol for 30 min with the shaking machine.
3. Allow the vial to stand for 10–15 min to settle the solid material.

4. Pipette 10 mL of water, 100 µL of methanol extract, and 5 µL of the working internal standard solution into a headspace vial.
5. Place the vial in the headspace system and heat the sample at 80 °C for 1 h.
6. Use headspace injection for gas chromatographic analysis.
7. Detect the compounds with the mass selective detector (MSD).
8. Identify the peaks of the internal standards by using the absolute retention times.
9. Determine the relative retention times for all the other relevant peaks in the gas chromatogram. These retention times should be determined in relation to those of the internal standards.
10. Determine the dry mass content, e.g., by using the method described in ISO 11465 (Chapt. 2)
11. Calculate the concentrations of the analytes.

To prepare a calibration curve, treat the calibration standards as the soil samples:

1. Add 100 µL of calibration solution to a headspace vial containing 10 mL of water.
2. Add a known amount of working internal standard solution into the vial.
3. Close the vial and treat it according to the procedure.

Quality Control

1. Procedural blank determination: add 100 µL of methanol and 5 µL of the working internal standard solution to 10 mL of water. Treat this mixture as the soil sample.
2. Control sample determination: add a known amount of working standard solution to a pristine soil sample that contains neither VOCs nor methanol. Treat the control sample as the soil sample and calculate the recovery (%) of the analytes. Mark the recovery on the quality-control chart.

Calculation

Concentration of analytes is quantified with respect to the internal standard using the following formula:

$$c_{\mathrm{m,i}} = \frac{c_{\mathrm{iw}} \times V_{\mathrm{te}} \times V_{\mathrm{w}}}{m_{\mathrm{dm}} \times V_{\mathrm{a}}} \tag{3.1}$$

$c_{m,i}$ content of the analyte "i" in the sample (mg/kg soil dry mass)

c_{iw} mass concentration of the analyte "i" in the spiked water sample obtained from the calibration curve (µg/L)

V_{te} total volume of the extract (methanol added to the soil sample + water in the sample obtained from the determination of dry mass content; mL)

V_w volume of the spiked water sample for headspace measurement (mL)

m_{dm} dry mass of the test sample used for extraction (g)

V_a volume of the aliquot of methanol extract used for the spiking of water sample for headspace measurement (µL)

Notes and Points to Watch

- Assure that compounds do not evaporate during sample handling.
- Exposure of samples to air, even during sampling, shall be avoided as far as possible.
- The use of plastics, other than PTFE, shall be avoided.
- Samples shall be analyzed as soon as possible.
- Store the samples in the dark at 4 ± 2 °C no longer than 4 days.
- The standard and calibration solutions can be stored for 1 year at −18 °C.
- The internal standard solutions can be stored for several years at −18 °C.
- Avoid direct skin contact and inhalation of vapors from standards and samples.

3.3 Hydrocarbons in the Range C_{10} to C_{40}

Introduction

Objectives. Petroleum derivatives such as diesel fuel, heating oil, and lubrication oil are widely used in human activities and thus are common pollutants in the soil environment. These petroleum products are complex mixtures of hundreds of various hydrocarbons. The analytical method described here (modified ISO 16703 2004 Salminen et al. 2004) allows a quantitative and a composition pattern determination of all hydrocar-

bons (that is, *n*-alkanes from $C_{11}H_{22}$ to $C_{39}H_{80}$, isoalkanes, cycloalkanes, alkyl benzenes, and alkyl naphthalenes) with a boiling range of 196 to 518 °C. Gasolines cannot be quantified using this method. Furthermore, high concentrations of polyaromatic hydrocarbons (PAHs) may interfere with the analysis.

Principle. A soil sample is extracted by sonication with *n*-heptane-acetone including the internal standards (*n*-decane and *n*-tetracontane). To separate the organic phase, water is subsequently added. The extract is washed with water and the polar constituents and water are removed from the extract with Florisil (U.S. Silica Co., Berkeley Springs WV, USA) and sodium sulfate, respectively. Hydrocarbons in the range from C_{10} to C_{40} are determined from an aliquot of the purified extract with a gas chromatograph equipped with a flame ionization detector (FID). For the quantification of all the hydrocarbons in this range, the total peak area between the internal standards *n*-decane and *n*-tetracontane is measured.

Theory. Petroleum derivatives are complex mixtures of various hydrocarbons with different characteristics (e.g., volatility, water solubility, biodegradability). In the assessment of petroleum hydrocarbon contamination and the effects of microbial activity (past, present, or future) on the fate of these contaminants in soil, it is essential to know the quantity and the composition of the contaminating agents. This information is of high value when, for instance, a bioremediation process is followed over a span of time. Moreover, as hydrocarbons differ in their amenability to microbial degradation, this information is of a remarkable value.

In the past, gravimetric or infrared spectrometric methods have been extensively used for the determination of hydrocarbons in soil. While these methods can be used for quantification of a range of hydrocarbons, they do not provide any information of the their quality, that is, of their compound composition pattern. To obtain this information, more sophisticated methods such as gas chromatographic analyses, are employed.

The extraction of hydrocarbons shall be performed in such a manner that the broad spectrum of the compounds of interest is included in the analysis. Moreover, it is essential that the extraction procedure is suitable for field-moist soil samples in which hydrocarbons may be attached to soil particles, and in which soil water present in the samples may impede the extraction of the non-polar hydrocarbons. Thus, a mixture of polar (acetone) and non-polar (*n*-heptane) solvents is used. On the other hand, polar compounds have to be removed from the extract as they interfere with the gas chromatographic analysis, and to avoid the inclusion of polar compounds other than petroleum hydrocarbons in the analysis. It is to be noted that PAHs and volatile compounds have to be analyzed separately.

Equipment

- Usual laboratory glassware free of interfering compounds
- Sonicator
- Laboratory centrifuge
- Gas chromatograph (GC) with a non-discriminating injection system and a flame ionization detector (FID), helium as a carrier gas
- Pre-column (in case on-column injection is used)
- Capillary column specifications: 5% phenyl polysilphenylene-siloxane stationary phase, e.g., SGE BPX5 capillary column, 5 m length and 1.4-μm film thickness

Reagents

- *n*-Heptane
- Acetone
- Ion-exchanged water
- *n*-Decane ($C_{10}H_{22}$), *n*-eicosane ($C_{20}H_{42}$), *n*-triacontane ($C_{30}H_{62}$), *n*-pentatriacontane ($C_{35}H_{72}$), and *n*-tetracontane ($C_{40}H_{82}$) – *n*-decane and *n*-tetracontane being used as the integration window and the latter also as an internal standard
- Florisil (150–250 μm, 60–100 mesh) (Activated Florisil is stored in a desiccator and is usable for a week after the activation. Note: the activity of Florisil will gradually decrease after the activation.)
- Anhydrous sodium sulfate (Na_2SO_4) must be kept at 550 °C for at least 2 h prior to its use
- Diesel fuel and lubrication oil standards free of additives
- Helium
- Hydrogen
- Synthetic air
- Control soil sample
- Standard stock solutions
  - Standard extraction solution (0.15 mg/mL of $C_{10}H_{22}$ and 0.20 mg/mL of $C_{40}H_{82}$): weigh 20 μL of *n*-decane and 20 mg of *n*-tetracontane and dissolve in 100 mL of *n*-heptane. Prepare the solution in a volumetric

flask by weighing and calculate the accurate concentrations of the internal standards *n*-decane and *n*-tetracontane in the solution. Store the solution at 4 °C in the dark. The solution is usable for at least 6 months if stored in a tightly closed (Teflon-capped) glass vial.

- Working standard extraction solution: dilute the standard extraction solution 1:9 (v/v) in *n*-heptane. Prepare the solution in a volumetric flask by weighing and calculate the accurate concentrations of the internal standards *n*-decane and *n*-tetracontane in the solution. The solution is usable for 1 week if stored in a tightly closed (Teflon-capped) glass vial.
- Calibration stock solution (20 mg hydrocarbons/mL): Weigh 100 mg of diesel fuel and 100 mg of lubrication oil and dissolve in 10 mL of *n*-heptane. Prepare the solution in a volumetric flask by weighing and store the solution at 4 °C in the dark. The solution is usable for at least 6 months if stored in a tightly closed (Teflon-capped) glass vial.
- Working calibration solutions: prepare at least five solutions with final hydrocarbon concentration ranging from 0.1 to 2–3 mg/mL. Prepare the solution by diluting the calibration stock solution with *n*-heptane to obtain a final volume of 10 mL. Weigh the amounts of solutions used to calculate the exact hydrocarbon concentrations in the working calibration solutions. The solution is usable for at least 6 months if stored in a tightly closed (Teflon-capped) glass vial.
- Stock solution for testing the performance of the gas chromatograph: weigh 5.0 mg each of *n*-decane ($C_{10}H_{22}$), *n*-eicosane ($C_{20}H_{42}$), *n*-triacontane ($C_{30}H_{62}$), *n*-pentatriacontane ($C_{35}H_{72}$), and *n*-tetracontane ($C_{40}H_{82}$) and dissolve them in 10 mL of heptane. Prepare the solution in a volumetric flask by weighing the mass of the added heptane to calculate the exact concentration of the individual *n*-alkanes in the solution. Store the solution at 4 °C in the dark. The solution is usable for at least 6 months if stored in a tightly closed (Teflon capped) glass vial.
- Working solution for testing the performance of the gas chromatograph: dilute the test stock solution in *n*-heptane in a ratio of 1:9 (v/v). Prepare the solution in a volumetric flask by weighing to calculate the exact concentration of the individual *n*-alkanes in the solution.

Sample Preparation

Sampling should be performed according to good practices (ISO 10381–1 1994; ISO 10381–2 1994). For the analysis, a homogenized field-moist soil

sample is used (ISO 14507 2003). However, if the water content of the sample is extraordinarily high, separation of the organic phase may occur prior to the extraction (that is, at the time of the introduction of the sample into the extraction solution). In such case, the sample has to be pre-dried overnight at room temperature prior to the extraction.

Procedure

Prior to Analysis

1. Calibrate the gas chromatograph by running aliquots of the working standard solutions.
2. An aliquot of the working test solution should be run on the GC and the yields of the individual *n*-alkanes calculated. The ratio between $C_{20}H_{42}$ and $C_{40}H_{82}$ should not exceed 1.2.

Analytical Procedure

1. Weigh 10 g of a sample into an extraction vial.
2. Weigh 5–10 g of a control sample with a known concentration into a separate vial.
3. Add 10 mL of working standard solution and 20 mL of acetone into each of these vials.
4. Prepare a blank determination: add 10 mL of working standard solution and 20 mL of acetone but omit the sample. The blank and the control sample are treated in a similar manner to the (unknown) samples.
5. Mix the samples gently and sonicate for 30 min. Add ice into the sonicator to keep the samples cool.
6. Add 30 mL of water and shake for 1 min.
7. Centrifuge the samples (2,500 rpm, 5 min).
8. Transfer the organic phase into a 25-mL test tube with a Teflon-lined screw cap, add 10 mL of water, and shake for 1 min.
9. Transfer the organic phase into another test tube with a Teflon-lined screw cap and add approx. 0.5 g of Na_2SO_4 and shake.
10. Add approx. 1.5 g of Florisil into the tube and shake for 10 min in a mechanical shaker.
11. Centrifuge the tubes (2,000 rpm, 1 min).
12. Transfer an aliquot of the purified extract into a GC vial. Avoid the introduction of Florisil into the GC vial.

13. Run all the samples by GC.
14. Solvent blank should be subtracted from the sample chromatogram. Integrate the total area between the peaks of $C_{10}H_{22}$ and $C_{40}H_{82}$ to obtain the hydrocarbon concentration of the extracts from the calibration extracts.
15. Integrate the total area of the $C_{40}H_{82}$ peak to obtain the recovery of $C_{40}H_{82}$ in the analysis.

Quality Assurance

1. The hydrocarbon concentration in the blank extract should be below 0.025 mg/mL.
2. The recovery of the internal standard *n*-tetracontane should be calculated in each extract. The yield should be 100 ± 20% of the theoretical value of $C_{40}H_{82}$ in the extraction solution.
3. The hydrocarbon content of the control soil sample should be monitored over time and the results ought to be analyzed according to general good quality procedures.

Calculation

The concentration of hydrocarbons in the range from $C_{10}H_{22}$ to $C_{40}H_{82}$ (c_{HC}) in the sample is calculated as follows:

$$c_{HC} = \frac{c_{gc} \times 10 \times 1000 \times f}{m \times d_s} \tag{3.2}$$

c_{HC} concentration of hydrocarbons in the range from $C_{10}H_{22}$ to $C_{40}H_{82}$ in the sample (mg/kg dry mass)

c_{gc} hydrocarbon concentration of the extract calculated from the calibration equation (mg/mL)

10 volume of the organic solvent used in the extraction (10 mL of heptane)

1,000 conversion factor of the soil mass (1 kg = 1,000 g)

f dilution factor (if applicable)

m wet mass of the sample (g)

d_s content of dry substance in the field-moist sample (g/g), determined according to ISO 11465 (1993)

Notes and Points to Watch

- The samples should be analyzed as soon as possible. If this is not feasible, the samples should be stored at −20 °C.
- Hydrocarbons are subjected to biodegradation both under aerobic and anaerobic conditions. Therefore, storage of the samples at temperatures above 0 °C should be avoided (Salminen et al. 2004).
- The efficacy of each Florisil stock has to be tested prior to its use in the analysis.
- Weighing of the liquid, viscous standard compounds gives very precise solutions.
- Avoid skin contact and inhalation of vapors from standards and samples.

3.4 Polyaromatic Hydrocarbons (PAHs)

Introduction

Objectives. Polycyclic aromatic hydrocarbons (PAHs) are often found at contaminated sites, particularly in connection with tar contamination at former gasworks. They also exist as diffuse contamination in urban areas and alongside roads. Furthermore, wood impregnation with creosote and incomplete combustion of hydrocarbons are major sources of PAHs in soil. Polyaromatic hydrocarbons are a group of more than 100 different compounds. This method describes the determination of a small selection of the many PAHs found in the environment. The US Environmental Protection Agency (EPA) has chosen 16 of these PAHs to be the most important ones to be analyzed (EPA priority list 1982).

Principle. A field-moist sample is extracted twice with acetone, and then hexane is added to the acetone extract. The extract is washed twice with water and the organic layer is dried with anhydrous sodium sulfate. When necessary, the extract is cleaned up by adsorption chromatography on a silica gel. The (purified) extract is analyzed by capillary gas chromatography with mass selective detection, using appropriate deuterated PAHs as internal standards.

Theory. Polycyclic aromatic hydrocarbons occur ubiquitously in the environment. Sixteen PAHs (Table 3.1) were chosen by the US EPA to be analyzed in environmental samples because they are the most abundant at hazardous waste sites and more information is available on these than on other PAHs. Moreover, the chosen compounds exhibit harmful effects that

Table 3.1. Native and deuterated PAHs with their specific ions (target ion with qualifier ion in parentheses)

Native PAH	Mass number (amu)	Deuterated PAH	Mass number (amu)
Naphthalene	128 (129)	D_8-Naphthalene	136
Acenaphthene	154 (153)	D_{10}-Acenaphthene	164
Acenaphthylene	152 (151)		
Fluorene	166 (165)		
Anthracene	178 (89)	D_{10}-phenanthrene	188
Phenanthrene	178 (179)		
Fluoranthene	202 (101)		
Pyrene	202 (101)		
Benz(a)anthracene	228 (114)	D_{12}-chrysene	240
Chrysene	228 (114)		
Benzo(b)fluoranthene	252 (253)	D_{12}-perylene	264
Benzo(k)fluoranthene	252 (253)		
Benzo(a)pyrene	252 (253)		
Indeno(1,2,3-cd) pyrene	276 (138)		
Dibenzo(ah)anthracene	278 (139)		
Benzo(ghi)perylene	276 (138)		

are representative of PAHs and exposure to these is more frequent than that to other PAHs.

The most common analytical methods are based on liquid chromatography with fluorescence detection or UV detection (HPLC/FL or UV), or on gas chromatography with mass selective detection (GC/MSD). Both techniques have their benefits. Generally, HPLC has less resolution, which is problematic when the studied samples contain complex PAH mixtures. On the other hand, the UV and the fluorescence detection are highly sensitive and specific. Mass spectrometry is a powerful tool for identifying individual compounds. The sensitivity of the GC/MSD can be increased if the mass spectrometer is operated in selected ion monitoring (SIM) mode. In the literature, there are numerous applications available for analyzing PAHs that differ as to, e.g., extraction techniques, solvents used, and clean-up methods. The method presented here is based on GC/MSD technology and on the draft international standard method (ISO/DIS 18287 in prep.), but uses hexane instead of petroleum ether as the solvent. The method is relatively fast and is applicable to all types of soils, covering a wide range of PAH contamination. With this method, other PAHs than those in the Table 3.1

can be determined as well. A detection limit of 0.01 mg/kg dry mass can be ensured for each PAH.

Equipment

- Usual laboratory glassware free of interfering compounds
- Shaking machine
- Laboratory centrifuge
- Gas chromatograph (GC) with a mass selective detector (MSD)
  - Oven temperature program: maintain 60 °C for 2 min, then steadily raise by 20 °C/min up to 180 °C, then raise by 8 °C/min up to 280 °C and keep at that temperature for 10 min.
  - Splitless injection (split closed for 2 min) of 1 µL.
  - Carrier gas: helium 1 mL/min.
- Capillary column specifications: medium polar stationary phase, e.g., HP-5MS, film thickness 0.25 µm, length 30 m, internal diameter 0.25 mm.

Reagents

- Acetone
- *n*-Hexane
- Ion-exchanged water
- Anhydrous sodium sulfate (Na_2SO_4), must be kept at 550 °C for at least 2 h prior to its use
- Silica gel 60 (particle size 60–200 µm), deactivated (Heat silica gel 60 for 5 h at 130 °C in a drying oven. Allow to cool down in a desiccator and add 10% water (w/w) in a flask. Shake for 5 min by hand until all lumps have disappeared, and then shake for 2 h in a shaking machine. Store deactivated silica gel in absence of air. It can be used for 1 week.)
- Helium
- Nitrogen
- Quality control soil sample (e.g., certified reference material or in-house reference material)
- Calibration stock solutions
  - Native PAHs (PAHs to be determined): commercially available certified standard stock solution can be used with a concentration of approx. 100 µg/mL for each native PAH (e.g., Dr. Ehrenstorfer PAH Mix 9, X20950900CY).

  - Deuterated PAHs (internal standards): commercially available certified standard stock solution can be used with a concentration of approx. 1,000 µg/mL for each deuterated PAH (e.g., Dr. Ehrenstorfer PAH Mix 31, YA20953100TO). It is recommended that at least five deuterated PAHs be used as internal standards. The internal standards are chosen to resemble the physical and chemical properties of the compounds to be analyzed (see Table 3.1).

- Calibration working solution
  - Native PAHs: transfer 5 mL of the calibration stock solution containing the native PAHs stock solution into a 25-mL volumetric flask and fill up to the mark with hexane (20 µg/mL)
  - Deuterated PAHs: transfer 1 mL of the calibration stock solution containing the deuterated PAHs stock solution to a 25-mL volumetric flask and fill up to the mark with hexane (40 µg/mL)
- Calibration standard solutions: prepare a series of calibration standards over a suitable range (e.g., 0.2–10 µg/mL) by transferring 0.1–5 mL of the native PAH calibration working solution into a 10-mL volumetric flask and fill up to the mark with hexane. Transfer 1 mL of the standard solution into a GC vial and add 100 µL of the deuterated PAH calibration working solution. Each of the calibration standards nominally contains 4 µg/mL of each of the deuterated PAHs. However, laboratories should determine their own concentration range depending on the samples to be analyzed.

Sample Preparation

Sampling should be performed according to good practices (ISO 10381–1 1994, ISO 10381–2 1994). For the analysis, a homogenized field-moist soil sample is used (ISO 14507 2003). Stones and other bigger materials obviously not contaminated should not be analyzed. Large particles with expected contamination should be reduced in size and analyzed with the finer sample material.

Procedure

Extraction Procedure

1. Weigh 10 g of a field-moist (or air-dried-overnight) sample into an extraction flask equipped with a Teflon inlay (a conical flask or a centrifuge tube with a capacity of 100 mL).
2. Add 25 mL of acetone.

3. Close the flask with a screw cap and extract by shaking for 15 min in a shaking machine.
4. After settling, separate the organic phase into a shaking funnel of 500 mL either by decanting or by using a centrifuge (2,500 rpm, 5 min).
5. Repeat the extraction with 25 mL of acetone.
6. Add 50 mL of hexane to the combined acetone extracts, and remove the acetone and other polar compounds by shaking with 100 mL of water. Discard the water and perform another wash in the same manner.
7. If necessary, concentrate the extract on a water bath at 40 °C to about 10 mL using a gentle stream of nitrogen at room temperature. Record the final volume of the extract and dry the concentrated extract over anhydrous sodium sulfate.
8. Transfer 1 mL of the dried extract into a GC vial and add 100 µL of the deuterated PAH calibration working solution. The sample then nominally contains 4 µg/mL of each of the deuterated PAHs.
9. Prepare a blank determination in a similar manner but without any soil sample.
10. Perform an extraction of the quality control soil sample in the same manner as of the test sample.

Clean-Up Procedure

1. If necessary, the extract can be cleaned with a silica gel adsorption column. Prepare the column by placing a small plug of glass wool on the bottom of the column, add 4 g of deactivated silica gel and then about 1 cm of anhydrous sodium sulfate to the top.
2. Condition the column by eluting 10 mL of hexane. When the eluant reaches the top of the column packing, transfer an aliquot (1 mL) of the concentrated extract containing the internal standards to the top of the column. Elute with 50 mL of hexane and collect the extract in a point-shaped test tube.
3. Concentrate the purified extract in a water bath at 40 °C to about 1 mL using a gentle stream of nitrogen at room temperature.
4. Transfer the purified extract into a GC vial.

Gas Chromatographic Analysis

1. Set the gas chromatograph in such a manner that optimum separation of the PAHs is achieved. Special attention should be paid to benzo(b)fluoranthene and benzo(k)fluoranthene separation.

2. Run the working standard solutions and all the samples by a GC with mass selective detection in the scan mode (mass range from 50 to 300 amu).

Quality Assurance

1. The blank measurement of the total method should be carried out with each series of soil samples. The PAH concentration in the blank should be carefully studied, and if traces of contamination are found, the source of contamination should be investigated.
2. The quality control sample should also be analyzed with each series of soil samples. The results should be monitored over time and the results treated statistically.

Calculation

For the quantitative analysis, a calibration curve of the ratio of the PAH determined to the internal standard peak area against the mass of PAH in the sample injected is constructed using the data handling system. Prepare these calibration curves for each native PAH using the specific ions (target ion as the quantitation ion and another ion as the qualifier ion), and the appropriate deuterated PAH as an internal standard (see Table 3.1).

The amount of PAH in the GC vial (A_{PAH} in µg/mL) can be obtained from the calibration curve. Hence, the concentration of the native PAH in the soil sample can be calculated by the following equation:

$$c_n = \frac{A_{\mathrm{PAH}}}{m \times d_{\mathrm{s}}} \times V \times f \tag{3.3}$$

c_n content of an individual PAH in the sample (mg/kg soil dry mass)

A_{PAH} amount of PAH in the GC vial, obtained from the calibration curve (µg/mL)

V volume of the concentrated extract (mL)

f dilution factor

m mass of the sample (g wet mass)

d_{s} content of dry mass in the field-moist sample, determined according to ISO 11465 (g dry mass/g wet mass)

Notes and Points to Watch

- The samples should be analyzed as soon as possible. If not feasible, the samples should be stored at −20 °C.

- Certain PAHs are carcinogenic and all the samples and standard solutions should be handled with extreme care.
- The efficacy of each silica gel batch has to be tested prior to its use in the analysis.
- For highly polluted soil samples, clean-up and concentration steps may not be necessary.

3.5 Heavy Metals

Introduction

Objectives. Most heavy metals are of geological origin, but contamination with them may be due to industrial, mining, agricultural, waste handling or other activity. Often a mixture of such metals occurs. The most common contaminants are arsenic, cadmium, chromium, copper, cobalt, lead, mercury, nickel, uranium, and zinc. In contrast to organic contaminants, heavy metals cannot be degraded by microbes or plants. Thus the bioremediation strategy is based on the movement of metals, e.g., from soil to plants as in phytoremediation, or on bioloeaching (see Chapt. 6). Some metals can undergo microbial oxidation–reduction or become methylated. Different ionic species of a heavy metal may have different toxicity, e.g., As^{3+} is much more toxic than As^{5+}. The method described here gives total concentration of each metal, but does not give any information on the speciation. For that purpose separation of the ionic species may be achieved, e.g., by ion chromatography, followed by induced plasma mass spectrometry (ICP-MS).

Principle. Soil samples are freeze dried, homogenized, sieved, digested in conc. HNO_3 in a microwave oven, and analyzed using ICP-MS.

Theory. Traditional methods for heavy metals' extraction have been based on digestion in aqua regia (ISO 11466 1995) before determination by atomic absorption spectrometry (AAS), or more recently by ICP-MS. Destruction with hydrofluoric acid (ISO 14869–1 2001) is being used for some metal samples, e.g., in geological research. These extraction procedures give the highest yield of the metal content in a soil sample. However, these agents pose occupational health risks and alternative digestion using HNO_3 has become common. The yield obtained using this method has been considered sufficient in many countries for the determination of contamination with heavy metals (Karstensen et al. 1998). The method described here employs digestion with HNO_3 and analysis by ICP-MS and has earlier been described by Salminen et al. (2004).

ICP-MS is a multi-element analytical technique that can be used to measure the concentration of several elements simultaneously. The sample solution is nebulized into the plasma. A large percentage of atoms are ionized and a fraction of these ions are captured in the interface region of the system and channeled into the mass spectrometer. The mass spectrometer serves as a mass filter, and selectively transmits ions according their mass-to-charge ratio.

The common elements to be analyzed by ICP-MS in soils are Al, As, Cd, Cr, Cu, Mn, Ni, Pb, Zn, B, Ba, Cs, Fe, Se, Sr, Ti, U, and V. Mercury is best determined by using the technique of direct combustion, which decomposes the sample in an oxygen-rich environment and removes interfering elements. A dual-path-length cuvette/spectrophotometer specifically determines mercury over a wide dynamic range. The method for mercury requires no pretreatment other than freeze-drying, but a special piece of equipment is needed (e.g., an AMA254 Advanced Mercury analyzer); it is not described here in further detail.

Equipment

- Freeze drier
- Microwave oven with Teflon tubes and a cooling system
- Inductively coupled plasma mass spectrometer (ICP-MS)
- Centrifuge and centrifuge tubes
- Polystyrene tubes

Reagents

- Water: grade 1 as specified in ISO 3696
- Digestion solution: conc. HNO_3 density 1.42 kg/L (69%)
- Calibration standards: Single or multi-element (SPEX CertiPrep, Metuchen, NJ, USA)
  - Multi-element solution 2 (10 mg/L; Ag, Al, As, Ba, Be, Bi, Ca, Cd, Co, Cr, Cs, Cu, Fe, Ga, In, K, Li, Mg, Mn, Na, Ni, Pb, Rb, Se, Sr, Tl, U, V, Zn). Standards are commercially available in 5% HNO_3.
  - Multi-element solution 4 (10 mg/L; B, Ge, Mo, Nb, P, Re, S, Si, Ta, Ti, W, Zr)
- Optimization solution: Mg, Ba, Rh, Pb, and Ce in 1% HNO_3 (10 µg/L)
- Internal standard: rhodium (1 mg/L)

- Control material (NIST, Gaithersburg, MD, USA; SRM NIST 2709 San Joaquin Soil: Al, Ca, Fe, Mg, P, K, Si, Na, S, Ti, Sb, As, Ba, Cd, Cr, Co, Cu, Pb, Mn, Hg, Ni, Se, Ag, Sr, Th, V, Zn)

Sample Preparation

Use freeze-dried, homogenized, and sieved (< 2 mm) soil samples.

Procedure

1. Dry a frozen sample in a freeze-drier.
2. Homogenize the dried sample manually.
3. Sieve the sample (< 2 mm).
4. Weigh accurately 0.25–0.5 g of the dried sample into a digestion tube.
5. Add 5 mL of conc. nitric acid.
6. Set one blank, one reference sample, and one duplicate sample to each batch.
7. Digestion program: step 1: 250 W, 5 min; step 2: 400 W, 5 min; step 3: 500 W, 10 min.
8. Cool the digestion tubes to room temperature in a water bath.
9. Open the tubes and transfer each digested solution quantitatively to a 30 mL plastic tube and dilute with water to a volume of 25 mL.
10. If the samples are not clear, centrifuge at 3,000 rpm for 3 min.
11. Dilute each sample (1:10 or 1:100) to a volume of 10 mL with water and add the internal standard (100 µL of rhodium solution).
12. The sample is ready for analysis.

To perform a calibration, proceed as follows:

1. Calibrate the instrument using two calibration solutions, namely, blank and 50 µg/L of standard solution. Normally multi-element standard solutions are used.
2. Prepare 10 mL of the calibration solution and add the internal standard as described for soil samples.
3. Perform the calibration and analyze the samples.

Calculation

The mass concentration for each element is determined with the aid of the instrument's software. Enter the value of the dry mass of each sample into this software, and it calculates results directly in mg/kg soil dry mass.

Notes and Points to Watch

- Pay attention to the interference between/among different metals.
- See that the acid concentration is same in the calibration and the sample solutions.
- The instrument must be located in a laboratory free of contaminants.

References

Douglas DJ, Houk RS (1985) Inductively Coupled Plasma Mass Spectrometry. Prog Anal. Atom Spectrosc 8:1

EPA Method 3015 (1994) Microwave assisted acid digestion of aqueous samples and extracts for total metals analysis by FLAA, Furnace AA, ICP Spectrometry and ICP Mass Spectrometry

ISO 10381–1 (1994) Soil quality – Sampling – Part 1: Guidance on the design of sampling programmes

ISO 10381–2 (1994) Soil quality – Sampling – Part 2: Guidance on the design of sampling techniques

ISO 11465 (1993) Soil quality – Determination of dry matter and water content on a mass basis – Gravimetric method

ISO 11466 (1995) Soil quality – Extraction of trace elements soluble in aqua regia

ISO 13877 (1998) Soil quality – Determination of polynuclear aromatic hydrocarbons (PAH) – Method using high performance liquid chromatography

ISO 14507 (2003) Soil quality – Pretreatment of samples for determination of organic contaminants

ISO 14869–1 (2001) Soil quality – Dissolution for the determination of total element content – Part 1: Dissolution with hydrofluoric and perchloric acids

ISO 15009 (2002) Soil quality – Gas chromatographic determination of the content of volatile aromatic hydrocarbons, naphthalene and volatile halogenated hydrocarbons – Purge-and-trap method with thermal desorption

ISO 15587–2 (2002) Water quality – Digestion for the determination of selected elements in water – Part 2: Nitric acid digestion

ISO 16703 (2004) Soil quality – Determination of content of hydrocarbon in the range C_{10} to C_{40} by gas chromatography

ISO 17294–1 (2004) Water quality – Application of inductively coupled plasma mass spectrometry (ICP-MS) – Part 1: General guidelines

ISO 17294–2 (2003) Water quality – Application of inductively coupled plasma mass spectrometry (ICP-MS) – Part 2: Determination of 62 elements

ISO 3696 (1992) Water Quality – Water for analytical laboratory use – Specification and test methods

ISO/DIS 18287 (in preparation) Soil quality – Determination of polycyclic aromatic hydrocarbons (PAH) – Gas chromatographic method with mass spectrometric detection (GC-MS)
ISO/PRF 22155 (in preparation) Soil quality – Gas chromatographic determination of the content of volatile aromatic and halogenated hydrocarbons and selected ethers – static headspace method
ISO/DIS 22892 (in preparation) Soil quality – Guideline for GC/MS identification of target compounds
ISO/TR 11046 (1994) Soil quality – Determination of mineral oil content – method by infrared spectrometry and gas chromatographic method
Jørgensen KS, Puustinen J, Suortti A-M (2000) Bioremediation of petroleum hydrocarbon-contaminated soil by composting in biopiles. Environ Pollut 107:245–254
Karstensen KH, Ringstad O, Rustad I, Kalevi K, Jørgensen K, Nylund K, Alsberg T, Ólafsdóttir K, Heidenstam O, Solberg H (1998) Methods for chemical analysis of contaminated soil samples – tests of their reproducibility between Nordic laboratories. Talanta 46:423–437
Laine MM, Jørgensen KS (1997) Effective and safe composting of chlorophenol-contaminated soil in pilot scale. Environ Sci Technol 31:371–378
MLS-1200 MEGA Microwave digestion system with MDR Technology; Operator Manual (1992) Milestone, Sorisole, Italy
Owen S, Whittle P (2003) Volatile organic compounds. In: Thompson KC, Nathanail CP (eds) Chemical analysis of contaminated land. Blackwell Publ, CRC Press, pp 177–188
Reference Manual Elan 5000 (1992) Perkin Elmer, Norwalk, Connecticut, USA
Salminen JM, Tuomi PM, Suortti A-M, Jørgensen KS (2004) Potential for aerobic and anaerobic biodegradation of petroleum hydrocarbons in boreal subsurface. Biodegradation 15:29–39
User's Manual Elan 5000 (1992) Perkin Elmer, Norwalk, Connecticut, USA

4 Immunotechniques as a Tool for Detection of Hydrocarbons

Grażyna A. Płaza, Krzysztof Ulfig, Albert J. Tien

4.1 RaPID Assay Test System

Introduction

Objectives. Immunoassays (IMAs) are now being seen as useful analytical tools, and supplement to conventional analytical methods such as gas chromatography and high performance liquid chromatography. The main IMA principle can be illustrated by the following reaction: $Ab + Ag + Ag^* \leftrightarrow AbAg + AbAg^*$ (Ab = antibody, Ag = antigen, Ag^* = labeled antigen).

Immunochemical methods provide rapid, sensitive, and cost-effective analyses for a variety of environmental contaminants (van Emon and Mumma 1990; van Emon and Lopez-Avila 1992; Marco et al. 1995). The driving force in the development of immunochemical methods is the need for rapid, simple, sensitive, and cheap tests that can be performed on-site without requiring sample transfer to an analytical laboratory. The increasing popularity of field IMA analyses can, in large part, be ascribed to portable equipment and minimal set-up requirements (van Emon and Gerlach 1995). Table 4.1 shows advantages and disadvantages of IMAs for environmental analyses.

The following IMA techniques can be used in environmental studies: D TECH (Strategic Diagnostics, Newark, DE, USA), PETRORISC (EnSys, Research Triangle Park, NC, USA), EnviroGard (Millipore, Billerica, MA, USA), and RaPID (Ohmicron, Newtown, PA, USA) assays. Table 4.2 presents the properties of these systems and their application matrices and detection limits.

Principle. The RaPID assay uses magnetic particles as the solid-support component of the ELISA (enzyme-linked immunosorbent assay). Attaching antibodies to microscopically small magnetic particles facilitates the chemical reaction between antibody and contaminant. The concentration of the compound to be detected is quantified after a color reaction.

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Table 4.1. Some advantages and disadvantages of IMAs for environmental analysis (according to Sherry 1992; Sherry 1997)

Advantages	Disadvantages
• Wide applicability • Sensitive, specific, and highly selective • Rapid and easy to use • Reduced preparation • Rapid with high sample throughput • Ideal for large sample loads; easily automated • Suited to laboratory and field use • Cost-effective analysis of small-volume samples	• Development costs • Hapten synthesis can be difficult • Can be vulnerable to cross reacting compounds and non-specific interferences • Requires independent confirmation • Not suited to small sample loads or multi-residue determinations • Lack of acceptance

Theory. One of the most common enzyme immunoassay (EIA) modifications, sometimes termed "double antibody sandwich techniques," is ELISA (van Emon and Mumma 1990). ELISA is based on combining selective antibodies with sensitive enzyme reactions to produce analytical systems capable of detecting very low levels of chemicals. The RaPID system utilizes covalent binding of antibodies to magnetic particles that are made of silanized iron oxide (Fig. 4.1). The first stage is the immunochemical reaction between antibodies/magnetic particles and a chemical compound as antigen. The second stage is separation of magnetic particles from the antigen by applying a magnetic field. After washing, the color reagent is added and the concentration of the colored product is measured (RaPID assay environmental user's guide 1996; Płaza et al. 1999). The assay steps are presented in Fig. 4.2.

Equipment

- RPA-I RaPID analyzer (spectrophotometer): laboratory bench-top-based, single wavelength, microprocessor-controlled analyzer
- Magnetic rack composed of two parts: the top rack holds the test tubes in place and the bottom base contains the magnets
- Portable balance
- Test tubes
- Vortex mixer
- Timer

Table 4.2. A comparison of immunological test systems (EnviroGard Protocol 2004b, www.sdix.com)

	D TECH	EnSys	EnviroGard	RaPID Assay
Technology	Latex particle	Coated tube	Coated tube	Magnetic particle
Result type	Qualitative and semi-quantitative	Qualitative and semi-quantitative	Qualitative and semi-quantitative	Qualitative and semi-quantitative
Sample throughput	1–4 samples/h	1–10 samples/h	1–17 samples/h	1–50 samples/h
Analysis time	20 min/run	30 min/run	30 min/run	60 min/run
EPA SW-846 method	4030, 4035	4030, 4035	4030, 4035	4030, 4035
Storage shelf life	Ambient 1 year	Ambient 1 year	Refrigerated 1 year	Refrigerated 1 year
Training level	Low; no training required	Medium; training recommended	Medium; training recommended	Medium; training recommended
Instrument	DTECHTOR Analyzer or color card	Photometer	Photometer	RPA-1 Analyzer
	Application matrix and detection limits			
Analyte	*Application*			
BTEX	Soil	2.5–35 ppm	–	2 ppm	0.9 ppm
	Water	0.6–10 ppm	–	0.1 ppm	0.09 ppm
TPH	Soil	–	10 ppm	5 ppm	10 ppm
	Water	–	–	0.1 ppm	1 ppm
PAH	Soil	–	1 ppm	1 ppm	0.2 ppm
	Water	–	15 ppb	2 ppb	0.9 ppb
Carcino-genic PAH	Soil	–	–	–	10 ppb
	Water	–	–	–	0.2 ppb

Reagents

- All the reagents (Extraction Solution, Enzyme Conjugate, Antibody-Coupled Magnetic Particles, Color Reagent, Washing Solution, and Stopping Solution) are supplied by Ohmicron, Newtown, PA, USA, and their composition is under protection.

Sampling and Sample Preparation

- Collect water and soil samples from the contaminated area in 500 mL wide-mouth bottles (Nalgene; Nalge Nunc, Naperville, IL, USA).

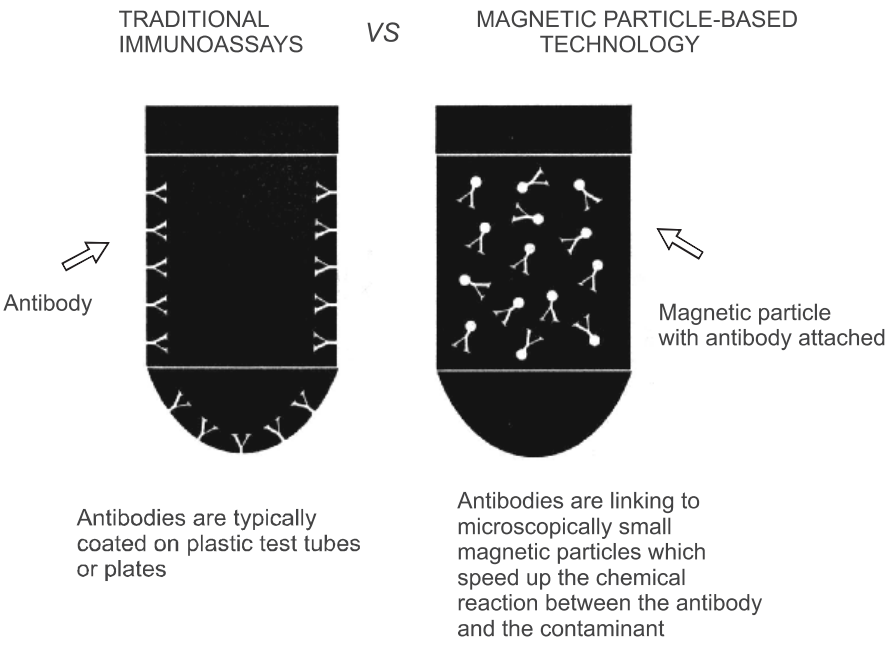

Fig. 4.1. Comparison of traditional IMAs and magnetic-particle-based technology (RaPID assay environmental user's guide 1996)

- Test directly samples or store them at 4 °C; water content in soil samples should not be more than 20–25%.
- Extract soil samples before testing:
  - Weigh 10 g of soil into the soil collection tube (Fig. 4.3) and add 20 mL of the extraction solution; screw the cap on tightly.
  - Shake vigorously and continuously for at least 60 s.
  - Remove the screw cap and attach the filter cap, then attach the plunger rod to the plunger of the soil collector, and filter the extract into the Extract Collection Vial.
  - Fill with 0.5–1 mL of the filtrate and cap the vial.

Procedure

1. Mix 200 µL of soil extract or water sample with 250 µL of the Enzyme Conjugate and 500 µL of antibody-coupled magnetic particles; incubate the mixture for 15 min at room temperature.
2. Put all tubes into the magnetic rack and wait 2 min for the particles to separate.

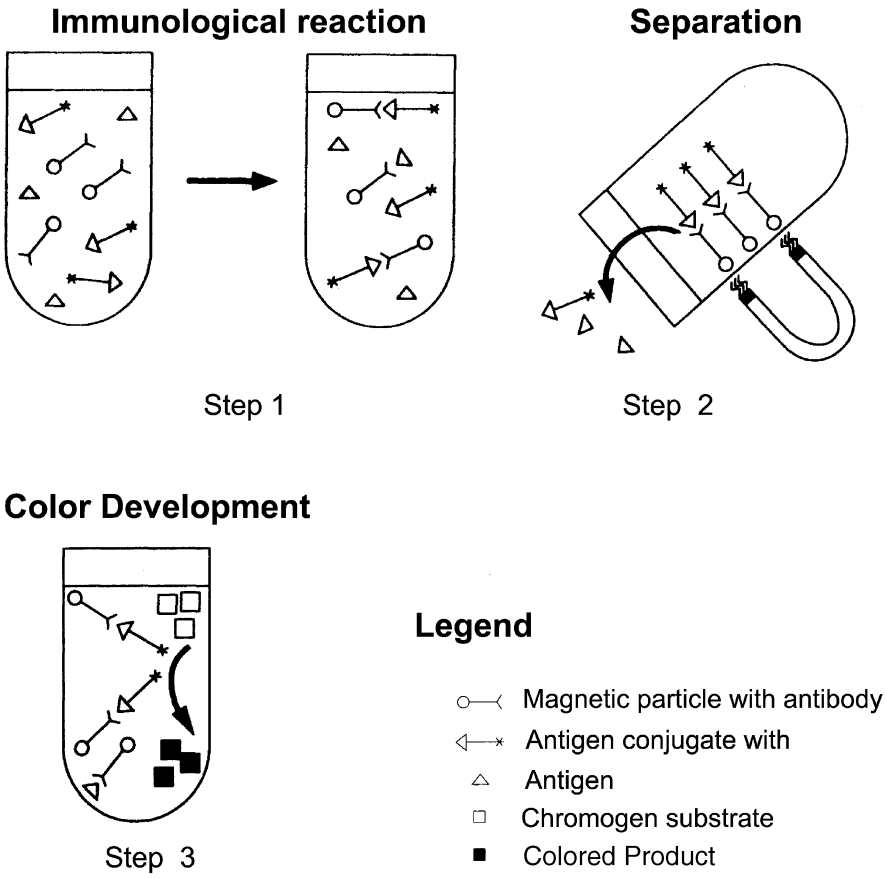

Fig. 4.2. Principle of RaPID assay (RaPID assay environmental user's guide1996; Plaza et al. 1999)

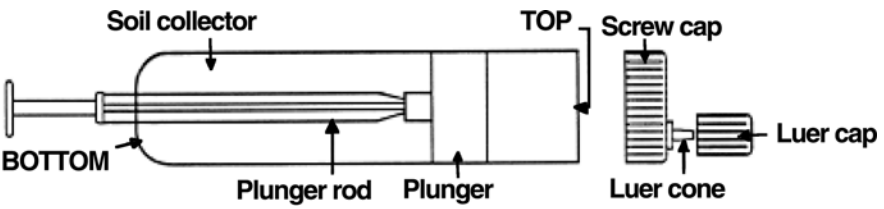

Fig. 4.3. Soil collection tube (RaPID assay environmental user's guide 1996)

3. Add 1 mL of washing solution, vortex each tube and wait 2 min. Repeat this step.
4. Add 500 µL of color reagent. 3,3′,5,5′–tetramethylbenzidine is used as the chromogen. Incubate for 20 min at room temperature.
5. Add 500 µL of stopping solution (0.5% sulfuric acid).
6. Within 15 min after adding the stopping solution, transfer 1 mL of solution to cuvettes and read absorbance of standard solutions and samples at 450 nm using the RPA-I analyzer.

Calculation

1. The concentration of the colored product is directly proportional to the concentration of the labeled compounds.
2. The RPA-I analyzer can perform mathematical computations, and sample concentrations with statistics are obtained. Results are directly reported in ppb (ng/g) or ppm (µg/g).
3. The hydrocarbon concentration in soil is calculated according to the following formula, taking into account the concentration calculated by the analyzer (ppb or ppm), the volume of extraction solution (20 mL), and the mass of soil used for extraction (g dry mass):

$$\text{Hydrocarbon concentration (ppb or ppm)} = \frac{\text{concentration} \times \text{volume}}{\text{soil mass}}$$

Notes and Points to Watch

- Temperature control is required for reagent storage (4–8 °C) and during the performance of the assay (room temperature: 15–30 °C).
- Use specific test kits for specific hydrocarbons; do not mix the reagents.
- All the reaction steps should be done exactly according to the assay protocol.
- Do not use test kit components after the expiration date.

4.2 EnviroGard Test System

Introduction

Objectives. Environmental IMAs have been developed and evaluated for analyses including major classes of pesticides, organic compounds such

as polychlorinated biphenyls (PCB), polyaromatic hydrocarbons (PAH), pentachlorophenols (PCP), benzene/toluene/ethylbenzene/xylene (BTEX), total petroleum hydrocarbons (TPH), dioxins and furans, microbial toxins, as well as inorganic compounds such as cadmium, lead, and mercury (Vanderlaan et al. 1988; van Emon and Mumma 1990; Sherry 1992; van Emon and Lopez-Avila 1992; Knopp 1995; van Emon and Gerlach 1995; Gerlach et al. 1997). The EnviroGard test systems are quick and reliable with semi-quantitative results allowing screening at various levels of contamination (Table 4.2). They can be used during site remediation to detect contaminants and monitor the cleanup and industrial processes.

Principle. EnviroGard uses coated polystyrene test tubes as the solid support component of the ELISA. The system is based on the use of polyclonal antibodies, immobilized on the test tube walls, that can bind specific contaminants.

Theory. The EnviroGard IMA kit uses 12 × 75 mm polystyrene test tubes coated with an antibody against the target contaminant (analyte). Coated polystyrene test tubes allow screening for various contaminations like PCB, PAH, TPH, BTEX, and PCP. When analytes are present in the sample, they compete with the specific Enzyme Conjugate (labeled analyte) for a limited number of binding sites on the antibodies (EnviroGard Protocol 2004a, 2004b). According to the principles of competitive IMAs, the absorbance signal (or optical density) of the final reaction mixture is inversely proportional to the concentration of the contaminant (analyte) present in the test sample. After the immunological reaction, the unbound molecules are washed away and a chromogenic substance is added to the test tube. In the presence of bound specific enzyme conjugate, the clear substance is converted to a blue color.

Equipment

- SDI Sample Extraction Kit contains devices to process 12 samples, i.e., 12 each: extraction jars with screw caps, filter modules, ampule crackers, wooden spatulas, weigh canoes, disposable transfer pipettes, and ampules each containing 10 mL of 100% methanol
- 20 antibody-coated test tubes (12 × 75 mm), 1 vial of negative control (methanol), calibrator vials, 1 vial of Hydrocarbons-Enzyme Conjugate, 1 vial of Substrate Solution, 1 vial of Stop Solution, pipettes and tips
- Portable balance
- Spectrophotometer

- Test tube rack
- Timer

Reagents

- Methanol (100%)
- Stop solution: 1 N HCl

Sampling and Sample Preparation

- Collect soil without excess twigs, rocks, or pebbles in labeled 500 mL plastic containers.
- Dry soil samples with a water content of 30% or more (by mass) before testing.
- Store soil samples at 4 °C in tightly sealed containers to avoid evaporative losses.
- Use 10 mL of methanol to extract hydrocarbons from 10 g soil. When extracting clay samples add an additional 10 mL of methanol to the sample and shake vigorously for 1–2 min.
- Filter the extract using Whatman #1 filter paper. A clay sample may soak up all the methanol, leaving little or no excess liquid to filter.

Procedure

1. Incubate all reagents at room temperature (at least 1 h) before use.
2. Remove the antibody-coated test tubes (20 tubes/assay) from the foil pouch and label as negative control (NC), calibrators (C1, C2, etc.), and samples (S1, S2, etc.).
3. Place the test tubes in the test tube rack, and add 100 µL of sample extract.
4. Add 200 µL of specific enzyme conjugate to all test tubes.
5. Gently shake the rack to mix for 10–15 s, and leave the tubes undisturbed for 10 min.
6. Shake out the test tube contents vigorously into a sink, and wash the test tubes with double distilled water. Repeat this wash step three times.
7. Add 500 µL of substrate solution to all test tubes, briefly shake, and then incubate for 5 min at room temperature.
8. Add 500 µL of stop solution to all test tubes.

9. The results can be interpreted visually within 5 min after adding the substrate solution, or can be obtained more precisely with a spectrophotometer.

Calculation

1. One enzyme molecule can convert many substrate molecules. Every test tube has the same number of antibody-binding sites and receives the same number of the specific enzyme conjugate molecules. Color development is inversely proportional to the hydrocarbon concentration, i.e., dark color indicates low concentration and light color indicates high concentration.
2. Visual interpretation: compare the sample test tube to the calibrator test tubes against a white background. If a sample test tube contains a darker color than the calibrator test tube, the sample contains hydrocarbons at a concentration lower than the calibrator. If the sample test tube is less colored than the highest calibrator test tube, the soil extract should be diluted in methanol and the assay performed again.
3. Photometric interpretation: place the negative control test tube into the spectrophotometer. Record the optical density (OD) at 450 nm for the negative control, and then measure the OD of calibrator #1. Repeat this step to determine the OD for each of the remaining calibrators and for samples.

   An example of data is given here:

Tube	OD 450 nm	Interpretation
NC (negative control)	1.00	
C1 (2 ppm)	0.87	
C2 (10 ppm)	0.52	
C3 (50 ppm)	0.25	
S1 (sample 1)	0.45	$>$ 10 ppm, $<$ 50 ppm
S2 (sample 2)	0.72	$>$ 2 ppm, $<$ 10 ppm

Notes and Points to Watch

- The EnviroGard test is a screening test only.
- The test system can be used in the laboratory and in the field.
- Store all test kit components at 4–8 °C when not in use.
- Storage all reagents at ambient temperature (18–27 °C) on the day before using.

- Use only reagents or test tubes from one kit; do not mix the components from different test kits.
- Do not expose substrate to direct sunlight.
- Do not freeze test kit components or expose them to temperature greater than 37 °C.
- Use gloves and protective clothing during the experiment.

References

EnviroGard™ Protocol (2004a) Petroleum fuels in soil. Test kit 70040000. Strategic Diagnostics Inc, USA

EnviroGard™ Protocol (2004b) Remediation assessment and industrial testing. Strategic Diagnostics Inc, USA (www.sdix.com)

Gerlach RW, White RJ, O'Leary WFD, van Emon JM (1997) Field evaluation of an immunoassay for benzene, toluene and xylene (BTX). Wat Res 31:941–945

Knopp D (1995) Application of immunological methods for the determination of environmental pollutants in human biomonitoring. A review. Anal Chem Acta 311:383–392

Marco MP, Gee S, Hammock BD (1995) Immunochemical techniques for environmental analysis. II Antibodies production and immunoassay development. Trends Anal Chem 14:415–428

Płaza G, Ulfig K, Bevolo AJ (1999) Application of the immunoassay techniques for the determination of PAHs and BTEX in soil. Int Environ Tech 9: 9–10

RaPID assay environmental user's guide (1996), OHMICRON, USA

Sherry J (1992) Environmental chemistry. The immunoassay option. Crit Rev Anal Chem 23:217–300

Sherry J (1997) Environmental immunoassays and other bioanalytical methods. Overview and update. Chemosphere 34:1011–1025

Van Emon JM, Gerlach CL (1995) A status report on field-portable immunoassay. Environ Sci Technol 29:312–317

Van Emon JM, Lopez-Avila V (1992) Immunochemical methods for environmental analysis. Anal Chem 64:79–88

Van Emon JM, Mumma RQ (1990) Immunochemical methods for environmental analysis. ACS Symp Series 442, Am Chem Soc, Washington, DC

Vanderlaan M, Watkins BE, Stanker L (1988) Environmental monitoring by immunoassay. Environ Sci Technol 11:247–254

5 Feasibility Studies for Microbial Remediation Hydrocarbon-Contaminated Soil

Ajay Singh, Owen P. Ward, Ramesh C. Kuhad

5.1 Introduction

While bioremediation processes are considered to be advantageous in terms of their relatively low cost, process flexibility, benign nature environmentally, and on-site utility, there have also been many instances where the processes have failed to achieve the required low contaminant concentration criteria (Mandelbaum et al. 1995; Iwamoto and Nasu 2001; Grommen and Verstraete 2002). These failures have reduced consumer confidence in bioremediation and consequently the technology only garners a small portion of the US$ 7–8 billion US annual remediation market (Srinivasan 2003). Bioremediation processes must comply with generally accepted good operating principles and have predictable end-points. The processes must be validated in advance such that they do not fail (Ward 2004). Feasibility studies are therefore critical for the implementation of a successful bioremediation technology.

Contaminated sites never exhibit identical characteristics and the experience from one site can only be exploited at another to a limited extent. The biodegradation process in soil is complex, involving diffusion of contaminants in the soil matrix, adsorption to the surface of soil particles, and biodegradation in the biofilms existing on the soil particles, in pores, and in the bound and free water after desorption from the soil surfaces. A variety of complex biodegradation patterns result from physical interactions between pollutants and soil matrix and from biological interactions among different organisms. Numerous factors such as soil moisture, pH, temperature, aeration, nutrient sources, type of soil, type(s) of contaminant(s), and interplay between these factors, affect the ecology of the microbial population and degradation of hydrocarbons in contaminated soil.

For full-scale bioremediation applications, several important points need to be considered. In particular, how low can the concentration of the con-

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taminant be obtained during treatment considering: bioavailability and microbial activity, the fate of the contaminant in terms of mineralization, biotransformation, evaporation, build-up of microbial biomass, sorption to soil – and also considering indicators of time needed to obtain the set goal such as degradation rate for achieving the target level of contaminant, and finally the capital and operating costs?

Both slurry bioreactors and land treatment technologies have successfully been used to remove hydrocarbons from petroleum-contaminated soils. Naturally occurring or introduced microbial populations convert hydrocarbons to carbon dioxide, water, biomass, and humic material. For a successful bioremedial treatment of soil, it is important to consider several factors, including type and extent of contamination, bacterial population present, duration since contamination, optimal microbiological conditions, soil characteristics, proper bioremediation technique, and appropriate analytical method. Proper planning for the execution of a bioremediation technology based on the above criteria is of utmost importance to minimize risk of failure in terms of effort, time, and money. Protocols for conducting feasibility studies are, therefore, required to evaluate the effectiveness of a planned treatment method.

In this Chapter, methods commonly used for determination of biodegradation potential in feasibility or biotreatability studies on bioremediation of contaminated soils and sludges are discussed. During feasibility studies certain environmental and nutritional parameters are optimized for achieving accelerated and complete biodegradation of hydrocarbons.

5.2 Determination of Biodegradation Potential

The data from the feasibility studies of hydrocarbon-contaminated soil is used to design a suitable full-scale bioremediation technology. Hence, it is important to carefully plan the biotreatability studies. Successive stages of planning and execution of feasibility studies are shown in Fig. 5.1.

5.2.1 Sampling and Soil Preparation

Preparation of soil samples for site characterization or biodegradation experiments can alter the physico-chemical and biological properties of the original soil present in the field. In order to assess the extent of the overall soil contamination, proper field sampling procedures are required to obtain representative samples. The spatial variability of soil characteristics may be

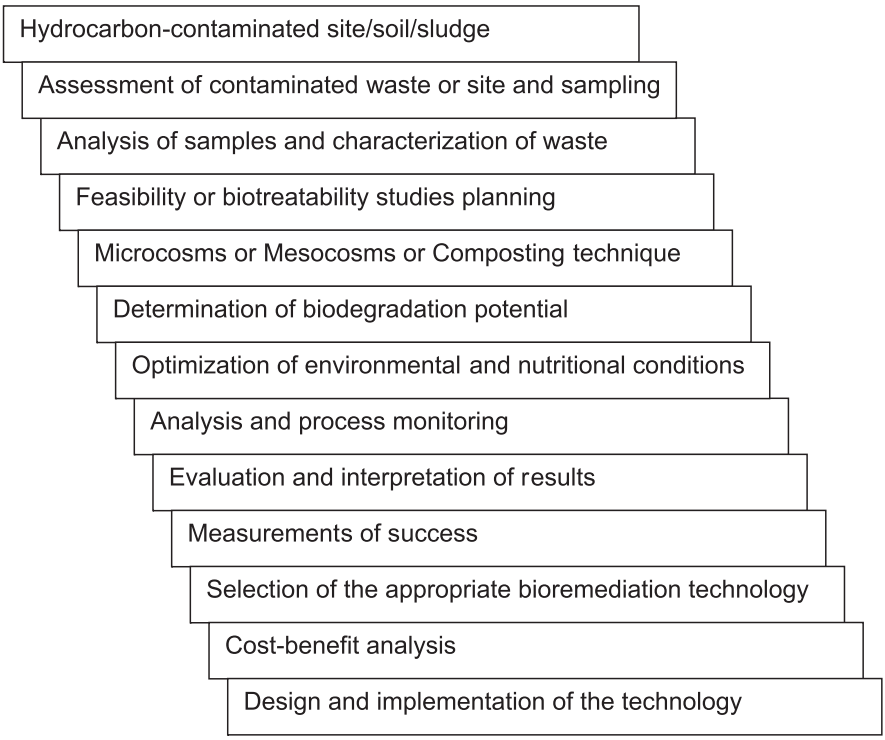

Fig. 5.1. Successive stages for planning and implementation of feasibility studies

considered for determination of the location and number of representative samples in heterogeneous and very large sites. The number of samples to be collected and analyzed usually depends on the overall sampling objective, contaminant distribution, size of the contaminated site and sampling and analytical costs (Huesemann 1994a).

1. Samples may be collected from a uniform depth, using proper soil sampling equipment such as a core or a split-spoon sampler at 20–30 locations and pooled. Top 10–15 cm of soil is often sampled. There are many procedures for choosing a sampling location. The most popular ones are either randomly selecting field locations for periodic sampling or placing a hypothetical square or rectangular grid over the site and taking samples at the center of each grid. Samples should be homogenized prior to subsampling and submission for analysis (see also Chapt. 1).
2. Soil samples are stored in either plastic bags or in glass or other nonreactive containers in a cooler on ice for immediate analysis. For long term storage, the samples should be at the field moisture levels and

stored in glass or other non-reactive container around 4 °C. Air drying of samples should be avoided.

3. For biodegradation experiments, a relatively homogeneous sample should be prepared by sieving soil through a 2–4 mm sieve and soil moisture level determined so that all the experiments are carried out using the same conditions. Depending on the aim of the experiment, contaminant level, water content, pH, organic matter, nutrient levels, and type of soil are determined by standard methods (see Chapt. 2).

5.2.2 Selective Microbial Enrichment

Successful application of a bioremediation technique requires the identification of favorable conditions for the useful hydrocarbon-degrading microorganisms to actively grow and metabolize contaminants. Classical isolation methods, selective enrichment of specific microbes, and genetic approaches can be used to obtain a single microbial species or a group of different microorganisms (a consortium; see Chapt. 13). Selective enrichment is the most practical approach for large scale applications where the enrichment process is designed to increase the population of specific microorganisms. Suitable conditions and selective pressures are applied to encourage growth of microbes capable of degrading a particular substrate or a mixture of potential contaminants, in the growth medium, that are the targets for biodegradation or bioremediation as the sole sources of carbon (Vecchili et al. 1990).

The initial inoculum can be obtained from the contaminated soil, sludge, or wastewater with known degradative activity. A consortium of hydrocarbon-degrading microorganisms can be obtained by adding about 1 or 2 g of hydrocarbon-contaminated soil or sludge to 100 mL mineral medium in Erlenmeyer flasks. Culture can be further maintained in a flask by routinely transferring a 2% inoculum into a fresh medium at weekly intervals.

Cyclone fermenters (Liu 1989) with a 1-L working volume can also be used to develop and maintain hydrocarbon-degrading cultures (Fig. 5.2). The cultures obtained by selective enrichment in the flasks can be inoculated into a liter of mineral medium in a cyclone fermenter. The culture is maintained once weekly by removing about 50% of the volume of the culture and replacing with fresh medium and a known amount of the hydrocarbon as a sole carbon source.

Soil columns may be used to enrich for hydrocarbon-degrading cultures. Glass soil columns with an inner diameter of 40 mm and length of 350 mm packed with 50 g air-dried soil and slightly moist pre-washed quartz sand between two layers of glass wool can be used to enrich desired strains

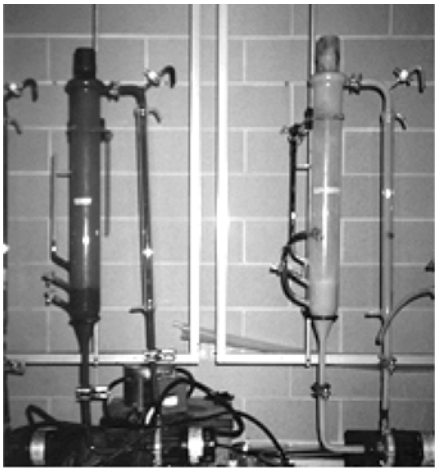

Fig. 5.2. Cyclone fermenters used for maintenance of hydrocarbon-degrading microorganisms

(Pfarl et al. 1990). The columns are rinsed with mineral medium several times prior to enrichment process and air is provided by using compressed air bubbled through distilled water at 1–2 L/h.

5.2.3 Controls

In bioremediation experiments, sterile controls should frequently be used to demonstrate the biological activity and biodegradation process, since abiotic loss mechanisms such as adsorption and volatilization can occur simultaneously. Soil sterilization can be achieved by various physical and chemical methods. Methods include using chemicals such as mercuric chloride and sodium azide, as well by as autoclaving and providing gamma radiation. For inoculation of sterile soil, autoclave and gamma radiation are the most suitable because no residual chemical is left after sterilization. However, any sterilization method will alter the soil properties. Wolf et al. (1989) found that mercuric chloride had the least effect on soil properties such as pH, surface area, and release of Mn among various sterilization methods/agents tested and compared such as gamma radiation, microwave, dry heat, propylene oxide, sodium azide, mercuric chloride, chloroform, and antibiotics.

In bioaugmentation studies, where the experiments are conducted with added inoculum to the soil, a killed-culture inoculum treatment should be included as control for possible nutrient effects of dead microbial cells. Mass balance of the existing or spiked contaminant should be determined at the conclusion of the experiment to evaluate contaminant disappearance due to biodegradation or abiotic mechanism.

5.2.4 Soil Microcosms

One of the simplest methods requiring minimal equipment for soil biodegradation studies is with use of a biometer flask (Bellco, Vineland, NJ, USA). The United Sates Environmental Protection Agency (US EPA) and Organization of Economic Cooperation and Development (OCED) have also recommended this method (OECD 1981; McFarland et al. 1991; Skladany and Baker 1994). Biodegradation activity can be evaluated by directly monitoring the loss of the target compounds or indirectly by measuring by-products of biodegradation or electron acceptor consumption.

A biometer flask, a 250-mL Erlenmeyer flask with a side arm containing potassium hydroxide to trap CO_2 evolved during biodegradation, is used in batch experiments to monitor degradation of the target compound present in or added to the contaminated soil. For biodegradation feasibilities studies, around 20% (w/v) aqueous soil suspension is recommended. Flasks are incubated with or without CO_2-free air and periodically KOH solution is withdrawn and titrated with a standard acid solution to determine the amount of CO_2 produced. The matrix can be analyzed at the end of the test for organic and inorganic compounds. The biometer flasks can be modified to investigate specific problems related to specific types of contaminants and challenges in studying a given biodegradation. This flask system can be used to study biodegradation of both semi-volatile and volatile compounds, and to screen commercial inoculates as well.

An electrolytic respirometer, designed to measure the oxygen uptake or rate of respiration by microbes in soil and sludge has been used by the US EPA for evaluation of commercial products for use in Prince William Sound, Alaska (Venosa et al. 1992). The respirometer consists of a reactor module connected to an electrolytic oxygen generator. The depletion of oxygen by microbes creates a vacuum that triggers the oxygen generator. The electricity used to generate the oxygen is proportional to the amount of oxygen (mg/L), while the CO_2 produced by microbial activity is trapped in KOH solution. The decision to choose a better amendment is based on high oxygen uptake rate, growth of degraders, and significant degradation of aliphatic and aromatic hydrocarbons.

Another method to quickly determine biotreatability of hydrocarbon-contaminated soils and sludges is to simply use 250 mL Erlenmeyer flasks with working volumes of 50 mL containing 20% (w/v) soil or sludge slurry. For petroleum-contaminated soil or sludge samples, total petroleum hydrocarbon (TPH) content is determined as hexane-extractable material.

1. Set up at least 6 flasks for each test.

2. Add a known mass of sludge or contaminated soil to the flask in order

to obtain less than 20% solids and 10% TPH concentration in a total working volume of 50 mL.

3. Add 45 mL of the nutrient medium and 1.25 mL (0.25% final concentration) of a non-ionic surfactant (10% w/v stock solution).
4. Adjust pH of the contents to 6.8–7.2 using 5 N NaOH or 5 N HCl.
5. Inoculate the flask with 2.5 mL (5%, v/v) of a microbial inoculum.
6. Incubate the flasks at 30 °C for 14 days on a shaker (200 rpm).
7. Extract the whole contents of 2 flasks with equal volume of n-hexane at the starting time of the test to determine initial TPH content. Extract contents of 2 flasks each with hexane after 7 and 14 days to determine residual TPH contents.
8. After determination of TPH (Chapt. 3), dissolve the residue in a known volume of hexane for gas chromatographic analysis of hydrocarbons.

In the above method, at least duplicate flasks should be set up for each sampling point and the contents of whole flasks should be extracted to determine residual hydrocarbons. Appropriate, controls for abiotic losses should be also be set up as described above.

5.2.5 Slurry Bioreactors

The slurry bioreactor approach is to suspend and mechanically mix soil in aqueous solutions in a contained vessel or tank. Land-based systems usually require very long treatment times due to lack of control of environmental factors such as seasonal variation in temperature, pH, moisture, as well as of natural microbial activity, and mixing and circulation limitations. These problems can be eliminated in bioreactor systems, which are characterized by much higher rates and extents of degradation due to the minimization of mass-transfer, increased desorption of contaminants by continuous mixing, and control of environmental and nutritional factors such as pH, temperature, and moisture, bioavailability of nutrients and oxygen in order to promote rapid microbial growth and activity (Singh et al. 2001; Van Hamme et al. 2003).

Process conditions in bioreactors can be optimized for biodegradation depending on the nature of contaminant. Desired temperature and pH can be consistently maintained throughout the process and suitable amendments such as nutrients, surfactants, and microbial cultures can be supplied. Several examples of slurry reactors can be found in the literature.

A method developed in the authors' laboratory and successfully scaled up for field applications is described here.

1. Depending on the availability, use a 1–5 L or even larger volume bioreactor fitted with pH, temperature, and dissolved oxygen control for biotreatability studies. Alternatively, construct an inexpensive bioreactors by putting an air sparger in a glass or metal beaker or container.
2. For biotreatability studies in the bioreactor, and depending on the soil and sludge composition, mix a sample of about 20% solids by mass with aqueous nutrient medium.
3. Depending on the critical micellar concentration, add a non-ionic surfactant with a hydrophilic-lipophilic balance (HLB) value 12–13 to obtain final concentration of 0.05–0.25%.
4. Adjust the pH of the medium to around 7.0 using NaOH or HCl solutions.
5. Add the inoculum, prepared and maintained in a cyclone fermenter as described in Sect. 5.2.2, at the level of 10% (v/v) to the bioreactor.
6. Maintain an aeration level of 0.1–0.2 vvm (volume per volume per minute) during the process to avoid oxygen limitation in the system. Dissolved oxygen concentration should be maintained above 2 mg/L.
7. A small mixer can also be used at about 200–300 rpm to achieve better mixing of the reactor contents.
8. Keep the temperature at between 28 and 32 °C using a water bath or heater.
9. Monitor the pH regularly and maintain it between 6.5 and 7.5 throughout the process.
10. Compensate for any losses due to evaporation of water by adding water to the working volume level.
11. Total microbial count and hydrocarbon-degrading bacteria can be determined at regular intervals to monitor the progress of biodegradation.
12. Monitor biodegradation of hydrocarbons at periodic intervals for 2–4 weeks.

The experimental design and data analysis during a biotreatability study will depend on the specific aim of the study. The slurry reactor experiments should be repeated to ensure consistent results. While sub-sampling over an extended period in a bioreactor experiment, care should be taken to ensure that the volume in the reactor is not drastically reduced.

5.2.6 Land Treatment

A set of laboratory experiments using contaminated soil can be carried out in order to investigate the feasibility of land treatment of such soil. Biodegradation potential of a particular hydrocarbon waste can be determined by the extensive chemical characterization of the petroleum-contaminated soil. Huesemann (1994b) has provided useful guidelines on carrying out laboratory feasibility studies on potential of land treatment of petroleum-contaminated soil.

Laboratory mesocosms to study biodegradation of petroleum hydrocarbons in contaminated soil can be prepared in open glass or metal trays as follows:

1. Trays containing 5–10 kg of contaminated or spiked soil are prepared.
2. Oil and grease or TPH content is determined and adjusted in the range of 5–7% by diluting with clean soil.
3. To obtain optimal soil moisture content for the microbial activity, soil moisture is adjusted to between 50 and 80% of the field capacity (water-holding capacity), usually between 10 and 16 g of water per 100 g of dry soil.
4. Adjust the pH to around 7.0 using lime, caustic soda, elemental sulfur or ammonium sulfate.
5. The trays should be incubated at the optimum temperature range for microbial degradation of 25–35 °C.
6. For each 100 kg of oil to be degraded, 1 kg of nitrogen and 0.2 kg of phosphorus should be added as nutrient fertilizer to obtain an oil:N:P ratio of 100:1:0.2.
7. The duration of the biotreatability study depends on the overall objective of the project. In general, it is recommended to run for 3–6 months.
8. Oil and grease or TPH content, moisture and pH should be periodically monitored.
9. The soil should be lightly raked or mixed at 1–2-week intervals to provide proper aeration, mixing, and moisture control.
10. The moisture content should be monitored at 1- or 2-week intervals and the soil sprayed with water to adjust to the optimum moisture content.

Monitoring the disappearance of oil and grease or TPH, as well as moisture, pH, and nitrogen is important during the treatability studies. Total

heterotrophic or hydrocarbon-degrading microbial counts may also be monitored to evaluate the biodegradation process. It is important to use the same sampling strategy and methods throughout the treatment period.

5.2.7 Composting

While composting of yard and municipal wastes has been performed for decades, composting of hydrocarbon-contaminated soils represents an emerging ex-situ biological technology. Composting has been demonstrated to be effective in biodegrading explosives and polycyclic aromatic hydrocarbons (PAHs) in soils (USEPA 1996, 1998). In the composting of contaminated soil, organic amendments including manure, sewage sludge, compost, yard wastes, and food processing wastes are often added to supplement the amount of nutrients and readily degradable organic matter in soil. Sewage sludge and compost containing abundant nitrogen, organic matter, and high microbial diversity, with total microbial populations higher than fertile soils, have great potential in bioremediation. A small-scale biotreatability method (Van Gestel et al. 2003) for composting technology is described here:

1. Two insulated composting bins can be used, one filled with biowaste (vegetable, fruit, garden, and paper waste) only, and the other filled with a mixture of biowaste and petroleum-oil-contaminated soil at a 10:1 ratio (fresh mass).
2. Dewatered sewage sludge or matured compost can be used instead of biowaste.
3. Spruce bark can be used as a bulking agent at the ratio of soil to bulking agent, 1:3 on a volume basis.
4. The soil should be collected from the top 15 cm of the soil surface and air dried and sieved to pass a 2–4 mm sieve.
5. The soil can be spiked with commercial crude oil or diesel oil at a concentration to obtain a concentration of 5–10 g/kg after mixing with the biowaste.
6. The initial pH is adjusted about 7.0–7.4.
7. The composting process is controlled using airflow and moisture content.
8. Aerobic composting can be performed for 12 weeks.

9. At regular time intervals, the content should be turned to avoid preferential aeration pores.
10. Compost samples for chemical and microbiological analyses should be taken every time the compost is mixed.
11. Microbial counts, dry matter content, pH, temperature, electrical conductivity, and exhaust gas composition should be regularly monitored.
12. Microbial composition of the biowaste-only composting bin serves as a reference for the composting process of contaminated soil.
13. To investigate the degradation rate of oil in soil alone, a soil-only experiment (without organic amendments) should also be run as a control.

Composting technologies can be applied to cleanse contaminated soil ex situ. By adding an organic matrix to contaminated soil the general microbial activity is enhanced and also the activity of specific degraders, which may be found in the contaminated soil or introduced along with the organic material. Biodegradation rates in composting systems have been found to be slightly higher than in land treatment of hydrocarbons and lower than in slurry reactors.

5.2.8 Scale-Up

The data obtained from the small-scale biodegradation experiments can be used to design full-scale biotreatment systems. In most cases slurry bioreactors can be directly scaled up. The US EPA has suggested a three-tier approach before a full-scale application of the technology in the field (US EPA, 1991; McFarland et al. 1991):

1. Laboratory screening to establish the occurrence and rate of biodegradation and establishing optimum process parameters
2. Bench-scale testing to establish performance of the process parameters and cost estimate for the scale-up of appropriate technologies
3. Pilot testing on the most promising technology to establish system design and detailed cost structure

Land- or reactor-based full-scale bioremediation systems have been successfully used to clean up hydrocarbon-contaminated soils and sludges. More information on the scale-up of bioremediation technologies can be obtained in the literature (Huesemann 1994; Cutright 1995; Crawford and Crawford 1996; Loehr and Webster 1996; Von Fahnestock et al. 1998; Alleman and Leeson 1999; Stegmann et al. 2001; Singh and Ward 2004).

5.3 Process Monitoring and Evaluation

It is important to make sure that system operation and monitoring plans have been developed for the land treatment operation. Regular monitoring is necessary to ensure optimization of biodegradation rates, to track constituent concentration reductions, and to monitor vapor emissions, migration of constituents into soils beneath the landfarm (if unlined), and groundwater quality. If appropriate, ensure that monitoring to determine compliance with storm water discharge or air quality permits is also proposed.

1. Molecular composition of a petroleum contaminant can be useful in estimating the biodegradation potential of the contaminated soil. Gas chromatography (GC) analysis (Chapt. 3) may identify easily biodegradable compounds such as straight chain alkanes. GC analysis of various volatile (benzene, toluene, ethyl benzene, and xylenes) and semi-volatile (polynuclear aromatic hydrocarbons, PAHs) compounds are required by the regulatory agencies. However, gravimetric determination of oil and grease or TPH content following Soxhlet extraction can be used to design and optimize a reactor or land-based treatment process.

2. Since abiotic processes such as dilution, adsorption, and volatilization can be responsible for hydrocarbon disappearance, criteria other than simple hydrocarbon disappearance should be used to assess biodegradation by microorganisms. Increase in the number of hydrocarbon-degrading bacteria as the bioremediation progresses provides evidence of biodegradation. Formation of colonies on the surface of a solidified mineral salts medium with silica gel, incubated in vapors of volatile hydrocarbons (Walker and Coleman 1976), can be used to enumerate hydrocarbon-degrading bacteria. Bacteria capable of degrading semi-volatile hydrocarbons (e.g., PAHs) can be enumerated by examining colonies on agar plates for their ability to visibly alter a layer of precipitated insoluble hydrocarbon (Bogardt and Hemmingsen 1992). The modified most probable number (MPN) technique can be used for non-volatile hydrocarbons either by applying a floating sheen of oil to the surface of mineral medium or by placing hydrocarbons dissolved in a solvent in 24- or 96-well microtiter plates (Brown and Braddock 1990; Steiber et al. 1994; Haines et al. 1996). The presence of hydrocarbon-utilizing bacteria is detected by the emulsification or dispersion of sheen, by reduction of added iodonitrotetrazolium violet, or by the appearance of colored metabolites in the medium (see also Chapt. 13).

3. Since microbial communities play a significant role in biogeochemical cycles, it is important to analyze the community structure and its changes during bioremediation processes (Chaps. 10 and 12). The temporal and spatial changes in bacterial populations and the diversity of the microbial community during bioremediation can be determined using sophisticated molecular methods (van Elsas et al. 1998; Widada et al. 2002).
4. Biodegradation potential of a hydrocarbon-contaminated soil can be estimated by its chemical characterization and the relative biodegradability of the contaminants. Monoaromatic compounds such as benzene and alkyl benzene and low molecular weight n-alkanes are easily biodegradable as compared to high molecular weight and highly branched molecules. While PAHs with four or more rings are considered recalcitrant, two or three ring PAHs can be degraded by different microbial species.
5. The volatile constituents present in petroleum-contaminated soils tend to evaporate during biotreatment, particularly during tilling or plowing operations in land treatment and aeration of the bioreactors, rather than being biodegraded by bacteria. For compliance with air quality regulations, the volatile organic emissions should be estimated based on initial concentrations of the petroleum constituents present. Depending upon specific regulations for air emissions, control of VOC emissions may be required. Control involves capturing vapors and then passing them through an appropriate treatment process before being vented to the atmosphere. Control devices range from an erected structure such as a greenhouse or plastic tunnel to a simple cover such as a plastic sheet for land treatment and a carbon filter or biofilter for a slurry reactor.
6. Solid-phase microextraction (SPME) has been used to monitor biodegradation of semivolatile hydrocarbons in diesel-fuel-contaminated water and soil (Eriksson et al. 1998) and of volatile hydrocarbons during bacterial growth on crude oil (Van Hamme and Ward 2000). Although the method requires external calibration with several standard calibration curves, SPME was proven to be a rapid and accurate method for monitoring volatile and semivolatile hydrocarbons in petroleum biodegradation systems.

5.4 Bioaugmentation

Bioaugmentation can be defined as the introduction of a large number of exogenous microorganisms into the environment of a biotreatment sys-

tem. Diverse microorganisms, including many species of bacteria and fungi are known to degrade hydrocarbons. The most prevalent bacterial hydrocarbon degraders belong to the genera *Pseudomonas*, *Achromobacter*, *Flavobacterium*, *Rhodococcus,* and *Acinetobacter*. *Penicillium*, *Aspergillus*, *Fusarium,* and *Cladosporium* are most frequently isolated hydrocarbon degrading filamentous fungi. Among the yeasts *Candida*, *Rhodotorula*, *Aureobasidium*, and *Sporobolomyces* are the hydrocarbon degraders most often reported (Van Hamme et al. 2003). Environmental and nutritional factors influence the presence, survival, or activity of microorganisms in contaminated soils.

There are at least four different routes that result in the development of microbes capable of degradation of hydrocarbons at a certain site:

1. The indigenous microflora are exposed to the contaminant long enough for genetic evolution to create a capacity to degrade the compound(s).
2. The indigenous microflora, adapted to the local conditions, are exposed to one or more contaminating xenobiotic compounds. The bacteria acquire genes and degradation pathways from bacterial cells immigrating from elsewhere.
3. The indigenous, well-adapted microflora are maintained ex-situ and then artificially supplied with the required degradative capacity.
4. A bacterium that is thought to be competitive at the contaminated site is chosen. This may be a strain that is known to degrade the contaminant or one that is specifically constructed for this purpose.

Bioaugmentation-related experiments can be conducted in slurry bioreactors described above. Bioaugmentation studies can be carried out either using mixed cultures or individual pure strains. The effect of initial population size on biodegradation of contaminants can be determined by varying inoculum densities. The inoculum size can be varied from 10^5 to 10^9 CFU/g of soil in the bioaugmentation studies. The effect of a commercial or selectively developed inoculum on the rate of biodegradation, CO_2 evolution, time of lag phase after inoculation, and microbial population dynamics during biodegradation process can be monitored.

5.5 Effect of Surfactants

The biodegradation rate of a contaminant depends on the rate of contaminant bioavailability, uptake, and mass transfer. Bioavailability of a contaminant in soil is influenced by a number of factors such as desorption,

diffusion, and dissolution. Use of chemical- or bio-surfactants in contaminated soil can help overcome bioavailability problems and accelerate the biodegradation process.

Biosurfactants, surface-active substances synthesized by living cells, have the properties of reducing surface tension, enhancing the emulsification of hydrocarbons, stabilizing emulsions, and solubilizing hydrocarbon contaminants to increase their availability for microbial degradation. Biosurfactant-producing microbes play an important role in the accelerated bioremediation of hydrocarbon-contaminated sites (Rahman et al. 2003; Shin et al. 2004). The low-molecular-weight biosurfactants (glycolipids, lipopeptides) are more effective than those of high molecular weight (amphipathic polysaccharides, proteins, lipopolysaccharides, lipoproteins) in lowering the interfacial and surface tensions (Mulligan 2005).

Some simple laboratory experiments to study biosurfactant production and application in bioremediation are described here.

5.5.1 Screening of Microbial Cultures for Biosurfactant Production

Different microbial cultures can be screened for biosurfactant production using the following method:

1. Prepare a series of 250-mL flasks containing 50 mL of sterile YPG medium (composition per L: 5 g peptone, 5 g yeast extract, 10 g glucose, pH 7.0) and incubate on a shaker (200 rpm) at 30 °C after inoculation with individual cultures.
2. Add 1% glycerol after 24 h.
3. Measure biomass content, biosurfactant production, surface tension, and emulsification activity at 12–24 h intervals.
4. For biomass determination, filter the culture broth using GF/C filters, place the filters at 110 °C for 24 h, and weigh to calculate biomass (dry mass).
5. Surface-active compounds can be extracted by liquid-liquid extraction using 10 mL of chloroform:methanol (2:1) mixture from 10 mL of the cell-free culture broth acidified with 1 N HCl to pH 2. Concentrate the organic extracts by drying them overnight in a drying chamber at a temperature around 44 °C, and measure the mass of the biosurfactant.

For purification of the biosurfactant to determine its properties and application, the culture broth is filtered through a centrifuge filter with 10 kDa molecular weight cut-off at 6,000 *g* until the minimal amount of

retentate is achieved. The retentate is diluted in 50% methanol in order to dissociate the micelles and filtered at 6000 *g* again. After collection of filtrate, methanol is evaporated under vacuum in a rotary evaporator at 65 °C and the aqueous solution of the purified biosurfactant is lyophilized.

Surface tension (mN/m) can be measured using a standard commercial tensiometer. The emulsification activity can be determined by adding a hydrocarbon (xylene, benzene, *n*-hexane, kerosene, gasoline, diesel fuel, or crude oil) to the same volume of cell-free culture broth, vortexing for 2 min and letting stand for 24 h. The emulsification activity is determined as the percentage of height of the emulsified layer divided by the total height of the liquid column (Rahman et al. 2003).

A blood agar lysis method can also be used for screening cultures for their biosurfactant-producing capabilities (Youssef et al. 2004). Culture is streaked onto blood agar plates and incubated for 48 h at 37 °C. The zones of clearing around the colonies indicate biosurfactant production. The diameter of the clear zones depends on the concentration of the biosurfactant.

5.5.2 Effect of Biosurfactants

Biosurfactant preparations can be purchased from a commercial chemical supplier or purified from the culture broth as described above. For different hydrocarbons, a biosurfactant is added to the cultures to obtain concentrations above and below the critical micelle concentration (CMC). The CMC value is determined by measuring surface tension in different dilutions of a 4 g/L solution of the biosurfactant. The value of CMC, expressed in mg/L, is obtained from the plot of the surface tension versus the logarithm of the concentration. A rhamnolipid biosurfactant concentration of 50–2,000 mg/L is generally useful in biodegradation studies.

The biodegradation experiment to study effect of biosurfactants can be conducted in 250 mL Erlenmeyer flasks containing 50 mL of the culture medium described before. Appropriate controls, such as no-biosurfactant and abiotic controls, are run along with the flasks containing different concentrations of the biosurfactant. Cultures are incubated on a shaker for 7–14 days at 30 °C.

5.5.3 Effect of Chemical Surfactants

Properties of chemical surfactants that influence their efficacy include charge (nonionic, anionic, or cationic), hydrophilic-lipophilic balance (HLB, a measure of surfactant lipophilicity), and CMC (the concentration

at which surface tension reaches a minimum and surfactant monomers aggregate into micelles). However, there is always a concern that the surfactant may get used preferentially as a carbon source instead of the contaminant. Hence, there is a need to provide a perspective as to when or how surfactants may be exploited in petroleum hydrocarbon degradation processes to improve rates and extents of degradation.

Typical surfactant concentrations for washing of contaminant soil are 1–2%, whereas the same contaminants may be solubilized in an aqueous solution at a surfactant concentration of 0.1–0.2%. Non-ionic surfactants within the HLB range of 11 to 15 can optimally support microbial degradation of hydrophobic contaminants. Nonylphenol ethoxylated surfactants with HLB 12 and 13 can substantially enhance biodegradation of hydrocarbons at surfactant concentrations greater than CMC value. Different groups of nonionic surfactants should be tested at different concentrations greater than their CMC during feasibility studies in soil microcosms or slurry reactors.

5.6 Optimization of Environmental Conditions

The procedures described in the previous sections on different technologies can be used in studies of the factors affecting biodegradation rates and determining appropriate biotreatment strategy for contaminated soil.

1. The optimum soil pH for hydrocarbon bioremediation in soil ranges from 6 to 8. Methods for adjusting pH usually include periodic application of lime and/or sulfur. The requirement of acid or alkaline solutions/solids for pH control is developed in biotreatability studies and the frequency of their application is modified during land treatment or slurry reactor operation as needed. In case of acidic soil (pH < 6), lime or calcium carbonate may be added to increase the pH to the required optimum range. For alkaline soil (pH > 8), elemental sulfur, ammonium sulfate, or aluminum sulfate may be added to lower the pH.

2. Optimum temperature range for microbial degradation is 25 to 35 °C. Biodegradation rates are expected to slow considerably below 15 °C or above 40 °C. However, temperature cannot be maintained for land application. Land treatment of hydrocarbon-contaminated soils is difficult to operate in temperate and arid zones. Slurry bioreactors are always more useful in such places because environmental conditions can be more precisely maintained and with relative ease.

3. During land treatment, soil microorganisms can only biodegrade petroleum hydrocarbons within a limited range of favorable soil moisture

conditions. If the soil is too dry, bacterial growth and metabolisms will be greatly reduced or even inhibited. Alternatively, if the soil is too wet or flooded, soil aeration will be greatly impaired which, in turn, will result in anaerobic conditions that are not conducive to hydrocarbon biodegradation. Since the moisture content at field capacity is strongly dependent on the soil type (clay and high organic matter soils retain comparatively higher moisture content), it is important to determine the moisture retention profile for each soil to be studied. The optimum moisture content for stimulating petroleum hydrocarbon biodegradation ranges from 50 to 80% of the moisture content at field capacity. For example, if the soil moisture at field capacity was determined to be 20 g of water per 100 g of dry soil, the soil moisture content should be maintained between 10 and 16 g of water per 100 g of dry soil.

4. In order to limit the demand of oxygen by soil bacteria, it is important not to overload the soil with too high levels of oil contamination during land application. As outlined below, the optimum contaminant loading level for land treatment is about 5% (by weight) of oil. Maximum degradation rates are typically observed in the 10–15 cm upper plow layer if hydrocarbon concentrations are maintained around 5%. Addition of peroxygen compounds may also help slowly release oxygen into the soil and thereby enhance the aerobic biodegradation of petroleum hydrocarbons.

5. There are other processes such as volatilization, leaching, sorption and photo-oxidation that may cause the removal of certain hydrocarbon compounds or classes during biotreatment. It has been estimated that between 15 and 60% of fuel hydrocarbons (diesel, jet fuel, and heating oil) can be lost during soil bioremediation by land treatment solely due to evaporation (Salanitro 2001). At room temperature (20 °C), most hydrocarbons with carbon numbers up to C_{15} or C_{16} readily evaporate from soil if in free contact with air. Even heavier hydrocarbons ($> C_{16}$) including three- and four-ring PAHs are likely to volatilize in intense sunshine. These competing loss mechanisms during field or laboratory bioremediation studies should be measured or estimated either by calculating a complete mass balance or by carrying out proper microbial control experiments.

5.7 Optimization of Nutritional Factors

For biotreatment of petroleum hydrocarbons, bacteria that are both aerobic and heterotrophic are the most important in the biodegradation process.

Since microorganisms require organic and inorganic nutrients such as nitrogen, phosphorus, magnesium, calcium, iron, and trace metals to support cell growth and sustain biodegradation processes, nutrients need to be supplemented during biotreatment in bioreactors or land in order to maintain active bacterial populations. However, excessive amounts of certain nutrients such as phosphate and sulfate can repress microbial metabolism. Important nutrient sources for biotreatability or feasibility studies are shown Table 5.1.

1. Nutrients are added for the growth and maintenance of microorganisms. By providing an appropriate balance of nutrients it is possible to achieve high level of growth of hydrocarbon-degrading bacteria and thus accelerated rates of hydrocarbon degradation. The typical non-carbon elemental composition of major bacterial components is nitrogen 12.5%; phosphorus 2.5%; potassium 2.5%; sodium 0.8%; sulphur 0.6%; calcium 0.6%; magnesium 0.3%; copper 0.02%; manganese 0.01%, and iron 0.01% (Rehm 1993). Use of appropriate concentrations and ratios of nutrients can avoid a situation where growth is limited by depletion of one essential nutrient while all other nutrients may be present in excess.
2. An oil carbon content of 80% can be assumed for the purpose of calculating C:N or C:P ratio. Although a wide range of C:N and C:P ratios has been recommended in the literature, an oil:N:P ratio of 100:1:0.2 can be used for the feasibility studies. Thus, for each 100 kg of oil to be degraded, 1 kg of nitrogen and 0.2 kg of phosphorus can be added as nutrient fertilizer in the preliminary studies. Optimum C:N ratio can

Table 5.1. Important nutrient sources for biotreatability or feasibility studies

Nutrient source	Examples
Defined medium	
Nitrogen	KNO_3, NH_4NO_3, NH_4Cl
Phosphorus	KH_2PO_4, sodium tripolyphosphate ($Na_5P_3O_{10}$)
Potassium	KNO_3
Calcium	$CaCl_2 \cdot 2H_2O$
Magnesium	$MgSO_4 \cdot 7H_2O$
Iron	$FeCl_3 \cdot 6H_2O$
Trace metals	$MnSO_4 \cdot H_2O$, $CuSO_4 \cdot 5H_2O$, $ZnCl_3 \cdot 4H_2O$, H_3BO_3, $CoCl_2 \cdot 6H_2O$, $Na_2MoO_4 \cdot 2H_2O$
Complex medium	
Nitrogen	Yeast extract, Peptone, Urea, NPK fertilizer
Phosphorus	NPK fertilizer
Magnesium	NPK fertilizer
Trace metals	Yeast extract, NPK fertilizer

determined in microcosms or slurry bioreactors by varying C:N ratio from 10 to 100.

3. For land treatment, nutrient supply methods usually include periodic application of solid fertilizers, while tilling to blend soils with the solid amendments, or applying liquid nutrients using a sprayer. For bioslurry reactors, a blend of solid nutrients can be added, which is quickly dissolved in the medium due to continuous mixing. The composition of nutrients is developed in lab treatability studies.
4. The inability of microbes to completely mineralize a contaminant and transform it to other organic compounds means that these organisms require other substrates to support their growth. The contaminants are transformed by "co-metabolic" processes, where a second substrate serves as primary energy or carbon source.
5. Using a naturally selected and acclimated indigenous bacterial culture originating from the sludge is supplemented with a carefully designed blend of nutrients containing sources of nitrogen, phosphate, a complex protein, essential minerals, and a surfactant. The bioslurry reactor system can promote growth of a highly active microbial population and rapid conversion of the petroleum hydrocarbons at the rate of about 1% petroleum hydrocarbons degraded per day (Ward et al. 2003).
6. Generally high molecular weight PAHs (five-ring) are only biodegraded in the presence of other hydrocarbons such as lower molecular weight PAHs or complex hydrocarbon mixtures such as crude oil. If these necessary co-substrates are absent, the co-metabolic biodegradation of higher molecular weight PAHs cannot proceed.

5.8 Conclusions

Bioremediation is a cost effective and environmentally friendly hydrocarbon-contaminated soil remediation technology. The successful bioremediation of contaminated soils depends on numerous environmental parameters and operational factors, which need to be optimized in order to achieve maximum treatment benefits. Even under optimal conditions it is unlikely that all contaminants will be removed from the soil. This incomplete biodegradation may be acceptable if the residual hydrocarbons can be shown to have no significant impact on ecological receptors and do not pose a risk to groundwater resources.

The effectiveness of bioremediation depends on the success in identifying the rate-limiting factors and optimizing them in the feasibility and

biotreatability studies. Feasibility studies are essential and may have enormous impact on the cost of the full-scale operation. Depending on the site, nature of contamination, and type of soil, various methods for feasibility studies are currently available. These methods can be modified to accommodate the lab facilities and equipment availability. Sometimes it is difficult to extrapolate the results directly from the laboratory to the field. Nevertheless, successful bench- or pilot-scale test results are mostly useful in designing the full-scale bioprocessing system for bioremediation of hydrocarbon-contaminated soil.

One of the main barriers to greater effective adoption of bioremediation technologies is the perception that the processes are very project-specific, requiring much customization. There is a need to develop more robust and technologically versatile processes that do not require significant research and development for each project (Ward 2004). Government funding initiatives and the market favor use of more controlled and accelerated processes and that are typically more predictable (Srinivasan 2003). Hughes et al. (2000) have provided guidance with regard to selection of bioremediation configuration for treatment of different classes of chemicals.

The choice of technology configuration based on application of such principles should precede the design of a feasibility study, and the latter then used to confirm and validate the effectiveness of the technology.

References

Alleman BC, Leeson A (eds) (1999) Bioreactor and ex situ biological treatment technologies. Battelle Press, Columbus, pp 31–36

Bogardt AH, Hemmingsen BB (1992) Enumeration of phenanthrene-degrading bacteria by an overlayer technique and its use in evaluation of petroleum contaminated sites. Appl Environ Microbiol 58:2579–2582

Brown EJ, Braddock JF (1990) Sheen screen, a miniaturized most-probable number method for enumeration of oil-degrading microorganisms. Appl Environ Microbiol 56:3895–3896

Crawford RL, Crawford DL (eds) (1996) Bioremediation: Principles and Applications. Cambridge Univ Press, Cambridge, UK

Cutright (1995) A feasible approach to the bioremediation of contaminated soil: from lab scale to field test. Fresenius Environ Bull 4:67–73

DECHEMA (1992) Laboratory methods for the evaluation of biological soil clean-up processes. Interdisciplinary Working Group, Environmental Biotechnology – Soil. Deutsche Gesellschaft fuer Chemisches Apparatewesen, Chemische Technik und Biotechnologie, Germany

Eriksson M, Swartling A, Dalhammer G (1998) Biological degradation of diesel fuel in water and soil monitored with solid-phase micro-extraction and GC-MS. Appl Microbiol Biotechnol 50:129–134

Grommen R, Verstraete W (2002) Environmental biotechnology: the ongoing quest. J Biotechnol 98:113–123

Haines JR, Wrenn BA, Holder EL, Strohmeier KL, Herrington RT, Venosa AD (1996) Measurement of hydrocarbon-degrading microbial populations by a 96-well plate most-probable number procedure. J Ind Microbiol 16:36–41

Huesemann MH (1994a) Guidelines for the development of effective statistical soil sampling strategies for environmental applications. In: Calabrese EJ, Kostecki PT (eds) Hydrocarbon-contaminated soils and groundwater. Lewis, Boca Raton, pp 47–96

Huesemann MH (1994b) Guidelines for land-treating petroleum hydrocarbon contaminated soils. J Soil Contamin 3:299–318

Hughes JB, Neale CN, Ward CH (2002) Bioremediation. Encyclopedia of Microbiology, 2nd edn, Academic Press, New York, pp 587–610

Iwamoto T, Nasu M (2001) Current bioremediation practice and perspective. J Biosci Bioeng 92:1–8

Jurgensen KS, Puustinen1 J, Suortti A-M (2000) Bioremediation of petroleum hydrocarbon-contaminated soil by composting in biopiles. Environ Pollut 107:245–254

Lang S, Wullbrandt D (1999) Rhamnose lipids – biosynthesis, microbial production and application potential. Appl Microbiol Biotechnol 51:22–32

Liu D (1989) Biodegradation of pentachlorophenol and its commercial formulation. Bull Environ Contam Toxicol 4: 115–127

Loehr RC, Webster MT (1996) Performance of long-term, field-scale bioremediation processes. J Haz Mat 50:105–128

Mandelbaum RT, Allan DL, Wackett LP (1995) Isolation and characterization of a *Pseudomonas* sp that mineralizes the s-triazine herbicide atrazine. Appl Environ Microbiol 61: 1451–1457

McFarland MJ, Sims RC, Blackburn JW (1991) Use of treatability studies in developing remediation strategies for contaminated soils. In: Sayler GS, Fox R, Blackburn JW (eds) Environmental biotechnology for waste treatment. Plenum, New York, pp 163–174

Mulligan CN (2005) Environmental applications for biosurfactants. Environ Pollut 133: 183–198

Noha H, Youssef NH, Duncana KE, Naglea DP, Savagea KN, Knappb RM, McInerneya MJ (2004) Comparison of methods to detect biosurfactant production by diverse microorganisms. J Microbiol Meth 56:339– 347

OECD (1981) Inherent biodegradability in soil. In: OECD Guidelines for testing of chemicals, Part 304A, Organization of Economic Co-operation and Development, Paris, France, pp 1–11

Pfarl C, Ditzelmuller M, Loidle M, Streichsbier F (1990) Microbial degradation of xenobiotic compounds. FEMS Microbiol Ecol 73: 255–262

Rahman KSM, Rahman TJ, Lakshamanaperumalsamy P, Marchant R, Banat IM (2003) The potential of bacterial isolates for emulsification with a range of hydrocarbons. Acta Biotechnol 23: 335–345

Rehm HJ (1993) Biotechnology Vol 3, VCH, Weinheim, pp 131

Salanitro JP (2001) Bioremediation of petroleum hydrocarbons in soil. Adv Agron 72:53–105

Shin K-H, Kim K-W, Seagren EA (2004) Combined effects of pH and biosurfactant addition on solubilization and biodegradation of phenanthrene. Appl Microbiol Biotechnol 65: 336–343

Shulga A, Karpenko E, Vildanova-Martsishin R, Turovsky A, Soltys M (1999) Biosurfactant enhanced remediation of oil-contaminated environments. Adsorpt Sci Technol 18: 171–176.

Singh A, Mullin B, Ward OP (2001) Reactor-based process for the biological treatment of petroleum wastes, PN # 200. Proceedings, Middle East Petrotech 2001 Conference, Bahrain, pp 1–13

Singh A, Ward OP (eds) (2004) Applied bioremediation and phytoremediation. Soil Biology Series, Volume 1, Springer-Verlag, Germany
Skladany GJ, Baker KH (1994) Laboratory biotreatability studies. In: Baker KH and Herson DS (eds) Bioremediation, McGraw-Hill, New York, pp 97–172
Srinivasan U (2003) US Bioremediation Markets. Frost and Sullivan Research Report No 7857. Palo Alto California, pp 1–300
Stegmann R, Brunner G, Calmano W, Matz G (2001) Treatment of contaminated soil: fundamentals, analysis, applications. Springer, Berlin
Steiber M, Haesler F, Werner P, Frimmel FH (1994) A rapid screening method for microorganisms degrading polycyclic aromatic hydrocarbons in microplates. Appl Microbiol Biotechnol 40: 753–755
USEPA (1991) Guide for conducting treatability studies under CERCLA: aerobic biodegradation remedy screening – interim guidance, USEPA publication no. EPA/540/2–91/013A
USEPA (1996) Engineering bulletin: Composting, EPA/540/S-96/502
USEPA (1998) An analysis of composting as an environmental remediation technology, EPA530-R-98–008
van Elsas JD, Duarte GF, Rosado AS, Smalla K (1998) Microbiological and molecular biological methods for monitoring microbial inoculants and their effects in the soil environment. J Microbiol Meth 32:133–154
Van Gestela K, Mergaertb J, Swings J, Coosemansa J, Ryckeboer J (2003) Bioremediation of diesel oil-contaminated soil by composting with biowaste. Environ Poll 125: 361–368
Van Hamme J, Singh A, Ward OP (2003) Recent advances in petroleum microbiology. Microbiol Mol Biol Rev 67:503–549
Van Hamme JD, Ward OP (2000) Development of a method for the application of solid-phase microextraction to monitor biodegradation of volatile hydrocarbons during bacterial growth on crude oil. J Ind Microbiol Biotechnol 25:155–162
Vecchili GI, Delpanno MT, Painceira MT (1990) Use of selected autochthonus soil bacteria to enhance degradation of hydrocarbons in soils. Environ Pollut 67:249–258
Venosa AD, Haines JR, Nisasamaneepong W, Gouind R, Pradhan S, Siddique (1992) Efficacy of commercial products in enhancing oil biodegradation in closed laboratory reactor. J Ind Microbiol 10:13–23
Von Fahnestock FM, Wickramanayake GB, Kratzke RJ, Major WR (1998) Biopile design, operation, and maintenance handbook for treating hydrocarbon-contaminated soils, Battelle Press, Columbus, Ohio
Walker JD, Colwell RR (1976) Enumeration of petroleum-degrading microorganisms. Appl Environ Microbiol 31:198–207
Ward OP (2004) The industrial sustainability of bioremediation processes. J Ind Microbiol Biotechnol 31:1–4
Ward OP, Singh A, Van Hamme JD (2003) Accelerated biodegradation of petroleum waste. J Ind Microbiol Biotechnol 30: 260–270
Widada J, Nojiri H, Omori T (2002) Recent developments in molecular techniques for identification and monitoring of xenobiotic-degrading bacteria and their catabolic genes in bioremediation. Appl Microbiol Biotechnol 60:45–59
Wolf DC, Dao TH, Scott HD, Lavy TL (1989) Influence of sterilization method on selected soil microbiological, physical and chemical properties. J Environ Qual 18:39–44
Youssef NH, Duncan KE, Nagle DP, Savage KN, Roy M, Knapp RM, McInerney MJ (2004) Comparison of methods to detect biosurfactant production by diverse microorganisms. J Microbiol Meth 56:339– 347

6 Feasibility Studies for Microbial Remediation of Metal-Contaminated Soil

Franz Schinner, Thomas Klauser

Introduction

Objectives. Heavy metal contamination of soil is widespread due to metal processing industries, tannery, combustion of wood, coal and mineral oil, traffic, and plant protection. The toxic effects of heavy metals result mainly from the interaction of metals with proteins (enzymes) and inhibition of metabolic processes. In contrast to organic pollutants, metals are not mineralized by microorganisms but can be oxidized or reduced, transformed to different redox stages, or complexed by organic metabolites.

Besides excavation and deposition, a conventional treatment for decontamination of metal-polluted soil is extraction using mineral acids. The disadvantages of such a treatment are the destruction of soil, high costs of acids, and low acceptance. Alternative remediation strategies to reduce bioavailability of metals are: (1) immobilization with repeated addition of substances such as carbonate, phosphate, apatite, zeolite, clay minerals, peat, or humus: and (2) bioleaching with heterotrophic microorganisms, preferably fungi. The latter method represents a sustainable remediation treatment of metal-polluted soils (Wasay et al. 1998, Schinner et al. 2000). Autotrophic bacteria as known from ore leaching cannot be recommended for this treatment due to the high buffer capacity of soil compared to ore. The most limiting factor of heterotrophic leaching is the availability of inexpensive carbohydrates, such as molasses or the refuse from processing sugar, fruits, or white wine. Heterotrophic leaching can be done off site, on site, and in situ, and is an alternative treatment for the decontamination of metal-containing filter dusts (Schinner and Burgstaller 1989; Burgstaller et al. 1992) and industrial sludges.

Principle. Metal-contaminated soil is supplemented with carbohydrates to increase the excretion of organic acids by autochthonous (procedure A) or inoculated (procedure B) fungi. Organic acids mobilize metals that are eluted from soil by percolation with water.

Theory. In natural ecosystems fungi play an important role in the mobilization of nutrients and trace elements from soils. Autochthonous soil fungi

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but also inoculated single strains or mixed populations increase organic acid production after the addition of carbohydrates. Fungi mobilize metals from mineral soils by excretion of acidifying protons (acidolysis), by excretion of organic acids forming cyclic organometal complexes (complexolysis), or by redoxolysis with organic acids. Some of the Deuteromycetes, especially members of the genera *Aspergillus* and *Penicillium*, produce various organic acids, such as citric, oxalic, tartaric, gluconic, succinic, formic, and amino acids (Burgstaller and Schinner 1993; Gadd 1999). The metal-containing eluate obtained after percolation of soil with water can be regenerated by conventional metal recovery, such as precipitation, ion exchange, or biosorption.

Equipment

- Percolator: a filtration unit (funnel and suction flask; e.g., Nalgene, 500 mL; Nalge Nunc, Naperville, IL, USA). with gauze mat (e.g., Schleicher & Schüll TG 100; Schleicher & Schüll, Dassel, Germany) instead of filter
- Vacuum pump
- Multichannel peristaltic pump
- Timer (for circuit switching peristaltic pump and vacuum pump)
- Atomic absorption spectrometry (AAS) or inductively-coupled plasma-atomic emission spectrometry (ICP-AES) for metal analyses
- High performance liquid chromatography (HPLC) for analyses of organic acids (optional): e.g., column AMINEX-HPX 87H (Bio-Rad, Hercules, CA, USA); flow rate 0.6 mL/min; column temperature 41 °C, wavelength 210 nm, eluant 4 mM H_2SO_4 (Womersley et al. 1985)

Materials and Reagents

- Quartz sand
- Sawdust
- Grain (e.g., barley, wheat, rye)
- Organic-acid-producing fungi, e.g., *Aspergillus* sp. or *Penicillium* sp.
- Complex substrate: e.g., dried refuse of sugar production, fruit, or white wine processing
- Molasses solution: 150 g (< 75% dry mass)/L of water

- Sterile KCl solution: 10 g/L
- Deionized water

Sample Preparation

Use air-dried, sieved (< 5 mm) soil.

Procedure

Procedure A: Metal Leaching with Autochthonous Microorganisms

1. Assemble percolation units, pour about 10 mm quartz sand onto the gauze mat. Connect tubes to flasks containing molasses and water, and install pumps and timer. Prepare 3–4 replicates.
2. Weigh 150 g of sieved soil, 15 g of sawdust, and 15 g of complex substrate into a beaker, mix, and pour it into the percolator.
3. Add 60 mL of molasses solution, 50 mL of KCl solution and 70 mL of water to rewet substrate.
4. Start percolation with molasses solution, use the peristaltic pump at a flow rate of 20 mL/h for 15 h every day. Repeat this percolation on days 2, 3, and 6.
5. On days 3, 5, and 7, use water instead of molasses solution as percolation fluid.
6. For the suction of the fluid substrate and water and for additional aeration, start the vacuum pump every 90 min for 25 min without interrupting the percolation (from the beginning to the end of experiment).
7. To measure the leaching efficiency, take daily samples for metal detection. After 15 h of percolation with molasses or water add 120 mL of water onto the soil, suck off each percolator for 5 min, centrifuge 5 mL of eluate for 15 min at 10,000 *g*. The supernatant is used for quantification of metals and organic acids. Repeat this procedure every day in the same way.
8. After 3 days add 15 g of complex substrate and mix it into the topsoil of each percolator.

Procedure B: Metal Leaching with Bioaugmentation

1. Prepare the inoculum for bioaugmentation as follows:
   1.1. Sterilize 30 g of grain together with 30 mL of water in a 500-mL Erlenmeyer flask for 35 min at 121 °C.

1.2. Inoculate the substrate with organic-acid-producing fungi. Use 5 mL of spore suspension or collect spores from a stock culture to which sterile ringer solution is added to wash away spores.

1.3. Incubate the inoculated grain 7–10 days at 25 °C to produce spores.

1.4. Add 200–300 mL of sterile KCl solution and shake thoroughly. Transfer the spore-containing suspension to a sterile flask. About 10^8 spores/mL are required.

1.5. Store spore suspensions for the preparation of further inoculates at −20 °C.

2. Perform the procedure for metal leaching as described for procedure A, except for step 3: To rewet and inoculate soil and substrate, add 70 mL of spore suspension instead of 70 mL of water to 60 mL of molasses solution and 50 mL of KCl solution. After 3 days (step 8) add 20 mL of spore suspension together with 15 g complex substrate and mix it into the topsoil of each percolator.

Monitoring Metal Remediation

Determine the pH value (Chapt. 2), the heavy metal content (using AAS or ICP; Chapt. 3), and eventually organic acids in the eluate using HPLC (Womersley et al. 1985). Additionally, soil enzyme activity (Chapt. 17), microbial biomass (Chapt. 14), or fungal biomass of soil (Rössner 1996) can be analyzed.

Calculation

Calculate the amount of leached metals from the sum of metal contents measured each day, considering soil dry mass and extraction volume.

Notes and Points to Watch

- Metal-leaching experiments with soil percolation attain decontamination rates of 40–80%.
- Inoculation with organic-acid-producing strains can result in a higher leaching efficiency.
- Mixed cultures of fungi are more efficient than single strains.
- The clay fraction of soils contains more metals than the silt and sand fraction.
- The leaching efficiency of soil microorganisms depends strongly on the soil buffer capacity. Acidification of soil (pH < 6) may be necessary.

- The leaching efficiency of soil microorganisms depends on the quality and quantity of soil organic matter, and on the aeration of soil.
- The duration of percolation and the addition of molasses and water must be optimized for each soil.
- In situ bioleaching of metal-contaminated soils needs effective drainage systems.
- Depending on the soil material needed for monitoring analyses, a larger volume of soil (for example, 300 g soil in a 1 L percolator) can be used.

References

Burgstaller W, Schinner F (1993) Leaching of metals with fungi. J Biotechnol 27:91–116

Burgstaller W, Straßer H, Wöbking H, Schinner F (1992) Solubilization of zinc from filter dust with *Penicillium simplicissimum*: Bioreactor leaching and stoichiometry. Environ Sci Technol 26:340–346

Gadd G.M (1999) Fungal Production of citric and oxalic acid: Importance in metal speciation, physiology and biogeochemical processes. Adv Microb Phys 41:47–92

Rössner H (1996) Fungal biomass by ergosterol content. In: Schinner F, Kandeler E, Öhlinger R, Margesin R (eds) Methods in soil biology. Springer, Berlin Heidelberg New York, pp 49–51

Schinner F, Burgstaller W (1989) Extraction of zinc from industrial waste by a *Penicillium* sp. Appl Environ Microbiol 55:1153–1156

Schinner F, Huber W, Atzwanger M, Klauser T (2000) Metodo per il trattamento di suoli inquinati da composti da metalli pesanti (Method for the treatment of soils polluted by heavy metal compounds and related plant of treatment). Patents: IT/01.12.00/IT RM000631, IT/01.12.00/IT RM000632

Wasay S.A, Barrington S.F, Tokunaga S (1998) Using *Aspergillus niger* to bioremediate soil contaminated by heavy metals. Bioremediation J 2:183–190

Womersley C, Drinkwater L, Crowe JH (1985) Separation of tricarboxylic acid cycle acids and other related organic acids in insect haemolymph by high-performance liquid chromatography. J Chromatogr 318:112–116

7 Feasibility Studies for Phytoremediation of Metal-Contaminated Soil

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7.1 Introduction

Phytoremediation, which is the use of herbaceous plants and trees to stabilize, recover, or volatilize pollutants in contaminated soil, is considered an emerging new technology. The application of phytoremediation is said to be environmentally friendly, relatively low in cost, and high in public acceptance. However, there are still a number of limitations that affect its implementation on a large scale. The most considerable limitations are: narrow range of contaminant concentrations within which the method can be applied (potential of plant toxicity), dependence on weather, time-dependent growing season, and requirement for management of by-products. Until recently, the most commonly applied phytoremediation methods have been phytoextraction and phytostabilization, particularly for soils polluted with heavy metals.

Phytoremediation is more a biological than a technical approach, and it is difficult to create a definitive protocol that could be applied to any polluted site. The limiting factors differ from site to site, and therefore each project protocol, must be customized to site-specific conditions.

7.2 Phytoextraction

Phytoextraction is a biological method that utilizes properties of specific species of plants to take up and accumulate pollutants from soil. Certain species, called hyperaccumulators, may accumulate metals up to several percent of their dry mass (Brooks 1998; McGrath et al. 2000). Unfortunately, the practical use of these plants for phytoextraction is limited due

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to sparse production of biomass and problems with mechanical harvesting. Nevertheless, genetic research is being conducted to increase biomass production.

A compromise to the problem of low accumulation properties is to use plant species with extensive biomass production to compensate for the lower metal accumulation rates. Such plants remove certain amounts of metals, but the process is very slow. The addition of chelators to contaminated soil enhances metal uptake, an approach known as "induced phytoextraction" (Salt et al. 1998). Depending on local climate and chemistry of pollutants being removed, the most commonly used species for heavy-metal extraction are *Brassica* and *Helianthus*.

A schematic diagram of the processes in an induced phytoextraction project aimed at cleaning up soils moderately contaminated with lead, cadmium, and/or zinc is presented in Fig. 7.1. The description contains step-by-step procedures necessary to perform a phytoextraction project including theory, legal considerations, technical aspects, as well as logistic issues and equipment.

7.2.1 Treatability Study

Site Characterization

Site characterization includes the following information:

- Site contaminants (targets: Pb, Zn, and/or Cd)
- Existing vegetation (indicating potential for plant growth)
- Proximity to water (for irrigation)
- Proximity to electrical supply
- Site accessibility for vehicles and farm equipment
- Field observations
- Historical site activities
- Summary of regional hydrology/geology

The purpose of a treatability study is to identify optimal conditions for metal uptake into the aboveground portion of the plants and to determine if the soil to be treated will support plant growth. The further objectives are to evaluate and select the appropriate plant species and soil amendments, and to optimize plant growth for maximal removal of metals from soils. Treatability studies include short-term investigations for evaluating the growth and metal uptake potential of selected plant species under controlled conditions in a growth chamber or greenhouse.

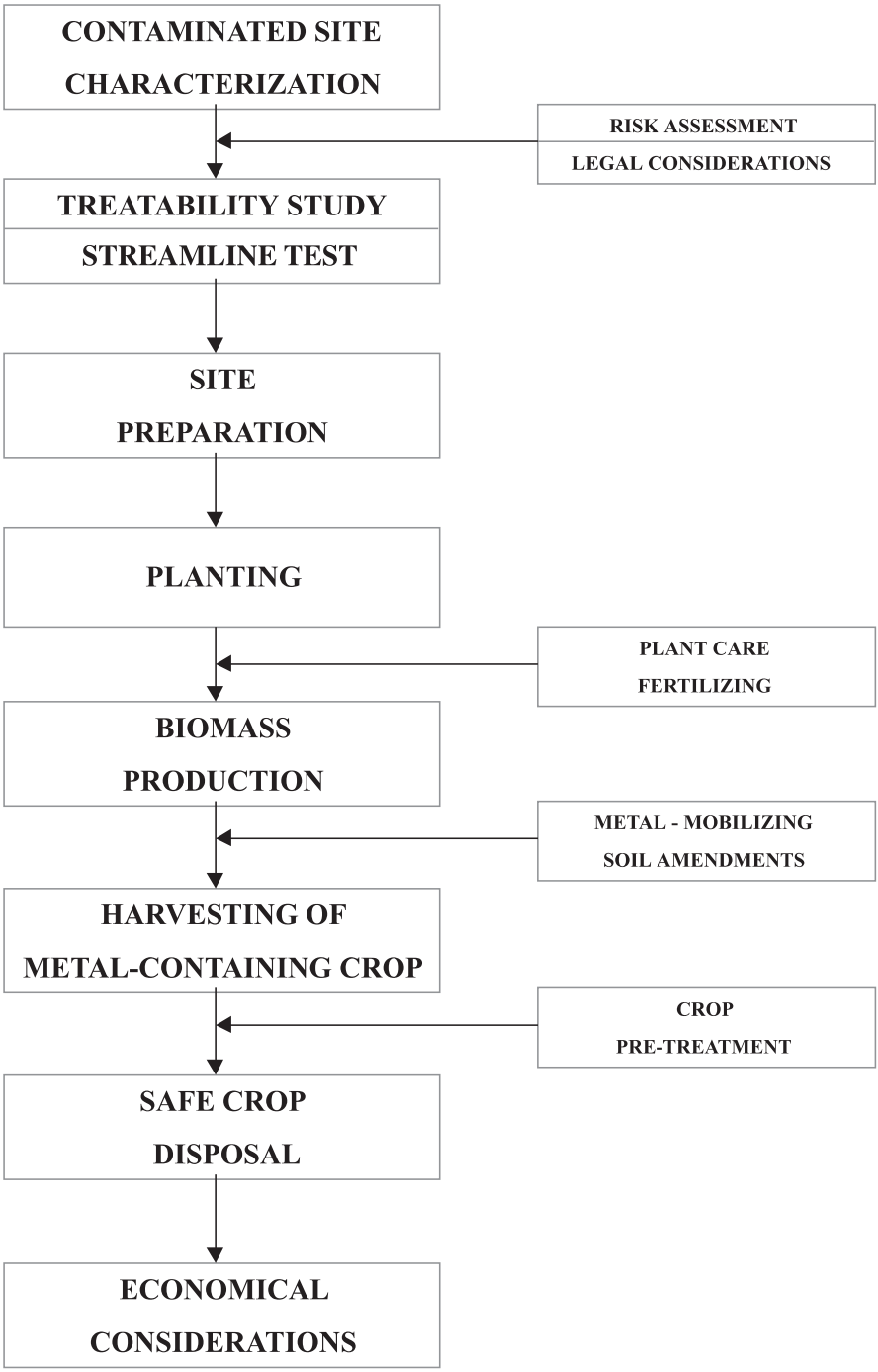

Fig. 7.1. Induced phytoextraction process

A bridge between routine treatability studies and full-scale field applications is the Streamline Test (Sas-Nowosielska et al. 2001), which in combination with a routine laboratory study allows for a rapid and inexpensive assessment of soil features across the entire site to be treated.

Performance of Treatability Study

1. Soil from the site being investigated should be collected from the top 0–25 cm depth horizon and be well homogenized and sieved to pass a 4 mm sieve. The soil is placed inside 400-cm^3 plastic pots filled previously with drainage of approx. 100 g of clean pea/river gravel (2–8 mm diameter) placed in the bottom. Each pot should be filled with 300 g of the sieved soil and watered with 100 mL of distilled water prior to planting the seeds. The seeds should be placed on the surface of the soil in a circular pattern, covered with a thin layer of soil and moistened with an additional 40 mL of water.
2. Pots are then placed inside the growth chamber or greenhouse on separate plastic saucers in order to prevent leaching of metals and to avoid cross contamination. The seeds should be kept wet during germination to avoid additional compaction of the soil surface and exposure of seeds.
3. Plants should be adequately fertilized 10 days after germination using commercial mixtures, and 14–16 days after germination the seedlings should be thinned as needed.
4. Application of soil amendments should be completed approx. 1 week prior to harvest. Amendments should be administered in a single dose, or in three doses if necessary.

Five replicates of each treatment are recommended.

Sampling and Analytical Procedures

An accurate chemical analysis of soil and plants by an accredited laboratory is a key requirement for conducting a successful treatability study. The results should be reliable, as they will indicate the effectiveness of the process and what changes may be needed to enhance the phytoremediation process. For most of the sampling and analytical activities described in this chapter, ISO Standards are recommended (see References and Chapts. 1–3).

Samples should be collected to determine the concentration (spatial variation) of the target metals at the site, initially and during the phytoremediation activities. The actual number and location of samples should be based on the final layout of the field. Samples collected should be extracted and analyzed following the ISO Standards (see References).

Plant material should be washed with tap water in an ultrasonic washer to remove soil particles and then dried at 70 °C. Approximately 1 g of dried ground material should be wet-ashed using concentrated nitric acid in a microwave system. Concentrations of metals should be analyzed by flame atomic absorption spectrophotometer (FAAS) or by inductively coupled plasma spectroscopy (ICP-AES).

A chain of custody should be maintained during sampling activities and Quality Assurance/Quality Control procedures are to be followed. Analytical work in phytoremediation projects focuses on soil and plant analyses, preceded by the sampling process. The related regulations are listed below. In the case of soil, the following information on the investigated material is required:

- Soil texture (hydrometric method)
- Soil pH in 1 N KCl, H_2O, and 0.01 M $CaCl_2$; soil-to-solution ratio of 1:2.5 (Chapt. 2)
- Soil electroconductivity; soil to solution ratio of 1:2.5
- Soil organic matter content by loss on ignition (Chapt. 2; Houba et al. 1995)
- Cation exchange capacity (CEC), according to ISO 13536 (1995)
- Content of NO_3^- and NH_4^+ (Chapt. 2; Houba et al. 1995)
- P content in water extract and in an ammonium lactate-acetic acid extract (Houba et al. 1995)
- K content soluble in 8 M KCl (Houba et al. 1995)
- Amorphous Al and Fe content, using an ammonium oxalate-oxalic acid extract method (Houba et al. 1995), and obtaining the concentration of Al and Fe with ICP analysis (Houba et al. 1995)
- Metal extraction and determination (optional):
  - Total heavy-metal and other major cation concentrations are determined after extraction with aqua regia. Soil should be ground to pass a 0.25 mm sieve; concentrations of metals is analyzed by FAAS or ICP-AES (Chapt. 3).
  - Bioavailable fraction: 5 g of air-dried soil ground to < 0.25 mm is extracted with 50 mL of 0.01 M $CaCl_2$ for 5 h and the concentration of metals is analyzed in the extract using FAAS or ICP-AES.
  - Potentially available metal fraction: 4 g of air-dried soil ground to pass a < 2-mm sieve are extracted with 40 mL of 0.43 N HNO_3 for 4 h.

The concentration of metals is analyzed in the extract using FAAS or ICP-AES.

– Exchangeable cations: extraction is according to ISO 13536 (1995) as for CEC; concentration is measured by AAS.

Streamline Test

Site characterization and treatability studies currently are conducted sequentially, prior to the initiation of full-scale planting. The purpose of these activities is to describe the nature and extent of contamination at the target site, and to determine if, and under what conditions, proposed plant species will extract the target contaminants. The present approach is time consuming, expensive, and may not lead to a successful scale-up for field scale application of phytoextraction. The traditional treatability study is conducted in greenhouse conditions with controlled air temperature, light, water regime, and homogenized soil. These carefully controlled conditions often do not mimic real world conditions. A streamline test (ST) is an attempt to combine the treatability study and site characterization into an integrated single effort (Sas-Nowosielska et al. 2001). The concept of the ST was based on a geostatistical assumption that an adequately distributed number of soil samples may describe the distribution of metals across an investigated site. The variability of lead and cadmium contents in soil was estimated in previous field scale phytoextraction experiments (Kucharski et al. 1998). Based on these findings, it was assumed that two crossed strips covering approx. 20% of the total site surface would be sufficient to represent the entire area for site characterization purposes. Topsoil samples were taken outside and inside the strips, and analyzed for contents of metal. Comparison of average concentrations of lead, cadmium, and zinc in the soil inside and outside showed no significant differences. It was concluded that the ST better reflects the "real world" conditions as compared to the usual treatability study. The ST provides an early indication/screening of the suitability of the site for the application of phytoremediation.

7.2.2 Full-Scale Application

Seedbed Preparation and Plant Protection

Plant protection consists of applying herbicides for weed control and insecticides to combat herbivore insects. The principle of application should follow the rules of good agriculture practice. Once the fertilizer and insecticides have been applied, the seedbed will be prepared for planting.

The site preparation activities in general require the site to be cleared, cleaned and the soil developed into a condition that will allow planting. The following are examples of the kinds of obstacles that should be taken into consideration:

- Quantity and extent of surface debris
- Depth to water table
- Potential for flooding from off the site
- Location of depressions in the soil that will collect water and drown plants
- Location of a reliable water source for irrigation

Fertilization

Most plant species have varying nutritional needs. Fertilization protocols should be prepared individually after a soil analysis indicating the required nutrients for the species used. An example of this was demonstrated when *Brassica* was used to clean lead-contaminated soil. *Brassica* sp. have lower phosphorus and potassium requirements than other species, but require abundant nitrogen and supplemental sulfur to promote rapid vegetative growth. Thus, in accord with the findings of the soil fertility analysis, the site had to be fertilized with nitrogen, phosphorus, potassium, and sulfur. Generally, other nutrients are not found to be deficient. Fertilizer type, placement, and quality play an important role in the success of the crop development. For example, *Brassica* sp. are very sensitive to salt damage from fertilizer placed too close to the seed or with the seed.

Irrigation

Irrigation is used to achieve maximum plant growth pertaining to soil moisture. The objective is to maintain the recommended soil moisture levels for each individual crop (depending on plant species and/or cultivar) during the project's first and second crops. The soil moisture has to be kept at the optimum level. According to local conditions, various irrigation systems can be used (dripping, overhead sprinkling, wand-style spraying).

The initial irrigation after planting should wet the soil profile to a depth of 15 cm. Care should be taken to not apply too much water. *Brassica* sp. do not respond well to standing water. The soil should be kept damp but not saturated until the seedlings emerge. This may require irrigation every day for sandy soils and every 5–7 days for heavy soil types. The site should be checked daily to determine if the plants need irrigation. Timing of irrigations will depend on many variables, such as the size of the plants,

rainfall, temperature, soil type, and the rate of evapotranspiration, to name a few. A simple tool that can be used to aid a project's irrigation needs is a tensiometer.

Soil Amendments

- For *Brassica* sp.: K_3EDTA (2.5 mmol/kg of soil; stock solution concentration 50%) and acetic acid (5 mmol/kg of soil; stock solution concentration 80%).
- For *Helianthus* sp.: K_3EDTA (5 mmol/kg of soil; stock solution concentration 50%) and acetic acid (5 mmol/kg of soil; stock solution concentration 80%).

Doses of amendments should be calculated individually for each kind of soil and applied as a diluted solution through the irrigation system or tractor driven sprayer.

Species Used for Phytoextraction

- *Brassica juncea*, the species commonly used for lead phytoextraction, grows well on fertile, well-drained soils. Successful *B. juncea* establishment requires a fine, firm seedbed that is free of weeds and rubble. All vegetation that will compete with the phytoremediation crop should be removed. The objective is to produce a seedbed that will give the newly seeded crop maximum opportunity to germinate and grow. The addition of high quality clean organic matter should be made only when it is essential. *B. juncea* has a low tolerance for high salinity and poorly aerated soils. A well-drained soil provides optimum conditions for rapid germination and uniform emergence. The seeding rate for *B. juncea* is 15 kg/ha, and the ideal plant population is 110–160 plants/m^2, which produces 1.5−3.0 g seeds/m^2. Planting depth will depend on soil moisture. The seeds must have good contact with moist soil to achieve maximum germination and emergence. Ideal planting depth is 1−2 cm. Under dry sandy conditions the depth may have to be adjusted to 2.5 cm, but planting deeper than 2.5 cm can result in poor emergence and reduced plant population.
- Other plant species, such as sunflower, can be used for a phytoextraction process. This plant can produce a high amount of biomass (60 t/ha), but heavy-metal accumulation is rather low (about 100 mg Pb/kg dry soil).
- Some plants, termed "hyperaccumulators," take up toxic elements in substantial amounts, resulting in concentrations in aboveground biomass over 100 times of those observed in conventional plants. It is economically hard to grow plants hyperaccumulating toxic metals because of

their very low biomass production and difficulty of harvesting (Blaylock et al. 1997).

Harvesting

All aboveground biomass should be removed at harvest at the right time of plant maturity, after adding the chelating amendment. Application of the soil amendment should be made in a manner so that as little of the amendment as possible contacts the plant. This can be done with a hand-held application wand that directs the liquid amendment at the soil below the plants leaves, or by using an automatic dispenser (Kucharski et al. 2000).

The physical removal of the plants can be started 7–10 days after the application of the amendment. The plants should be cut as close to the ground as possible. This can be accomplished with a fodder harvester, which cuts plants into 3–5 cm pieces loaded onto the adjacent trailer.

After harvest, the soil must be retilled and prepared for the next crop's planting. In general, the techniques for the first crop are used for the subsequent crops, but less fertilizer should be used for them. The recommended rates must be determined.

Crop Disposal

Important steps after harvest are reduction of the crop volume and removal of excess water. These will improve technical parameters of harvested biomass in terms of further processing and reduce transport costs to the treatment or disposal site. Volume reduction of contaminated plant material can be achieved by composting, compaction, or pyrolysis processes.

Composting and compaction should be considered as pre-treatment steps, since a large volume of contaminated biomass will still exist after both processes. Total dry-mass loss of contaminated plant biomass is an advantage of composting as a pretreatment step. It will reduce costs of transportation to a hazardous waste disposal facility and of deposition, or of transportation to other facilities where final crop disposal is to take place. Compaction does not result in total dry mass loss of plant biomass but works faster than composting. Pyrolysis is also considered a pretreatment step, since metal-contaminated material (coke breeze) is one of the end-products. Significant more volume/mass reduction of contaminated plant biomass is observed than from composting or compaction. Moreover, pyrolytic gas is recovered in the process (Sas-Nowosielska et al. 2004).

For final disposal, incineration of contaminated plant material in non-ferrous or cement rotary kilns or in a municipal waste incineration plant is considered the most promising method because it significantly reduces the biomass of harvested plant material. Deposition in hazardous waste disposal facilities seems to be the simplest way to dispose of contaminated

crops. However, it is not completely adequate since significant amount of heavy-metal contaminated material will remain in the environment and costs of its disposal are high (Sas-Nowosielska et al. 2004).

Monitoring

Routine monitoring should include collection and analysis of the following: soil, airborne deposition, plants, vadose zone moisture, ground water, and irrigation water. In addition, routine soil chemistry and weather monitoring should be conducted. Weekly visits to the site are recommended to examine the growth of plants, soil moisture, appearance of pests, etc.

7.2.3 Conclusions

The following remarks attempt to summarize the up-to-date observations concerning phytoextraction:

- The method is applicable to cleansing sites contaminated to a moderate or medium degree.
- Although described in literature as a lead-extraction method, significant amounts of zinc and cadmium can thus be extracted. High concentrations of zinc in soil can impair plant growth.
- Results of laboratory and bench studies should be cautiously transferred to the field; conclusions carelessly drawn from experiments on pollutant-spiked soil can be a source of very serious errors.
- Each new clean-up project has to be custom-tailored due to significantly varying soil conditions and pollutant distribution.
- Induced phytoextraction, where costs of reagents contribute to the major portion of all project expenditures, should for economic reasons only be used on particularly valuable areas.
- Continuous phytoextraction, which may be considered a type of natural contaminant attenuation by selected species of plants, is applicable to the sites where time is not a driving factor.
- It is highly recommended to use indigenous species for phytoextraction, as they are considerable cheaper than exotic species, and do not create adaptation problems.
- Although EDTA, a commonly used metal-chelating agent, was found not to exert any adverse effects on soil bacterial and fungal life, it should be very carefully applied considering its potential for mobilization of metal into other compartments of the environment.

7.3 Phytostabilization Potential for Soils Highly Contaminated with Lead, Cadmium and Zinc

The use of certain plant species to immobilize contaminants in the soil and ground water through accumulation and absorption by roots, adsorption onto roots' epidermis, or precipitation within the root zone is called phytostabilization. This term further infers a physical stabilization of soil. Phytostabilization does not remove contaminants from the soil, but reduces the hazards to human health and environment.

Plants are used to cover the soil surface to prevent erosion, reduce water percolation, serve as a barrier to prevent direct contact with the soil-immobilized contaminants, and to control soil pH, gases, and redox conditions (Vangronsveld et al. 1995). Plant roots may change soil pH by release of exudates or through the production of CO_2 during root respiration.

Phytostabilization is a site stabilization technique that reduces the risk of soil contaminants through the use of soil amendments that induce the formation of insoluble contaminant species. The method essentially consists of a combination of the use of immobilizing soil additives to reduce the bioavailability of heavy metals in contaminated soil and the creation of a dense vegetation cover. Bioavailability as a function of remediation treatment can be quantified based on the contaminant enrichment factor (EF), which is the ratio of contaminant metal concentration in plant tissue to the total concentration in the soil.

7.3.1 Evaluation of Site Contaminants

The investigator must establish a procedure, using less aggressive extraction, for evaluating the level of contaminant initially associated with the solid phase. The relative extractability of the contaminant of interest is then evaluated before and after a given stabilization treatment. In essence, such techniques are an abbreviated sequential extraction and subjected to the same empirical limitations with respect to interpretation as is the full procedure.

Sampling Setup

The first sampling takes place before the technology is started. All contaminated areas should be sampled by using the procedures described in ISO Standards. The following general soil parameters are quantified:

- Soil texture (hydrometric method)
- Soil pH in 1 N KCl, H_2O and 0.01 M $CaCl_2$; soil to solution ratio of 1:2.5 (Chapt. 2)
- Soil electroconductivity; soil to solution ratio of 1:2.5
- Soil organic matter content by loss on ignition (Chapt. 2; Houba et al. 1995);
- Cation exchange capacity (CEC), according to ISO 13536 (1995);
- Content of NO_3^- and NH_4^+ (Chapt. 2; Houba et al. 1995);
- P content in water extract and in an ammonium lactate-acetic acid extract (Houba et al. 1995);
- K content soluble in 8 M KCl (Houba et al. 1995);
- Amorphous Al and Fe content, by an ammonium oxalate-oxalic acid extract method (Houba et al. 1995), t concentrations of Al and Fe being analyzed with ICP (Houba et al. 1995)
- Metal extraction and determination (optional; see Sect. 7.2.1)

7.3.2 Logistic Considerations

As in case of phytoextraction, the following is to be examined before the technical activity in the project is started:

- Existing vegetation (indicating potential for plant growth)
- Proximity to water (for installation of irrigation system)
- Site accessibility for vehicles and farm equipment
- Field observations
- Historical site activities
- Summary of regional hydrology/geology

7.3.3 Additives

Soil amendments for phytostabilization should inactivate metal contaminants, reducing their bioavailability, and preventing leaching and plant uptake. Some amendments, e.g., phosphate fertilizer, have secondary benefits such as supplying plant nutrients.

Limestone and organic materials (Li and Chaney 1998), natural and synthetic zeolites, phosphate minerals (apatite, calcium phosphate, ammonium polyphosphate, etc.), iron and manganese oxides, are suggested for metal stabilization (Knox et al. 2000, 2001)

The application rates of the additives generally range from 0.5–5% by soil mass. The largest amounts of additives are generally needed for highly contaminated areas with high a percentage of bioavailable forms of contaminant.

7.3.4 Plants

Desirable features of species used for land phytostabilization are as follows (Berti et al. 1998; Vangronsveld and Cunningham 1998):

- Tolerance to high concentrations of pollutant (Li and Chaney 1998)
- Ability to create a dense root mat
- Ability to accumulate pollutants in a non-edible underground part
- Low maintenance requirements (watering, pest and weed control)
- Resistance to the local climatic extremes

In the course of investigation of very highly metal-polluted areas it has been found that *Deschampsia caespitosa* ecotype *Warynski*, a weed growing spontaneously on the soil close to zinc smelter dumps, creates very dense and durable plant cover upon being supplied with necessary nutrients. This ecotype has appeared to be relatively strong and healthy, in spite of poor soil conditions and inadequate watering. Species such as *Brassica juncea*, some cultivars of grasses (*Agrostis tenuis*, *Festuca rubra*) and some cultivars of hybrid poplars are also suggested for this purpose.

7.3.5 Full-Scale Application

The methodology presented has focused on highly metal-contaminated areas with poor plant cover. Chemostabilization combined with phytostabilization is meant to prevent pollutant migration via wind, water erosion, and leaching.

Agronomic input includes the nutrients necessary for vigorous growth of vegetation and rhizosphere microbes. It should be done based on a local Good Agriculture Practice. Before the soil contaminants are to be stabi-

lized, inorganic (nitrogen, phosphorus, potassium), and organic fertilizers (manure, compost, etc.) have to be applied.

For using 5% superphosphate, lime at the rate of about 12 t/ha should be applied and mixed to a depth of 20 cm. After liming, the amendment should be mixed with the upper 10-cm layer of soil. Two weeks after amendment application, about 60 kg/ha of *D. caespitosa* seeds should be planted. This concentration eliminates creation of tufts and increases density of plant cover.

7.3.6 Effectiveness of Technology

Phytochemostabilization can be achieved by creation of appropriate plant cover in combination with soil amendments. The approach we suggest is suitable for areas heavily polluted with bivalent heavy metals, where commercially available species for revegetation cannot survive. The overall goal is complete coverage of the contaminated surface with a plant canopy, whose growth is enhanced by chemicals, and which will simultaneously immobilize pollutants and continue to support plant growth.

In particularly complex deterioration of soil, where chemical soil damages are followed by its mechanical destruction, the concerted action of phyto- and chemostabilization may yield positive results. Evaluation of hazard reduction must be made to validate the effectiveness of phytostabilization.

7.3.7 Monitoring

Phytostabilization does not remove contaminants from the soil. There are two objectives of monitoring during a phytostabilization process:

- To evaluate the long term effectiveness of immobilization in revegetated areas, which leads to an estimation of the reduction of the long term leaching potential and the influence of vegetation on leaching risks
- To evaluate plant uptake potential in relation to potential transfer of heavy metals into the food chain

Monitoring the fate of contaminants, as well as the presence of additives over a long period of time is required, particularly in areas with strong impact from acid rain and possible changes in soil redox potential. Immobilization effectiveness and plant uptake determinations should be carried out during the growing seasons of several years.

7.3.8 Conclusions

The combined chemo-phytostabilization method has the following advantages:

- Phosphate as used in the method decreases the concentration of bivalent heavy metals in roots and shoots, and their bioavailable fraction in leachates, and also improves plant cover density.
- Further, phosphate thus introduced in soil may facilitate the propagation of *Deschampsia* in the third year of growth by enhancing production of seeds, which germinate on bare soil between the tufts.
- The procedure supports the growth of the root system and makes it stronger, resulting in increases of up to 70% water retention and reduced metal migration.
- The growth of *D. caespitosa* is improved in the process at the expense of the growth rate of *Cardaminopsis sp*. This is a positive phenomenon, because high heavy-metal accumulation rates in *Cardaminopsis* sp. shoots results in a potential introduction of heavy metals into the food chain.
- Metal migration to lower soil levels is decreased by the procedure as a result of metal-chemical binding and the development of a strong plant cover.
- An optimization study to evaluate phosphorus addition to the soil and satisfactory plant growth remains to be done, and the price of the additive is also a matter of concern.
- Phosphate used as a fertilizer for metal contaminated soils in very high concentration is considered disadvantageous as it causes saturation with phosphate in the upper soil layers. This can lead to phosphate leaching. Phosphate use is therefore limited to areas with a deep water table where groundwater pollution by phosphate is unlikely, and where the greater benefit of obtaining healthy plant cover is unlikely to be achieved.
- Phosphate is not recommended for arsenic-polluted soils, as competition between arsenate and phosphate can provoke increased arsenic levels in plants, causing risks of food-chain propagation and accumulation.

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References

Berti WR, Cunningham SD, Cooper EM (1998) Case studies in the field – in-place inactivation and phytorestoration of Pb-contaminated sites. In: Vangronsveld J and Cunningham SD (eds) Metal-contaminated soils: in situ inactivation and phytorestoration. Springer-Verlag, Berlin Heidelberg and RG Landes Co, Georgetown, TX, USA, pp 235–248

Blaylock MJ, Salt DE, Dushenkov S, Zakharova O, Gussman C, Kapulnik Y, Ensley BD, Raskin I (1997) Enhanced accumulation of Pb in Indian mustard by soil-applied chelating agents. Environ Sci Technol 31:860–865

Brooks RR (1998) Phytochemistry of hyperaccumulators. In: Brooks RR (ed) Plants that hyperaccumulate heavy metals. Cab International, Wallingford, Oxon, UK, pp 15–53

Houba VJG, Van der Lee JJ, Novozamsky I (1995) Soil analysis procedures, other procedures (Soil and plant analysis, Part 5b). Dept Soil Sci Plant Nutr, Wageningen Agricultural University, pp 217

ISO 11265 (1994) Soil quality – Determination of the specific electric conductivity

ISO 11464 (1993) Soil quality – Pretreatment of samples for physico-chemical analyses

ISO 13536 (1995) Soil quality – Determination of the potential cation exchange capacity and exchangeable cations using barium chloride solution buffered at pH = 8.1

ISO 7888 (1985) Water quality – Determination of electrical conductivity

ISO/CD/10381–5 (1995) Soil quality – Sampling

ISO/DIS 10390 (1993) Soil quality – Determination of pH

ISO/DIS 11047 (1994) Soil quality – Determination of cadmium, chromium, cobalt, copper, lead, manganese, nickel and zinc. Flame and electromatic thermal atomic absorption spectrometric methods

ISO/DIS 11466 (1995) Soil quality – Extraction of trace metals and heavy metals soluble in aqua regia

Knox AS, Seaman J, Adriano DC, Pierzynski G (2000) Chemophytostabilization of metals in contaminated soils. In: Wise DL, Trantolo DJ, Cichon EJ, Inyang HI, Stottmeister U (eds) Bioremediation of contaminated soils. Marcel Dekker, Inc, New York, Basel, pp 811–836

Knox AS, Seaman JC, Mench MJ, Vangronsveld J (2001) Remediation of metal- and radionuclides-contaminated soils by in situ stabilization techniques. In: Iskandar IK (ed) Environmental restoration of metal-contaminated soils. Lewis Publ, Boca Raton, London, New York, Washington, DC, pp 21–60

Kucharski R, Sas-Nowosielska A, Dushenkov S, Kuperberg JM, Pogrzeba M, Malkowski E (1998) Technology of phytoextraction of lead and cadmium in Poland. Problems and achievements. In: Symposium Proceedings, Warsaw'98, Fourth Int Symposium and Exhibition on Environmental Contamination in Central and Eastern Europe, pp 55

Kucharski R, Sas-Nowosielska A, Kryñski K (2000) Amendment application technology for phytoextraction. In: Symposium Program, Prague 2000, Fifth Int Symposium and Exhibition on Environmental Contamination in Central and Eastern Europe, Abstract, p 376

Kucharski R, Sas-Nowosielska A, Kuperberg M, Bocian A (2004) Survey and assessment. How urbanization and industries influence water quality. In: Integrated watershed management – ecohydrology & phytotechnology, manual. UN Educational, Scientific and Cultural Organization, Venice, Italy, pp 45–60

Li YM, Chaney L (1998) Case studies in the field – industrial sites: phytostabilization of zinc smelter-contaminated sites: the Palmerton case. In: Vangronsveld J, Cunningham SD (eds) Metal-contaminated soils: in situ inactivation and phytorestoration. Springer-Verlag Berlin Heidelberg, and RG Landes Co, Georgetown, TX, USA, pp 197–210

McGrath SP, Dunham SJ, Correl RL (2000) Potential for phytoextraction of zinc and cadmium from soils using hyperaccumulator plants. In: Terry N, Banuelos G (eds) Phytoremediation of contaminated soil and water. Lewis Publ, Florida, pp 1–13

Salt DE, Smith RD, Raskin I (1998) Phytoremediation. Annu Rev Plant Physiol Plant Mol Biol 49:643–668

Sas-Nowosielska A, Kucharski R, Korcz M, Kuperberg M, Malkowski E (2001) Optimizing of land characterization for phytoextraction of heavy metals. In: Gworek B, Mocek A (eds) Element cycling in the environment, bioaccumulation – toxicity – prevention, Monograph, vol 1. Instytut Ochrony Œrodowiska, Warsaw, Poland, pp 345–348

Sas-Nowosielska A, Kucharski R, Malkowski E, Pogrzeba M, Kuperberg M, Kryñski K (2004) Phytoextraction crop disposal – an unsolved problem. Environ Pollution 128:373–379

Vangronsveld J, Cunningham SD (1998) Introduction to the concept. In: Vangronsveld J, Cunningham SD (eds) Metal-contaminated soils: in situ inactivation and phytorestoration. Springer-Verlag, Berlin Heidelberg, and RG Landes Co, Georgetown, TX, USA, pp 1–15

Vangronsveld J, Van Assche F, Clijsters H (1995) Reclamation of a bare industrial area contaminated by non-ferrous metals: in situ metal immobilization and revegetation. Environ Pollution 87:51–59

8 Quantification of Hydrocarbon Biodegradation Using Internal Markers

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Introduction

Objectives. Soil contamination is invariably heterogeneous, and monitoring the loss of contaminant during bioremediation is often frustrated by this heterogeneity. But if the initial source of contamination was relatively homogeneous, it is possible to identify biodegradation as the selective loss of the most biodegradable components, while more recalcitrant molecules are conserved. Measuring the concentrations of a series of compounds using gas chromatography (GC) coupled with mass spectrometry (MS), often in the selected ion monitoring (SIM) mode, allows this to be achieved with high precision.

Hopanes have proven to be useful conserved internal markers for following the biodegradation of crude oil contamination (Prince at al. 1994), trimethylphenanthrenes for following the biodegradation of diesel fuel (Douglas et al. 1992), and 2,2,3,3-tetramethylbutane and 1,1,3-trimethylcyclopentane for following the anaerobic biodegradation of gasoline and condensate (Townsend et al. 2004). Undoubtedly, there are many other compounds that could be used. Even if the "conserved" internal marker is itself eventually degraded, this will have the effect of underestimating the extent of biodegradation of compounds referred to it, making the approach a conservative one. The principal requirements are that the samples under consideration initially had the same contaminant, and that the compound chosen as the "conserved" internal standard be amongst the least degradable in the mixture under study, and be present at a high enough concentration to be measured with good precision.

Principle. Depending on the type of contamination, which can be determined from the hydrocarbons present (Stout et al. 2002), the least biodegraded sample is identified, and candidate conserved species are identified. The ratios of various analytes to these species are then followed over time, and biodegradation is identified from their coherent loss. The concentration of the conserved species (e.g., hopane) on an oil-weight basis may

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also be used to estimate the total quantity of oil that has been degraded (Douglas et al. 1994) within a sample.

Theory. The biodegradation of hydrocarbons has been studied for almost a century, and the overall process is quite well understood (Prince 2002). Under aerobic conditions, *n*-alkanes and simply substituted monoaromatic species are amongst the most readily biodegraded hydrocarbons, followed by the iso- and monocyclic alkanes, benzene and the simply alkylated two and three-ring aromatics (Solano-Serena et al. 1999). More highly alkylated species, four-ring and larger aromatics (Douglas et al. 1994), and compounds containing tertiary carbons are more resistant to biodegradation (Prince et al. 1994). Similar patterns are seen under methanogenic and sulfate-reducing conditions, with the apparent distinction that some cyclic alkanes are very readily degraded under these conditions (Townsend et al. 2004). The biodegradation of at least some hydrocarbons, e.g., toluene, occurs under other anaerobic conditions as well (Chakraborty and Coates 2004).

Inevitably some analyte in any complex mixture is its least biodegradable compound. Referring the concentrations of other analytes to this compound provides a ready index of the extent of biodegradation of that analyte, and removes much of the variability in the absolute concentration of the analyte in soil and sediment samples. This is shown graphically in the figures. Figure 8.1 shows the biodegradation of 2-methylhexane over 100 days in samples from a condensate-contaminated anaerobic aquifer amended with a small amount of gasoline and incubated under sulfate-reducing conditions (Townsend et al. 2004). The raw data are seen in Fig. 8.1A, the data referred to 1,1,3-trimethylcyclohexane as a conserved internal marker in Fig. 8.1B. Similarly, Fig. 8.2 shows the biodegradation of the sum of the USEPA priority pollutant polycyclic aromatic hydrocarbons (PAHs; Keith and Telliard 1979) in a historically contaminated refinery soil over a time span of 1.5 years (Prince et al. 1997). The raw data are seen in Fig. 8.2A, the data referred to 17α(H),21β(H)-hopane as a conserved internal marker in Fig. 8.2B. In both cases, the biodegradation of the target compound(s) is much more apparent in the B panels.

Procedure

The precise recipes for extracting and analyzing samples will depend on many site-specific variables, and we give only a broad description of the protocols involved. Measurements made for regulatory compliance are usually specifically mandated by the regulators involved, and we do not discuss them here. Rather we focus on measurements made to assess whether biodegradation is proceeding, and whether bioremediation protocols are

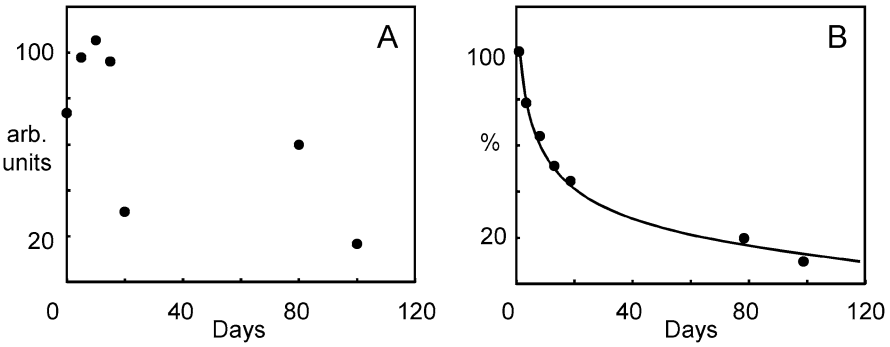

Fig. 8.1. A The biodegradation of 2-methylhexane under sulfate-reducing conditions in samples collected from a condensate-contaminated aquifer, amended with 1 μL of gasoline (per 50 g sediment, 75 mL groundwater) and incubated in the laboratory under sulfate-reducing conditions (Townsend et al. 2004). The individual incubations were carefully assembled with equal weights of sieved sediments in each bottle, yet the raw data are still very heterogeneous. **B** The data and referenced to the concentration of 1,1,3-trimethylcyclohexane in each sample

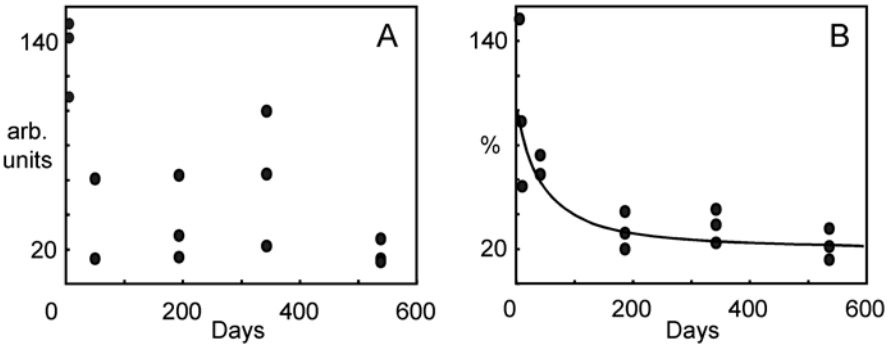

Fig. 8.2. Biodegradation of the 16 USEPA Priority Pollutant PAHs in a refinery soil. The data (the sum of the concentrations) were collected after a bioremediation protocol of adding slow release nutrients was initiated (Prince et al. 1997). **A** Although the soil was tilled during the treatment, and individual samples were sieved prior to analysis, the raw data are still very heterogeneous. **B** The data referenced to the concentration of 17α(H),21β(H)-hopane in each sample

indeed stimulating the process. This is best done by comparing samples from a site undergoing active bioremediation with samples from a similarly contaminated site with no intervention. Unfortunately, this is often impossible, and samples collected during active bioremediation protocols have to be compared with samples taken at the beginning of the remediation. In either case, absolute amounts of contaminants in "replicate" samples are likely to be log-normally distributed (Limpert et al. 2001), and changes due

to biodegradation will be difficult to detect unless the conserved-marker approach is used.

Sample Preparation

Sample preparation is fundamentally different if the compounds of concern are in the gasoline or diesel and higher range. For soils, sediments, and water samples contaminated with gasoline, the appropriate extraction procedure is "purge-and-trap" analysis (Uhler et al. 2003). For soils contaminated with kerosene, diesel, heating, or crude oil it is more appropriate to extract the hydrocarbons into a solvent and inject the solvent–hydrocarbon mixture directly into the GC (Douglas et al. 1992, 2004).

Internal Standards

Often it is appropriate to add surrogate internal standards prior to extraction. These may be added for two fundamentally distinct reasons. One is to assess the efficiency of the extraction protocol: fluorobenzene is often used for "purge-and-trap" analyses, while *o*-terphenyl is often used in solvent extractions. The second is to add compounds to check that the mass spectrometer is working correctly: deuterated compounds are often used (Uhler et al. 2003; Douglas et al. 1992, 1994, 2004).

"Purge-and-Trap"

"Purge-and-trap" protocols for the extraction of volatile hydrocarbons are described in USEPA methods 5030B: "Purge-and-Trap for Aqueous Samples," and 5035: "Closed-System Purge-and-Trap and Extraction for Volatile Organics in Soil and Waste" (USEPA 2003). Although the technical aspects are discussed in the EPA Method, the target analytes to which this method is applied includes only eight hydrocarbons present in gasoline (benzene, toluene, ethylbenzene, *m*-, *p*-, and *o*-xylene, styrene, and naphthalene). This is inadequate for detailed characterization of gasoline and other light hydrocarbon products and for measuring conserved species. Uhler et al. (2003) have modified Method 8260 to quantitatively measure more than 100 diagnostic gasoline-related compounds ranging from isopentane to dodecane in nonaqueous phase liquid products, water, and soil. Due to the wide range of solubilities and volatilities of these compounds (e.g., benzene versus dodecane), caution must be exercised when analyzing these additional compounds by the purge-and-trap methods and careful calibration and monitoring of analyte-recovery efficiencies should be performed (Uhler et al. 2003).

In essence, an appropriate amount of sample to give a response within the calibrated range of the GC system is flushed (purged) with an inert gas to transfer the analytes of interest to a trap. When the purging is complete,

which usually takes several minutes, the trap is rapidly heated to transfer the sample into the GC column. If the sample is a soil sample, sufficient clean water is added prior to the purging to make a fluid slurry. The initial sampling must be done rapidly and into tightly sealed vessels to prevent any loss of volatile components during sample collection and storage. In our hands, samples containing about 1 µL of gasoline are appropriate for analysis (Townsend et al. 2004).

Solvent Extraction

Solvent extraction protocols are described in USEPA method 3500B: "Organic extraction and sample preparation" (USEPA 2003). Soil or sediment samples are dried by mixing them with enough anhydrous sodium sulfate to make a freely flowing dry mixture. Typical samples may require an equal weight of sodium sulfate, and it is important to mix thoroughly and for some time (perhaps 20 min) to allow the drying agent to hydrate and dry the sample. Samples are then serially extracted, at least three times, with an appropriate solvent (e.g., methylene chloride or methylene chloride/acetone 1+1), perhaps in a Soxhlet extraction device, by accelerated solvent extraction (ASE), or by supercritical fluids.

The extracts are dried with sodium sulfate, filtered, and then concentrated as appropriate. It is important that this solvent-evaporation be done carefully to minimize the loss of lighter volatile components, such as the two-ring aromatics. Only in rare cases where it is known that there are no volatile compounds should it be allowed to proceed to dryness. Automated devices are available, but solvent-evaporation can be done manually under a gentle stream of dry nitrogen gas at ambient temperature.

Depending on the minimum detection limits required (Douglas et al. 2004), and the presence of interfering compounds, it may be appropriate to process the solvent extract on an alumina or silica column to isolate "clean" fractions of saturate, aromatic, and polar compounds. This is described in detail in USEPA method 3611: "Alumina column cleanup and separation of petroleum wastes" and USEPA method 3630 "Silica Gel Cleanup" (USEPA 2003). Often the two hydrocarbon fractions (saturate and aromatic hydrocarbons) are combined, concentrated to an appropriate volume, and amended with additional internal standards to allow quantitation; again deuterated compounds are often used. In our hands, 1 µL injections of samples containing about 5 mg of crude oil/mL solvent are appropriate for analysis (Douglas et al. 1992, 2004).

Gas Chromatography and Mass Spectrometry (GC/MS)

This requires an appropriate high-resolution capillary column equipped with a mass spectrometer (McMaster and McMaster 1998; Hubschmann

2000). USEPA methods 8260 and 8270D (USEPA 2003) provide GC/MS protocols for the measurement of volatile and semi-volatile hydrocarbons, respectively. As noted above, the EPA protocols are not designed for petroleum product analysis and have been modified by various investigators to increase the number of petroleum-specific target compounds (Douglas and Uhler 1993; Uhler et al. 2003) and improve the sensitivity of the methods (Douglas et al. 1994, 2004).

For the modified EPA Method 8260 (Uhler et al. 2003) compounds are identified and quantified using full-scan mass spectrometry (typically from m/z = 35–300) for the extended volatile hydrocarbon target analyte list (109 gasoline-specific compounds). The advantage of full-scan analysis is that additional compounds can always be evaluated, and extracted ion plots of compound classes (e.g., alkylcyclohexanes, Townsend et al. 2004) can be obtained to determine that the products are derived from the same source. Although the full-scan GC/MS approach is not as sensitive as selected ion monitoring (SIM), it is generally adequate for volatile hydrocarbon analysis.

In contrast, it is essential to use selected ion monitoring (SIM) in the modified EPA Method 8270 (Douglas et al. 1992, 2004). This protocol allows the measurement of the major paraffins and isoparaffins, the aromatics on the USEPA list of priority pollutants (Keith and Telliard, 1979) and their alkylated forms, and the steranes and hopanes that are so valuable in discriminating different crude oils (Peters et al. 2004). The most significant modifications of the USEPA Method are the inclusions of the dibenzothiophenes, alkylated PAHs, steranes and hopanes that provide petroleum source identification and bioremediation efficacy information (Douglas et al. 2002).

Analytes are identified by the retention times of authentic standard compounds, and by reference to mass spectral libraries such as those distributed by NIST/EPA/NIH (NIST 2004). It is always appropriate to use more than one ion to identify analytes in the initial samples to assess whether there are any interfering species present, and if so, how to account for them.

For research purposes it is usually possible to arrange the concentrations of analytes to fall into the linear range of detectability, which should be determined with a range of calibration standards. A lot of work has gone into optimizing detection limits for the analysis of complex environmental samples for forensic applications (Douglas et al. 2004), but only the simplest precautions are needed for most studies quantifying biodegradation. Certainly the mass spectrometer should be tuned with an appropriate standard, such as decafluorotriphenylphosphine, before every batch of samples, and standard samples and blanks should be included in every group of samples. Of course, if the analytical variability is large then the ability to detect an impact of a bioremediation protocol is reduced. Therefore, it is preferable to measure all the samples for a particular study at one time, or at least to

include control and reference samples with every batch. This may require that early samples be preserved until analysis; careful freezing or acidification to pH 2 with HCl both work well. Furthermore, it is appropriate to set some "quality control" values that the standard samples must satisfy before the data are considered suitable for analysis. Guidelines for suitable control values are given in USEPA method 8270D (USEPA 2003) and in Page et al. (1995).

Calculation

We can calculate the percent of an analyte remaining (Figs. 8.1 and 8.2) from the equation:

$$\%\,\text{Remaining} = \frac{(A_S/C_S)}{(A_0/C_0)} \times 100 \tag{8.1}$$

A_S concentration of the target analyte in the sample

C_S concentration of the conserved compound in the sample

A_0 concentration of the target analyte in the initial sample

C_0 concentration of the conserved compound in the sample

Alternatively the percent depletion of biodegradable analytes within the oil (Fig. 8.3) can be calculated using the equation:

$$\%\,\text{Loss} = \frac{(A_0/C_0) - (A_S/C_S)}{(A_0/C_0)} \times 100 \tag{8.2}$$

Note that these equations work equally well in absolute concentration terms, or in arbitrary units, as long as the latter are obtained under identical conditions for all samples.

Notes and Points to Watch

- The approach outlined here relies on the initial source of contamination being reasonably homogeneous. This is readily achieved in laboratory studies, and often pertains to acute contamination accidents such as oil spills. But chronic contamination may prove too heterogeneous for this approach to work without subdividing areas under consideration (e.g., Prince et al. 1997). For example, the composition of gasoline has changed over the years as more effective refinery processes have been introduced, and as the molecular composition has come under regulatory oversight.

Similarly, contamination at town gas sites and refineries may be from a mixture of sources. It is thus essential to take enough samples of the contamination prior to any remediation activities to delineate areas of similar and distinctly different contamination.

- It is important to minimize evaporative losses prior to analysis. This means carefully sealed sample vials for "purge-and-trap" analyses, and care during evaporative solvent removal from extracts. Including appropriate surrogate compounds in the analysis can assess such losses.
- Biochemical intuition and published work will help identify potential analytes to be used as conserved internal compounds. Consistently negative values for the % depletion of other analytes with respect to the "conserved" one will indicate that the "conserved" compound is in fact more degradable than the other analytes, and allow selection of a better standard compound (e.g., see Fig. 8.3)
- The simple analysis of Figs. 8.1 and 8.2 may be all that is needed to demonstrate that biodegradation is occurring, but more complicated models for biodegradation, taking into account the amount of oil, its

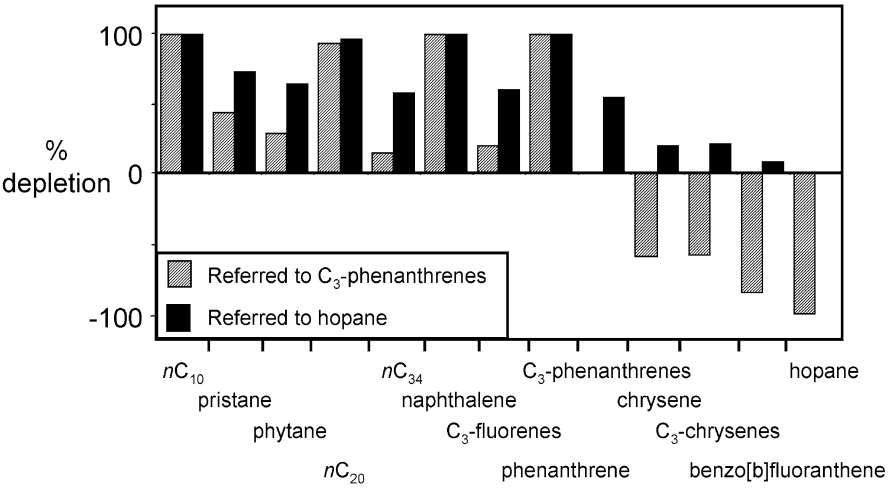

Fig. 8.3. Percent depletion plot for some alkanes, PAHs, and hopane in a degraded Alaskan North Slope crude oil (Douglas et al. 1994). The *hatched* series represents the percent depletion of each analyte based on the C_3-phenanthrenes (the trimethyl, methyl-ethyl, propyl and isopropylphenanthrenes) as the conserved internal marker. Note that some compounds have a negative apparent depletion, indicating that the C_3-phenanthrenes are less conserved than those analytes. The *solid* series represents the percent depletion based on the more biodegradation resistant 17α(H),21β(H)-hopane. (Prince et al. 1994)

prior weathering, and the amount of available fertilizer, have been used to demonstrate the effectiveness of bioremediation in the field (Bragg et al. 1994).

- Biodegradation can be identified by the loss of biodegradable compounds, as discussed above. The loss of photochemically labile species can also be followed (Garrett et al. 1998; Douglas et al. 2002), as can the loss following extensive washing and evaporation (Douglas et al. 2002; Prince et al. 2002) and the increase of pyrogenic compounds following partial oil combustion (Garrett et al. 2000). Providing a sample of the initially spilled oil is available, these environmental processes can then be identified in samples collected from historical spills (Prince et al. 2003).
- The general approach can also be used to follow the biodegradation of any complex mixture of contaminants, such as polychlorinated biphenyls (Abramowicz 1995).

References

Abramowicz DA (1995) Aerobic and anaerobic PCB biodegradation in the environment. Environ Health Perspect 103 Suppl 5:97–99

Bragg JR, Prince RC, Harner EJ, Atlas RM (1994) Effectiveness of bioremediation for the *Exxon Valdez* oil spill. Nature 368:413–418

Chakraborty R, Coates JD (2004) Anaerobic degradation of monoaromatic hydrocarbons. Appl. Microbiol. Biotechnol. 64:437–446

Douglas GS, Burns WA, Bence AE, Page DS, Boehm P (2004) Optimizing detection limits for the analysis of petroleum hydrocarbons in complex environmental samples. Environ Sci Technol 38:3958–3964

Douglas GS, McCarthy KJ, Dahlen DT, Seavey JA, Steinhauer WG, Prince RC, Elmendorf DL (1992) The use of hydrocarbon analyses for environmental assessment and remediation. J Soil Contam 1:197–216

Douglas GS, Owens EH, Hardenstine J, Prince RC (2002) The OSSAII pipeline spill: the character and weathering of the spilled oil. Spill Sci Technol Bull 7:135–148

Douglas GS, Prince RC, Butler EL, Steinhauer WG (1994) The use of internal chemical indicators in petroleum and refined products to evaluate the extent of biodegradation. In: Hinchee RE, Alleman BC, Hoeppel RE, Miller RN (eds) Hydrocarbon remediation. Lewis Publ, Boca Raton, FL, pp 219–236

Douglas GS, Uhler AD (1993) Optimizing EPA methods for petroleum contaminated site assessments. Environ Test Anal 2:46–53

Garrett RM, Guénette CC, Haith CE, Prince RC (2000) Pyrogenic polycyclic aromatic hydrocarbons in oil burn residues. Environ Sci Technol 34:1934–1937

Garrett RM, Pickering IJ, Haith CE, Prince RC (1998) Photooxidation of crude oils. Environ. Sci. Technol. 32:3719–3723

Hubschmann, H.-J. (2000) Handbook of GC/MS: fundamentals and applications. Wiley-VCH, Weinheim, Germany

Keith LH, Telliard WA, (1979) Priority pollutants I. – a perspective view. Environ Sci Technol 13:416–423

Limpert E, Stahel WA, Abbt M (2001) Log-normal distributions across the sciences: Keys and clues. Bioscience 51:341–352

McMaster M, McMaster C (1998) GC/MS: A practical user's guide. Wiley-VCH, New York

NIST (2004) NIST/EPA/NIH mass spectral library www.nist.gov/srd/mslist.htm

Page DS, Boehm PD, Douglas GS, Bence AE (1995) Identification of hydrocarbon sources in benthic sediments of Prince William Sound and the Gulf of Alaska following the *Exxon Valdez* oil spill. In: Wells PG, Butler JN, Hughes JS (eds) Exxon oil spill: Fate and effects in Alaskan waters, ASTM Special Technical Publication #1219, American Society for Testing and Materials, Philadelphia, pp 41–83

Peters KE, Walters CC, Moldowan JM (2004) The Biomarker guide, biomarkers and isotopes in petroleum exploration and earth history, vol 1–2, 2nd edn. Cambridge Univ Press, New York

Prince RC (2002) Biodegradation of petroleum and other hydrocarbons. In: Bitton G (ed) Encyclopedia of environmental microbiology. Wiley, New York, pp 2402–2416

Prince RC, Drake EN, Madden PC, Douglas GS (1997) Biodegradation of polycyclic aromatic hydrocarbons in a historically contaminated site. in: Alleman BC, Leeson A (eds) In situ and on-site bioremediation 2. Battelle Press, Columbus, OH, pp 205–210

Prince RC, Elmendorf DL, Lute JR, Hsu CS, Haith CE, Senius JD, Dechert GJ, Douglas GS, Butler EL (1994) 17α(H),21β(H)-hopane as a conserved internal marker for estimating the biodegradation of crude oil. Environ Sci Technol 28:142–145

Prince RC, Garrett RM, Bare RE, Grossman MJ, Townsend GT, Suflita JM, Lee K, Owens EH, Sergy GA, Braddock JF, Lindstrom JE, Lessard RR (2003) The roles of photooxidation and biodegradation in long-term weathering of crude and heavy fuel oils. Spill Sci Technol Bull 8:145–156

Prince RC, Stibrany RT, Hardenstine J, Douglas GS, Owens EH (2002) Aqueous vapor extraction: a previously unrecognized weathering process affecting oil spills in vigorously aerated water. Environ Sci Technol 36:2822–2825

Solano-Serena F, Marchal R, Ropars M, Lebeault JM, Vandecasteele JP (1999) Biodegradation of gasoline: kinetics, mass balance, and fate of individual hydrocarbons. J Appl Microbiol 86:1008–1016

Stout SA, Uhler AD, McCarthy KJ, Emsbo-Mattingly S (2002) Chemical fingerprinting of hydrocarbon. In: Murphy B, Morrison R (eds) Introduction to environmental forensics. Academic Press, New York, pp 135–260

Townsend GT, Prince RC, Suflita JM (2004) Anaerobic biodegradation of alicyclic constituents of gasoline and natural gas condensate by bacteria from an anoxic aquifer. FEMS Microbiol Ecol 49:129–135

Uhler RM, Healey EM, McCarthy KJ, Uhler AD, Stout, SA (2003) Molecular fingerprinting of gasoline by a modified EPA 8260 gas chromatography-mass spectrometry method. Int J Environ Anal Chem 83:1–20

USEPA (2003) Index to EPA test methods. http://www.epa.gov/epahome/index/

9 Assessment of Hydrocarbon Biodegradation Potential Using Radiorespirometry

Jon E. Lindstrom, Joan F. Braddock

Introduction

Objectives. Following environmental exposure to petroleum, acclimation of microbial communities to hydrocarbon metabolism may occur through selective enrichment of member populations possessing hydrocarbon catabolic pathways, induction or repression of enzymes, or genetic mutations resulting in new metabolic capabilities (Leahy and Colwell 1990). Measurements of carbon substrate mineralization in vitro can be used to assess the hydrocarbon biodegradative potential of microbial communities in environmental samples previously exposed to oil contamination in situ (Walker and Colwell 1976; Lindstrom et al. 1991; Børresen et al. 2003).

Using ^{14}C-labeled hydrocarbon substrates, mineralization of specific hydrocarbon compounds can be tracked, and low levels of mineralization activity are detectable if sufficiently high specific activity substrates are employed. Model compounds can indicate the degree of a community's acclimation to various hydrocarbon classes (e.g., hexadecane for linear alkanes, toluene for monoaromatic hydrocarbons, or phenanthrene for polycyclic aromatic hydrocarbons (PAHs; Bauer and Capone 1988). By appropriately manipulating experimental conditions, this method may be used to assess the prior exposure of environmental samples to hydrocarbon contamination (Braddock et al. 1996; Braddock et al. 2003), or the effects of fertilization or other field treatments used to enhance in situ hydrocarbon degradation (Lindstrom et al. 1991). In addition, manipulation of nutrient levels or other amendments in the assay may be used in bench-scale treatability studies prior to initiating field-scale bioremediation efforts.

Principle. A ^{14}C-labeled hydrocarbon substrate is added to a soil sample suspended in sterile diluent contained in a sealed volatile organic analysis (VOA) vial. The sample is incubated under appropriate conditions (dictated by the experimental question), and microbial metabolism of the added substrate is measured by recovery of ^{14}C-labeled CO_2 evolved during

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incubation. Microbial activity is halted by adding a strong base at the end of the incubation period, which sequesters the CO_2 generated by microbial substrate mineralization as carbonates in solution. The ^{14}C-labeled CO_2 is subsequently recovered by acidifying the suspension, then stripping the CO_2 from solution with nitrogen gas, and capturing it in a basic scintillation cocktail. The $^{14}CO_2$ derived from mineralization of the added labeled substrate is counted by liquid scintillation, and its radioactivity compared to that added with the labeled substrate.

Theory. Petroleum is a complex mixture of hydrocarbons, and nitrogen-, sulfur- and oxygen-containing organic compounds; and the hydrocarbon fraction itself may be composed of hundreds of aliphatic, alicyclic, and aromatic compounds (National Research Council 1985). Heterotrophic biodegradation of the organic substrates in petroleum therefore occurs via a diversity of pathways, with metabolic intermediates funneled to central metabolic pathways leading to the production of microbial biomass and carbon dioxide (Wackett and Hershberger 2001). The fate of carbon in the substrate metabolized varies depending on the organism, the pathways used, and other factors. For example, biomass incorporation of glucose was approximately twice that of phenolic compounds in taiga forest floor samples, while respiration of CO_2 in these samples was significantly higher for phenolic compounds (Sugai and Schimel 1993). Despite the variation in carbon allocation among substrates and microbial communities, respiration of carbon dioxide is useful for monitoring biodegradation of organic substrates, particularly when the source of the carbon may be tracked by radioactive labeling.

The protocol described here assesses the respiration activity of organisms in environmental samples. The procedure is designed to minimize the many factors affecting the actual mineralization activity in situ, except for the in situ microbial biomass and its potential to biodegrade the hydrocarbons tested. The rate of $^{14}CO_2$ production (r^*, Bq/day) from a radiolabeled substrate is a function of the overall rate of CO_2 production (R) and the specific activity of the added label (Brown et al. 1991):

$$r^* = \frac{A^*}{(Sn + A)} \times R \tag{9.1}$$

A^* radioactivity of the labeled substrate added to the sample (Bq/g soil)

S_n in situ substrate concentration (μg/g soil)

A concentration of substrate added with the radiolabeled substrate (μg/g soil)

R rate of CO_2 production (μg/day) from carbon sources in the sample

By adding to the sample an amount of the tested substrate (A) that is large compared to S_n, the value of r* will mainly depend on A, rather than S_n (Brown et al. 1991). As the amount of substrate added to the sample must be greater than the in situ concentration, and conditions in vitro are designed to minimize the various other factors affecting in situ mineralization rates, the value of r* reflects the microbial community's biodegradation potential only and is not a measure of in situ mineralization rates.

The choice of incubation conditions may be used to assess the degree of a microbial community's acclimation to a given hydrocarbon substrate in the environmental sample, evaluate the effectiveness of field treatments, or establish optimum growth conditions for the community being studied. As the in situ mineralization rate may be attenuated due to nutrient deficiencies or other environmental factors, radiorespirometric assays conducted with added nutrients or other amendments are useful for assessing the degree of community acclimation (suggesting prior exposure; Braddock et al. 1996; Braddock et al. 2003) to the hydrocarbon substrate or class of substrates (e.g., alkanes, monoaromatics, PAHs) being tested, since such environmental limitations are removed.

A lag period following substrate addition is observed in the assay, with its duration commonly varying as a function of the solubility and molecular structure of the substrate (Brown et al. 1991). To measure the activity of the extant biomass present in the sample on collection, an appropriate incubation period must be chosen that is short enough to avoid in vitro acclimation of the native biomass to the added substrate, but long enough to detect its mineralization (see below).

Equipment

- Incubators equilibrated to temperatures dictated by experimental requirements
- Apparatus for collecting CO_2 evolved from the soil suspension following incubation and liquid scintillation counter to detect the radioactivity associated with mineralization of the added labeled substrate. [A schematic of an apparatus suitable for stripping and capturing CO_2 evolved from the soil suspension is shown in Fig. 9.1: Nitrogen gas is bubbled through the acidified soil suspension via a spinal needle (10-cm, 18-gauge deflected-point, non-coring, septum-penetrating needle with standard hub and stainless steel cannula; Popper and Sons, New Hyde, NY, USA) that pierces the silicone septum of the VOA vial. The gas stream strips the CO_2 from the suspension, and is conveyed to a Harvey trap (R.J. Harvey Instruments, Hillsdale, NJ, USA) containing acidified toluene via Tygon tubing attached to a 1-mL syringe sleeve cut to fit in

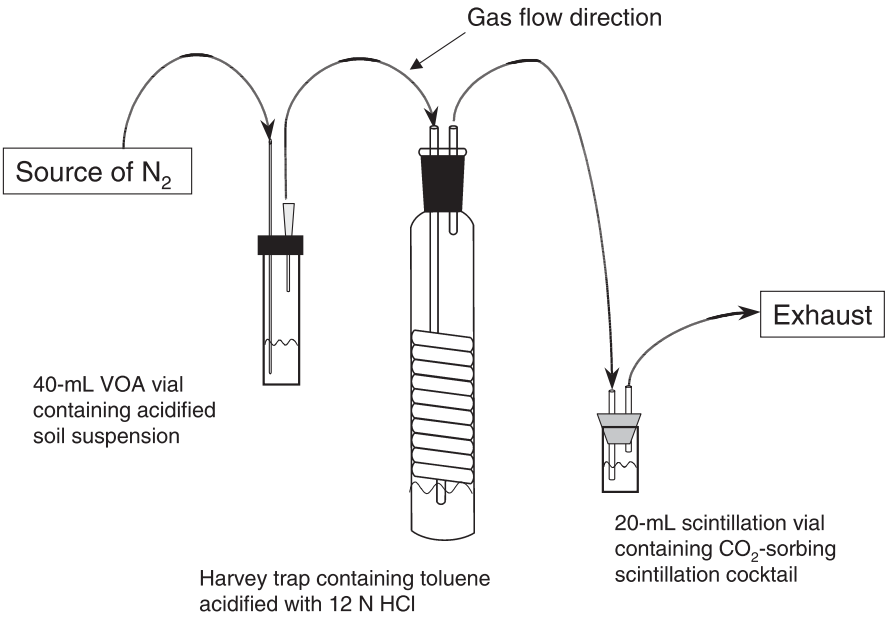

Fig. 9.1. Schematic diagram of stripping apparatus used to collect $^{14}CO_2$ from samples following incubation. Nitrogen gas is bubbled through the sample, and the gas stream flows through a Harvey trap containing acidified toluene to trap any volatile hydrocarbons in the gas stream. Finally, $^{14}CO_2$ is collected in a vial containing a CO_2-sorbing scintillation cocktail

the tubing and equipped with a 16-gauge needle that pierces the VOA vial septum. The gas stream is bubbled through the acidified toluene in the Harvey trap to capture any labeled organic substrate that may have been stripped from the soil suspension. The gas stream containing the labeled CO_2 is then conveyed to a 20-mL scintillation vial fitted with a two-hole rubber stopper and glass tubing (a 1-mL glass pipette cut to a 5 cm length works well here for the glass tubing, as it provides a tapered and polished tip). The influent gas stream is bubbled through a 10 mL scintillation cocktail containing β-phenylethylamine (PEA) to capture the CO_2. Following a 15-min stripping period, the gas flow is stopped, the rubber stopper removed, and the scintillation vial capped and placed in a scintillation counter to determine the amount of recovered radioactivity. The stripping apparatus may be modified so that a number of samples may be run simultaneously. This requires a manifold equipped with valves and multiple sets of the apparatus described above. A single nitrogen tank can be connected to the manifold and used to strip $^{14}CO_2$ evolved from several soil suspensions in parallel.]

- Sterile and pre-cleaned or combusted 40 mL borosilicate VOA vials equipped with Teflon-lined, 0.125-mm-thick, silicone septa (e.g., I-Chem Brand; Nalge Nunc, Rochester, NY, USA)
- Sterile 10-mL pipettes
- 100-µL syringe (Hamilton, Reno, NV, USA)
- Syringes fitted with an 18-gauge needle

Reagents

- Sterile diluent: modified Bushnell-Haas broth (mineral nutrient; from Atlas 1993, but modified to contain 1/10th strength $FeCl_3$) or Ringer's solution (Collins et al. 1989)
- Hydrocarbon test substrate: Prepare a solution of non-labeled hydrocarbon substrate (hexadecane, benzene, phenanthrene, etc.) in acetone (2 g/L). Then add ^{14}C-labeled hydrocarbon substrate with sufficient specific activity to obtain a final radioactivity of about 20 Bq/µL.
- Toluene, acidified by adding HCl: Approximately 5-mL aliquots of toluene are used in the Harvey trap of the stripping apparatus (Fig. 9.1); add 0.1 mL of 12 N HCl to 5 mL of toluene placed in the trap.
- Scintillation cocktail (Cytoscint ES; MP Biomedicals, Irvine, CA, USA) containing PEA to sorb CO_2. Add 2.5 mL PEA to 7.5 mL Cytoscint and shake to mix; the PEA cocktail needs to be mixed within about 1 h of use.
- 10 N NaOH to terminate incubation, and sequester evolved $^{14}CO_2$ in solution
- 12 N HCl to release $^{14}CO_2$ for recovery and counting

Sample Preparation

Use fresh soil samples. If samples must be stored, refrigerate them following collection. Sieve soil samples (2-mm mesh) to homogenize.

Procedure

Assay Preparation

Soil samples are prepared as a suspension in a sterile aqueous diluent, determined by the experimental question. Modified Bushnell-Haas broth is used as diluent if assaying nutrient-optimized mineralization potential (to assess acclimation of the microbial population to the target substrate). Ringer's solution is used as diluent if assaying the mineralization potentials

of field-treated soils (e.g., fertilized versus unfertilized). Ringer's solution may also be amended with macronutrients (N, P), vitamins, or anaerobic terminal electron acceptors for bench-scale treatability studies.

1. Prepare a nominal 1:10 dilution (w/v) of soil in diluent based on soil wet mass, preparing a volume sufficient for distribution into several assay vials. For example, if three or four replicates are desired per sample, add 5 g soil to 45 mL diluent. Collect a portion of the soil sample for a dry mass determination. The final measured potential will be adjusted per gram dry mass accordingly.
2. Distribute 10 mL of the soil suspension into VOA vials. Prepare a minimum of three replicates for each substrate/treatment combination to obtain a mean value for the sample's mineralization potential. Securely replace the caps on the vials to avoid gas leakage during incubation and CO_2 recovery.
3. Prepare killed controls ("time zero" samples) to be used for subtracting background radioactivity counts from assay samples. Inject 1 mL 10 N NaOH solution through the septum of each control vial. This should result in a solution pH above 12 in the vial, halting microbial activity. At least three controls should be prepared for each substrate/treatment combination, and the mean value is used to "correct" the final result, as described below.
4. Accurately inject 50 µL radiolabeled substrate solution though the septum of each vial. Careful measurement is required at this step to assure reproducibility of the assay. The injection results in addition of 100 µg substrate to the soil suspension. Briefly swirl or shake the vial to mix, and incubate under conditions dictated by the experimental design.
5. Sample microbial activity is terminated at the end of the incubation period (determined from time-course experiments, described below) by injecting 1 mL 10 N NaOH through the septum of each sample vial, as described for the time zero controls. Swirl the sample vial to distribute the NaOH. Samples may be stored after treatment with NaOH; the high pH conditions in the vial sequester the carbon dioxide generated by microbial mineralization as carbonates in aqueous solution, preventing loss of CO_2 from the vial pending processing to recover the ^{14}C-labeled CO_2. The samples can be stored for at least a month after this step if necessary.

Recovery of Evolved $^{14}CO_2$

Radiolabeled CO_2 evolved from the soil suspension during the incubation period is captured by stripping it from solution and capturing it in a basic medium. PEA is used to trap the CO_2.

1. Following the incubation period, the soil suspension is acidified by adding HCl to release the CO_2 previously sequestered in solution by addition of NaOH. Inject 1.5 mL 12 N HCl through the septum into the VOA vial, and swirl briefly to distribute into solution.
2. Using the apparatus shown in Fig. 9.1, place the two-hole rubber stopper with glass tubing and Tygon on a scintillation vial containing 10 mL PEA scintillation cocktail.
3. Place the influent tubing attached to the scintillation vial on the effluent side of the Harvey trap containing acidified toluene.
4. Attach Tygon tubing to the influent side of the Harvey trap, and attach a needle to the other end of the Tygon.
5. Pierce the septum of the VOA vial with the needle, making certain the tip of the needle is above the liquid level in the VOA vial.
6. Pierce the VOA vial septum with the spinal needle attached to a source of N_2 gas. There should be no gas flow until all connections have been checked for tightness.
7. Turn on the N_2 gas source and adjust the gas flow rate to approx. 10 mL/min.
8. Strip the CO_2 from the soil suspension for 15 min, then stop the gas flow through the apparatus.
9. Remove the stopper from the scintillation vial, place a cap on the vial, and determine the amount of radioactivity using a liquid scintillation counter.
10. Rinse all glass tubing tips that contacted scintillation cocktail by dipping in distilled water several times and wiping clean with a lab wipe; follow with an acetone rinse.
11. Periodically check for radioactivity carryover between samples by running method blanks (distilled water in VOA vials) treated as though they were samples, except without addition of NaOH or HCl.
12. After running each sample, check for clogged needles. Spinal needles can be cleared with a fine-gauge wire.

Volatile Versus Nonvolatile Substrates

If assaying the mineralization potential of a volatile substrate (e.g., benzene, toluene, etc.), it is necessary to remove unmetabolized substrate from the suspension prior to recovering the CO_2. This is accomplished by bubbling N_2 gas through the suspension *after* adding the NaOH, but *before* adding

the HCl. The CO_2 will still be sequestered in solution in carbonate form, and will not be lost while volatilizing the substrate from the suspension. After removing the volatile substrate from the suspension, the CO_2-stripping apparatus is assembled, the sample is acidified, and CO_2 recovery proceeds as described.

Determining Incubation Period

Relatively high concentrations of both labeled and non-labeled substrate are added to the soil suspensions in this assay to avoid interferences from field-derived hydrocarbon substrates (Brown et al. 1991). It is therefore necessary to detect significant substrate mineralization in a reasonable time frame, while avoiding artifacts associated with in vitro acclimation of the microbial community assayed. This is accomplished by conducting time-course assays with samples prepared as described above. A minimum of three replicate assays should be conducted for each incubation period.

Depending on the substrate chosen, sample incubation times should be distributed evenly from time zero to the longest reasonable incubation time. Relatively labile substrates (e.g., linear alkanes up to C_{16}, low molecular weight aromatics up to naphthalenes) may be incubated up to 2 weeks, with incubations of, e.g., 0, 3, 7, 10, and 14 days. Anaerobic incubations, more recalcitrant substrates, colder temperatures, etc., may dictate time courses of longer duration.

Following completion of the time series, plot and inspect the data. Choose an incubation time longer than the observed lag period, but the shortest possible time that yields $^{14}CO_2$ recoveries significantly above background (time zero data).

Calculation

The radioactivity recovered from each vial is normalized to a dry soil basis, using the data from the portion of soil sample collected for dry mass determination. The mean value of the radioactivity recovered from the time zero control samples prepared at the beginning of the incubation period is then determined, and subtracted from the associated treatment samples to obtain a "corrected" radioactivity value for each vial. Note that 1 g wet mass of soil is added per vial; thus, each vial represents 1 g wet mass of soil.

$$X_{(\text{corrected})} = \frac{X_{(\text{sample})} - X_{(\text{time zero controls})}}{\text{soil dry mass}} \tag{9.2}$$

$X_{(\text{corrected})}$ sample radioactivity corrected (Bq/g soil dry mass)

$X_{(\text{sample})}$ sample radioactivity recovered as CO_2 (Bq)

$X_{(\text{time zero controls})}$ mean radioactivity of controls recovered as CO_2 (Bq)

Soil dry mass (g dry soil/g wet soil)

A mean value of radioactivity recovered as $^{14}CO_2$ for each sample can be calculated from the corrected Bq/g dry soil data. The radioactivity recovered as $^{14}CO_2$ is then compared to that supplied with the added labeled substrate. The results may be expressed as a percentage of substrate added that was mineralized in the assay by the formula:

$$S_1 = \frac{X_{(\text{corrected})}}{X_{(\text{substrate})}} \times 100 \tag{9.3}$$

S_1 substrate mineralized (%/g soil dry mass)

$X_{(\text{corrected})}$ radioactivity corrected (Bq/g soil dry mass)

$X_{(\text{substrate})}$ total radioactivity added to microcosms (Bq)

Alternatively, the data may be converted to μg substrate mineralized. The addition of 50 μL of the substrate solution (2 g/L) results in 100 μg substrate being added to the microcosms. The mass of substrate mineralized per gram dry soil may be calculated by the formula:

$$S_2 = \frac{S_0 \times S_1}{100} \tag{9.4}$$

S_2 substrate mineralized (μg/g soil dry mass)

S_0 initial substrate concentration (100 μg)

S_1 substrate mineralized (%/g soil dry mass)

Depending on the experimental question, results among field treatments may be assessed for significant treatment effects (using unamended diluent in the assay). Nutrient-amended assays can be used to demonstrate the prior acclimation of microbial communities to hydrocarbon degradation, as nutrient limitations potentially present in the field are removed in the laboratory assay. Alternatively, a comparison between nutrient-amended assays and unamended assays, or among various amendments, may be conducted as a treatability study prior to implementing field treatment.

Notes and Points to Watch

- To assure no gas leakage occurs from the stripping apparatus, all Tygon tubing connections (i.e., to glass tubing, Harvey trap, 16-gauge and spinal needles) should be secured with several wraps of wire. Tygon tubing can be protected from being cut by the wire by wrapping the tubing with a piece of laboratory tape before securing with wire.

- As noted above, it is important to periodically check for obstructions in the various needles and glass tubing used in the stripping apparatus, and to clean the glass tubing that comes in contact with scintillation cocktail to prevent carryover of radioactivity from previously stripped samples.
- Carryover of radioactivity from previous samples run on the stripping apparatus should be checked periodically by running distilled water method blanks. If excessive radioactivity (i.e., significantly above background) is recovered from the method blank, change the toluene in the Harvey trap, and rerun a method blank. If excessive radioactivity persists, it may be necessary to change the Tygon tubing.
- When using volatile substrates in the assay, the volatile compounds require removal prior to recovering the $^{14}CO_2$, as described. As the volatile substrate is radioactive, the exhaust gas from this process must be properly captured (e.g., activated carbon filter) and disposed.
- It is not uncommon to observe substantial variance among samples using this assay; careful adherence to the protocol will reduce the variance substantially. We recommend preparing as many replicate assays as possible in order to obtain a lower standard error for the mean mineralization potentials determined.

References

Atlas RM (1993) Handbook of microbiological media. CRC Press, Boca Raton, FL

Bauer JE, Capone DG (1988) Effects of co-occurring aromatic hydrocarbons on degradation of individual polycyclic aromatic hydrocarbons in marine sediment slurries. Appl Environ Microbiol 54:1649–1655

Børresen M, Breedveld GB, Rike AG (2003) Assessment of the biodegradation potential of hydrocarbons in contaminated soil from a permafrost site. Cold Regions Sci Technol 37:137–149

Braddock JF, Lindstrom JE, Prince RC (2003) Weathering of a subarctic oil spill over 25 years: the Caribou-Poker Creeks Research Watershed experiment. Cold Regions Sci Technol 36:11–23

Braddock JF, Lindstrom JE, Yeager TR, Rasley BT, Brown EJ (1996) Patterns of microbial activity in oiled and unoiled sediments in Prince William Sound. Proceedings of the Exxon Valdez Oil Spill Symposium, Feb. 1993. Am Fish Soc Symp 18:94–108

Brown EJ, Resnick SM, Rebstock C, Luong HV, Lindstrom J (1991) UAF radiorespirometric protocol for assessing hydrocarbon mineralization potential in environmental samples. Biodegradation 2:121–127

Collins CH, Lyne PM, Grange JM (1989) Collins and Lyne's microbiological methods, 6th edn, Butterworths, London

Leahy JG, Colwell RR (1990) Microbial degradation of hydrocarbons in the environment. Microbiol Rev 54:305–315

Lindstrom JE, Prince RC, Clark JC, Grossman MJ, Yeager TR, Braddock JF, Brown EJ (1991) Microbial populations and hydrocarbon biodegradation potentials in fertilized shore-

line sediments affected by the T/V Exxon Valdez oil spill. Appl Environ Microbiol 57:2514–2522
National Research Council (1985) Oil in the sea: inputs, fates, and effects. National Academy Press, Washington, DC
Sugai SF, Schimel JP (1993) Decomposition and biomass incorporation of ^{14}C-labeled glucose and phenolics in taiga forest floor: effect of substrate quality, successional state, and season. Soil Biol Biochem 25:1379–1389
Wackett LP, Hershberger CD (2001) Biocatalysis and biodegradation: microbial transformation of organic compounds. ASM Press, Washington, DC
Walker JD, Colwell RR (1976) Measuring the potential activity of hydrocarbon-degrading bacteria. Appl Environ Microbiol 31:189–197

10 Molecular Techniques for Monitoring and Assessing Soil Bioremediation

Lyle G. Whyte, Charles W. Greer

10.1 General Introduction

Classical culture-dependent microbiological methods have succeeded in culturing ~1% of the microbial species in a given environmental sample. In reality, this is due to the fact that most isolation procedures are too general, and a wider variety of methods must be developed to recover a larger representation of microorganisms from most natural environments. Nevertheless, our knowledge of microorganisms is largely based on the representatives that have been cultured in the laboratory and studied in vitro. Since approx. 1990, significant advances in molecular biology techniques have transformed environmental microbiology and microbial ecology. These techniques bypass the major limitations of culture-dependent microbiological methods by extracting nucleic acids directly (DNA and RNA) from terrestrial or aquatic samples (soils, waters, wastewaters, etc.) and which theoretically represent 100% of the microbial species in a given sample. A variety of techniques are then used to manipulate and subsequently characterize individual DNA and RNA molecules from complex microbial communities with a relatively high degree of sensitivity and specificity. These techniques have been applied to contaminated soil and aquatic systems and have greatly aided in characterizing and monitoring pollutant biodegrading microbial populations within these systems. In addition, the knowledge gained from using these molecular techniques has helped identify novel biodegradation pathways and opened up new perspectives in bioremediation processes and pollution abatement. The following survey presents an overview of the prominent molecular techniques that are currently being utilized for environmental microbiology with a specific focus on soil microbiology. The overview is summarized in Fig. 10.1. Several specific techniques that include total DNA extraction, polymerase chain

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reaction (PCR) analyses, and community characterization using denaturing gradient gel electrophoresis, are covered in detail in this chapter.

10.2 Extraction and Purification of Nucleic Acids (DNA) from Soil

Introduction

Objectives. All of the current molecular methods *crucially* rely on the successful extraction and purification of sufficient amounts of nucleic acids from environmental samples. Consequently, many methodologies have been and continue to be developed for extracting nucleic acids from soils and sediments, and improvements are constantly being reported in the literature. The soil-DNA-isolation methodologies vary considerably with respect to reliability, yield, purity, and degree of shearing, and only recently have some of these methods been compared (Yeates et al. 1998; Martin-Laurent et al. 2001; Schneegurt et al. 2003; Kauffmann et al. 2004; Mumy and Findlay 2004). Several commercial extraction kits are also available such as MoBio Laboratories Ultraclean Soil DNA Kit (Mobio Laboratories, Solona Beach, CA, USA) and the Bio101 FastDNA soil kit (La Jolla, CA, USA); they feature short extraction times and the potential for reduced variability (Mumy and Findlay 2004) and have become quite popular despite their relatively high cost and limitation to the extraction of nucleic acids from 1 g or less of sample. In addition, extraction efficiency and resulting nucleic acid quality are strongly influenced by the source of the sample, and there are numerous co-extracted interfering substances (humics, pollutants, heavy metals, etc.). The method described below is routinely used in our laboratories for isolating nucleic acids from soil and sediments from both contaminated and pristine environments, and has yielded fairly uniform quantities and qualities of nucleic acids.

Principle. An initial soil washing step prior to DNA extraction helps solubilize and reduce contaminants when high quality DNA is required (Fortin et al. 2004). Total community DNA is isolated from soil and sediments using a direct DNA extraction procedure based on chemical/enzymatic lysis (lysozyme and proteinase K in combination with SDS). Released microbial DNA is ethanol precipitated and the total community DNA is purified by a polyvinylpolypyrrolidone (PVPP) spin column filtration step which removes contaminating soil organic compounds such as fulvic and humic acids (Fortin et al. 2004).

Theory. The starting point in the analysis of total community DNA from environmental samples is the efficient extraction of nucleic acids of suf-

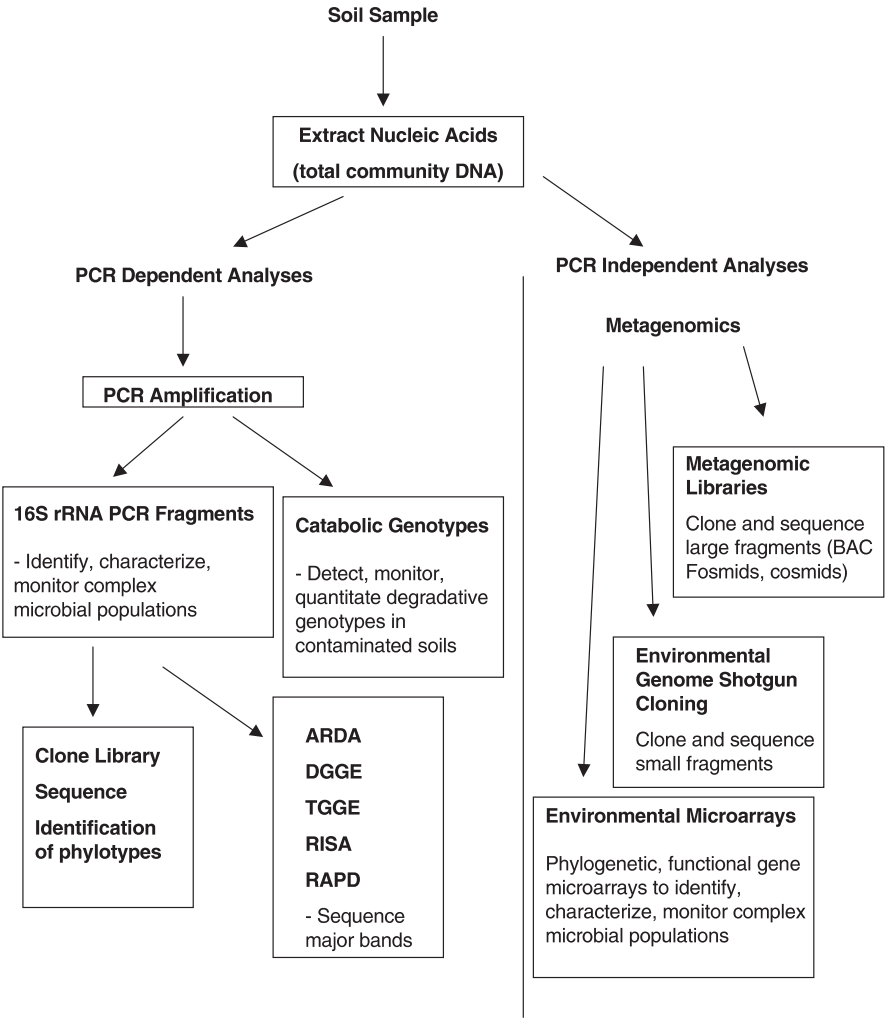

Fig. 10.1. Molecular techniques used in environmental microbiology

ficient quality for subsequent molecular analyses. Two general methodologies are commonly employed: (1) direct lysis of microbial cells within the environmental sample by chemical treatments, sonication, freeze-thaw, or bead beating protocols; or (2) extraction of the bacterial cells from the environmental sample followed by cell lysis. Humic and fulvic acids are often co-extracted from soil with the nucleic acids; they must be removed as they can seriously interfere with subsequent molecular reactions (DNA polymerase amplification, DNA-DNA hybridizations, DNA labeling, restriction nuclease digestion). The released soil DNA usually can be puri-

fied by a variety of methods/techniques such as chromatography and silica gel or PVPP spin-filter columns. The quality and quantity of the purified soil DNA extract is generally verified by agarose gel electrophoresis and/or spectrophotometry (Abs. 260/280 nm).

Equipment

- Water bath incubators (30 °C, 37 °C, 85 °C)
- Microcentrifuge (13,600–15,800 *g*) at 4 °C
- Platform shaker
- Speedvacuum (optional)
- Gel electrophoresis apparatus, ultraviolet light source, and camera or gel documentation system
- Spectrophotometer, quartz cuvettes
- Vortex
- Pipettors (10–20, 100, 1,000 µL) and tips
- Microcentrifuge tubes (0.5, 1.5, 2.0 mL)
- MicroSpin columns (Amersham Biosciences, Baie d'Urfe, Que., Canada)

Reagents

- Buffer 1: 50 mM Tris-HCl, pH 8.3, containing 200 mM NaCl, 5 mM EDTA, and 0.05% Triton X-100 (Fisher Scientific, Nepean, Ont. Canada)
- Buffer 2: 50 mM Tris-HCl, pH 8.3, containing 200 mM NaCl and 5 mM EDTA
- Buffer 3: 10 mM Tris-HCl, pH 8.3, containing 0.1 mM EDTA
- Distilled water
- 250 mM Tris-HCl, pH 8.0, with 5 mg/mL lysozyme (freshly prepared)
- Proteinase K (20 mg/mL)
- 20% SDS
- 7.5 M ammonium acetate
- 2-propanol
- 70% ethanol
- TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0

- TAE buffer: 40 mM Tris-acetate, 1 mM EDTA, pH 8.0
- Acid-washed PVPP spin columns (preparation below)
- 200 mM potassium phosphate buffer, pH 7.0
- Conc. HCl

Note: All solutions should be sterilized by filtration or autoclave. The recipes for many of the solutions described throughout this chapter can be found in Sambrook and Russell (2001).

Sample Preparation

Soil samples to be extracted should be frozen (−20 or −80 °C) as soon as possible upon collection and stored frozen to minimize changes in microbial communities because of the sampling process and/or degradation of nucleic acids.

Procedure

Soil-Washing Step

1. Add 1 mL of buffer 1 to 0.5 g of soil or sediment in a microcentrifuge tube. Mix by vortexing (speed 4) for 30 s, then by inverting for 1.5 min.
2. Centrifuge 5 min at 4 °C at 3,000 *g*. Remove the supernatant with a pipette.
3. Add 1 mL of buffer 2 to sediment. Mix by vortexing (speed 4) for 30 s, then by inverting for 1.5 min.
4. Centrifuge 5 min at 4 °C at 3,000 *g*. Remove the supernatant with a pipette.
5. Add 1 mL of buffer 3 to sediment. Mix by vortexing (speed 4) for 30 s, then by inverting for 1.5 min.
6. Centrifuge 5 min at 4 °C at 3,000 *g*. Remove the supernatant with a pipette.

Nucleic Acid Extraction Step

1. Add 450 µL of sterilized distilled water to the 0.5 g of sample in a 1.5 mL microcentrifuge tube. Vortex (moderate speed) for approx. 2 s to dislodge the pellet.
2. Add 50 µL of 250 mM Tris-HCl, pH 8.0, containing lysozyme (5 mg/mL).
3. Incubate at 30 °C, mixing by inversion, for 30 min.
4. Transfer to a 37 °C water bath and incubate for another 30 min, mixing by inversion every 10 min.

5. Add 5 µL of proteinase K (20 mg/mL). Incubate for 1 h at 37 °C, mixing by inversion every 10 min.
6. Add 50 µL of 20% SDS. Incubate at 85 °C for 30 min, mixing *gently* by inversion every 10 min.
7. Centrifuge 10 min at room temperature at 13,600 *g*. Transfer the supernatant to a fresh 1.5 mL microcentrifuge tube.
8. Add 1/2 volume of 7.5 M ammonium acetate. Mix *gently* by inversion, and incubate on ice for 15 min.
9. Centrifuge 5 min at 4 °C, at 13,600 *g*. Transfer and split the supernatant in two fresh 1.5 mL microcentrifuge tubes and treat each tube separately.
10. Add 1 volume of cold 2-propanol, and precipitate DNA overnight at −20 °C.
11. Centrifuge 15 min at 4 °C at 15,800 *g*. Discard the supernatant.
12. Wash the pellet with 500 µL of cold 70% ethanol. Gently tap the tube or mix by inversion.
13. Centrifuge 5 min at 4 °C at 15,800 *g*. Discard the supernatant.
14. Dry the pellet in speedvacuum for 5 min or air dry the pellet (approx. 30 min).
15. Add 100 µL of TE buffer to the pellet in each tube, place the sample on ice on a shaking platform and let the pellet slowly dissolve (approx. 1 h). Combine the DNA extracts from the two tubes.
16. Warm up the DNA combined extract for 10 min at 37 °C.
17. Purify 50 µL of total community DNA on a PVPP column.

DNA Purification with PVPP Columns (Modified from Berthelet et al. 1996)

1. Prepare acid washed PVPP. Pour 1,034 mL of conc. HCl (11.6 M) slowly with stirring into 2,966 mL of MilliQ (Qiagen Inc., Hilden, Germany) water; this will result in ca. 4 L of 3 M HCl. Add 150 g PVPP and suspend with stirring at room temperature for 12–16 h. Leave the suspension to settle for 30–60 min, then aspirate or decant the supernatant. Again suspend the PVPP, now in approx. 3.5 L of 200 mM potassium phosphate buffer (pH 7.0) and stir 1–2 h. Repeat the aspiration/decant and suspension twice more until the supernatant pH is close to 7.0 (check aliquot with pH meter). Then repeat the aspiration/decant and suspension two more times with approx. 3.5 L of 20 mM potassium phosphate buffer

(pH 7.0). Aliquot the final suspension into small bottles and autoclave (15–20 min, 121 °C). Store at 4 °C.

2. Acid washed PVPP (0.9 mL) slurried in 20 mM potassium phosphate (pH 7.0) is added to empty sterile MicroSpin columns placed inside 2.0-mL tubes and centrifuged for 3 min at 735 *g* at room temperature. If the top of the column is still immersed following centrifugation, remove the liquid from the collection tube, and re-spin the column.
3. Load the "crude" DNA extract (50–100 µL) onto the center of the column being careful not to touch the side of the column. This ensures that all of the sample will pass through the column and be cleaned, and not run down the side of the column.
4. Place the loaded columns in the microcentrifuge, ensuring that the sloping face of the packed column is facing the middle of the centrifuge. Centrifuge the columns for 3 min at 735 *g* at room temperature and collect the filtrate.
5. The "clean" DNA extract is then stored at −20 °C and is ready for PCR. The used PVPP is discarded and the MicroSpin columns washed for reuse.

Agarose Gel Electrophoresis

We check the quality and quantity of ca. 5 µL of purified soil DNA extract by both agarose gel electrophoresis in 0.7% gels in TAE buffer (stained with ethidium bromide and visualized by ultraviolet light) and spectrophotometry (Abs. 260/280 nm) using standard methods as described by Sambrook and Russell (2001).

Notes and Points to Watch

- This methodology can be readily scaled up to 10 g soil samples as described in Fortin et al. (2004).
- All molecular biology methodologies are notoriously variable. Incubation temperatures and durations, volumes, centrifugation parameters, etc. should be vigorously adhered to.
- Lysis of the microbial cells during DNA extraction represents a critical step in PCR-mediated approaches (von Wintzingerode et al. 1997). Each physical, chemical, and biological step involved in the preparation and analysis of an environmental sample is a source of bias which might give a distorted view of a given ecological niche (von Wintzingerode et al. 1997). It is often a question of whether there was sufficient or preferential

disruption of microbial cells. Rigorous conditions may be required to lyse Gram-positive cells but also may cause excessive shearing of nucleic acids of the Gram-negative cells, potentially biasing the reported diversity of the sample as well as possibly creating artifacts and chimeric PCR products (Liesack et al. 1991). Therefore, checking the soil DNA extract by agarose gel electrophoresis will indicate the quality of the extracted DNA and the extent of shearing.

- Quantification of the soil DNA extract by spectrophotometry is often inaccurate and does not appear to correlate with other methods such as agarose gel electrophoresis using known concentrations of DNA standards or PicoGreen (Molecular Probes, Leiden, Netherlands).

10.3 Amplification of Catabolic Genotypes and 16S rDNA Genotypes by PCR

Introduction

Objectives. Many of the molecular methodologies used in environmental microbiology rely on a PCR amplification step and are therefore considered PCR dependent. The objective of PCR is to amplify target gene sequences from total community DNA extracted from an environmental sample. The amplified sequences can then be characterized by a variety of molecular methodologies as shown in Fig. 10.1. In soil biodegradation studies, the target gene sequences can be either catabolic (biodegradative) genes of interest or a phylogenetic gene (almost always the 16S rDNA gene). In the case of catabolic genes, the PCR amplification step is generally used to detect the presence or absence of various catabolic genotypes in contaminated soils. Determining the prevalence and composition of specific biodegradative genotypes, and hence microbial populations, in contaminated soils significantly aids in assessing the feasibility of using biotreatment and in developing appropriate bioremediation strategies for a particular contaminated site, as well as in monitoring the effects on specific populations during bioremediation operations. For example, we routinely use PCR screening for hydrocarbon-degradative genotypes to perform biotreatability assessments of contaminated soils (Whyte et al. 1999; Soloway et al. 2001; Whyte et al. 2001) and to monitor bioremediation treatments (Whyte et al. 2003). The prevalence of various alkane monooxygenase genotypes and other degradative genotypes in hydrocarbon-contaminated and pristine soils from a variety of Arctic, Antarctic, and alpine contaminated soils was also determined by PCR screening (Whyte et al. 2002; Margesin et al. 2003; Luz et al. 2004).

In comparison, PCR amplification is currently the most widely used method to obtain 16S rDNA genotypes for detailed characterization of microbial communities. Unlike PCR amplification of degradative genotypes, however, PCR amplified 16S rDNA genes from a total community DNA extract must be further characterized by one or more of the molecular methodologies [clone and sequencing, denaturing gradient gel electrophoresis (DGGE; see Sect. 10.4, below), temperature gradient gel electrophoresis (TGGE), etc.], shown in Fig. 10.1, to obtain meaningful information on the amplified 16S rDNA PCR product. These molecular methods have been used to characterize cold-adapted microbial populations in hydrocarbon-contaminated soils originating in northern Canada (Juck et al. 2000), to determine the effect of oil contamination and a biostimulation treatment on *Pseudomonas* diversity in soil microcosms (Evans et al. 2004), to monitor microbial population changes in beach sediments during an experimental oil spill (Macnaughton et al. 1999), and to monitor the impact on microbial community composition during the bioremediation of hydrocarbon-contaminated soils (Mills et al. 2003).

Principle. During PCR, double-stranded DNA (from total community DNA soil extracts) is separated into single strands at high temperature (denaturation). Two oligonucleotide primers then anneal (at a lower annealing temperature) to complementary regions (which flank the target sequence) of the single-stranded DNA. A heat-stable DNA polymerase synthesizes a new strand of DNA by extending the primer using the complementary strand as a template, thus creating a duplicate copy of the target sequence. This cycle is repeated 20–30 times resulting in an exponential amplification (2^{20}–2^{30} fold) of the target sequence.

Theory. PCR is the simplest and currently the most widely used method to detect/obtain catabolic genotypes or 16S rDNA genotypes for detailed downstream characterization of soil microbial communities. These procedures are increasingly being utilized to perform biotreatability assessments of contaminated soils, to monitor the effects of soil bioremediation treatments on microbial populations, and to identify and characterize important and/or novel biodegradative microbial strains or groups of microorganisms in contaminated soils. This Section describes PCR procedures for the amplification of catabolic genotypes and 16S rDNA genes for cloning and sequencing.

Equipment

- PCR work station chamber (UV hood; optional but recommended for 16S rDNA PCR)

- Microcentrifuge
- Thin walled 0.2-mL PCR tubes
- Pipettes (10, 100 µL) and tips (filtered tips are recommended for 16S rDNA PCR)
- PCR thermocycler
- Gel electrophoresis apparatus, ultraviolet light source, camera or gel documentation system
- PCR cleanup kit (QIAquick PCR Purification Kit; Qiagen Inc.)
- PCR cloning kit (Promega pGem-T Easy Cloning Kit; Promega Corporation, Madison, WI, USA)
- Petri dishes
- Incubator (37 °C)
- Water bath incubators (37, 42 °C)

Reagents

Catabolic Genotype PCR Amplification

- Catabolic target gene forward and reverse oligonucleotide primers (forward and reverse; 0.4–0.8 mM stock solutions)
- Soil DNA extract
- DNA from reference organisms
- 100 bp DNA ladder (Fermentas, SM0241, Invitrogen, Carlsbad, CA, USA)
- DNA polymerase (*Taq* DNA polymerase is often used)
- DNA polymerase buffer: 10 mM Tris-HCl, pH 9.0, containing 50 mM KCl and 15 mM $MgCl_2$
- 25 mM $MgCl_2$
- 1.25 mM stock solution of each deoxynucleoside triphosphate (dNTP), namely dATP, dCTP, dGTP, dTTP

16S rDNA PCR Amplification

- General ("universal") Bacteria primers:
  - 27F (10 µM) 5′–GGTTACCTTGTTACGACTT
  - 758R (10 µM) 5′–CTACCAGGGTATCTAATCC

- Soil DNA extract
- DNA from reference organisms (control DNA)
- 100 bp DNA ladder (Fermentas, SM0241)
- *Taq* DNA polymerase (5 U/μL; Invitrogen)
- 10× PCR buffer: 200 mM Tris-HCl/500 mM KCl
- 50 mM $MgCl_2$
- 10 mM stock solution of each dNTP (dATP, dCTP, dGTP, dTTP)
- Bovine serum albumin (BSA; 10 mg/mL in sterile UV irradiated ddH_2O)
- Sterile UV irradiated H_2O

Store all PCR reagents at −20 °C.

Cloning and Sequencing of 16S rDNA PCR Amplicons: Transformation

- *Lac*$^-$ competent *E. coli* cells (DH5α)
- Isopropyl-B-D-thiogalactopyranoside (IPTG) solution: 0.1 M, filter sterilized (store at 4 °C)
- Ampicillin (Amp) solution: 10 mg/mL, filter sterilized (store at 4 °C)
- 5-bromo-4-chloro-3-indoyl-β-d-galactoside (X-Gal) solution: 50 mg/mL in N, N′-dimethyl-formamide, make fresh for each transformation (store at −20 °C)
- Luria broth (LB) medium (per L): 10 g tryptone, 5 g yeast extract, 5 g NaCl, adjust to pH 7.0 with NaOH
- LB plates with Amp/IPTG/X-Gal: To 1 L of autoclaved LB medium, add 5 ml of IPTG solution, 10 ml of ampicillin solution, and 1.6 mL of X-Gal solution.

Note: All solutions should be sterilized by filtration or autoclave. The recipes for many of the solutions described throughout this chapter can be found in Sambrook and Russell (2001).

Sample Preparation

Total community DNA is extracted and purified as described in Sect. 10.2. The soil DNA extracts should always be stored frozen at −20 °C or −80 °C and kept on ice during the procedure to minimize nucleic acid degradation, which will occur at greater rates at higher temperatures.

Procedure

Primer Design

An important key to PCR is optimal design of oligonucleotide primers specific to the desired gene target of interest. It is the specificity of the primers that allows PCR to amplify catabolic or 16S rDNA genes that can be in low abundance in complex environmental samples.

Catabolic Gene PCR Primers

We have designed and utilized a variety of primers for PCR amplification of catabolic genes; the specific oligonucleotide sequences of these primers are available for a variety of hydrocarbon degradative genes (Whyte et al. 2002; Margesin et al. 2003; Luz et al. 2004) and dehalogenation of chlorinated organics (Fortin et al. 1998). In general, we design PCR primers as follows. Gene sequences for key enzymes from known bacterial biodegradative pathways are identified and searched for in databases such as the nucleotide database (GenBank) at the NCBI web site (www.ncbi.nlm.nih.gov). The DNA sequences of all corresponding genes encoding the key enzyme are retrieved for comparative DNA and protein alignments using appropriate molecular biology sequence software. PCR forward and reverse primers for each catabolic gene sequence are then selected from the alignments by PCR primer software and/or manually by identifying homologous regions shared by the selected DNA sequences. For construction of the oligonucleotide primers, long sequences originating from within the coding region of the catabolic genes and having a high G+C content are preferred to ensure specificity. In addition, we use the following general criteria for designing catabolic gene primers:

- Generally 20–30 nt in length.
- At least 5 nt at both ends of the primer exhibiting exact match pairing with the target DNA sequence,
- Ideally, if there are mismatches, they should be in the middle of the primer sequence.
- Ideally, the ends of the primer should terminate with 2–3 G or C.
- Both forward and reverse primers should possess similar G+C content, with an average G+C content of 40–60%, with limited stretches of polypurines or polypyrimidines to ensure specificity.
- The PCR products generated should be ca. 200–1,000 nt in length and thus produce an easily detectable band by agarose gel electrophoresis.

Specificity of the selected primer sequences for the gene of interest is then verified by Fasta and blastn search programs available at the NCBI website.

16S rDNA PCR Primers

Given the varying degrees of conservation of the16S rDNA gene for Bacteria and Archaea, 16S rDNA primers or probes can be designed with any degree of specificity for groups, ranging from both domains (Ward et al. 1992), to a single domain (Battin et al. 2001), or to various subgroups all the way down to the species and sub-species level (Ahn et al. 2002). Generally, most 16S rDNA PCR-based studies rely on using a set of general or "universal" primers specific for the *Bacterial* domain and/or less often, the *Archaeal* domain. These general primer sets are readily available in the literature but should be updated periodically as the 16S rDNA database grows daily. One can also design new conserved 16S rDNA primer sets by accessing the Ribosomal Database Project (RDP) website (http://rdp.cme.msu.edu/index.jsp) algorithm that can be used in choosing the proper primer set for the PCR amplification in question.

PCR Amplification, Cloning, and Sequencing

PCR Amplification of Catabolic Genotypes

1. For each PCR reaction, set up the following reaction in a 0.2-mL microcentrifuge tube:
   - 1–5 µL of total community DNA soil extract (ca. 100 ng of DNA)
   - 2 µL of *each* oligonucleotide primer (0.4–0.8 mM stock; final concentration 0.2 µM)
   - 5 µL of DNA polymerase buffer
   - 2 µL of 25 mM $MgCl_2$
   - 8 µL of 1.25 mM dNTP (200 µM of each dATP, dCTP, dGTP, and dTTP solution)
   - 2.0–5.0 U of DNA polymerase
2. The final volume in the tube is brought to 50 µL with sterile distilled water. Prior to the addition of DNA polymerase, the samples are boiled for 2 min and then transferred to ice.
3. *Negative control*: The same mixture is used for the negative control except that the total community DNA soil extract is replaced with sterile distilled water.
   Positive Control: The same mixture is used for the positive control except that the total community DNA soil extract is replaced with a genomic DNA extract from the appropriate control reference organism. Template DNA for PCR from the reference organism can be obtained by resuspending 2–3 colonies in 500 µL of sterile distilled water and boiling for

10 min. The sample is cooled on ice, centrifuged in a microcentrifuge for 2 min at 12,000 *g*, the supernatant collected, and stored at −20 °C.

4. PCR is conducted using an appropriate PCR thermal cycler. We generally are successful using the following PCR parameters:
   - 30 cycles of 1 min at 94 °C (denature)
   - 1 min at 60 °C (anneal)
   - 1 min at 72 °C (extend)
   - A final extension of 3 min at 72 °C.
5. To determine the presence or absence of the appropriately sized PCR fragment, ca. 5–10 µL of the PCR reaction mixture of soil DNA extracts and the corresponding positive and negative controls, and a 100 bp DNA ladder are analysed by agarose gel electrophoresis (1–1.4% agarose gels using TAE buffer) and visualized by ethidium bromide staining essentially as described by Sambrook and Russell (2001). There should be a single band of the same size in both the positive and sample lanes, with no band in the negative control lane.
6. To confirm that DNA had been successfully extracted from the soils and could be amplified by PCR, general ("universal") 16S rDNA bacterial primers are used as a positive PCR amplification control for all soil DNA extracts (Whyte et al. 2002).

PCR Amplification of 16S rRNA for Downstream Cloning and Sequencing

The protocol given is to PCR amplify 16S rDNA from an environmental sample.

1. All subsequent work should be conducted in an enclosed PCR work station chamber. This will help eliminate contamination of plastic ware with extraneous 16S rDNA that is present ubiquitously. Latex gloves should be worn throughout the procedure.
2. A PCR master mix (enough for three PCR reactions) is prepared in a 1.5 mL microcentrifuge tube by combining the following reagents:
   - 7.5 µL of *each* oligonucleotide primer (10 µM stock; final concentration 0.5 µM)
   - 1 µL of 10× PCR buffer
   - 4.5 µL of 50 mM $MgCl_2$ (final concentration 1.5 mM)
   - 3 µL of 10 mM dNTP stock solution (final concentration 200 µM of each dATP, dCTP, dGTP, and dTTP)

- 1.9 µL of BSA (10 mg/mL)
- 92.6 µL of sterile, irradiated water

3. Briefly microcentrifuge the PCR master mix tube at 15,000 *g* for ca. 10 s.
4. Add 3 µL of *Taq* polymerase to the PCR master mix tube, briefly vortex (2–4 s) to mix and microcentrifuge at 15,000 *g* for ca. 10 s.
5. Label the following 0.2 mL PCR reaction tubes: positive control, negative control, sample. To each reaction tube add 45 µL of master mix.
6. Add 5 µL of *E. coli* (or other positive control) genomic DNA (ca. 50–100 ng) to the positive control. To the negative control, add 5 µl of water. To the sample tube, add 1–5 µL of total community DNA soil extract (ca. 100 ng of DNA).
7. Briefly vortex (2–4 s) the PCR reaction tubes to mix. Microcentrifuge the tubes at 15,000 *g* for ca. 10 s.
8. PCR is conducted in an appropriate thermal cycler. We are generally successful using the following PCR parameters:
   The first 10 cycles are conducted using a "touchdown protocol" from 65–55 °C, with the annealing temperature decreasing by 1 °C at each cycle.
   - 1 min at 94 °C
   - 1 min at 65–55 °C
   - 3 min at 72 °C

   The subsequent 20 cycles are performed with an annealing temperature of 55 °C.
9. The presence or absence of the appropriately sized PCR fragment (731 bp for Bacteria 16S rDNA) is determined by agarose gel electrophoresis (0.8%) of 5 µL of the PCR reaction mixture of soil DNA extracts, positive and negative controls, and a 100 bp DNA ladder, and visualized by ethidium bromide staining essentially as described by Sambrook and Russell (2001). There should be a single band of the same size in both the positive and sample lanes, with no band in the negative control lane.

Cloning and Sequencing of 16S rDNA PCR Products

1. We generally use a spin column purification system such as the Qiaquick PCR purification kit to clean the PCR reaction prior to cloning. It is important to purify PCR reaction products as unbound primers and unincorporated nucleotides can be inhibitory to the ligation reaction.
2. The purified PCR product is quantified by spectrophotometry (Abs. 260/280 nm; Sambrook and Russell 2001).

3. Because of their ease of use and reliability, commercial PCR cloning kits, such as the pGEM-T Easy Vector (Promega Corp.), are commonly used for ligating 16S rDNA PCR amplicon libraries into a cloning vector. A ligation reaction is set up as described in the pGEM-T Easy Vector technical manual. If this is a first-time attempt at cloning with this PCR product, it may be necessary to optimize the vector:insert molar ratio. We generally optimize the reaction with 1:3, 1:1, and 3:1 ratios. To calculate the amount of PCR product to include in the reaction use the following formula:

$$\text{ng insert} = \frac{(50\,\text{ng of vector}) \times (\text{size of PCR product}) \times (\text{insert:vector ratio})}{\text{kb size of vector (3.0 kb for pGEM-T Easy)}}$$

4. Incubate the ligation reaction at 4 °C overnight.
5. Transformation protocols of *E. coli* competent cells with recombinant vector (16S rDNA inserted into the pGEM-T Easy Vector) can be found in the pGEM-T Easy technical manual or Sambrook and Russell (2001). Competent cells can be provided with the pGEM-T Easy Vector system; we have had success using *E. coli* DH5α (made chemically competent as described by Sambrook and Russell 2001). We generally follow the protocol provided in the manual. Always ensure that positive and negative controls are included in the analysis. The positive control consists of cells transformed with the vector DNA alone; the negative control cells are treated the same as cells being transformed, but with no added DNA.
6. Spread plate 100 µL of each transformation (in duplicate) and controls onto appropriately labeled LB/Amp/X-Gal/IPTG plates.
7. Incubate plates overnight at 37 °C.
8. Score blue and white colonies. White colonies arise from insertion of a cloned product into the pGEM-T Easy Vector. More than 60% white colonies should be observed.
9. White colonies are either directly sequenced or screened for unique clones prior to sequencing by amplified ribosomal DNA restriction analysis (ARDRA; sometimes called restriction fragment length polymorphism, RFLP; see Massol-Deya et al. 1997 for a typical ARDRA protocol for 16S rDNA amplicons). Sequencing of the 16S rDNA inserts in the pGEM-T Easy Vector system is conducted with primers as described by that system's technical manual; ensure that when reamplifying the cloned inserts to use the pGemT-Easy primers T7 and Sp6

to avoid amplifying *E. coli* 16S rDNA genes. Sequencing is usually performed by commercial laboratories or by in-house sequencing facilities commonly found in most large research institutions.

10. Sequences are submitted for comparison and identification to the GenBank databases using the NCBI Blastn algorithm, the EMBL databases using the Fasta algorithm (http://www.ebi.ac.uk/fasta33/nucleotide.htmL) and/or the Ribosomal Database Project (RDP) using its Sequence Match. Sequences that demonstrate strong homology are then aligned to reference sequences and phylogenetic trees commonly constructed (Juck et al. 2000). Sequences that demonstrate uncertain alignments are checked for chimeras using the CHECK_CHIMERA software program function at the RDP site.

Notes and Points to Watch

- A key limitation to 16S rDNA PCR amplifications is contamination of DNA introduced by unintentional tube-to-tube contamination or contaminated reagents. For this reason, false-positive signals and false-negative amplifications are not uncommon due to the extreme sensitivity of the 16S rDNA PCR reaction, and the ubiquity of 16S rDNA genes in almost all biological materials. Fortunately, this problem can be avoided simply by using good laboratory techniques as indicated above.
- We often perform PCR amplification on both undiluted soil DNA extracts (as described here) and diluted extracts (1/10, 1/100). Diluting the DNA extract can result in the parallel dilution of undesired contaminants that inhibit the PCR reaction; it is not uncommon to observe successful PCR amplification from the diluted samples but not the undiluted sample.
- To minimize the loss of nucleic acids from small sample volumes, additives such as BSA and T4 gene 32 (gp32) can be used to reduce the inhibitory effect of contaminants (Kreader 1996).
- The PCR procedures described here should be considered qualitative rather than quantitative. Differences in band intensity do suggest differences in the relative amounts of the genotypes in the original samples, but keep in mind that PCR reactions are very sensitive to reaction conditions. Quantitative PCR protocols (real-time quantitative PCR or RT-qPCR) have been recently developed and are being applied to contaminated soils.
- Very similar nucleic acid sequences can also affect amplification of total community DNA, especially during 16S rDNA PCR amplifications. Chimeric sequences result from the heterologous combination of two

non-identical but similar strands of DNA, but do not generally exist in the sample being investigated. However, chimeric sequences can be formed at frequencies of several percent during PCR (Liesack et al. 1991). The resultant PCR artifacts can affect subsequent analyses by erroneously suggesting the existence of novel taxa from these hybrid sequences. The binding of heterologous DNA into chimeric structures has also been shown to compete with the binding of specific primers during the annealing step (Meyerhans et al. 1990; Ford et al. 1994; Wang and Wang 1996). As well, DNA damage such as that caused by mechanical and chemical shearing has been suggested to contribute to the formation of chimeric DNA during PCR (Paabo et al. 1990).

- Another pitfall of PCR is the production of minute errors by *Taq* DNA polymerase, which lacks the ability to proofread. (Ford et al. 1994). However, this is only a potential problem when sequencing the resulting PCR products.
- For cloning into the pGEM-T Easy Vector system, it is essential to use a thermostable polymerase that lacks 3′-5′ exonuclease activity in the initial 16S rDNA amplification step of soil DNA extracts. This will insure that a 3′A overhang is present on the PCR product and will greatly improve the efficiency of the ligation process, as well as avoiding circularization of PCR products. Common polymerases that lack the 3′-5′ exonuclease activity are *Taq*, *Tfl*, and *Tth*.

10.4 DGGE Analysis Soil Microbial Communities

Introduction

Objectives. Denaturing gradient gel electrophoresis (DGGE) is a very versatile method for screening the total microbial community DNA from a complex sample. Our limited knowledge of the total microbial community composition and function in complex environmental samples has necessitated the development of techniques like DGGE to enable us to look more directly at the representative microorganisms, independent of the biases introduced by culturing. In the last 10 years, more than 1,000 articles have been published using DGGE for the analysis of various environmental samples.

DGGE analysis of microbial communities produces a complex profile or banding pattern, which can be quite sensitive to spatial and temporal sampling variations (Murray et al. 1998). The classic means of analyzing this variability has been visual, reporting differences between samples in band

intensity, or the presence or absence of specific bands. However, a recent study suggests that the results of denaturing gradient methods are readily amenable to statistical analysis, provided there is sufficient standardization of analytical procedures (Fromin et al. 2002). This would provide the rigor of statistical validation of observations and permit a broader range of comparisons to be made between different samples and between different experimental or environmental parameters.

DGGE is a useful method for visualizing the major members of a microbial community, but several factors must be considered when interpreting the data. The limit of resolution of this method is about 1% of the total community population (Muyzer et al. 1993; Murray et al. 1996), and in very complex samples, more bands may be produced than can be resolved. Initial calibration to ensure optimal gradient and electrophoretic conditions is also important (Muyzer et al. 1993; Muyzer and Smalla 1998). DGGE requires rather large quantities of DNA for reliable visualization, possibly as much as 500 ng for environmental samples (Nakagawa and Fukui 2002). Also, DGGE is typically limited to fragments of no more than 500 bp (Myers et al. 1985), which limits the amount of sequence information that can ultimately be retrieved. Some ambiguity can exist in associating a single band in a DGGE profile with a single microbial species, since it is possible that multiple amplicons co-migrate to the same location in the gel, and similarly, multiple bands may be produced by a single species since multiple copies of 16S rDNA do exist in the same microorganism (Nübel et al. 1997).

Principle. DGGE separates a mixture of PCR-amplified DNA fragments according to differences in sequence G-C content, based on their differential mobility through a DNA-denaturing gel. Once separated, the individual fragments can be recovered from the gel and the nucleotide sequences determined and compared against existing databases (GenBank, Ribosome Database Project) to identity microorganisms in the sample.

Theory. DGGE, which is based on the early work of Fischer and Lerman (1979), is one of the most commonly used methods for the characterization of complex microbial communities, and was pioneered by Muyzer et al. (1993) for environmental samples. In a manner similar to the other PCR-based characterization techniques, samples for DGGE analysis are prepared either directly from PCR-amplified environmental DNA (Ahn et al. 2002; Ibekwe et al. 2002), from clone libraries constructed from PCR-amplified environmental samples (Liu et al. 2002), or in some cases from colonies obtained from enrichment cultures (Bonin et al. 2002). Total community DNA is extracted, purified and used as a PCR template for the amplification of specific target molecules. The most common target molecule is the 16S rDNA gene which is used as a phylogenetic marker to assess biodiversity

and eventually to identify individual members within the community. General 16S rDNA primers, often referred to as universal primers, are used to amplify the total community DNA. This produces a mixture of fragments derived from the individual microorganisms in the sample. Because each fragment has a different internal sequence, the fragments can subsequently be separated based on their melting behavior in a denaturing gradient, usually composed of urea and formamide. As the double-stranded PCR fragments move through the gel from low to high denaturant concentration, they begin to separate into single strands, which reduces their mobility. Complete strand separation is prevented by incorporating a GC rich region (ca. 40 bases), referred to as a GC clamp, at the 5′-end of one of the PCR primers. The DNA comes to rest when it is almost fully denatured. The position along the gradient at which the DNA stops is determined primarily by the relative proportions of G+C and A+T in a given amplicon, since G-C bonds are more difficult to denature than A-T bonds. Properly calibrated, DGGE is sensitive enough to detect even single base-pair differences between amplicons (Miller et al. 1999). The result in complex samples is typically a banding pattern that is representative of the molecular diversity in the sample. The individual bands can subsequently be extracted from the DGGE gel and sequenced to potentially identify individual microorganisms.

Equipment

- See Sect. 10.3 for equipment for PCR amplification
- Gradient mixer (BioRad Model 385 Gradient Former, BioRad Laboratories Inc., Mississauga, Ont. Canada)
- BioRad Dcode Universal Mutation Detection System (BioRad Labs.; or equivalent)
- FluorImager system, model 595 (Molecular Dynamics Inc., Sunnyvale, CA, USA; or equivalent)
- PCR clean up kit (QIAquick PCR Purification Kit, Qiagen Inc.; containing PB, EB, PE buffer, and column-collection tubes)

Reagents

- 50× TAE (per L): 242 g Tris base, 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA, pH 8.0. To prepare 1× TAE, dilute 1:50 with distilled water.
- Acrylamide-denaturant solutions: the acrylamide solutions are only stable for 1 month. All glassware should be rinsed with ultrapure water.

  - 8% acrylamide/0% denaturant: To make 100 mL of solution, mix 20 mL of 40% Acrylamide/Bisacrylamide (37.5:1; BioRad); 2 mL of 50× TAE buffer prepared with ultrapure water and ultrapure reagents, and 78 mL ultrapure water. Filter through a 0.22-µm filter. Mix and degas for 10–15 min. Store at 4 °C in a brown bottle for approx. 1 month.
  - 8% acrylamide/80% denaturant: To make 100 mL of solution mix 20 mL of 40% acrylamide/bisacrylamide (37.5:1); 2 mL of 50× TAE buffer prepared with ultrapure water and ultrapure reagents; 32 mL deionized formamide; 33.6 g ultrapure urea, and adjust volume to 100 mL. Filter through a 0.22-µm filter. Mix and degas for 10–15 min. Store at 4 °C in a brown bottle for approx. 1 month.

- Ammonium persulfate (APS) 10% (w/v) solution: Add 100 mg of dry APS to 1 mL of distilled water, vortex to dissolve. This is used immediately and then discarded.
- TEMED
- Gel Loading Dye 2X (BioRad's recipe, final concentration): 0.05% bromophenol blue/0.05% xylene cyanol/70% glycerol. Prepare a 2% bromophenol blue and a 2% xylene cyanol solution. Mix 0.25 mL of each solution with 7.0 mL of 100% glycerol, add 2.5 mL of distilled water to make volume up to 10.0 mL. Store at room temperature.
- Glycogen solution (20 mg/mL; Roche Diagnostics 901393, Laval, Que., Canada)
- 3 M sodium acetate (pH 5.2)
- 100% ethanol
- Vistra Green (Amersham Biosciences) solution: Dissolve 25 µL of Vistra Green in 250 mL of 1× TAE buffer (1:10,000 dilution). Store solution at 4 °C for 3–4 days.
- 100 bp molecular weight ladder (Fermentas SM0241)

Sample Preparation

1. PCR amplification of extracted and purified total community DNA: It may be necessary to dilute the soil DNA extract (preparation see Sect. 10.2) 1:10 or 1:100 to optimize PCR yield.
2. A typical PCR reaction (total volume 50 µL) is composed of the following:
   - 1.0 µL of template DNA (or dilution)

- 1.0 µL U341GC#2 primer (25 µmol); sequence: $5'_{341-357}$-GCGGGCGGGGCGGGGGCACGGGGGGCGCGGCGGGC GGGGCGGGGGCCTACGGGAGGCAGCAG-3′ (GC clamp underlined)
- 1.0 µL of U758 primer (25 µmol); sequence: $5'_{758-740}$-CTACCAGGGTATCTAATCC-3′
- 0.5 µL of 100 mM $MgCl_2$
- 8.0 µL of 1.25 mM dNTPs
- 32.4 µL of sterile deionized water
- 0.625 µL of BSA (10 mg/mL; optional, but often improves the PCR when using DNA recovered from soils with high organic content)

3. In a separate tube add 10x DNA polymerase buffer (5 µL per reaction) and DNA polymerase (0.5 µL per reaction). We typically use r*Taq* polymerase for this work. It is easier to prepare this mixture to accommodate all planned reaction tubes, and add 5.5 µL of the mixture to each reaction.
4. For a "hot start" the tubes are put in the thermal cycler and heated to 96 °C for 5 min. The temperature is then reduced to 80 °C and the DNA polymerase buffer/DNA polymerase mix is added to each tube.
5. PCR is conducted using the following conditions:
   The first ten cycles use a "Touchdown protocol" from 65–55 °C, with the annealing temperature decreased by 1 °C at each cycle.
   - 1 min at 94 °C
   - 1 min at 65–55 °C
   - 3 min at 72 °C

   The subsequent 20 cycles are performed with an annealing temperature of 55 °C.
7. The PCR reactions are analyzed by agarose gel electrophoresis using 5–10 µL of reaction in a 1.4% agarose gel using TAE buffer (Sambrook and Russell 2001). Several dilutions (i.e., 1, 2, and 4 µL of a 1:10 dilution) of a 100 bp molecular weight ladder (Fermentas SM0241) are electrophoresed in the gel as well to quantify the amount of PCR product. For complex environmental samples it is advisable to prepare up to 500 ng of PCR product to apply to each lane of the DGGE.

Procedure

Denaturant Gradient Gel (after Fortin et al. 2004)

1. Assemble the glass plates with spacers and clamps and secure to the casting stand.

2. Clamp (or tape) needle outlet from the gradient mixer between the glass plates (middle/top) so that it will inject the gel solution between the plates.
3. To prepare a 30–70% gradient, add 7.2 mL of 8% acrylamide/0% denaturant solution and 4.3 mL of 8% acrylamide/80% denaturant solution to one 50-mL Falcon tubes (Fisher Scientific; label "Low") and add 1.4 mL of 8% acrylamide/0% denaturant solution and 10.1 mL of 8% acrylamide/80% denaturant solution to another 50 mL Falcon tubes (label "High").
4. Add 115 µL of 10% fresh APS solution to each tube. Mix gently by inversion. Be careful not to introduce air into the solution.
5. Add 11.5 µL of TEMED to each tube. Mix gently by inversion. Be careful not to introduce air.
6. Add the low denaturant solution gently to the left chamber (Low) of the gradient mixer. Remove air bubble from transfer tube by opening the valve stem quickly until the transfer tube between the two chambers is just full of low denaturant solution.
7. Add the high denaturant solution gently to the right chamber (High) of the gradient mixer, turn on the mixer and the pump, open the out valve on the right side, and transfer the entire solution to the plates.
8. Gently layer 1 mL of water on top of the gel to stop it from drying out.
9. Let the gel polymerize for 1.5 h at room temperature.

Buffer

10. Add 6 L of 1× TAE to gel tank (i.e., fill to the FILL line).
11. Insert the lid and turn on. Let the buffer warm up until the temperature reaches 60 °C. This takes more than 1 h, so you should do this 30 min after pouring the gel.

Spacer Gel

12. Using filter paper, remove the water on top of the polymerized gel.
13. Insert gel comb fully.
14. Mix 3.75 mL of 8% acrylamide/0% denaturant with 1.25 mL of 1× TAE and with 45 µL of 10% (w/v) APS and 4.5 µL TEMED.
15. Add this to the top of the denaturant gradient with a pipette.
16. Let polymerize for 0.5 h.

Loading and Running Gel

17. Remove the comb and any excess polyacrylamide from the gel.
18. Assemble the plates on the core. Pour approx. 350 mL of 1× TAE in the upper chamber to check the integrity of the seal. If buffer is leaking, discard the buffer, disassemble, lubricate the gasket, reassemble the plates onto the core and test again. Insert into tank containing 1× TAE buffer prewarmed to 60 °C.
19. Let equilibrate for 15 min.
20. Wash wells with syringe using the 1× TAE buffer from the tank.
21. Load wells with samples diluted in 2× gel-loading buffer.
22. Run gel at 80 V for 16 h at 60 °C.

Staining Gel

23. Stain gel for 0.5 h in 1:10,000 dilution of Vistra Green solution with gentle shaking.
23. De-stain for 0.5 h in 250 mL of 1× TAE with gentle shaking.
24. Scan the gel on a glass plate using a FluorImager, save an image of the gel for printing (image the same size as the gel) to use as a template for selecting bands for excision and sequencing.

Excising DGGE Bands and Purification for Nucleotide Sequencing

1. Transfer the gel onto a sheet of Plexiglas under which has been placed the printed image of the stained gel.
2. Cut bands of interest from gel using a scalpel or razor blade, and transfer into microcentrifuge tubes.
3. Add 60 µL of sterile deionized water to each DGGE fragment and elute overnight in a 37 °C incubator.
4. Centrifuge at 16,000 *g* at room temperature for 1 min and transfer supernatant to a fresh tube. Purify 50 µL with the QIAquick PCR purification kit.
5. Add 5 volumes of Qiagen PB buffer to the 50 µL of supernatant. Vortex, and apply the sample to the QIAquick column-collection-tube assembly.
6. Centrifuge at 16,000 *g* for 1 min (binding step).
7. Discard flow-through. Place the column back in the collection tube.

8. To wash, add 750 µL of Qiagen PE buffer to the column, and centrifuge at 16,000 *g* for 1 min.
9. Discard flow-through. Place the column back in the collection tube.
10. Centrifuge again at 16,000 *g* for 1 min, and transfer the column to a 1.5 mL microcentrifuge tube.
11. Add 50 µL of prewarmed (5 min at 50 °C) Qiagen elution buffer (EB; 10 mM Tris-HCl, pH 8.5) to the center of the membrane.
12. Incubate for 5 min at room temperature.
13. Centrifuge at 16,000 *g* for 1 min (elution step).
14. Store the purified DNA at −20 °C.
15. Re-amplify the purified DNA: It is advisable to prepare 200 ng for sequencing both strands. (To obtain good sequence results it is important to optimize PCR conditions to obtain a single band, in an agarose gel, of re-amplified product.) Several PCR reactions can be pooled and precipitated: Add 1 µl of glycogen solution, 1/10 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol. Precipitate at −20 °C for 1.5 h or overnight to accumulate sufficient product.
16. Purify the re-amplified product using a QIAquick PCR purification kit or GENE CLEAN II (Qbiogene) or a GFX punification kit (Amersham Biosciences).
17. Quantify the product on an agarose gel with a dilution series of a molecular standard, as described above, prior to submitting for sequencing.

Notes and Points to Watch

- The DGGE gel plates should be carefully assembled and checked for leaks.
- The minimum and maximum denaturant solutions can be varied in concentration to change the resolution of the gel. We have found that a gradient from 35 to 65% denaturant gives the best resolution for many environmental samples.
- When preparing denaturant solutions and adding the APS and TEMED, care should be taken not to introduce air. This can be accomplished by adding all the ingredients to separate 50-mL Falcon tubes, and gently mixing by inversion before adding to the gradient mixer.
- It is important to ensure that the printed image of the scanned gel is identical in dimensions to the gel itself, since bands are being excised "in

the blind" and the image is the template for removing the bands. When scanning the gel ensure that all four corners of the gel are scanned to facilitate subsequent alignment with the printed image. After removing the bands, the gel is re-scanned to ensure that the correct bands have been successfully recovered.

10.5 Genomics in Environmental Microbiology

Environmental microbiology has only recently entered the genomics era. Genomics in its broadest sense entails the complete sequencing of an organism's entire complement of DNA (made up of the 4 bases, A, T, C, and G). The sequence of DNA for a particular gene is the genetic code, or blueprint, that is translated into specific proteins, the key components in assembling all the organism's structures, regulating its functions, and consequently its behavior and physiology. Since the cost of genome sequencing has decreased substantially, an ever increasing number of microbial genomes are being sequenced, including important microorganisms from industrial or environmental perspectives. Many of the publicly available genome sequencing projects directed towards these latter organisms are sponsored by the US Department of Energy's (DOE) Microbial Genome Project (www.sc.doe.gov/production/ober/microbial.html) in collaboration with other partners. The DOE projects are targeting microorganisms involved in, for example, bioremediation, carbon sequestering, energy production, cellulose degradation biotechnology, and technology development. Presently 43 microorganisms with biodegradation capabilities have been/are being fully sequenced to hopefully identify new microbial processes involved in bioremediation and lead to the development of novel technologies and methodologies (i.e., genomic approaches, molecular monitoring tools) for studying the structure and function of complex microbial communities associated with contaminated environments.

As sequencing costs have diminished, PCR-independent methodologies (Fig. 10.1), including *metagenomic libraries* and *environmental genome shotgun cloning approaches*, have also emerged as novel ultra-high throughput methods to characterize complex environmental microbial communities. Although still quite expensive, the PCR-independent methodologies overcome some of the limitations of the PCR-dependent methodologies, including the inherent bias of primer specificity in PCR amplification and the relatively limited amount of sequence information obtained from the small PCR gene targets amplified and sequenced (ca. 300–1,000 nt). Metagenomic libraries are created by extracting total genomic DNA from an environment and cloning relatively large fragments (5,000–300,000 nt) into

lambda, cosmid, fosmid, or bacterial artificial chromosome (BAC) vectors. The metagenomic libraries created are then screened for functional and/or genetic diversity that allows for clones of interest to be singled out and sequenced (Eyers et al. 2004). For example, Rondon et al. (2000) constructed metagenomic libraries from total DNA extracted from two soils that contained more than 1 Gbp of DNA. Shotgun cloning is the process by which total community DNA is extracted from a sample, broken up to reduce the size, and the fragments (ca. 2,000–6,000 nt) then ligated into cloning vectors. Each cloning vector and the fragment of community DNA it carries is then amplified separately by growth in a bacterial host. The entire assemblage of the clone library is randomly sequenced and then reassembled in a procedure termed direct shotgun sequencing. Venter et al. (2004) performed direct shotgun sequencing (1.045 billion base pairs) of the microorganisms in the Sargasso Sea and identified hundreds of new bacterial species and 1.2 million new genes! These studies have clearly demonstrated the enormous biodiversity present in the environment, and that we have only begun to identify the vast majority of microorganisms out there.

Environmental microarrays are considered an emerging technology with tremendous potential in the field of environmental genomics (Greer et al. 2001; Rhee et al. 2004; Stahl 2004; Zhou and Thomson 2002). Successful application of microarray technology, which uses high-density, high-throughput techniques, promises to revolutionize our understanding of microbial diversity and microbial ecology, as thousands of potential gene probes can be printed on an array and hybridized to labeled total nucleic acids extracted from environmental samples. Environmental microarray technology is at a developmental stage where significant problems regarding specificity, sensitivity, and quantitation remain to be resolved (Eyers et al. 2004; Rhee et al. 2004; Stahl 2004). Nevertheless, application-specific environmental microarrays were recently used to detect sulfate-reducing bacteria (Loy et al. 2002), methanotrophs (Bodrossy et al. 2003), and biodegradative populations (Rhee et al. 2004) in environmental samples. Two types of environmental microarrays are presently being developed. Functional gene microarrays (FGMA) contain a variety of catabolic, biogeochemical cycling, heavy metal transformation genes, etc., as gene targets. Phylogenetic gene microarrays (PGMA) contain taxonomic gene targets, usually the 16S rDNA genes representing most genera of *Bacteria* and *Archaea*. Environmental microarrays will be increasingly used to detect and characterize complex microbial communities in contaminated soils as well as to monitor degradative populations during bioremediation treatments. This will lead to a better understanding of important processes such as biogeochemical cycles and bioremediation in soils that are associated with mixed microbial populations in natural environments.

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References

Ahn J, Daidou T, Tsuneda S, Hirata A (2002) Characterization of denitrifying phosphate-accumulating organisms cultivated under different electron acceptor conditions using polymerase chain reaction-denaturing gradient gel electrophoresis ASSA Water Res 36:403–412

Battin TJ, Wille A, Sattler B, Psenner R (2001) Phylogenetic and functional heterogeneity of sediment biofilms along environmental gradients in a glacial stream. Appl Environ Microbiol 67:799–807

Berthelet M, Whyte LG, Greer CW (1996) Rapid, direct extraction of DNA from soils for PCR analysis using polyvinylpolypyrrolidone spin columns. FEMS Microbiol Lett 138:17–22

Bodrossy L, Stralis-Pavese N, Murrell JC, Radajewski S, Weilharter A, Sessitsch A (2003) Development and validation of a diagnostic microbial microarray for methanotrophs. Environ Microbiol 5:566–82

Bonin PC, Michotey VD, Mouzdahir A, Rontani J-F (2002) Anaerobic biodegradation of squalene: Using DGGE to monitor the isolation of denitrifying bacteria taken from enrichment cultures. FEMS Microbiol Ecol 42:37–49

Evans FF, Seldin L, Sebastian GV, Kjelleberg S, Holmstrom C, Rosado AS (2004) Influence of petroleum contamination and biostimulation treatment on the diversity of *Pseudomonas* spp. in soil microcosms as evaluated by 16S rRNA based-PCR and DGGE. Lett Appl Microbiol 38:93–98

Eyers L, George I, Schuler L, Stenuit B, Agathos SN, El Fantroussi S (2004) Environmental genomics: exploring the unmined richness of microbes to degrade xenobiotics. Appl Microbiol Biotechnol 66:123–130

Fisher SG, Lerman LS (1979) Length-independent separation of DNA restriction fragments in two dimensional gel electrophoresis. Cell 16:191–200

Ford JE, McHeyzer-Williams MG, Lieber, MR (1994) Chimeric molecules created by gene amplification interfere with the analysis of somatic hypermutation of murine immunoglobulin genes. Gene 142:279–283

Fortin N, Beaumier D, Lee K, Greer CW (2004) Soil washing improves the recovery of total community DNA from polluted and high organic content sediments. J Microbiol Meth 56:181–191

Fortin N, Fulthorpe RR, Allen DG, Greer CW (1998) Molecular analysis of bacterial isolates and total community DNA from Kraft pulp mill effluent treatment systems. Can J Microbiol 44:537–546

Fromin N, Hamelin J, Tarnawski S, Roesti D, Jourdain-Miserez K, Forestier N, Teyssier-Cuvelle S, Gillet F, Aragno M (2002) Statistical analysis of denaturing gel electrophoresis (DGE) fingerprinting patterns. Environ Microbiol 4:634–643

Greer CW, Whyte LG, Lawrence JR, Masson L, Brousseau R (2001) Current and future impact of genomics-based technologies on environmental science. Environ Sci Technol 35:360A–366A

Ibekwe AM, Kennedy AC, Frohne PS, Papiernik SK, Yang C-H, Crowley DE (2002) Microbial diversity along a transect of agronomic zones. FEMS Microbiol Ecol 39:183–191

Juck D, Charles T, Whyte LG, Greer CW (2000) Polyphasic microbial community analysis of petroleum hydrocarbon contaminated soils originating from 2 northern Canadian communities. FEMS Microbiol Ecol 33:241–249

Kauffmann IM, Schmitt J, Schmid RD. (2004) DNA isolation from soil samples for cloning in different hosts. Appl Microbiol Biotechnol 64:665–70

Kreader CA (1996) Relief of amplification in PCR with bovine serum albumin or T4 gene 32 protein. Appl Environ Microbiol 62:1102–1106

Liesack W, Stackebrandt E (1992) Occurrence of novel groups of the domain Bacteria as revealed by analysis of genetic material from an Australian terrestrial environment. J Bacteriol 174:5072–5078

Liesack W, Weyland H, Stackebrandt E (1991) Potential risks of gene amplification by PCR as determined by 16S rDNA analysis of a mixed culture of strict barophilic bacteria. Microb Ecol 21:191–198

Liu W-T, Chanc O-C, Fang HP (2002) Characterization of microbial community in granular sludge treating brewery wastewater. Water Res 36:1767–1775

Loy A, Lehner A, Lee N, Adamczyk J, Meier H, Ernst J, Schleifer KH, Wagner M (2002) Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. Appl Environ Microbiol 68:5064–5081

Luz AP, Pellizari VH, Whyte L.G, Greer CW (2004) A survey of indigenous microbial hydrocarbon degradation genes in soils from Antarctica and Brazil. Can J Microbiol 50:323–333

Macnaughton S, Stephen JR, Chang YG, Peacock A, Flemming AM, Leung KT, White DC (1999) Characterization of metal-resistant soil eubacteria by polymerase chain reaction in denaturing gradient gel electrophoresis with isolation of resistant strains. Can J Microbiol 45:116–124

Margesin R, Labbé D, Schinner F, Greer CW, Whyte LG (2003) Characterization of hydrocarbon-degradative microbial populations in contaminated and pristine alpine soils. Appl Environ Microbiol 69:3985–3092

Martin-Laurent F, Philippot L, Hallet S, Chaussod R, Germon JC, Soulas G, Catroux G (2001) DNA extraction from soils: old bias for new microbial diversity analysis methods. Appl Environ Microbiol 67:2354–2359

Massol-Deya A, Weller R, Rios-Hernandez L, Zhou JZ, Hickey RF, Tiedje JM (1997) Succession and convergence of biofilm communities in fixed-film reactors treating aromatic hydrocarbons in groundwater. Appl Environ Microbiol 63:270–276

Meyerhans A, Vartanian JP, Wain-Hobson SW (1990) DNA recombination during PCR Nucleic Acids Res 18:1697–1691

Miller KM, Ming TJ, Schulze AD, Withler RE (1999) Denaturing gradient gel electrophoresis (DGGE): a rapid and sensitive technique to screen nucleotide sequence variation in populations. BioTechniques 27:1016–1024

Mills DK, Fitzgerald K, Litchfield CD, Gillevet PM (2003) A comparison of DNA profiling techniques for monitoring nutrient impact on microbial community composition during bioremediation of petroleum-contaminated soils. J Microbiol Methods 54:57–74

Mumy KL, Findlay RH (2004) Convenient determination of DNA extraction efficiency using an external DNA recovery standard and quantitative-competitive PCR. J Microbiol Methods 57:259–68

Murray AE, Preston CM, Massana R, Taylor LT, Blakis A, Wu K, DeLong EF (1998) Seasonal and spatial variability of bacterial and archaeal assemblages in the coastal waters near Anvers Island, Antarctica. Appl Environ Microbiol 64:2585–95

Muyzer G, Smalla K (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. Antonie van Leeuwenhoek 73:127–141

Muyzer G, Waal EC, Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. Appl Environ Microbiol 59:695–700

Myers RM, Fischer SG, Lerman LS, Maniatis T (1985) Nearly all single base substitutions in DNA fragments joined to a GC-clamp can be detected by denaturing gradient gel electrophoresis. Nucleic Acids Res 13:3131–3145

Nakagawa T, Fukui M (2002) Phylogenic characterization of microbial mats and streamers from a Japanese alkaline hot spring with a thermal gradient. J Gen Appl Microbiol 48:211–222

Nübel U, Garcia-Pichel F, Muyzer G (1997) PCR primers to amplify 16S rRNA genes from cyanobacteria. Appl Environ Microbiol 63:3327–3332

Paabo S, Irwin DM, Wilson AC (1990) DNA damage promotes jumping between templates during enzymatic amplification. J Biol Chem 265:4718–4721

Rondon MR, August PR, Bettermann AD, Brady SF, Grossman TH, Liles MR, Loiacono KA, Lynch BA, MacNeil IA, Minor C, Tiong CL, Gilman M, Osburne MS, Clardy J, Handelsman J, Goodman RM. (2000) Cloning the soil metagenome: a strategy for accessing the genetic and functional diversity of uncultured microorganisms. Appl Environ Microbiol 66:2541–2547

Sambrook J, Russell DW (2001) Molecular Cloning: A laboratory manual. Cold Spring Harbor Laboratory Press, New York

Schneegurt MA, Dore SY, Kulpa CF Jr. (2003) Direct extraction of DNA from soils for studies in microbial ecology. Curr Issues Mol Biol 5:1–8

Soloway DA, Nahir M, Billowits ME, Whyte LG (2001) In-situ bioremediation of diesel-contaminated soil in Canada's Arctic territory: a case study at the Whitehorse International Airport, Yukon Territory. Polar Record 202:267–272

Stahl DA (2004) High-throughput techniques for analyzing complex bacterial communities. Adv Exp Med Biol 547:5–17

Venter JC, Remington K, Heidelberg JF, Halpern AL, Rusch D, Eisen JA, Wu D, Paulsen I, Nelson KE, Nelson W, Fouts DE, Levy S, Knap AH, Lomas MW, Nealson K, White O, Peterson J, Hoffman J, Parsons R, Baden-Tillson H, Pfannkoch C, Rogers YH, Smith HO (2004) Environmental genome shotgun sequencing of the Sargasso Sea. Science 304:66–74

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

Wang GC, Wang Y (1996) The frequency of chimeric molecules as a consequence of PCR co-amplification of 16S rRNA genes from different bacterial species. Microbiology 142:1107–1114

Ward DM, Bateson MM, Weller R, Ruff-Roberts AL (1992) Ribosomal RNA analysis for microorganisms as they occur in nature. Adv Microbiol Ecol 12:219–286

Whyte LG, Bourbonniere L, Bellerose C, Greer CW (1999) Bioremediation assessment of hydrocarbon-contaminated soils from the high Arctic. Bioremediation J 3:69–79

Whyte LG, Goalen B, Hawari J, Labbé D, Greer CW, Nahir M (2001) Bioremediation treatability assessment of hydrocarbon contaminated soils from Eureka, Nunavut. Cold Regions Sci Technol 32:121–132

Whyte LG, Labbé D, Goalen B, Buchko J, Nahir M, Billowits M, Greer CW (2003) In-situ bioremediation of hydrocarbon contaminated soils in the high Arctic. ARCSACC Conference. Edmonton, May 4–6, pp 245–256

Whyte LG, Schultz A, van Beilen JB, Luz AP, Pellizari D, Labbé D, Greer CW (2002) Prevalence of alkane monooxygenase genes in Arctic and Antarctic hydrocarbon-contaminated and pristine Soils. FEMS Microbiol Ecol 41:141–150

Zhou J, Thompson, DK (2002) Challenges in applying microarrays to environmental studies. Curr Opin Biotechnol 13:204–207

11 Bioreporter Technology for Monitoring Soil Bioremediation

Steven Ripp

11.1 General Introduction

Bioreporters refer to intact, living cells that have been genetically engineered to produce a measurable signal transcriptionally induced in response to a specific chemical or physical agent in their environment. Bioreporters contain three essential genetic elements, a promoter sequence, a regulatory gene, and a reporter gene. In the wild-type cell, the promoter gene is transcribed upon exposure to an inducing agent, leading to subsequent transcription of downstream genes that encode for proteins that aid the cell in either adapting to or combating the agent to which it has been exposed. In the bioreporter, the downstream genes, or portions thereof, have been removed and replaced with a reporter gene. Consequently, transcription of the promoter gene activates the reporter gene, reporter proteins are produced, and some type of measurable signal is generated. These signals can be categorized as either colorimetric, fluorescent, luminescent, chemiluminescent, electrochemical, or amperometric. Although each bioreporter functions differently, the end product is always the same – a measurable signal that is, ideally, proportional to the concentration of the specific chemical or physical agent to which they have been exposed (Fig. 11.1).

Bioreporters can also be constructed without such inherent specificity. These bioreporters rely on reporter genes that are induced by a group of substances rather than just one or a few. Their primary use is for the detection of toxic substances, which, upon exposure to the bioreporter, induce a stress-response gene that is fused to a reporter gene. Thus, an increase in signal intensity indicates toxicity, but the substance that initiated the signal cannot be uniquely identified. Reporter systems can also be designed to operate in the reverse, where a decrease in signal intensity indicates toxicity. These bioreporters contain a constitutively expressed reporter gene that always remains on. Upon toxin exposure, the bioreporters either die or their metabolic activities are severely reduced, thereby causing a reduction in signal strength.

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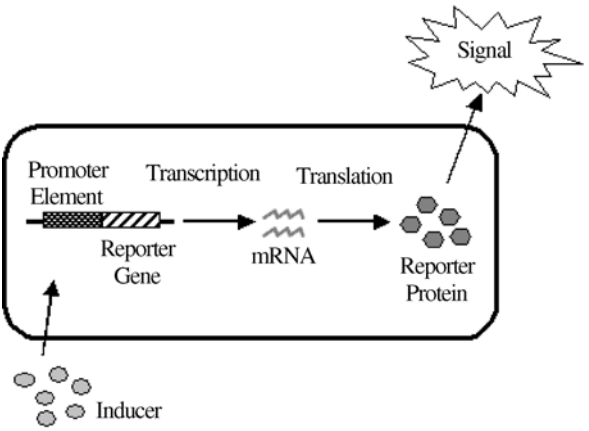

Fig. 11.1. Anatomy of a bioreporter organism. Upon exposure to a specific inducer, the promoter/reporter gene complex is transcribed into messenger RNA (mRNA) and then translated into a reporter protein that is ultimately responsible for signal generation

Although all data generated by a bioreporter can be obtained much more accurately using conventional analytical techniques such as gas chromatography and mass spectrometry (GC/MS), bioreporters offer a distinct advantage in that they report not only on a chemical's presence but on its bioavailability and overall effect on a living system. Bioreporters are also significantly cheaper, faster, and easier to use than typical analytical methods. Additionally, for some select bioreporter systems, the bioassay can be performed in situ, continuously, on-line, and in real time. Such traits make bioreporters particularly well suited for bioremediation application. Typically, in any bioremediative design, the first step is to identify and quantify the contaminants present, which is best achieved using analytical techniques such as GC/MS. But after site characterization, bioreporters can play useful roles in the frequent monitoring that ensues, for example, when mapping the site to assess contaminant distribution prior to and during the remediation process, as well as in post-closure monitoring. Bioreporters can also be used to report on the status of environmental parameters important to successful bioremediation, such as nutrient levels, pH, and dissolved oxygen; or they can provide general biomass measurements (via ATP quantification) as an indication of overall microbial activity. As well, where the bioremediation strategy entails using an enhanced, bioengineered microorganism, the bioremediation practitioner must provide a means of tracking the microbe to ensure containment and monitor potential recombinant gene transfer events in the indigenous microbial population. This visual tagging can often be provided through bioreporter technology. A series of excellent reviews on bioreporter systems are available (Daunert et al. 2000; Keane et al. 2002; Belkin 2003).

11.2 An Overview of Reporter Systems for Soil Bioremediation Application

β-Galactosidase (*lacZ*)

The *lacZ* gene derived from *Escherichia coli* encodes a *β*-galactosidase (*β*-gal) that catalyzes the hydrolysis of *β*-galactosides. Traditional *lacZ* bioreporters are assayed colorimetrically. The substrate *o*-nitrophenyl-*β*-D-galactoside (ONPG) is added to permeabilized bioreporter cells, after inducer exposure, to generate a yellow by-product whose intensity correlates with *β*-gal activity to provide an estimate of target chemical concentration. The assay is simple and highly reliable, and has become integral to commercially available genotoxicity test kits such as the SOS Chromotest (Institute Pasteur, Paris). Due to low sensitivities and narrow dynamic ranges, however, the colorimetric test is largely being replaced by other detection methods. By simply using different *β*-galactoside substrates, fluorescent, luminescent, or chemiluminescent assays are possible. A major disadvantage remains, however, in that the reporter cells must be lysed or undergo a membrane disruption step in order to quantify *β*-gal activity. Thus, data is obtained only incrementally and results are delayed, sometimes by several hours, in relation to the time required to complete the *β*-gal assay. Newer electrochemical and amperometric assays are beginning to solve this problem by measuring *β*-gal activity either directly or indirectly in an on-line, near real-time format. However, the endogenous presence of *β*-gal in natural environments and its potentially high background activity must always be taken into account when performing any of these assays. Table 11.1 provides examples of *lacZ*-based bioreporters for environmental monitoring.

Catechol 2,3-Dioxygenase (*xylE*)

The *xylE* encoded catechol 2,3-dioxygenase is part of the pWWO plasmid of *Pseudomonas putida* and is involved in the degradation of aromatic compounds. Catechol 2,3-dioxygenase catalyzes the cleavage of colorless catechol to produce the yellow compound 2-hydroxymuconic semialdehyde, forming the basis of this reporter assay. Reporter systems for *xylE* have been developed primarily for studying gene regulation, and their utility as environmental reporters is rather limited to the tagging of microorganisms destined for environmental release. In this task *xylE* serves well since its endogenous activity in environmental systems is extremely low, as compared to *lacZ*.

Table 11.1. The *lacZ*-based bioreporters

Analyte	Reporter gene	Time for induction	Concentration
Antimonite, arsenite	*arsR*	30 min	10^{-15} M
Biphenyls	*bphA*	3 h	1 mM
Cadmium	*zntA*	< 1 h	25 nM
Chlorocatechol	*clcR*	5 min	10^{-8} M
Chromate	*chr*	8 h	1 µM
Copper	*pcoE*	1 h	0.01 mM
	*CUP*1	25 min	0.5–2 mM
	Salmonella sulA	2 h	0.025 µg/mL
	dinA, *B*, *D*	< 30 min	1 µg/mL
	umuC	3 h	0.05 µg/mL
	recA	< 10 min	1 µg/mL
	sfiA	2 h	< 1 ng/mL
Mercury	*mer*	4 h	0.2 ng/mL
Nickel	*cnr*	8 h	128 µM
Pesticide toxicity	*HSP104*	1.5 h	0.1 mg/L
Phenols	*dmpR*	4 h	0.5 mM
Zinc	*smtA*	2 h	12 µM

β-Lactamase (*bla*)

β-Lactamase cleaves β-lactam rings in certain antibiotics. Synthetic substrates have been developed that can also be cleaved by β-lactamase to form colorimetric or fluorescent products. As with catechol 2,3-dioxygenase, β-lactamase is routinely used for gene regulation studies but rarely as a reporter for environmental assessment. However, β-lactamase-derived reporters for mercury, arsenic, and cadmium are available, although their operational capacity under environmental conditions is unknown.

Green Fluorescent Protein (GFP)

Green fluorescent protein (GFP) is a photoprotein isolated and cloned from the jellyfish *Aequorea victoria* (Misteli and Spector 1997). Variants have also been isolated from the sea pansy *Renilla reniformis*. GFP produces a blue fluorescent signal without the addition of an exogenous substrate. All that is required is an ultraviolet light source to activate the fluorescent properties of the photoprotein. This ability to autofluoresce makes GFP highly desirable in biosensing assays since it can be used on-line and in real time to monitor intact, living cells. Additionally, the ability to alter GFP to produce light emissions besides blue (i.e., cyan, red, and yellow) allows it to be used as a multianalyte detector. Consequently, GFP has been incorporated into bioreporters for the detection

Table 11.2. GFP-based bioreporters

Analyte	Reporter gene	Time for induction	Concentration
Arsenic	*arsR*	6 h	1 ppb
Benzene derivatives, branched alkenes	*tbu*	3 h	3.3 µM
Biocides	*TEF*	25 min	100 µg/mL
Cadmium	Cd-binding peptide	3 h	0.5 µM
Iron	*pvd*	Unknown	10^{-4} M
Mercury	*mer*	16 h	< 50 ng/mL
Nitrate	*nar*	4 h	0.05 mM
Octane	*alkB*	1–2.5 h	0.01–0.1 µM
Tetracyclines	*tetR*	50 min	< 10 ng/mL
Toluene	*tbuA1*	1 h	0.2 µM

Table 11.3. Representative examples of GFP used as a visual tag

Application	Matrix
Monitoring *Arthrobacter*	4-Chlorophenol-contaminated soil
Monitoring *Pseudomonas pseudoalcaligenes*	PCB-contaminated soil
Monitoring *Alcaligenes faecalis*	Phenol-contaminated soil
Monitoring *Pseudomonas fluorescens*	3-Chlorobiphenyl-contaminated root rhizosphere
Monitoring *Pseudomonas putida*	Activated sludge
Monitoring *Pseudomonas* sp.	PAH-contaminated soil
Survival of *Pseudomonas* sp.	2,3-Dichlorobiphenyl-contaminated soil
Survival of *Pseudomonas resinovorans*	2,3-Dichlorodobenzo-*p*-dioxin-contaminated soil
Survival of *Moraxella* sp.	*p*-Nitrophenol-contaminated soil
Temperature effects on *Arthrobacter chlorophenolicus*	Agricultural soil
TOL plasmid expression	Biofilm
Transport of *Pseudomonas putida*	Groundwater

of various heavy metals (Table 11.2) and as a visual tag within bacterial, yeast, nematode, plant, and mammalian hosts for monitoring purposes (Table 11.3).

Uroporphyrinogen (Urogen) III Methyltransferase (UMT)

UMT catalyzes a reaction that yields two fluorescent products that produce a red-orange fluorescence in the 590–770 nm range when illuminated with ultraviolet light (Sattler et al. 1995). So as with GFP, no addition of exogenous substrates is required. UMT has been used for whole-cell sensing of antimonite, arsenite, and arsenate (Feliciano et al. 2000).

Luciferases

Insect Luciferase (*luc*). Firefly luciferase catalyzes a reaction that produces visible light in the 550–575 nm range. A click-beetle luciferase is also available that produces light at a peak closer to 595 nm. Both luciferases require the addition of an exogenous substrate (luciferin) for the light reaction to occur. Examples of *luc*-based bioreporters constructed for the detection of inorganic and organic compounds of environmental concern are presented in Table 11.4. Visual tagging of microorganisms with *luc* has also been performed in, for example, 4-chlorophenol-contaminated soils to track bioremediation progress.

Bacterial Luciferase (*lux*). Luciferase is a generic name for an enzyme that catalyzes a light-emitting reaction. Luciferases can be found in bacteria, algae, fungi, jellyfish, insects, shrimp, and squid, and the resulting light that these organisms produce is termed bioluminescence. In bacteria, the genes responsible for the light-emitting reaction (the *lux* genes) have been isolated and used extensively in the construction of bioreporters that emit a blue-green light with a maximum intensity at 490 nm (Meighen 1994). Three variants of *lux* are available, one that functions at $< 30\,^{\circ}\mathrm{C}$, another at $< 37\,^{\circ}\mathrm{C}$, and a third at $< 45\,^{\circ}\mathrm{C}$. The *lux* genetic system consists of five genes, *luxA*, *luxB*, *luxC*, *luxD*, and *luxE*. Depending on the combination of these genes used, several different types of bioluminescent bioreporters can be constructed.

Table 11.4. The *luc*-based bioreporters

Analyte	Reporter Gene	Time for Induction	Concentration
Arsenite	*ars*	2 h	10 nM
Arsenite, antimonite, cadmium	*ars*	2 h	33 nM (antimonite)
Benzene, toluene, xylene	*xylR*	30 min	3 µM (xylene)
Cadmium, lead, antimony	*cadA*	2–3 h	1 nM (antimony)
Chromate	*chr*	2 h	50 nM
Copper, lead, mercury	*Drosophila* Mtn promoter	48 h	3–19 ppm
Environmental estrogens	ERE	10–12 h	10^{-7} M (DDT)
Herbicides	*tac-luc-luxAB-aphII*	$\geq$ 30 min	ppm levels
Mercury	*mer*	2 h	100 nM
Organomercurials	*mer*	2 h	0.2 nM (methyl-mercury chloride)
Zinc	*znt*	2 h	40 µM

Luciferase AB (*luxAB*). The *luxAB* bioreporters contain only the *luxA* and *luxB* genes, which together are responsible for generating the light signal. However, to fully complete the light-emitting reaction, a substrate must be supplied to the cell. Typically, this occurs through the addition of the chemical decanal at some point during the bioassay procedure. Numerous *luxAB* bioreporters have been constructed within bacterial, yeast, insect, nematode, plant, and mammalian cell systems and have been applied toward detection of various environmental contaminants, monitor and control of bioremediation process, assays of toxicity, application of visual tags, and estimation of microbial biomass.

Luciferase CDABE (*luxCDABE*). Instead of containing only the *luxA* and *luxB* genes, bioreporters can contain all five genes of the *lux* cassette, thereby allowing for a completely independent light generating system that requires no extraneous additions of substrate nor any excitation by an external light source. In these bioassays, the bioreporter is simply exposed to a target analyte and a quantitative increase in bioluminescence results, often within less than 1 h. Due to their rapidity and ease of use, along with the ability to perform the bioassay repetitively in real time and on-line, *luxCDABE* bioreporters have become extremely attractive for environmental monitoring. Additionally, the recent development of microluminometers for detecting the bioluminescent signal reduces this assay down to a miniaturized format (Nivens et al. 2004). Table 11.5 illustrates the widespread application of *luxCDABE*-based bioreporters.

Non-specific *luxCDABE*. Nonspecific *lux* bioreporters are typically used for the detection of chemical toxins. They are usually designed to continuously bioluminesce. Upon exposure to a chemical toxin, either the cell dies or its metabolic activity is retarded, leading to a decrease in bioluminescent light levels. Their most familiar application is in the Microtox assay (Azur Environmental, Newark, DE, USA) where, following a short exposure to several concentrations of the sample, decreased bioluminescence can be correlated to relative levels of toxicity (Hermens et al. 1985). The Vitotox test (Flemish Institute for Technological Research, Mol, Belgium) operates similarly (Verschaeve et al. 1999).

Mini-Transposons as Genetic Tools in Bioreporter Constructions

A transposon is a discrete genetic element capable of translocating from a donor site within the DNA molecule into one of many non-homologous target sites under the assistance of a transposase enzyme. Their use as reporter elements was first applied in gene regulation studies using a phage Mu transposable element containing a promoterless *lac* gene. This was followed by similar constructs using primarily the Tn5 and Tn10 family

Table 11.5. The *luxCDABE*-based bioreporters

Analyte	Reporter	Time for induction	Concentration
2,3-Dichlorophenol	*recA* (stress promoter)	2 h	50 mg/L
2,4,6-Trichlorophenol	*recA* (stress promoter)	2 h	10 mg/L
2,4-D	*tfdRP*	20–60 min	2 µM–5 mM
3-Xylene	*xyl*	hours	3 µM
4-Chlorobenzoate	*fcbA*	1 h	380 µM–6.5 mM
4-Nitrophenol	*recA* (stress promoter)	2 h	0.25 mg/L
Aflatoxin B1	Various stress promoters	45 min	1.2 ppm
Ammonia	*hao*	30 min	20 µM
BTEX (benzene, toluene, ethylbenzene, xylene)	*tod*	1–4 h	0.03–50 mg/L
Cadmium	*cupS*	4 h	19 mg/kg
Chlorodibromo-methane	*recA* (stress promoter)	2 h	20 mg/L
Chloroform	*recA* (stress promoter)	2 h	300 mg/L
Chromate	*chrA*	1 h	10 µM
Cobalt	*cnr*	4–6 h	9 µM
Copper	Not specified	1 h	1 µM–1 mM
Hydrogen peroxide	*katG*	20 min	0.1 mg/L
Iron	*pupA*	hours	10 nM–1 µM
Isopropyl benzene	*ipb*	1–4 h	1–100 µM
Lead	*pbr*	4 h	4,036 mg/kg
Mercury	*mer*	70 min	0.025 nM
Naphthalene	*nahG*	8–24 min	12–120 µM
Nickel	*cnr*	4–6 h	0.1 µM
Nitrate	*narG*	4 h	0.05–50 µM
Organic peroxides	*katG*	20 min	Not specified
PCBs	*bph*	1–3 h	0.8 µM
p-Chlorobenzoic acid	*fcbA*	40 min	0.06 g/L
Pentachlorophenol	*recA* (stress promoter)	2 h	0.008 mg/L
Phenol	*recA* (stress promoter)	2 h	16 mg/L
Salicylate	*nahG*	15 min	36 µM
Silver	*zntAp*	1 h	0.1 µM
Tetracycline	*tet*	50 min	< 10 ng/mL
Trichloroethylene	*tod*	1–1.5 h	5–80 µM
Zinc	*smtA*	4 h	0.5–4 µM

of transposons as well as a variety of others such as Tn3/Tn1, Tn916/917, and Tn1000. Although powerful mutagenic tools, natural transposons had several disadvantages, especially in environmental applications; they required an antibiotic resistance marker for selection and were composed of

inverted repeat elements that promoted unwanted genetic rearrangements and inherent instability (secondary transposition). They were also large and difficult to work with genetically and were subject to transposition immunity, which prevented multiple transposon insertions within the same bacterial strain, severely limiting their cloning value. The development of mini-transposons solved many of these problems. Mini-transposons are shortened hybrids of natural transposons, usually Tn5 and Tn10, in which the transposase gene is placed outside the boundaries of the inverted repeats. In this formation, the mobile element undergoes insertion into the target site but the transposase does not, thus preventing any further rearrangements. Mini-transposons are also not affected by transposition immunity, thereby allowing for multiple insertions of foreign inserts in the same strain, provided that each insert has its own unique selectable marker. Additionally, mini-transposons typically maintain an origin of replication that allows for delivery into a broad range of hosts. Various mini-transposons customized with reporter genes have been developed for simplified construction of bioreporter organisms. By inserting a genetic promoter element into a unique cloning site within the mini-transposon vector, one can theoretically engineer any of the bioreporter classes discussed above. Furthermore, the ability to stably insert the mini-transposon into the host chromosome makes these systems ideal for environmental applications, since the necessity for antibiotic selection can be reduced. Newer mini-transposons based on heavy-metal-resistance determinants make antibiotic selection obsolete. Methods for constructing and using mini-transposons are expertly described by de Lorenzo and Timmis (1994).

11.3 Single Point Measurements of Soil Contaminants

Introduction

Objectives. The application of bioreporters to soil bioremediation monitoring can be applied in several different formats. This can range from simply adding bioreporters to soil extracts to detect chemical presence to being so multifaceted as to use in flow-cell formats for on-line, continuous monitoring. With the numerous types of bioreporter systems available, the bioremediation practitioner has a wide range of options to choose from for the particular monitoring needs being addressed. For the sake of simplicity, the protocols described below will relate to a *luxCDABE*-based bioreporter system, but other systems can be substituted with corresponding substitutions in growth conditions, types of substrate added (if required), and monitoring instrumentation. The reviews by Belkin (2003), Daunert et al. (2000), and Keane et al. (2002) should be addressed for further direction.

Principle. Bioreporter cells containing all five genes of the *lux* cassette are exposed to a soil suspension containing the target analyte, and a quantitative increase in bioluminescence is measured.

Equipment

- Centrifuge and Corex glass centrifuge tubes (Corning Inc., Corning, NY, USA) with Teflon screw cap lids
- 25 mL mineralization vials with Teflon screw cap lids
- Rotating shaker
- Instrument capable of monitoring bioluminescence [Perkin-Elmer Victor Multilabel reader (Wellesley, MA, USA), Azur Environmental Deltatox, Zylux Femtomaster (Oak Ridge, TN, USA), Wallac Microbeta (Wellesley, MA, USA), etc.]

Reagents

- YEPG medium (per L): 0.2 g yeast extract, 2 g polypeptone, 1 g glucose, 0.2 g NH_4NO_3, pH 7.0
- Mineral salts medium (MSM; per L): 0.1 g $MgSO_4 \times 7H_2O$, 0.2 g NH_4NO_3, 100 mL phosphate buffer, 0.1 mL trace elements solution
  - Phosphate buffer: 0.5 M K_2HPO_4/NaH_2PO_4 mixture, pH 7.0; added to MSM after autoclaving separately
  - Trace elements (per L distilled water): 10.0 g MgO, 2.94 g $CaCl_2$, 5.4 g $FeCl_3 \times 6H_2O$, 1.44 g $ZnSO_4 \times 7H_2O$, 0.25 g $CuSO_4$, 0.062 g H_3BO_4, 0.49 g $Na_2MoO_4 \times H_2O$; added to MSM after filter sterilizing

Sample Preparation

Obtain field-moist soil samples or soil cores from test site.

Procedure

1. Preparation of bioreporter (description is for the *luxCDABE* bioreporter *Pseudomonas fluorescens* HK44 (Ripp et al. 2000); other bioreporter growth conditions will differ):
   1.1. Inoculate 100 mL of YEPG medium from a frozen stock of bioreporter cells. Grow overnight at 30 °C, with shaking at 200 rpm.
   1.2. The next day, inoculate 1:10 into 100 mL fresh YEPG medium. Grow at 30 °C with shaking to an optical density at 546 nm of 0.35.

2. Soil preparation
   2.1. Divide soil into 10 g portions into clean 25 mL Corex glass centrifuge tubes. Perform in triplicate.
   2.2. Add 7 mL MSM and shake at room temperature and 200 rpm for 1 h.
   2.3. Centrifuge at 7,500 *g* and 25 °C for 10 min to remove large particulates.
   2.4. Remove 2 mL of supernatant into a 25 mL mineralization vial.
3. Bioluminescent assay
   3.1. Add 2 mL of bioreporter culture (OD_{546} = 0.35) to 2 mL soil extract in a mineralization vial.
   3.2. Transfer bioreporter/soil mixture to an appropriate holding device based on type of light reader being used (microtiter plate, glass vial, cuvette, etc.). Monitor light output for approximately 1 h.
4. Preparation of standard curve
   4.1. Perform assay as described above with sterilized, uncontaminated soil to which known concentrations of contaminant have been added.
5. Controls
   5.1. Perform assay as described above with sterilized, uncontaminated soil. Bioreporters will generate a background level of signal that must be subtracted from signals obtained in test samples.

Calculation

Subtract background light levels from test sample light levels and plot results on the standard curve to determine contaminant concentrations. Light levels are expressed using the arbitrary unit of relative light unit (RLU).

Notes and Points to Watch

- The optimal temperature for *lux* bioluminescent activity can be modified by using different *lux* cassettes. *Vibrio fischeri lux* functions at 30 °C, *V. harveyi* at 37 °C, and *Photorhabdus luminescens* at 42 °C.
- The *lux* reaction requires oxygen and will not operate efficiently under anaerobic or low oxygen conditions.

- Any of the bioreporter systems described above could generate false positive signals due to non-specific induction by non-target inducers. Appropriate controls must be incorporated into experimental protocols.
- For statistical purposes, assays should be performed in triplicate.

11.4 Continuous On-Line Vapor Phase Sensing of Soil Contaminants

Introduction

Objectives. Bioremediation processes often require a quick "snapshot" of soil contaminant concentrations to verify that environmental conditions are conducive for optimal bioremediation and to provide an overall assessment of where the contaminants are located. Bioreporters offer a very rapid assessment technology that can be economically applied to contaminated sites of interest.

Principle. A flow-through chamber containing alginate-encapsulated bioreporter cells is inserted into a borehole and the presence of contaminants in the vapor phase is continuously monitored via bioluminescent signals.

Equipment

- Flow-through bioreporter chamber (Fig. 11.2): The chamber consists of a porous stainless steel tube (10 cm long × 2.3 cm diameter). Into the bottom of the tube are packed bioreporter cells encapsulated in alginate. Into the top of the tube is inserted a 1-mm diameter fiber-optic cable. The other end of the cable truncates into a photomultiplier tube [PMT; Hamamatsu model R-4632 Hamamatsu, Hamamatsu City, Japan), for example] that measures the bioluminescent signals. For complete details, see Ripp et al. (2000).
- 26-gauge needle
- Disposable 10-mL syringes

Reagents

- YEPG medium (Sect. 11.3)
- Sterile saline solution: 0.85% NaCl

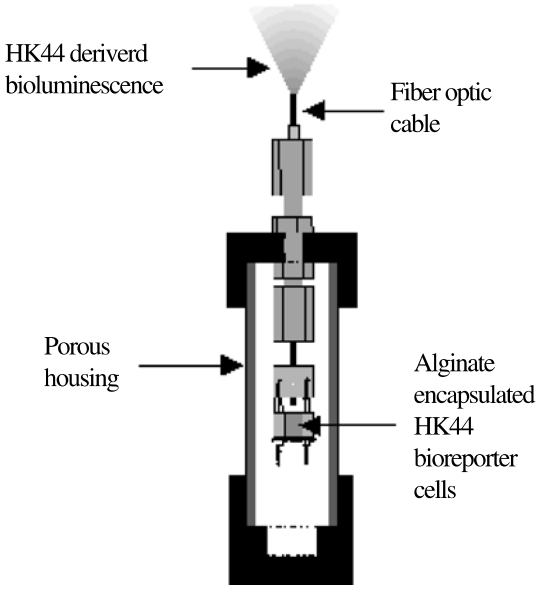

Fig. 11.2. Device for monitoring volatile PAHs using immobilized *P. fluorescens* HK44 bioreporters. The HK44 bioreporters bioluminesce in response to PAH exposure and the bioluminescent signal is transduced through a fiber-optic cable to an external light detector

- Sterile 3.5% (w/v) low viscosity alginic acid (Sigma-Aldrich, St. Louis) solution in distilled water (this must be stirred overnight, then autoclaved the next day)
- Sterile 0.1 M $SrCl_2$ solution

Sample Preparation

Holes (approx. 4-cm diameter) need to be bored on-site, to extend into the zone of suspected contamination, for insertion of flow-through bioreporter chambers. A control hole should also be bored in a known uncontaminated area.

Procedure

1. Preparation of alginate-encapsulated bioreporter cells
   1.1. Grow bioreporter culture in YEPG to $OD_{546} = 0.35$, as described in Sect. 11.3.
   1.2. Centrifuge (ca. 10 min at 3,000 *g*) cells and wash with an equal volume of saline solution. Recentrifuge and again suspend in an equal volume of saline solution.

1.3. Gently mix one part of cell suspension with two parts of alginic acid solution. Hold on ice.

1.4. Gently stir 1 L of refrigerated $SrCl_2$ solution in a sterile 1.5-L beaker on a stirplate.

1.5. Transfer the cell/alginic acid mixture to a 10-mL disposable syringe. Attach a 26-gauge needle and gently push mixture through needle in dropwise fashion, allowing drops to fall into the beaker with the $SrCl_2$ solution. Drops will solidify into beads upon contact with $SrCl_2$. To ensure complete solidification, allow beads to gently stir in $SrCl_2$ solution for approx. 45 min.

1.6. Remove the beads by decanting through sterile cheesecloth.

1.7. Store in a closed container at 4.0 °C for up to 3 months.

2. Preparation of flow-through bioreporter chamber

2.1. Pack 5 g of alginate-encapsulated cells into the bottom of the chamber.

2.2. Insert fiber-optic cable directly above the encapsulated cells.

2.3. The chamber, suspended by the fiber-optic cable, can now be dropped into the boreholes. Vapor phase detection of contaminant presence is then monitored continuously by the PMT and data downloaded to a laptop computer. Encapsulated cells must be replenished on a weekly basis. Newer generation sensors have reduced the bulky PMT modules down to an integrated circuit format, and it is now possible to construct wireless chip-based devices, to wit, bioluminescent bioreporter integrated circuits (BBICs), for remote sentinel detection of target analytes (Nivens et al. 2004).

2.4. Background light levels should be obtained from a bioreporter chamber installed in a control borehole.

Calculation

Subtract light levels obtained from the control bore hole to correct for background base levels of bioluminescence emanating from the bioreporter. Plot bioluminescence (RLU) versus time to illustrate trends in bioluminescence output. Based on these trends, the overall bioremediation process can be evaluated and monitored to determine the effectiveness of the bioremediation program (i.e., low light levels will indicate unfavorable growth conditions for the bioremediative microbes, which should result in the implementation of analytical and microbiological assays and/or localized treatments to diagnose and correct the existing problem).

Notes and Points to Watch

- See Sect. 11.3
- Avoid using phosphate buffers since they will degrade the alginate matrix.
- The target contaminant(s) must produce an adequate vapor phase to be detected.

11.5 Quantification of Soil-Borne *lux*-Tagged Microbial Populations Using Most-Probable-Number (MPN) Analysis

Introduction

Objectives. Microorganisms genetically engineered for optimal biodegradation of target contaminants can be introduced at a contaminated site to enhance the bioremediation process. After release, these microbes must be monitored to ensure that they remain within site boundaries. As well, their population numbers must be monitored to verify that they remain viable and metabolically active. The *lux* genes serve as excellent markers here because their endogenous presence in soil ecosystems is negligible, thus negating background interferences. The *lux* bioluminescent signal is also easily measured. The *lux*-MPN assay described below requires insertion of the *luxCDABE* cassette within the bioremediative microbe. For examples on how this is accomplished, see King et al. (1990). Other bioreporter systems are applicable as well, but heightened background interferences will occur. For information on the introduction and release of engineered microbes into soil and other environmental ecosystems, see Sayler and Ripp (2000).

Principle. Soil inoculated with a *lux*-tagged bacterium is serially diluted. The addition of sodium salicylate results in the induction of the *lux* operon, and the intensity of bioluminescence can be correlated to cell numbers.

Equipment

- PMT-based light reader capable of monitoring in 96-well microtiter plate formats [Perkin-Elmer Victor Multilabel reader, Wallac Microbeta, BMG Labtech Lumistar (Offenburg, Germany), etc.]

Reagents

- Sterile sodium pyrophosphate solution: 0.1% (w/v)
- Sterile saline solution: 0.85% NaCl
- Sterile sodium salicylate solution: 6 mg/mL

Sample Preparation

The contaminated soil is first inoculated with the bioremediation-enhanced, *lux*-tagged microbe. There are several methods for accomplishing this, and one can refer to Sayler and Ripp (2000) for general guidelines. After inoculation, soil samples (> 1 g) are removed from within areas and at depths that received inoculant and transported to the lab on ice. Again, *P. fluorescens* HK44 (Ripp et al. 2000) is used as an example.

Procedure

1. In a sterile test tube, add 1 g of soil to 9 mL sodium pyrophosphate solution and vortex 1 min at top speed to remove microbes from soil particles.
2. Add 100 µl of soil suspension to the first column of a 96-well black, solid bottom microtiter plate (Dynex Technologies, Chantilly, VA, USA).
3. Dilute 1:2 in 100 µL saline solution throughout columns 2 through 12.
4. Add 20 µL of sodium salicylate solution to all wells. In this example, sodium salicylate serves as the inducer of the *lux* operon in *P. fluorescens* HK44. Other bioreporters will use different inducers, but all act similarly to turn on the bioreporter signal.
5. Prepare a duplicate control plate containing saline dilutions from 1 g of sterile soil mixed with sodium pyrophosphate solution. Add 20 µL of sodium salicylate solution to all wells. This plate will provide a measure of background bioluminescence, if any, that needs to be subtracted from bioluminescence counts in the sample plate.
6. Seal plates with transparent plate sealer (Perkin-Elmer Topseal) and incubate at room temperature (23–28 °C) with gentle shaking for 16 h. This permits maximum induction of bioluminescence from HK44 cells. Use of other bioreporters will require optimization of incubation times.
7. Measure photon emission from wells with a microtiter-based light reader, such as the Perkin-Elmer Victor instrument. Read each plate in triplicate for statistical verification.

Calculation

Input data into any variety of MPN software programs (see, for example, Klee 1993). These programs will use Poisson statistics to estimate cell numbers based on where bioluminescence is first observed within the dilution series.

Notes and Points to Watch

- See Sect. 11.3.
- Accurate MPN population estimates require accurate dilutions. Ensure that all dilution series are performed as carefully as possible.

References

Belkin S (2003) Microbial whole-cell sensing systems of environmental pollutants. Curr Opin Microbiol 6:206–212

Daunert S, Barrett G, Feliciano JS, Shetty RS, Shrestha S, Smith-Spencer W (2000) Genetically engineered whole-cell sensing systems: coupling biological recognition with reporter genes. Chem Rev 100:2705–2738

de Lorenzo V, Timmis KN (1994) Analysis and construction of stable phenotypes in gram-negative bacteria with Tn5- and Tn10-derived minitransposons. Methods Enzymol 235:386–405

Feliciano J, Liu Y, Ramanathan S, Daunert S (2000) Fluorescence-based sensing system for antimonite and arsenite using *cobA* as the reporter gene. 219th ACS National Meeting

Hermens J, Busser F, Leeuwangh P, Musch A (1985) Quantitative structure-activity relationships and mixture toxicity of organic chemicals in *Photobacterium phosphoreum*: the Microtox test. Ecotoxicol Environ Saf 9:17–25

Keane A, Phoenix P, Ghoshal S, Lau PCK (2002) Exposing culprit organic pollutants: a review. J Microbiol Meth 49:103–119

King JMH, DiGrazia PM, Applegate B, Burlage R, Sanseverino J, Dunbar P, Larimer F, Sayler GS (1990) Rapid, sensitive bioluminescence reporter technology for naphthalene exposure and biodegradation. Science 249:778–781

Klee AJ (1993) A computer program for the determination of most probable number and its confidence limits. J Microbiol Meth 18:91–98

Meighen EA (1994) Genetics of bacterial bioluminescence. Annu Rev Genet 28:117–139

Misteli T, Spector DL (1997) Application of the green fluorescent protein in cell biology and biotechnology. Nat Biotechnol 15:961–964

Nivens DE, McKnight TE, Moser SA, Osbourn SJ, Simpson ML, Sayler GS (2004) Bioluminescent bioreporter integrated circuits: potentially small, rugged and inexpensive whole-cell biosensors for remote environmental monitoring. J Appl Microbiol 96:33–46

Ripp S, Nivens DE, Ahn Y, Werner C, Jarrell J, Easter JP, Cox CD, Burlage RS, Sayler GS (2000) Controlled field release of a bioluminescent genetically engineered microorganism for bioremediation process monitoring and control. Environ Sci Technol 34:846–853

Sattler I, Roessner CA, Stolowich NJ, Hardin SH, Harris-Haller LW, Yokubaitis NT, Murooka Y, Hashimoto Y, Scott AI (1995) Cloning, sequencing, and expression of the uroporphyrinogen-III methyltransferase *cobA* gene of *Propionibacterium freudenreichii* (*shermanii*). J Bacteriol 177:1564–1569

Sayler GS, Ripp S (2000) Field applications of genetically engineered microorganisms for bioremediation processes. Curr Opin Biotechnol 11:286–289
Verschaeve L, Van Gompel J, Thilemans L, Regniers L, Vanparys P, van der Lelie D (1999) VITOTOX bacterial genotoxicity and toxicity test for the rapid screening of chemicals. Environ Mol Mutagen 33:240–248

12 Interpretation of Fatty Acid Profiles of Soil Microorganisms

David B. Hedrick, Aaron Peacock, David C. White

12.1 Obtaining Fatty Acid Profiles from Soil Samples

This work focuses on the calculations performed on the peak areas obtained by gas chromatography (GC). All the steps of soil sampling, lipid extraction and fractionation, derivatization, and capillary GC have been repeatedly reviewed, and will only be briefly mentioned (a bibliography of work done in this laboratory is available at http://cba.bio.utk.edu/director_peerfull.html, and an extensive bibliography of methods is provided by Dr. William Christie's group, Mylnefield Research Services Ltd. at http://www.lipidlibrary.co.uk/lit_surv.html).

Sampling is the most important step in sample analysis, and is often delegated to the most junior member of the lab or to site specialists not associated with the lipid laboratory, such as a subsurface sediment drilling crew. Besides sampling location, the sample's consistency, integrity, and appearance should be recorded. In order to obtain deep subsurface samples, the use of drilling equipment and drilling mud is usually required, and methods have been developed to prevent and detect drilling mud contamination of samples (Griffin et al. 1997; Phelps et al. 1989).

Capillary GC with flame ionization detection (FID) is a powerful analytical method – simpler in operation, of greater linear range, and more sensitive, reliable, and reproducible than most analytical instrumentation available. The users' manuals for the chromatograph and data system are the primary references for their operation. If you won't read the manual, you shouldn't touch the equipment. There are also many excellent reviews of capillary chromatography of polar lipid fatty acids (PLFA) available (for example, Grob and Barry 1995).

Capillary GC-MS is a necessary adjunct to GC-FID for the identification of fatty acid peaks (Christie 2003). Various chemical methods are also available to help with specific identification problems such as silver ion chromatography to separate saturates, monounsaturates, and polyunsaturates

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(Momchilova and Nikolova-Damyanova 2000), and special derivatization methods to determine the position and geometry of monounsaturation, such as MS of dimethyldisulfide adducts (Nichols et al. 1986). MS of picolinyl esters provides more informative fragmentations than GC-MS of the methyl ester (Christie et al. 1991; Harvey 1992).

This work presupposes some knowledge of Microsoft Excel (Microsoft Corp., Redmond, WA), which is used to manipulate chromatographic results in many laboratories. The on-line help system is the basic reference for Excel, such as it is. A novice user will benefit from one of the many introductory books available at a bookstore. Also assumed is some background in the statistical procedures commonly applied to PLFA data, including analysis of variance (ANOVA) and factor analysis.

12.2 Transforming Fatty Acid Peak Areas to Total Microbial Biomass

Gas chromatography provides a peak area proportional to the amount of the compound in the sample responsible for the peak. A known concentration of an internal standard, usually 19:0 or 21:0, is added to the sample before analysis to allow calculation of absolute amounts (see Sect. 12.5 for the naming of fatty acids). The equation used to calculate the total amount of fatty acids in a sample is,

$$FA = \frac{(sum\ A_{FA}/A_{IS}) \times IS \times X}{Y} \tag{12.1}$$

FA total picomoles of fatty acids per gram dry mass of sample (pmol/g dry mass)

sum A_{FA} sum of the areas of all identified fatty acid peaks excluding the internal standard

A_{IS} area of the internal standard peak

IS concentration of internal standard used (50 pmole/µL)

X volume of internal standard used to dilute the fatty acid methyl esters (µL)

Y mass of sample extracted (g soil dry mass). In some instances, rather than grams dry mass as the divisor, it will be volume of water (L), surface area in meters squared, or some other extensive variable.

Many analysts calculate the pmol/g dry mass for each fatty acid, then add them together to get the total pmole/g dry mass. This is not good practice, since the pmol/g dry mass for each fatty acid is not then of use in further analysis, and the more complicated calculation makes more work and opportunities for error.

The total moles of membrane fatty acids is proportional to the total microbial biomass. The constant of proportionality used in our laboratory is 2.5×10^4 cells/pmol PLFA (Balkwill et al. 1988; White et al. 1996 and references therein). This conversion factor was derived from measurements on laboratory cultures, so the number of cells will be underestimated for environments populated by smaller bacterial cells, such as oligotrophic environments.

Researchers who count cells, with automated cell counting instruments or by microscopy, are often uncomfortable with measurements of viable biomass expressed as moles of PLFA or grams dry mass of cells. In order to estimate cell counts from moles of PLFA requires knowledge of the distribution of cell sizes in the sample and the amount of PLFA per cell for different sizes, information which is not usually available. It makes more sense to transform cell counts to moles PLFA or from the latter to grams dry weight of cells, since the cell counting can provide the data on cell size distribution.

For most sample sets, the biomass will not be normally distributed, that is, a histogram of the biomass data will be skewed with a long tail toward the higher biomasses. This can be tested for by using the standard f-test for normality. Also, in most biomass data sets, the variance of biomass increases with the absolute value of the biomass. This violates the assumptions of parametric statistics, including ANOVA and factor analysis, and lowers the power of any statistical test employed. These problems can be solved by a $\log(X + A)$ transformation, where X is the mole percent of the fatty acid, and A is a small constant. The small constant is added so that zero values give a real solution when the log transform is applied. The most common value used for A is one, which gives a value of zero for the transform when X is zero, since $\log(0 + 1) = 0$.

There are two approaches to proving the value of applying a log transform to biomass data, the theoretical and the practical. The theoretical explanation involves the scaling of the forces affecting microbial biomass (Magurran 1988) and the fractal structure of microbial environments (Mandelbrot 1982), and is beyond the scope of this work. The practical reason for the log transform is that it works; applying a log transformation to the data is perfectly legitimate, and results in more significant differences on statistical tests.

12.3 Calculation and Interpretation of Community Structure

After the biomass, the next most important information to extract from a PLFA profile is the community structure. But where the biomass is a single value for each sample with a straightforward interpretation, the community structure data is multivariate with many options in its interpretation. A "standard" method for presenting community structure data, how to create a custom method for community structure, and factor analysis will be presented.

12.3.1 Standard Community Structure Method

In the standard method for community structure analysis of PLFA profiles, chemically related fatty acids are grouped as in Table 12.1. A PLFA profile may contain, for example, from 18 to 92 fatty acids. The standard community structure approach summarizes that in six variables, which are just the sum of the mole percents of each of the fatty acid groups. The use of a standard community structure analysis method allows comparison between/among experiments.

Table 12.1. Groups of chemically related fatty acids used in the standard community structure analysis

Group name	Rule	Examples	Microbiota represented
Saturates	Saturated straight-chain fatty acids	12:0, 13:0, 14:0, 15:0, 16:0, 17:0, 18:0	All organisms
Monounsaturates	Fatty acids with a single unsaturation plus cyclopropyls	14:1ω5c, 16:1ω7c, 16:1ω7t, 18:1ω7c	Proteobacteria
Mid-chain branched	Any mid-chain branched fatty acid	10Me16:0, 10Me18:0	Actinomycetes, sulfate-reducers
Terminally branched	*Iso*- and *anti-iso*-branched saturated fatty acids	i14:0, i15:0, a15:0, i16:0, i17:0, a17:0	Gram positive bacteria
Polyunsaturates	Any fatty acid with more than one unsaturation	18:2ω6c, 18:3ω3c	Eukaryotes
Branched unsaturates	Any branched monounsaturate	i17:1ω7c	Anaerobes

The standard community structure breakdown was originally developed on marine sediments, and has been successfully applied to microbial communities from many environments, including, for example, marine macrofaunal burrows (Marinelli et al. 2002), a subsurface zero-valent iron reactive barrier for bioremediation (Gu et al. 2002), marine gas hydrates (Zhang et al. 2002), soils contaminated with jet fuel (Stephen et al. 1999), and to a comparison of subsurface environments (Kieft et al. 1997).

12.3.2 Custom Community Structure Methods

When examination of the chromatograms or the mole percent table shows differences with treatment, but no significant differences are found in the standard community structure groups, some other way of grouping the fatty acids may be more useful. For example, if samples differ in the proportions of Cyanobacteria and Eukaryotic algae, it may be useful to separate the polyunsaturates with 18 or fewer carbons characteristic of Cyanobacteria (Øezanka et al. 2003) from those typical of Eukaryotic algae with 20 or more carbons (Erwin 1973).

There are several methods for developing alternative community structure groups. The manual method uses the pattern recognition power of the human eye. The PLFA chromatograms are printed on the same scale and spread out on a large table. Similar-looking chromatograms are grouped together and different-looking ones are placed in separate groups. While very low-tech, this works remarkably well. This same approach can be applied to a mole percent table by printing it out, cutting out a strip for each sample, and sorting the samples by similarity. Once the samples have been sorted into similar groups, the fatty acids responsible are summed to form new community structure groups.

Given access to statistical software, a triangular table of Pearson's r correlation coefficients is usually available as an output option. Visual examination of this table will locate fatty acids with high correlations, which are then grouped together to form new community structure groups.

12.3.3 Factor Analysis

Factor analysis includes several related methods, including principal-components analysis. The virtue of this method is that it automatically constructs fatty acid groups reflecting the differences in community structure, rather than applying a preconception of fatty acid groups. The data determines the fatty acid groups, rather than the analyst. Factor loadings greater

than 0.7 indicate fatty acids with "significant" effects on the results. The factor scores are new variables that are linear combinations of the original values. These new variables can be submitted to statistical tests such as ANOVA like any other variable. Examples of the application of factor analysis to PLFA profiles include storage perturbation of soil microbial communities (Haldeman et al. 1995; Brockman et al. 1997), soils at different temperatures (Zogg et al. 1997), and soils from different ecosystems (Myers et al. 2001).

The results of factor analysis are usually improved by applying the log(X+1) transformation to the mole percent data before factor analysis. A rough method to determine whether the mole percent data is normally distributed is to calculate the maximum, average, and the minimum not equal to zero for each fatty acid. The formulas for these in Excel are "= max(b2.b45)", "= average(b2.b45)", and "= min(if(b2.b45 = 0, 100, b2.b45))", where b2.b45 is the range containing the data. The formula for min 0 is what Excel terms an array formula; you have to hold down the Shift and Control keys while you press Enter to enter the formula. If the difference between the maximum and average is greater than the difference between the average and the minimum 0 for most of the fatty acids, then the data is not normally distributed and the log(X+1) transformation will probably improve results.

There are theoretical reasons to advocate the arcsin[square root(X)] transformation over the log(X+1) transformation, but very little difference is found in practice, and the log(X + 1) is simpler to apply and explain. Similarly, there are theoretical reasons to prefer factor analysis *sensu stricto* over principal components analysis, and vice versa, which can, and have been, argued for days to no conclusion. In practice, the two methods give very similar results.

12.4 Calculation and Interpretation of Metabolic Stress Biomarkers

The membrane of the bacterial cell handles all of its interactions with its environment, and bacteria have many strategies to deal with stressful environmental conditions, including modifying the fatty acids used in the membrane. This is illustrated in Eq. (12.2), where S stands for the substrate fatty acid and P for the product fatty acid induced by metabolic stress, namely, a *trans* monounsaturate or cyclopropyl fatty acid.

$$\begin{array}{rcl} & \mathrm{S} \rightarrow \mathrm{P} & \\ \textit{cis}\ \text{monounsaturate} & \rightarrow & \textit{trans}\ \text{monounsaturate} \\ \textit{cis}\ \text{monounsaturate} & \rightarrow & \text{cyclopropyl} \end{array} \qquad (12.2)$$

The stress biomarkers are then calculated as the ratio of the mole percents of the product to the substrate fatty acids, as in Eq. (12.3):

$$BM_{Stress} = P/S \tag{12.3}$$

where BM_{Stress} is the value of the stress biomarker. The most common transformations are 16:1ω7c→16:1ω7 t, 16:1ω7c→Cy17:0, 18:1ω7c→18:1ω7 t, and 18:1ω7c→Cy19:0.

There are problems with the application of the stress biomarkers. The first type of problem is when the stress-induced product fatty acid is only detected in a minority of the samples. This will most likely prevent detection of statistically significant differences. The second problem is when the substrate fatty acid is not detected, but the stress-induced fatty acid is; this has been seen in hot acid environments such as hydrothermal systems. Since division by zero is undefined in standard algebra, undefined results appear that standard statistical programs are unable to use. This problem can be solved by a modification of Eq. (12.3),

$$BM_{Stress} = P/(S + 1) \tag{12.4}$$

The metabolic stress biomarkers have been applied to, for example, tap water biofilms (White et al. 1999), and soils contaminated with jet fuel (Stephen et al. 1999).

12.5 Naming of Fatty Acids

Creating clear, consistent, and unambiguous names for microbial fatty acids is challenging due to the wide variety of possible structures. At the same time, it is essential for understanding the data and communicating results. The IUPAC rules for naming chemical compounds are supposed to provide unambiguous names, but there are problems with this approach. The most important is that IUPAC counts carbons from the opposite end of the fatty acid molecule from most of the enzymes that modify the fatty acid.

The need for a compact notation has led to the development of the omega system for naming fatty acids. Fatty acids are named according to the pattern of A:BωC. The A stands for the number of carbon atoms in the fatty acid backbone, B is the number of double bonds, and C is distance of the nearest unsaturation from the aliphatic (ω) end of the molecule. This can be followed by a "c" for cis or a "t" for trans configuration of the unsaturation. The prefixes "i," "a," and "br" stand for iso, anti-iso, and unknown branching position of the carbon chain, respectively. Mid-chain branching is noted by a prefix "10Me" for a 10-methyl fatty acid, and

cyclopropyl fatty acids by prefix "Cy." For example: 18:1ω7c is 18 carbons long with one double bond occurring at the 7th carbon atom from the ω end, and the unsaturation is in the cis conformation. Also, 16:0, i16:0, a16:0, and br16:0 are all 16-carbon fatty acids, while 10Me16:0 and Cy17:0 both contain a total of 17 carbons, not counting the carbon of the methyl ester moiety.

References

Balkwill DL, Leach FR, Wilson JT, McNabb JF, White DC (1988) Equivalence of microbial biomass measures based on membrane lipid and cell wall components, adenosine triphosphate, and direct counts in subsurface sediments. Microbial Ecol 16:73–84

Brockman FJ, Li SW, Fredrickson JK, Ringelberg DB, Kieft TL, Spadoni CS, White DC, McKinley JP (1997) Post-sampling changes in microbial community composition and activity in a subsurface paleosol. Microbial Ecol 36:152–164

Christie WW (2003) Lipid analysis; isolation, separation, identification and structural analysis of lipids, 3rd edn. Oily Press, Bridgwater, UK

Christie WW, Brechany EY, Lie Ken Jie MSF, Bakare O (1991) MS characterization of picolinyl and methyl ester derivatives of isomeric thia fatty acids. Biol Mass Spectrom 20:629–635

Erwin JA (1973) Fatty acids in eukaryotic microorganisms. In: Erwin JA (ed) Lipids and biomembranes of eukaryotic microorganisms. New York, Academic Press, pp 41–143

Griffin WT, Phelps TJ, Colwell FS, Fredrickson JK (1997) Methods for obtaining deep subsurface microbiological samples by drilling. In: Amy PS and Haldeman DL (eds) The microbiology of the terrestrial and deep subsurface. CRC Press, Boca Raton, pp 23–43

Grob RL Barry EF (1995) Modern practice of gas chromatography. Wiley, New York

Gu B, Zhou J-Z, Watson DB, Philips DH, Wu L, White DC (2001) Microbiological characterization in a zero-valent iron reactive barrier. Appl Environ Microbiol 77:293–309

Haldeman DL, Amy PS, Ringelberg DB, White DC (1994) Changes in bacteria recoverable from subsurface volcanic rock samples during storage at 4 °C. Appl Environ Microbiol 60:2679–2703

Harvey DJ (1992) Mass spectrometry of picolinyl and other nitrogen-containing derivatives of fatty acids. In: Christie WW (ed) Advances in lipid methodology, vol 1. Oily Press, Ayr, UK, pp 19–80

Kieft TL, Murphy EM, Amy PS, Haldeman DL, Ringelberg DB, White DC (1997) Laboratory and field evidence for long-term starvation survival of microorganisms in subsurface terrestrial environments. In: Proceed instruments, methods, and missions for the investigation of extraterrestrial organisms, 27 July to 1 August. Int Soc Optical Engin, San Diego, CA

Magurran AE (1988) Chapt. 2. Diversity indices and species abundance models. In: Magurran AE (ed) Ecological diversity and its measurement. Princeton Univ Press, Princeton, NJ

Mandelbrot B (1982) The fractal geometry of nature. Freeman, San Francisco, CA

Marinelli RL, Lovell CR, Wakeham SG, Ringelberg D, White DC (2000) An experimental investigation of the control of bacterial community composition in macrofaunal burrows. Marine Ecol Prog Series 235:1–13

Momchilova S, Nikolova-Damyanova B (2003) Stationary phases for silver ion chromatography of lipids: Preparation and properties. J Sep Sci 26:261–270

Myers RT, Zak DR, Peacock A, White DC (2001) Landscape-level patterns of microbial community composition and substrate. use in forest ecosystems. Soil Sci Soc Am J 65:359–367

Nichols PD, Guckert JB, White DC (1986) Determination of monounsaturated double bond position and geometry for microbial monocultures and complex consortia by capillary GC-MS of their dimethyl disulphide adducts. J Microbiol Meth 5:49–55

Phelps TJ, Fliermans CB, Garland TR, Pfiffner SM, White DC (1989) Recovery of deep subsurface sediments for microbiological studies. J Microbiol Meth 9:267–280

Øezanka T, Dor I, Prell A, Dembitsky VM (2003) Fatty acid composition of six freshwater wild cyanobacterial species. Folia Microbiol 48:71–75

Stephen JR, Chang Y-J, Gan YD, Peacock A, Pfiffner SM, Barcelona MJ, White DC, Macnaughton SJ (1999) Microbial characterization of JP-4 fuel contaminated-site using a combined lipid biomarker/PCR-DGGE based approach. Environ Microbiol 1:231–241

White DC, Kirkegaard RD, Palmer Jr. RJ, Flemming CA, Chen G, Leung KT, Phiefer CB, Arrage AA (1999) The biofilm ecology of microbial biofouling, biocide resistance and corrosion. In: Keevil CW, Godfree A, Holt D, Dow C (eds) Biofilms in the aquatic environment. Roy Soc Chem, Cambridge, UK, pp 120–130

White DC, Pinkart HC, Ringelberg DB (1996) Biomass measurements: biochemical approaches. In: Hurst CH, Knudsen GR, McInerney MJ, Stetzenbach LD, Walter MV (eds) Manual of environmental microbiology, 1st ed. ASM Press, Washington, DC, pp 91–101

Zhang CL, Li Y, Wall JD, Larsen L, Sassen R, Huang Y, Wang Y, Peacock A, White DC, Horita J, Cole DR (2001) Lipid and carbon isotopic evidence of methane-oxidizing and sulfate-reducing bacteria in association with gas hydrates from the Gulf of Mexico. Geology 30:239–242

Zogg GP, Zak DR, Ringelberg DB, MacDonald NW, Pregitzer KS, White DC (1997) Compositional and functional shifts in microbial communities related to soil warming. Soil Sci Soc Amer J 61:475–481

13 Enumeration of Soil Microorganisms

Julia Foght, Jackie Aislabie

13.1 Sample Preparation and Dilution

Introduction

Objectives. Soil is a heterogeneous matrix in which microbes are associated with organic and inorganic soil particles, forming aggregates. The goals of sample preparation for conventional enumeration techniques are to release the microbes from the matrix of a representative soil sample, then disperse them in a suitable diluent so that individual cells can be enumerated either by microscopic visualization or cultivation methods. The basic methods for soil aggregate disruption and dilution have been in common use for decades, but individual laboratories often develop variations to create their own empirical "standard methods." Different soil types may be more amenable to certain diluents or disruption techniques, so, if examining an unfamiliar soil type, it is wise to test combinations of methods to empirically optimize enumeration results. The presence of inorganic or organic contaminants (e.g., crude oil) may require adaptation of the basic methods to disperse the soil sample adequately or dilute a toxicant (e.g., heavy metal).

Principle. A suitable buffered diluent releases microbial cells from the soil matrix and is used to dilute the suspension to a cell density suitable for the enumeration method to be used. The dilution method must not compromise the structural integrity of cells to be enumerated by microscopy, nor the viability of cells for culture-based enumeration.

Theory. Microbes in soil are distributed heterogeneously in microenvironments of different scales and along depth profiles (Foster 1988; Ranjard and Richaume 2001). Therefore, representative samples of a suitable size must be collected for accurate enumeration. The number of individual samples theoretically required to represent the site can be calculated (Alef and Nannipieri 1995), but in practical terms the number of samples handled is

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dictated by the time and resources available. As a compromise, a composite sample can be prepared from several samples of equal mass or volume, but statistical evaluation of the data is relinquished. Commonly, at least 10 g wet mass of soil is used to prepare the first dilution, although the sample size may be adjusted according to the soil type and the organisms to be enumerated. Serial dilutions (commonly ten-fold) of soil suspensions are prepared with sufficient mixing to disrupt soil aggregates and release occluded microbes into suspension. Physical disruption of the soil aggregates can be enhanced by inclusion of small (2–3 mm) sterile glass beads in the diluent, at least in the first dilution. Suitable sterile diluents, of which many exist, aid the dispersion of soil aggregates. Diluents are often buffered (Strickland et al. 1988) and may contain proteins such as gelatin or tryptone to aid dispersion, glycerol to aid resuscitation of starved bacterial cells (Trevors and Cook 1992), or a surface active agent such as 0.1% Tween 80, although surfactants may reduce counts of sensitive Gram-negative cells (Koch 1994).

Equipment

- Top-loading balance capable of weighing to 0.1 g
- 150-mL glass dilution bottles and, optionally, approx. 20 g of 2–3 mm glass beads per bottle to aid in disruption of soil aggregates
- Spatula or small spoon, sterilized by autoclave or by flaming with ethanol
- Sterile pipettes for serial dilutions: 10-mL wide-mouth glass pipettes are less likely to plug during initial dilutions
- Optional mixing equipment: reciprocating or gyratory shaker for first dilution; vortex mixer; Waring blender

Reagents

- Suitable sterile, buffered diluent dispensed into dilution bottles, usually 90 or 99 mL each
- Suitable diluents include: 0.1% (w/v) sodium pyrophosphate with or without 1% glycerol (Trevors and Cook 1992); phosphate-buffered saline (0.85% (w/v) NaCl, 2.2 mM KH_2PO_4; 4.2 mM Na_2HPO_4, pH 7) with or without 0.01% gelatin or peptone (Koch 1994); 1–10 mM potassium phosphate (pH 7); or mineral salts medium lacking carbon source (Atlas 1995).

Sample Collection

Acceptable aseptic techniques for collection and storage of soil samples are given in Chapt. 1 in this volume. Soil intended for conventional enumeration

techniques should not be dried because this can reduce the microbial counts (Sparling and Cheshire 1979; van Elsas et al. 2002). Analyses should be conducted as soon as possible after sample collection.

Procedure

1. On a top-loading balance use sterile spatula to aseptically dispense 10 g of soil into the first dilution bottle containing 90 mL of diluent and record exact wet mass of sample added. This is the 10^{-1} dilution. Alef and Nannipieri (1995) recommend using 20 g soil in 180 mL of diluent to reduce the effects of sample heterogeneity.
2. To express the counts on the basis of soil dry mass, dispense a similar sample into a tared aluminum pan for determining dry mass (in triplicate for accuracy). Dry the sample at 105 °C to constant mass overnight, and record mass.
3. Shake or mix the dilution bottle vigorously manually or mechanically (using reciprocating shaker or Waring blender) to disrupt soil aggregates; recommended times vary from 1 min to 1 h and can be optimized empirically for different soils.
4. Perform ten-fold dilutions by transferring a 10.0-mL sample from the center of the dilution bottle to a fresh 90-mL dilution bottle, or hundred-fold dilutions with 1.0 mL transferred into 99 mL of diluent. Mixing between dilutions may be performed by hand by vigorously shaking the bottle 25 times between each transfer, or with a vortex mixer.
5. Continue with ten-fold serial dilutions appropriate to the enumeration method to be used, e.g., for aerobic heterotrophs in uncontaminated agricultural soils dilute to 10^{-9} for most probable number (Sect. 13.3) and 10^{-7} for plate counts (Sect. 13.4).

Calculation

1. Dilution factor (reciprocal of dilution) = (1/dilution)
2. Dry-mass correction factor = (wet mass of sample/dry mass of sample)

Notes and Points to Watch

- The initial sample(s) must be as representative of the soil as possible and analysis of replicates is recommended.
- Sample preparation and dilutions must be performed in a standardized manner that can be replicated, so that results from samples taken at

different times or from different sample sites can be compared with confidence.

- Soil dilutions should be used immediately after preparation, as storage of the cell suspension in buffer may decrease the counts observed (Koch 1994).
- The dilution volumes can be scaled down, using test tubes with 1 g of soil in 9 mL of diluent and mixing by vortex, but caution should be used because small sample sizes may not be representative.
- A sonicator bath or probe may be used for initial soil sample disruption (Strickland et al. 1988), but this equipment is not standard in all laboratories, and excess sonication will reduce counts.
- Aggregates in hydrocarbon-contaminated soils may be difficult to disperse, yielding inaccurate results. Similarly, microbes with highly hydrophobic cell surfaces, such as acid-fast hydrocarbon-degrading bacteria, may themselves aggregate and be difficult to disperse.
- If using sodium pyrophosphate as the diluent, adjust the pH to neutrality, as it is ca. pH 10 without adjustment (Trevors and Cook 1992).

13.2 Direct (Microscopic) Enumeration

Introduction

Objectives. It has long been known that enumeration techniques relying on cultivation of microbes in environmental samples can underestimate the total number of cells present by orders of magnitude (Skinner et al. 1952; Amann et al. 1995). This bias can be overcome in part by using molecular methods (Chapt. 10) or by using direct microscopic observation of cells where no cultivation is required. Direct enumeration methods can provide the total number of cells (live plus dead) or may discriminate between live and dead cells. Some stains differentiate cells based on phylogeny or the presence of functional genes, providing information about the types of cells as well as numbers. Microscopy is suitable for direct enumeration of both bacteria and fungi.

Principle. A known volume of a soil suspension is filtered through a 0.2 µm pore size filter. The microbes on the filter are stained with a fluorescent dye and counted by using an epifluorescence microscope. At least 20 fields each containing 20–50 cells are counted and the total count is calculated from the area observed and the volume of suspension filtered.

Theory. To reduce the bias inherent in culture-based enumeration methods, total counts of microbes in soil can be observed directly using microscopy (Fry 1990; Kepner and Pratt 1994; Bottomley 1994; Bloem 1995). Traditionally, to aid detection, the cells have been stained with fluorescent dyes (reviewed by Bölter et al. 2002) such as acridine orange (AO) or 4′,6-diamino-2-phenylindole (DAPI) which stain DNA-containing cells. Recently, emphasis has been put on differentiating between actively metabolizing cells and resting cells, or on discriminating between live and dead cells. Hence, new fluorescent dyes have been developed. The redox dye 5-cyano-2,3-ditolyl tetrazolium chloride (CTC), for example, is used to count active bacterial cells (Créach et al. 2003). CTC is a colorless membrane-permeable compound that produces a red-fluorescing precipitate in the cell wall when reduced by the electron transport system of active bacterial cells. Staining with a combination of propidium iodide (PI, which is excluded from cells with intact membranes) and thiazole orange (TO, which is taken up by both live and dead cells) provides a method for discriminating between live and dead cells. Numerous commercial stain kits are available with specific instructions for their use, such as Live/Dead BacLight kits (Molecular Probes, Invitrogen, Carlsbad, CA, USA). The fluorescent in situ hybridization (FISH) method, which detects hybridization of fluorescently-labeled oligonucleotide probes with target DNA or RNA sequences, can combine total counts with counts of specific phylogenetic groups (Amman et al. 1995) by detecting multiple overlapping fluorescent signals, but, like other microscopic methods, suffers from sensitivity biases (Bölter et al. 2002).

Potential problems encountered when enumerating microbes in soil include autofluorescence of soil matrix components, particularly in oil-contaminated soils, and occlusion of cells by soil particles, particularly clay-sized particles. In the latter case, methods have been developed to reduce interference by clays (Boenigk 2004) and confocal laser-scanning microscopy (CLSM) has been used to overcome problems of limited depth-of-focus in conventional microscopy.

Equipment

- Filter membranes (0.2 µm pore size) for sterilizing reagents
- Black polycarbonate filter membranes (0.2 µm pore size, 25 mm diameter, e.g., Millipore; Millipore Corp., Billerica, MA, USA)
- 25-mm filter holder unit consisting of a 15-mL glass reservoir and fritted glass base (wrapped and heat sterilized), clamp, and vacuum flask
- Blunt-tipped filter forceps for handling filter membranes

- Vacuum pump with fine control
- Glass microscope slides and coverslips, pre-cleaned
- Epifluorescence microscope with appropriate filters

Reagents

- All diluents and reagents sterile and particle-free by filtration through 0.2-μm pore size membrane filters
- Appropriate diluent for sample (Sect. 13.1)
- Fluorescent stains appropriate to target cells: e.g., DAPI stock solution (1 mg/mL) in deionized water, freshly diluted to a working concentration of 1 μg/mL in filtered deionized water, stains protected from light
- Suitable wash solution: e.g., phosphate wash solution (PWS) containing 10 mM KH_2PO_4, 0.85% NaCl and 5 mM $MgCl_2 \cdot 6H_2O$
- Non-fluorescent immersion oil

Sample Preparation

Prepare suitable dilutions of soil sample (Sect. 13.1) in sterile, particle-free diluent.

Procedure

1. Prepare dilution series as required in filter-sterilized diluent. Vigorously mix sample for 5 min and allow suspension to stand for approx. 30 min to let larger soil particles settle out. If the sample will be kept longer than 30 min before counting, add a preservative (e.g., filter-sterilized formaldehyde to final concentration 3.7% or electron-microscopy-grade glutaraldehyde to final concentration 2.5%).
2. Place black filter membrane in filter unit, add PWS (e.g., 4 ml) to column reservoir and known volume (e.g., 0.1 mL) of diluted soil suspension, avoiding settled soil particles. Perform subsequent steps under reduced lighting for light-sensitive stains like DAPI.
3. Add required volume of stain (e.g., 1 mL DAPI working solution) to sample in column reservoir and stain in the dark for 7–10 min.
4. Filter slowly through membrane under gentle vacuum. Rinse sides of column reservoir gently with diluent (two- to three-fold of initial volume) and allow filter to air dry.

5. Place a drop of immersion oil on a glass microscope slide, place the membrane filter on top, and cover with a coverslip. Follow with a drop of immersion oil and examine under an epifluorescence microscope at correct wavelength with appropriate filters.
6. Count at least 20 fields of view (FOV) each containing 20–50 cells. Count randomly located FOV covering a wide area of the filter, avoiding its edges.
7. Blanks consisting only of reagents should be performed at intervals, or at least at the beginning and end of sample enumeration. Blanks should be $< 5\%$ of the total cell densities in the samples and should be subtracted from sample counts before calculation of total numbers.

Calculation

Counts are calculated on the basis of wet mass of soil, corrected for background, and usually expressed on the basis of dry mass of soil.

- Cells/g soil wet mass =

$$\frac{\text{total no. of cells counted}}{\text{total no. of FOV}} \times \frac{\text{total stained area}}{\text{area of FOV}} \times \frac{1}{\text{mass of soil on filter}}$$

- Cells/g soil dry mass =

  (cells/g soil wet mass) × (dry-mass conversion factor)

  A specific example is given:

- Area of FOV = $0.01\ \text{mm}^2$
- Stained area of filter = $\pi r^2 = 176.8\ \text{mm}^2$
  (diameter of the filter area covered by filtrate = 15 mm)
- Total counts in 20 FOV for 0.1 mL of 10^{-3} dilution = 929
- Total counts in 20 FOV for reagent blanks = 40
- Mass of soil on filter = 0.1 mL of 10^{-3} dilution = 10^{-4} g soil wet mass
- Dry mass conversion factor (Sect. 13.1) = 1.18
  Cells/g soil wet mass =

$$\frac{(929 - 40)\ \text{cells}}{20\ \text{FOV}} \times \frac{176.8\ \text{mm}^2}{0.01\ \text{mm}^2} \times \frac{1}{10^{-4}\ \text{g}} = 7.9 \times 10^9$$

- Corrected count = $7.9 \times 10^9 \times 1.18 = 9.3 \times 10^9$ cells/g soil dry mass

Notes and Points to Watch

- An analysis of the sources of variation in the direct count method (Kirchman et al. 1982) emphasizes the importance of enumerating replicate filters to reduce error.
- Starving ("dwarf") cells and ultramicrobacteria (< 0.5 µm diameter) may not be not retained on the filter membrane or may not be detected by activity stains (Bölter et al. 2002).
- At low cell densities it is difficult to achieve statistically valid counts, and efforts must be made to concentrate the sample if possible.
- Hydrocarbon-contaminated samples may suffer from autofluorescence and poor disruption of aggregates.

13.3 Enumeration by Culture in Liquid Medium (Most Probable Number Technique)

Introduction

Objectives. The Most Probable Number (MPN) method uses statistics to infer the number of viable organisms in a sample that are able to grow or metabolize in a liquid medium under given incubation conditions. MPN tests can be carried out in large volumes in bottles or test tubes, or in microliter volumes in microtiter well plates, depending on the sample and the viability assay.

Different media can be used to enumerate both generalist and specialist microbes in the soil. Total heterotrophs (generalists) can be enumerated in complex medium, although full-strength medium such as trypticase soy broth may not be suitable for enumerating microbes in nutrient-poor soils; for such samples tenth-strength medium may be appropriate (Alef and Nannipieri 1995). The MPN method can be customized to differentiate among specialists by providing selective growth substrates. For example, mineral medium can be supplemented with filter-sterilized crude oil or refined product (e.g., diesel fuel) to enumerate "total hydrocarbon degraders" or amended with specific hydrocarbon substrates representing aliphatic and aromatic components (e.g., *n*-hexadecane and naphthalene, respectively). Liquid hydrocarbons can be added directly to broth whereas solid hydrocarbons can be provided as a fine suspension of crystals or dissolved in a non-metabolized water-immiscible carrier such as heptamethylnonane (Efroymson and Alexander 1991). Volatile hydrocarbons may be supplied in the vapor phase although this can be technically cumbersome.

Positive tubes may be identified by various criteria, including: increased turbidity due to growth; emulsification of crude oil (e.g., "Sheen Screen," Brown and Braddock 1990); production of colored metabolites, particularly from some aromatic substrates (Stieber et al. 1994; Wrenn and Venosa 1996); reduction of an iodonitrotetrazolium (INT) dye after incubation to indicate metabolism of substrates (Wrenn and Venosa 1996; Johnsen et al. 2002); or evolution of $^{14}CO_2$ from radiolabeled substrates (Carmichael and Pfaender 1997). It is important that both positive and negative controls be included with these tests.

Principle. The microorganisms in a soil sample are serially diluted to extinction, inoculated in replicate into a suitable medium, and incubated under appropriate conditions to yield a series of cultures that is scored according to pre-determined criteria. The combination of positive and negative cultures after incubation is evaluated by statistical methods to infer the MPN of viable cells in the undiluted sample.

Theory. Culture-based enumeration methods such as MPN and plate count assay (Sect. 13.4) are biased because only a small proportion of environmental microbes has been cultured (Amann et al. 1995). With improved culture-based studies (e.g., Connon and Giovannoni 2002), the bias imposed by growth-based methods will lessen, but it must be considered when interpreting results. The advantage to growth-based enumeration over molecular methods is that the former is technically simpler, usually easy to interpret, and can yield isolates for further investigation. The advantage over plate count methods is that MPN is suitable for particulate samples (such as soil dilutions) that would obscure plate counts at low dilutions, and can detect microbes that will not grow on solid medium or are a minor component of a mixed culture. The disadvantages of MPN are that it yields only a statistical estimate of the viable microbes present and it requires many tubes and manipulations compared with plate counts.

Typically a decimal dilution series is prepared in suitable diluent and a fixed volume of each dilution is inoculated into medium in replicate cultures, usually in multiples of 3, 5, or 10. MPN tests can be conducted in tubes, vials, or bottles, generally containing 7–10 mL medium per test tube, or in microtiter plates with 200 µL per well. After incubation the tubes are scored qualitatively for criteria such as growth, production of metabolites, or loss of substrate.

The combination of positive and negative cultures is converted to the MPN and confidence intervals either by consulting standard probability tables (e.g., Eaton et al. 1995; Alef and Nannipieri 1995) or using an algorithm (Koch 1994). The method assumes that (1) the microorganisms have been distributed into the cultures such that the highest dilution positive tubes were inoculated with a single organism, (2) culture tubes inoculated

with as few as one viable microbe will produce a positive result, and (3) the microbes have not been injured or rendered non-viable during sample handling.

Equipment

- Pipettes
- Sterile test tubes or microtiter plates
- Vortex mixer for mixing inoculum into medium (optional)
- Incubation chamber with suitable temperature control and headspace (e.g., for anaerobes)
- Microtiter plate reader for measuring color changes or optical density (optional)
- Solvent-resistant filters (e.g., Millex-FG, Millipore Corp.) for filter sterilizing hydrocarbon solutions (optional)

Reagents

- Appropriate diluent for sample (Sect. 13.1)
- Sterile liquid or semi-solid medium suitable for growth of target organism(s). For enumeration of generalists, standard or dilute liquid media (Alef and Nannipieri 1995, Atlas 1995) are appropriate; for enumeration of specialists, a mineral salts medium amended with selective carbon sources such as hydrocarbons may be used (Sect. 13.4).
- Specialty chemicals, depending on criteria for positive cultures, such as radiolabeled substrates, endpoint reagents, carrier solvents, etc.
- Filter-sterilized liquid hydrocarbons or stock solutions of solid hydrocarbons dissolved in ethanol or dimethylformamide, for use as selective carbon sources (optional)

Sample Preparation

Perform serial dilutions of a representative soil sample in appropriate diluent (Sect. 13.1), to exceed the expected viable number of cells by one or two orders of magnitude.

Procedure

1. Dispense replicate volumes of growth medium into suitable receptacles (e.g., 10 mL in test tubes, 200 µL per well for microtiter plates). Prepare

replicates (typically 3, 5, or 10) for each sample dilution to be tested. Medium must contain complete nutrients for growth including carbon source, and may contain indicators such as dyes or radiolabeled substrates.

2. Inoculate replicate tubes with fixed volume of diluted sample (e.g., 1.0 mL for tubes, 100 µL for microtiter wells) covering at least three decimal dilutions.
3. Include negative controls (uninoculated medium) and positive controls (medium inoculated with a culture known to produce a positive result) for reference.
4. Incubate 7–14 days or longer in the dark under suitable conditions, taking into account in situ conditions of temperature, O_2 levels, etc.
5. Score tubes at intervals for positive results. Continue to incubate until two successive readings give the same results. Positive indicators include turbidity (e.g., heterotrophs growing in complex medium), hydrocarbon emulsification, production of soluble or gaseous metabolic end products (e.g., $^{14}CO_2$ evolution from radiolabeled substrates, methane, colored metabolites), and changes in indicators (pH indicators, redox dyes).
6. Identify the highest dilution set with all tubes positive, and the next two higher dilution sets. Use the pattern of positive and negative tubes with standard probability tables (e.g., Alef and Nannipieri 1995, Eaton et al. 1995) to calculate the MPN from the dilution factor of the middle set. When non-standard patterns are encountered, follow the recommended variations provided with the tables for calculating the MPN.

Calculation

Published tables of statistical probability (Alef and Nannipieri 1995; Eaton et al. 1995; tables are also available on several internet sites such as US Food & Drug Administration) are used to convert the pattern of positive and negative tubes into the MPN of viable microbes in the original sample. The dilution factor and volume of sample used to inoculate the tubes are used in calculation but the volume of growth medium used in the tubes is not taken into consideration. Sample volumes reported in standard MPN tables are designed for water samples and are usually expressed per 100 mL of sample; therefore, they must be corrected for the actual volume of inoculum used in the test. Values are calculated as the MPN $\pm$ 95% confidence intervals (provided with the tables) and expressed on the basis of soil dry mass by multiplying the MPN by the dry-mass correction factor (Sect. 13.1).

The simple algorithm below (Eaton et al. 1995) can be used to calculate the MPN without consulting published tables but does not provide

confidence intervals.

$$\text{MPN/100 mL} = \frac{\text{number of positive tubes} \times 100}{\sqrt{(\text{mL sample in negative tubes})(\text{mL sample in all tubes})}}$$

Notes and Points to Watch

- Match the incubation conditions to in situ conditions when feasible. For example, select an appropriate culture incubation temperature (including temperature of the diluent and medium when inoculating), provide semi-solid medium for enumeration of microaerophiles, anaerobic medium and headspace for anaerobes, etc.
- If aerobic tubes are sealed, ensure that there is adequate headspace to maintain aerobic conditions if extended incubation will be required.
- To use a high proportion of sample to growth medium, increase the strength of the medium (e.g., use double strength medium for 100% (v/v) inoculum).
- When using turbidity as the criterion for growth, be aware of the turbidity contributed by soil particles at low dilutions, and by particulate substrates (e.g., suspensions of polycyclic aromatic hydrocarbon crystals).
- If providing low molecular mass hydrocarbons as a carbon source avoid toxicity to the inoculum by minimizing substrate volumes.
- Multiple MPN tests can be performed on a soil sample to enumerate different specialist components of the soil microbiota. If generalist MPN tests or direct counts (Sect. 13.2) are also performed, the specialists can be expressed as a proportion of the total viable numbers present in the sample.
- After incubation, the positive MPN tubes may be suitable to use as an inoculum for subsequent isolation of pure cultures.

13.4 Enumeration by Culture on Solid Medium (Plate Count Technique)

Introduction

Objectives. The plate count technique quantifies the viable microbes in a sample by counting the number of colonies that form on or in a solid

growth medium inoculated with dilutions of that sample. Each colony is assumed to have originated from a single propagule or "colony forming unit" (CFU), whether that be a bacterial cell, endospore, hyphal fragment, or spore. Non-selective growth medium may be used to cultivate generalists, or selective medium may be used to enumerate specialists such as hydrocarbon-degrading bacteria and fungi. Specific enumeration of actinomycetes, filamentous fungi, or yeasts usually requires specialized media to suppress unwanted soil microbes such as spreading or mucoid colonies that overgrow the slower-growing colonies on non-selective plates (Labeda 1990). Alternatively, a differential assay can be applied after the colonies have grown, to distinguish those possessing specific metabolic capabilities (e.g., production of colored metabolites; Kiyohara et al. 1982). Plates can be incubated under different atmospheres to enumerate aerobes, anaerobes, or microaerophiles, or at different temperatures to cultivate psychrotolerant, mesophilic, or thermophilic microbes. Plate counts may be performed using several media and incubation conditions to enumerate different subsets of the viable microbes in a soil sample.

Principle. Dilutions of a soil sample, performed in suitable diluent, are inoculated in replicate onto solid medium for cultivation with or without selection for specific metabolic types. Plates containing 30–300 colonies are selected and the colonies counted so that the CFU can be calculated for the original soil sample using dilution factors and dry-mass correction factors.

Theory. It has long been recognized that the plate count method underestimates the actual number of living cells in the sample by one or more orders of magnitude (Skinner et al. 1952) because soil organisms may not be viable or are not cultivable under the conditions employed (Amann et al. 1995). The proportion of viable cells enumerated will depend on the soil and on the growth medium and incubation conditions. New strategies for enumerating previously uncultured microbes are being devised to alleviate the cultivation bias (e.g., Joseph et al. 2003; Stevenson et al. 2004), but selectivity will always be a disadvantage of the plate count method (or any other cultivation-based enumeration method) compared with direct counts or molecular methods. The advantages are that the plate count method is relatively rapid and inexpensive and yields well-separated colonies suitable for subsequent purification and characterization.

The medium and incubation conditions used in the plate count method determine which metabolic types of microbes will be enumerated, but the primary assumption for all plate counts is that each colony arises from a single viable propagule, i.e., a colony forming unit (CFU). After incubation, colonies are counted only on those plates containing 30–300 colonies, for statistically valid enumeration (Koch 1994). Variations on the basic method exist, including spread plates appropriate for aerobic bacteria

Table 13.1. Recommended methods for providing hydrocarbons on solid medium

Substrate	Suitable for:			
	Spread	Vapor	Spray	Overlayer
Toluene, xylenes		√		
Naphthalene		√		
Phenanthrene			√	√
Dibenzothiophene			√	√
Pyrene			√	√
Octane		√		
Hexadecane	√			
Jet fuel		√		
Crude oil	√			

and yeast, and pour plates suitable for microbes that do not grow well on surfaces but form subsurface colonies at reduced oxygen tension. Pour plates also help reduce problems with spreading colonies. Several methods can be used to supply substrates to mineral salts agar, including overlayer plates, spray plates and vapor plates (Table 13.1). Plates are incubated in the dark under suitable conditions of temperature and aeration, often for extended periods of time (e.g., 2–3 months) to enumerate slow-growing species (Janssen et al. 2002).

Equipment

- Sterile bent glass rod spreaders ("hockey sticks") for inoculating plates
- Flame and beaker of ethanol to surface-sterilize spreaders
- Manual turntable for turning Petri plates while spreading inoculum (optional)
- Incubators with suitable atmosphere (aerobic or anaerobic) and temperature setting
- Water bath at 50 °C for pour plates or overlayer plates
- Aerosol spray apparatus (e.g., Jet-Pak, Sherwin-Williams Co., Cleveland) for applying ether solution of substrate to agar surface, or sealed containers for incubating vapor plates (optional)

Reagents

- Solid growth medium in Petri plates, sufficient to inoculate 3 or 5 plates per dilution. Generalist media for bacteria include Plate Count Agar, Nutrient Agar and R2A agar (Difco; Becton, Dickinson & Co., Sparks, MD,

USA) among many others. General mycological media include Czapek-Dox Agar (Difco), Malt Extract Agar (Difco) and Mycobiotic ("Mycosel") Agar (Acumedia, Neogen; Lansing, MI, USA), usually containing antibiotics (e.g., oxytetracycline at 100 mg/L and/or streptomycin at 30 mg/L) to suppress bacterial growth. Selective agar for hydrocarbon degraders is usually a mineral medium such as Bushnell Haas (Difco; Atlas 1995) solidified with 1.5% (w/v) Purified Agar (Oxoid, Basingstoke, UK) or Agar Noble (Difco) or 0.8% (w/v) gellan gum (Gelrite; Serva, Heidelberg) and amended with a specific carbon source.

- Overlayer medium is usually prepared with agarose or purified agar (Bogardt and Hemmingsen 1992).
- An ether or acetone solution of hydrocarbon substrate is used for spray plates.

Sample Preparation

Dilute sample appropriately in suitable diluent (Sect. 13.1) to exceed expected number by at least one order of magnitude.

Procedure

Spread Plates (Bacteria, Yeasts, or Filamentous Fungi)

1. Pipette a fixed volume of inoculum (typically 0.1 mL or 1.0 mL) from a range of dilutions onto three or five replicate agar plates and spread evenly using sterile bent glass rod. When inoculum has been absorbed into agar, invert plates and place in plastic bag to maintain humidity.
2. Incubate at suitable temperature (e.g., ca. 25 °C for temperate soils) and under appropriate atmosphere, noting the appearance of colonies on plates with 30–300 colonies. Continue incubating until the number of colonies is constant. This may take less than a week for fast-growing bacteria, or more than 2 months for slow-growers or plates incubated at low temperatures. For extended incubation periods, seal the edges of plates with laboratory film or tape to prevent drying.
3. Count the colonies on replicate plates having 30–300 colonies, determine the mean and calculate the CFU per gram dry mass in the original sample using the dilution factor and dry-mass correction factor.
4. To enumerate colonies that can grow on liquid hydrocarbons spread a small volume (e.g., 50 µL per plate) onto the surface of mineral medium agar either before or after inoculation, leaving small droplets on the agar surface. For solid hydrocarbons, see spray plate method below.

Pour Plates (Bacteria or Yeasts)

1. Prepare 20 mL aliquots of molten growth medium agar and bring to ca. 50 °C in a water bath.
2. Add 1.0 mL inoculum to agar, mix briefly, and immediately pour into an empty sterile Petri plate. Alternatively, add inoculum to an empty sterile Petri plate, pour molten agar on top and mix by rotating the plate on the bench top. Allow agar to solidify then incubate as for spread plates.
3. Count both surface and subsurface colonies.

Overlayer Plates (Bogardt and Hemmingsen 1992)

1. Prepare Petri plates containing 20 mL mineral medium with or without carbon source, depending on whether the overlayer contains a carbon source.
2. Prepare 5 mL sterile molten 1.5% (w/v) agarose or purified agar containing a suspension of particulate, insoluble substrate at a nominal concentration sufficient to provide an opaque suspension of fine crystals. It may be necessary to dissolve the substrate in a small volume of ethanol before adding to the agarose. Bring to 50 °C in a water bath.
3. Add inoculum to the molten agarose, mix briefly, and immediately pour onto the mineral medium base, tipping the plate to distribute the overlayer evenly. The overlayer should be somewhat opaque. Allow to solidify then incubate as for spread plates. Alternatively, carefully inoculate the surface of the overlay as for spread plates.
4. Count colonies that have a surrounding halo of clearing or colored metabolites (Fig. 13.1).

Spray Plates for Solid Hydrocarbons (Kiyohara et al. 1982)

1. Prepare a solution of crystalline hydrocarbon (e.g., phenanthrene or dibenzothiophene) in either acetone or anhydrous ethyl ether. CAUTION: Ethyl ether is highly flammable and explosive. All procedures must be carried out in a well-vented fume hood away from any sparks or flames. Protective clothing and gloves must be worn to prevent exposure to hydrocarbon mist and precautions, such as spraying inside a cardboard box in the hood, should be taken to prevent contaminating the fume hood with potentially carcinogenic compounds. The concentration of the solution does not need to be precise; approx. 10 mg of hydrocarbon dissolved in 2 mL of solvent should be sufficient to cover one plate.
2. Use an aerosol canister to deliver a fine spray of solution to the surface of an inoculated plate. The surface should become slightly opaque with

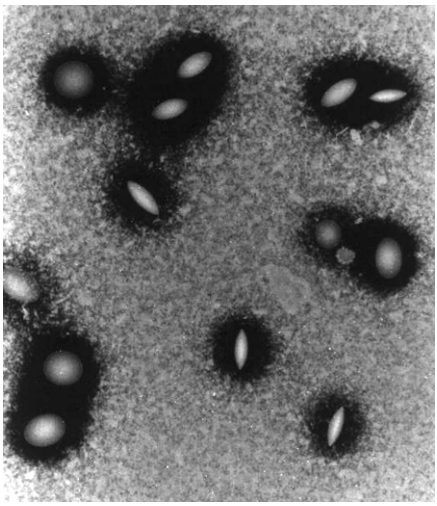

Fig. 13.1. Colonies capable of degrading carbazole form "haloes" of clearing in an overlayer plate prepared with carbazole. (From Shotbolt-Brown et al. 1996 with permission)

a thin, even layer of very fine crystals. Seal edges of plates with laboratory film and place in plastic bags to prevent cross-contamination by vapors.

3. Incubate and score for appearance of colonies. If the substrate can serve as a carbon source (e.g., phenanthrene), use mineral medium agar lacking a carbon source and score for colonies that are surrounded by zones of clearing and are larger than those observed on a parallel control plate lacking spray. If the substrate does not serve as a carbon source but can be co-metabolized (e.g., dibenzothiophene), use a low-nutrient agar that provides a carbon source and score for production of colored metabolites and/or zones of clearing around the colonies.

Vapor Plates for Volatile Hydrocarbons

1. Inoculate mineral medium agar by the spread plate method.
2. For volatile solid hydrocarbons such as naphthalene, add a few crystals (< 0.1 g) to the lid of each inverted Petri plate, seal the edges of the plate with laboratory film, and incubate in sealed plastic bags to prevent cross-contamination on vapors.
3. Volatile liquid hydrocarbons such as xylenes or jet fuel can be supplied to individual plates by placing a few drops of hydrocarbon into a plastic pipette tip stuffed with glass wool and placed on the lid of an inoculated, inverted mineral salts agar plate. Seal and incubate as above. To supply vapor to several plates at once, place inoculated plates into a sealable container with a small beaker containing a "wick" of glass wool or folded filter paper, and add a small amount of hydrocarbon, just sufficient to

saturate the headspace for a day or so, to reduce the chance of toxicity. Replenish hydrocarbon as necessary.

Calculation

1. Count the number of colonies arising on the plates, or the number showing the desired phenotype.
2. Calculate the mean value for replicates, correct for dilution and dry mass, and express as CFU or phenotype-positive colonies per gram dry mass of original soil.

Notes and Points to Watch

- When spreading inoculum on a plate, make sure that the glass rod is not too hot. Similarly, do not use pour plates or overlayer plates to enumerate psychrophiles.
- Incubate plates at temperatures close to those in situ when practical.
- When enumerating specialist populations (e.g., hydrocarbon degraders), positive and negative controls should be included.
- Antibiotics should be prepared as filter-sterilized concentrated solutions and added to cooled molten agar immediately before dispensing the agar into plates. Protect plates from the light before use and during incubation.
- The use of low-nutrient media for enumeration has been recommended by some researchers, as discussed in Sect. 13.3.
- Davis et al. (2005) suggest that plates with a minimum of ten colonies per plate rather than 30–300 colonies should be used for enumeration. This reduces depression in viable counts due to over-crowding of colonies on plates leading to inhibition of some species by others, or alternatively the depletion of nutrients by fast growing colonies that prevents slow growers from reaching a countable size.
- Problems may arise with long-term incubation of plates for enumeration of slow-growing bacteria and fungi, including drying of the plates, appearance of "spreading" or mucoid bacterial colonies or fungal colonies that obscure other colonies.
- Plate counts over-represent genera that sporulate profusely (e.g., *Penicillium, Trichoderma* spp., *Streptomyces* spp.), and under-estimate or exclude fastidious genera.
- Do not use too much solvent solution for spray plates, as the solvent can injure the inoculum.

References

Alef K, Nannipieri P (1995) Methods in applied soil microbiology and biochemistry. Academic Press, London

Amann RI, Ludwig W, Schleifer, K-H (1995) Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol Rev 59:143–169

Atlas RM (1995) Handbook of media for environmental microbiology. ASM Press, Washington DC

Bloem, J (1995) Fluorescent staining of microbes for total direct counts. In: Akkermans ADL, van Elsas JD, de Bruijn FJ (eds), Molecular microbial ecology manual. Kluwer Academic Publ, Dordrecht, The Netherlands, pp 1–12

Boenigk J (2004) A disintegration method for direct counting of bacteria in clay-dominated sediments: dissolving silicates and subsequent fluorescent staining of bacteria. J Microbiol Meth 56:151–159

Bogardt AH, Hemmingsen BB (1992) Enumeration of phenanthrene-degrading bacteria by the overlayer technique and its use in evaluation of petroleum-contaminated sites. Appl Environ Microbiol 58:2579–2582

Bölter M, Bloem J, Meiners K, Möller R (2002) Enumeration and biovolume determination of microbial cells – a methodological review and recommendations for applications in ecological research. Biol Fertil Soils 36:249–259

Bottomley PJ (1994) Light microscopic methods for studying soil microorganisms. In: Weaver RW, Angle S, Bottomley P, Bezdicek D, Smith S, Tabatabai A, Wollum A (eds) Methods of soil analysis, Part 2, Microbiological and biochemical properties. Soil Sci Soc Am Book Series No. 5, Madison WI, pp 81–105

Brown EJ, Braddock JF (1990) Sheen screen, a miniaturized most-probable-number method for enumeration of oil-degrading microorganisms. Appl Environ Microbiol 56:3895–3896

Carmichael LM, Pfaender FK (1997) Polynuclear aromatic hydrocarbon metabolism in soils: relationship to soil characteristics and preexposure. Environ Toxicol Chem 16:666–675

Connon SA and Giovannoni SJ (2002) High-throughput methods for culturing microorganisms in very-low-nutrient media yields diverse new marine isolates. Appl Environ Microbiol 68:3878–3885

Créach V, Baudoux A-C, Betru G, Le Rousiz B (2003) Direct estimate of active bacteria: CTC use and limitations. J Microbiol Meth 52:19–28

Davis KER, Joseph SJ, Janssen PH (2005) Effects of growth medium, inoculum size, and incubation time on the culturability and isolation of soil bacteria. Appl Environ Microbiol 71:826–834

Eaton AD, Clesceri LS, Greenberg AE (1995) Standard methods for the examination of water and wastewater, 19th Edition, American Public Health Association, Washington DC, Section 9221

Efroymson RA, Alexander M (1991) Biodegradation by an *Arthrobacter* species of hydrocarbons partitioned into an organic solvent. Appl Environ Microbiol 57:1441–1447

Foster RC (1988) Microenvironments of soil microorganisms. Biol Fertil Soils 6:189–203

Fry JC (1990) Direct methods and biomass estimation. In: Grigorova R, Norris JF (eds) Methods in Microbiology, vol 22, Techniques in microbiology. Academic Press, London, pp 41–85

Janssen PH, Yates PS, Grinton BE, Taylor PM, Sait M (2002) Improved culturability of soil bacteria and isolation in pure culture of novel members of the divisions *Acidobacteria*, *Actinobacteria*, *Proteobacteria*, and *Verrucomicrobia*. Appl Environ Microbiol 68:2391–2396

Johnsen AR, Bendixen K, Karlson U (2002). Detection of microbial growth on polycyclic aromatic hydrocarbons in microtiter plates by using the respiration indicator WST-1. Appl Environ Microbiol 68:2683–2689

Joseph SJ, Hugenholtz P, Sangwan P, Osborne CA, Janssen PH (2003) Laboratory cultivation of widespread and previously uncultured soil bacteria. Appl Environ Microbiol 69:7210–7215

Kepner RL, Pratt JR (1994) Use of fluorochromes for direct enumeration of total bacteria in environmental samples – past and present. Microbiol Rev 58:603–615

Kirchman D, Sigda J, Kapuscinski R, Mitchell R (1982) Statistical analysis of the direct count method for enumerating bacteria. Appl Environ Microbiol 44:376–382

Kiyohara H, Nagao K, Yano K (1982) Rapid screen for bacteria degrading water-insoluble, solid hydrocarbons on agar plates. Appl Environ Microbiol 43:454–457

Koch AL (1994) Growth measurement. In: Gerhardt P, Murray RGE, Wood WA, Krieg NR (eds) Methods for general and molecular bacteriology. ASM Press, Washington DC, pp 248–292

Labeda DP (1990) Isolation of biotechnological organisms from nature. The McGraw-Hill Environmental Biotechnology Series, McGraw-Hill, New York.

Ranjard L, Richaume A (2001) Quantitative and qualitative microscale distribution of bacteria in soil. Res Microbiol 152:707–716

Shotbolt-Brown J, Hunter DWF, Aislabie J (1996) Isolation and description of carbazole-degrading bacteria. Can J Microbiol 42:79–82

Skinner FA, Jones PCT, Mollison JE (1952) A comparison of a direct- and a plate-counting technique for the quantitative estimation of soil micro-organisms. J Gen Microbiol 6:261–271

Sparling GP, Cheshire MV (1979) Effects of soil drying and storage on subsequent microbial growth. Soil Biol Biochem 11:317–319

Stevenson BS, Eichorst SA, Wertz JT, Schmidt TM, Breznak JA (2004) New strategies for cultivation and detection of previously uncultured microbes. Appl Environ Microbiol 70:4748–4755

Stieber M, Haeseler F, Werner P, Frimmel FH (1994) A rapid screening method for microorganisms degrading polycyclic aromatic hydrocarbons in microplates. Appl Microbiol Biotechnol 40:753–755

Strickland TC, Sollins P, Schimel DS, Kerle EA (1988) Aggregation and aggregate stability in forest and range soils. Soil Sci Soc Am J 52:829–833

Trevors JT, Cook S (1992) A comparison of plating media and diluents for enumeration of aerobic bacteria in a loam soil. J Microbiol Meth 14:271–275

Van Elsas JD, Smalla K, Lilley AK, Bailey MJ (2002) Methods for sampling soil microbes In: Hurst CT, Crawford RL, Knudson GR, McInerney MJ, Stetzenbach LD (eds) Manual of environmental microbiology. 2nd Edition. ASM Press, Washington DC, pp 505–515

Wrenn BA, Venosa AD (1996) Selective enumeration of aromatic and aliphatic hydrocarbon degrading bacteria by a most-probable-number procedure. Can J Microbiol 42:252–258

14 Quantification of Soil Microbial Biomass by Fumigation-Extraction

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14.1 General Introduction

The soil microbial biomass responds much more quickly than most other soil fractions to changing environmental conditions, such as variations in substrate input (e.g., Powlson et al. 1987) or increases in heavy metal content (Brookes and McGrath 1984). Much research supports the original idea of Powlson and Jenkinson (1976) that the biomass is a much more sensitive indicator of changing soil conditions than, for example, the total soil organic matter content. Thus the biomass can serve as an "early warning" of such changes, long before they are detectable in other ways. Biomass measurements are certainly useful in studies of soil protection. They have the advantage that they are relatively cheap and simple as well as being rapid. There is now a considerable amount of literature to show that these measurements are useful in determining effects of stresses on the soil ecosystem. Measurements of the soil microbial biomass by the fumigation extraction method have been used to estimate the environmental effects of pesticides (Harden et al. 1993) and antibiotics (Castro et al. 2002). This method has been repeatedly used to monitor successfully the bioremediation process of fuel oil contaminated soil (Joergensen et al. 1994a, 1994b, 1995, 1997; Plante and Voroney 1998; Franco et al. 2004).

Linked parameters (e.g., biomass-specific respiration or biomass as a percentage of soil organic C) are also useful because they possess "internal controls" (see Barajas Aceves et al. 1999 for a discussion). This fact permits interpretation of measurements in the natural environment, where, unlike in controlled experiments, there may not be suitable non-contaminated soil (for example) to provide good "control" or "background" measurements (Brookes 1995).

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14.2 Fumigation and Extraction

Introduction

Objectives. The biomass of a microbial community can be quantitatively determined by fumigation and extraction in a large variety of soils developed under very different environmental conditions, especially in contaminated and remediated soils.

Principle. Soils are fumigated with chloroform, incubated for 24 h, and extracted. Different components can then be measured in the extracts, using various methods (Sects. 14.3–14.4). Non-fumigated soil is also extracted to correct for non-biomass soil organic matter.

Theory. Following chloroform fumigation of soil, there is an increase in the amount of various organic and inorganic components coming from the cells of soil microorganisms (Jenkinson and Powlson 1976). The membranes of living soil microorganisms are partially lysed by the fumigant chloroform. After a 24 h incubation period to allow autolysis, a large part of the soil microbial biomass can be extracted from fumigated soil. The amount additionally rendered extractable from killed microorganisms is proportional to the original microbial biomass. Organic C (Vance et al. 1987), total N and NH_4-N (Brookes et al. 1985), and ninhydrin-reactive N (Joergensen and Brookes 1990) can be measured in the same 0.5 M K_2SO_4 extract (Alef and Nannipieri 1995). Organic C (Joergensen 1995) and total S (Wu et al. 1993) can be measured after extraction with 0.01 M $CaCl_2$ and phosphate or total P after extraction with $NaHCO_3$ (Brookes et al. 1982).

Equipment

- Room, incubator, or water bath adjustable to 25 °C
- Implosion-protected desiccator
- Vacuum line (water pump or electric pump)
- Horizontal or overhead shaker
- Deep-freezer at −15 °C
- Folded filter papers (e.g., Whatman 42 or Schleicher & Schuell 595 1/2)
- Glass conical flasks (250 mL)

Reagents

- Ethanol-free chloroform ($CHCl_3$)
- Soda lime
- 0.5 M K_2SO_4

Sample Preparation

Use field-moist, sieved (between < 2 and < 5 mm) soil.

Procedure

1. Divide a moist soil sample of 50 g into two subsamples of 25 g.
2. Place the non-fumigated control samples in 250 mL conical flasks, extract immediately with 100 mL 0.5 M K_2SO_4 (extractant-to-soil ratio of 4:1) for 30 min by oscillating shaking at 200 rpm (or 45 min overhead shaking at 40 rpm), filter through a folded filter paper.
3. For the fumigation treatment, place 50-mL glass vials containing the moist soil into a desiccator containing wet tissue paper and a vial of soda lime, add a beaker containing 25 mL ethanol-free $CHCl_3$ and a few boiling chips and evacuate the desiccator until the $CHCl_3$ has boiled vigorously for 2 min.
4. Incubate the desiccator in the dark at 25 °C for 24 h. After fumigation, remove $CHCl_3$ by repeated (six-fold) evacuation and extract with 0.5 M K_2SO_4 as described above.
5. Store 0.5 M K_2SO_4 extracts at −15 °C prior to analysis of organic C, total N, or ninhydrin-reactive N.

Notes and Points to Watch

- The desiccator must be kept under vacuum for 24 h to ensure the presence of a $CHCl_3$ atmosphere, which kills virtually all soil microorganisms.
- Ethanol-free $CHCl_3$ must be used to measure microbial biomass C because ethanol cannot be completely removed from the soil after fumigation. Ethanol-stabilized $CHCl_3$ can be used if solely microbial biomass N or ninhydrin-reactive N will be measured (DeLuca and Keeney 1993).
- The soil must be sieved only if homogeneous samples are required (Ocio and Brookes 1990).

- Soil mass can range from 200 mg (Daniel and Anderson 1992) to 200 g (Ocio and Brookes 1990).
- Soil microbial biomass is extracted by 0.5 M K_2SO_4. The high potassium concentration flocculates the soil and prevents adsorption of NH_4^+ released by fumigation. The relatively high salt concentration also inhibits decomposition of the microbial material extracted after fumigation. However, if the extracts have to be stored for a long period, they must be frozen.
- Upon thawing of frozen K_2SO_4 soil extracts, a white precipitate of $CaSO_4$ occurs in near-neutral or alkaline soils. However, this causes no analytical problems in either method and may be safely ignored (Joergensen and Olfs 1998).
- Soil water content can fluctuate widely, but must be higher than 30% water-holding capacity (WHC). Microbial biomass C and biomass N of soils at 40–50% WHC have been found to be similar to those in saturated soils (Widmer et al. 1989; Mueller et al. 1992).
- Problems arise for fumigation and extraction in very compressed soils that cannot be dispersed.
- Young living root cells are also affected by $CHCl_3$ fumigation. Consequently, in soils containing large amounts of living roots, a pre-extraction procedure must be carried out (Mueller et al. 1992).
- In substrates containing more than 20% organic matter, e.g., compost, the ratio extractant-to-soil should be increased to 25:1 or more (Joergensen et al. 1997).

14.3 Biomass C

Introduction

Objectives. Very low concentrations of organic C can be measured in 0.5 M K_2SO_4 soil extracts of fumigated and non-fumigated soil samples for the quantitative determination of soil microbial biomass C.

14.3.1 Biomass C by Dichromate Oxidation

Principle. Organic C in the extracts is oxidized by dichromate digestion. The amount of dichromate left is determined after redox titration by the change in color from violet to dark green.

Theory. In the presence of a strong acid and dichromate, organic matter is oxidized and Cr(+VI) reduced to Cr(+III). The amount of dichromate left is back-titrated with an iron II ammonium sulfate complex solution (Kalembasa and Jenkinson 1973) and the amount of C oxidized is calculated.

Equipment

- Condenser
- 250-mL round-bottom flask
- Burette

Reagents

- $K_2Cr_2O_7$ solution (66.7 mM = 0.4 N)
- Digestion mixture: Mix two parts conc. H_2SO_4 with one part conc. H_3PO_4 (v/v)
- Indicator solution: 0.1% Aldrich (Milwaukee) ferroin solution (1,10-phenanthroline-iron II sulfate complex)
- Titration solution: 40 mM iron II ammonium sulfate $[(NH_4)_2Fe(SO_4)_2 \times 6H_2O]$ solution dissolved in distilled water, acidified with 20 mL conc. H_2SO_4, and made up to 1 L with distilled water

Sample Preparation

Use soil extract prepared as described in Sect. 14.2.

Procedure

1. Add 2 mL of $K_2Cr_2O_7$ solution and 15 mL of the digestion mixture to 8 mL soil extract in a 250-mL round-bottom flask.
2. Reflux the mixture gently for 30 min, allow to cool, and dilute with 20–25 mL water, added through the condenser as a rinse.
3. Back-titrate excess dichromate with titration solution after adding 5 drops of indicator solution to the digested soil extract.

Calculation

1. Calculation of extractable organic C

$$\text{C}\,(\mu\text{g/g soil}) = \frac{(HB - S) \times N \times E \times VD \times (VK + SW) \times 1000}{CB \times VS \times DM} \tag{14.1}$$

HB consumption of titration solution by the hot (refluxed) blank (mL)

S consumption of titration solution by the sample (mL)

N normality of the $K_2Cr_2O_7$ solution

E 3; conversion of Cr(+VI) to Cr(+III) assuming all organic C on average as [C(0)]

VD added volume of the $K_2Cr_2O_7$ solution (mL)

VS added volume of the sample (mL)

VK volume of K_2SO_4 extractant (mL)

CB consumption of titration solution by the cold (unrefluxed) blank (mL)

SW total amount of water in the soil sample (mL)

DM total mass of dry soil sample (g)

2. Calculation of microbial biomass C

$$\text{Biomass C} = E_C / k_{EC} \tag{14.2}$$

E_C (organic C extracted from fumigated soils) − (organic C extracted non-fumigated soils)

k_{EC} 0.38 (Vance et al. 1987)

Notes and Points to Watch

- Be careful when working with $K_2Cr_2O_7$!
- It is impossible to measure organic C with $K_2Cr_2O_7$ in the presence of high chloride concentrations.

14.3.2 Biomass C by UV-Persulfate Oxidation

Principle. After removal of inorganic C by acidification, organic C in the extracts is oxidized by UV light at 210–260 nm in the presence of $K_2S_2O_8$ to CO_2, which is measured using an infrared absorption detector.

Theory. The part of the microbial biomass rendered extractable after $CHCl_3$ fumigation is easily decomposable. For this reason, it is completely oxidized to CO_2 by UV-light in the presence of $K_2S_2O_8$. Infrared strongly absorbs CO_2.

Equipment

- Automatic carbon analyzer with IR-detection (e.g., Dohrman DC 80, Tekmar-Dohrmann, Cincinnati)

Reagents

- Acidification buffer: 50 g sodium hexametaphosphate [$((NaPO_4)_6)n$] dissolved in 900 mL distilled water, acidified to pH 2 with conc. H_3PO_4 and made up to 1 L
- Oxidation reagent: 20 g $K_2S_2O_8$ dissolved in 900 mL distilled water, acidified to pH 2 with conc. H_3PO_4 and made up to 1 L

Sample Preparation

Use soil extract prepared as described in Sect. 14.2.

Procedure

For the automated UV-persulfate oxidation method, mix 5 mL K_2SO_4 soil extract with 5 mL acidification buffer. Any precipitate of $CaSO_4$ in the soil extracts is dissolved by this procedure. The oxidation reagent is automatically fed into the UV oxidation chamber, where the oxidation to CO_2 is activated by UV light. The resulting CO_2 is measured by IR absorption. The IR detectors of Dimatec (Essen, Germany), for example, use a wavelength of 4.45 µm (80 nm width) with 3.95 µm as reference.

Calculation

1. Calculation of extractable organic C

$$\text{C}\,(\mu\text{g/g soil}) = \frac{[(S \times DS) - (B \times DB)] \times (VK + SW)}{DM} \tag{14.3}$$

S C in sample extract (µg/mL)
B C in blank extract (µg/mL)
DS dilution of sample with the acidification buffer
DB dilution of blank with the acidification buffer
VK volume of K_2SO_4 extractant (mL)
SW total amount of water in the soil sample (mL)
DM total mass of dry soil sample (g)

2. Calculation of microbial biomass C

$$\text{Biomass C} = E_C / k_{EC} \quad (14.4)$$

E_C (organic C extracted from fumigated soils) – (organic C extracted non-fumigated soils)

k_{EC} 0.45 (Wu et al. 1990; Joergensen 1996a)

Notes and Points to Watch

- It is impossible to measure organic C with the UV-persulphate oxidation method in the presence of high chloride concentrations because chloride absorbs a large amount of energy in the UV range.

14.3.3 Biomass C by Oven Oxidation

Principle. After removal of inorganic C by acidification, organic C in the extracts is oxidized at 850 °C in the presence of platinum catalyzer to CO_2, which is measured using an infrared absorption detector.

Theory. Easily decomposable material as the part of the soil microbial biomass extractable after $CHCl_3$ fumigation is completely oxidized at 850 °C in the presence of platinum catalyzer. Infrared strongly absorbs CO_2. The new auto analyzers with oven systems [Shimadzu 5050 (Shimadzu, Kyoto), Dimatoc 100 (Dimatec, Essen), multi N/C 2100 S (Analytik Jena, Jena, Germany), Maihack Tocor 4, Tocor 200 (SICK, Düsseldorf)] use small sample volumes so that they are able to measure C in extracts containing large amounts of salts (Joergensen and Olfs 1998).

Equipment

Automatic carbon analyzer with oven systems (for example Shimadzu 5050, Dimatoc 100, Dimatoc 2000, Analytik Jena multi N/C 2100 S; Analytik Jena multi N/C 3100), see manuals for detailed description.

Sample Preparation

Use soil extract prepared as described in Sect. 14.2.

Procedure

Dilute the samples to fit the calibration line and acidify using a few drops of HCl.

Calculation

See Sect. 14.3.1.

14.4 Biomass N

Introduction

Objectives. Low concentrations of ninhydrin-reactive N or total N can be measured in 0.5 M K_2SO_4 soil extracts of fumigated and non-fumigated soil samples without or with a digestion or oxidation step for the quantitative determination of soil microbial biomass N.

14.4.1 Ninhydrin-Reactive Nitrogen

Principle. The amount of ninhydrin-reactive compounds released from the microbial biomass during the $CHCl_3$ fumigation and extraction by 0.5 M K_2SO_4 is closely correlated to the initial soil microbial biomass C and biomass N content (Joergensen and Brookes 1991).

Theory. Ninhydrin forms a purple complex with molecules containing α-amino nitrogen and with NH_4^+ and other compounds with free α-amino groups such as amino acids, peptides, and proteins (Moore and Stein 1948). The presence of reduced ninhydrin (hydrindantin) is essential to obtain quantitative color development with NH_4^+.

Equipment

- Boiling water bath
- Spectrophotometer

Reagents

- Lithium acetate buffer (4 M, pH 5.2): 408 g lithium acetate dihydrate (for amino acid analysis) dissolved in water (400 mL), adjusted to pH 5.2 with 96% acetic acid, and finally made up to 1 L with water
- Citric acid buffer: citric acid monohydrate (42 g) and NaOH (16 g) dissolved in water (900 mL), adjusted to pH 5 with 10 M NaOH if required, then finally made up to 1 L with water

- Ninhydrin reagent: 2 g ninhydrin and 0.3 g hydrindantin dihydrate dissolved (for amino acid analysis) in 75 mL dimethylsulfoxide (DMSO), 25 mL of 4 M lithium acetate buffer then added (Moore 1968)
- Ethanol/water mixture (1 + 1, v/v)
- Standard solutions: 10 mM L-leucine prepared in 0.5 M K_2SO_4 and diluted within the range 0–1,000 µM

Sample Preparation

Use soil extract prepared as described in Sect. 14.2.

Procedure

1. Add 0.6 mL of standard solutions, K_2SO_4 soil extracts or blank, and 1.4 mL of citric acid buffer to 20 mL test tubes (Joergensen and Brookes 1990).
2. Add 1 mL of ninhydrin reagent slowly, mix thoroughly, and close with loose aluminum lids.
3. Heat the test tubes for 25 min in a vigorously boiling water bath; any precipitate formed during the addition of the reagents then dissolves.
4. After heating, add 4 mL of the ethanol-to-water mixture, mix the solutions thoroughly, and read the absorbance at 570 nm.

Calculation

1. Calculation of extracted ninhydrin-reactive N (N_{nin})

$$N_{nin}\,(\mu g/g\ soil) = \frac{(S - B) \times N \times (VK + SW)}{L \times DM} \tag{14.5}$$

S absorbance of the sample
B absorbance of the blank
N atomic mass of nitrogen (14)
VK volume of K_2SO_4 extractant (mL)
SW total amount of water in the soil sample (mL)
L millimolar absorbance coefficient of leucine
DM total mass of dry soil sample (g)

2. Calculation of microbial ninhydrin-reactive N

$$B_{nin} = \left(N_{nin} \text{ extracted from the fumigated soil}\right) - \left(N_{nin} \text{ extracted from the non-fumigated soil}\right) \quad (14.6)$$

3. Calculation of microbial biomass C

Biomass C = $B_{nin} \times 22$

(for soils with a $pH_{(H_2O)}$ above 5.0; Joergensen 1996b)

Biomass C = $B_{nin} \times 35$

(for soils with a $pH_{(H_2O)}$ of or below 5.0; Joergensen 1996b)

Notes and Points to Watch

- A reflux digestion is not required for ninhydrin N. This makes it very suitable for situations with minimal laboratory facilities.
- In both biomass C and N measurements the fraction coming from the biomass is determined following subtraction of an appropriate "control." With biomass C this value is often half of the total, while with biomass ninhydrin N it is commonly about 10% or less. This causes considerably less error in its determination.
- At 100 °C the reaction with free amino groups of proteins and amino acids is essentially complete within 15 min (e.g., leucine reaches the maximum optical density after approximately 5 min). However the reaction of hydrindantin with NH_4^+ requires 25 min.
- The ratio between the volume of the sample and that of citric acid should not be closer than 0.75:1.75 to avoid the formation of a precipitate after the addition of the ninhydrin reagent.
- The most common solvent in the ninhydrin method is 2-methoxyethanol (Amato and Ladd 1998). However, because it is an ether it tends to form peroxides that destroy ninhydrin and hydrindantin. Dimethylsulfoxide (DMSO) is peroxide free, has lower toxicity and a higher boiling point (189 °C), and gives a more stable color development than 2-methoxyethanol.
- The ninhydrin method proposed by Amato and Ladd (1988) for 2 M KCl extracts does not require the use of citric acid buffer. The optimum reagent-to-sample ratio is 1:2.

14.4.2 Total Nitrogen

Principle. Total nitrogen is measured under strong acidic conditions by Kjeldahl digestion. Ammonium can be measured by distillation (see Chapt. 16).

Theory. Ammonium is released from amines, peptides and amino acids in 0.5 M K_2SO_4 soil extracts of fumigated and non-fumigated soil samples. Nitrate is additionally reduced to ammonium under strong acidic conditions in the presence of $KCr(SO_4)_2$, Zn powder, and $CuSO_4$ as reducing agents.

Equipment

- Digestion block
- Steam distillation apparatus
- Burette or autotitrator

Reagents

- Reducing agent: 50 g of chromium(III) potassium sulfate dodecahydrate ($KCr(SO_4)_2 \times 12H_2O$) dissolved in approx. 700 mL deionized water, and after adding 200 mL conc. H_2SO_4, cooled and diluted to 1,000 mL
- Zn powder
- $CuSO_4$ solution (0.19 M)
- Conc. H_2SO_4
- 10 M NaOH
- 2% H_3BO_3
- 10 µM HCl

Sample Preparation

Use soil extract prepared as described in Sect. 14.2.

Procedure

1. Add 10 mL of the reducing agent and approx. 300 mg Zn powder to 30 mL of the K_2SO_4 soil extract and leave for at least 2 h at room temperature.
2. Add 0.6 mL of $CuSO_4$ solution, 8 mL of conc. H_2SO_4, heat gently for 2 h until all the water has disappeared, and then heat for 3 h at the maximum temperature.

3. Allow the digest to cool before distillation with 40 mL 10 M NaOH. The evolved NH_3 is adsorbed in 2% H_3BO_3.
4. Titrate the resulting solution with 10 µM HCl to pH 4.8.

Calculation

1. Calculation of extractable total N

$$\text{N}\,(\mu\text{g/g soil}) = \frac{(S - B) \times M \times N \times (VK + SW)}{A \times DM} \tag{14.7}$$

S HCl consumed by sample extract (µL)
B HCl consumed by blank extract (µL)
M molarity of HCl
N molecular mass of nitrogen (14)
VK volume of K_2SO_4 extractant (mL)
SW total amount of water in the soil sample (mL)
A sample aliquot (mL)
DM total mass of dry soil sample (g)

2. Calculation of microbial biomass N

$$\text{Biomass N} = E_N/k_{EN} \tag{14.8}$$

E_N (total N extracted from fumigated soils) – (total N extracted non-fumigated soils)
k_{EN} 0.54 (Brookes et al. 1985; Joergensen and Mueller 1996)

Notes and Points to Watch

- A method is available in which the extracted total N is oxidized to NO_3^-, which is then determined colorimetrically (Cabrera and Beare 1993).
- If losses of NO_3^- occur during the fumigation period, they can be corrected by considering the difference between the NO_3^- extracted initially and the NO_3^- extracted at the end of the fumigation period (Brookes et al. 1985).
- If (non-fumigated) soil samples contain large amounts of NO_3^- or NH_4^+ in the soil solution, a pre-extraction step should be carried out (Widmer et al. 1989; Mueller et al. 1992; Joergensen et al. 1995).

References

Alef K, Nannipieri P (1995) Methods in Applied Soil Microbiology and Biochemistry. Academic Press, London

Amato M, Ladd JN (1988) Assay for microbial biomass based on ninhydrin-reactive nitrogen in extracts of fumigated soils. Soil Biol Biochem 20:107–114

Barajas Aceves M, Grace C, Ansorena J, Dendooven L, Brookes PC (1999) Soil microbial biomass and organic C in a gradient of zinc concentrations around a spoil tip mine. Soil Biol Biochem 31:867–876

Brookes PC (1995) The use of microbial parameters in monitoring soil pollution by heavy metals. Biol Fertil Soils 19:269–279

Brookes PC, Landman A, Pruden G, Jenkinson DS (1985) Chloroform fumigation and the release of soil nitrogen: A rapid direct extraction method for measuring microbial biomass nitrogen in soil. Soil Biol Biochem 17:837–842

Brookes PC, McGrath SP (1984) The effects of metal toxicity on the soil microbial biomass. J Soil Sci 35:341–346

Cabrera ML, Beare MH (1993) Alkaline persulfate oxidation for determining total nitrogen in microbial biomass extracts. Soil Sci Soc Am J 57:1007–1012

Castro J, Sanchez-Brunete C, Rodriguez JA, Tadeo JL (2002) Persistence of chlorpyrifos and endosulfan in soil. Fres Environ Bull 11:578–582

Daniel O, Anderson JM (1992) Microbial biomass and activity in contrasting soil materials after passage through the gut of the earthworm *Lumbricus rubellus* Hoffmeister. Soil Biol Biochem 24:465–470

DeLuca TH, Keeney DR (1993) Ethanol-stabilized chloroform as fumigant for estimating microbial biomass by reaction with ninhydrin. Soil Biol Biochem 25:1297–1298

Franco I, Contin M, Bragato G, De Nobili M (2004) Microbiological resilience of soils contaminated with crude oil. Geoderma 121:17–30

Harden T, Joergensen RG, Meyer B, Wolters V (1993) Mineralization of straw and formation of soil microbial biomass in a soil treated with simazine and dinoterb. Soil Biol Biochem 25:1273–1276

Jenkinson DS, Powlson DS (1976) The effects of biocidal treatments on metabolism in soil – I. Fumigation with chloroform. Soil Biol Biochem 8:167–177

Joergensen RG (1995) The fumigation-extraction method to estimate soil microbial biomass: Extraction with 0.01 m $CaCl_2$. Agribiol Res 48:319–324

Joergensen RG (1996a) The fumigation-extraction method to estimate soil microbial biomass: Calibration of the k_{EC} value. Soil Biol Biochem 28:25–31

Joergensen RG (1996b) Quantification of the microbial biomass by determining ninhydrin-reactive N. Soil Biol Biochem 28:301–306

Joergensen RG, Brookes PC (1990) Ninhydrin-reactive nitrogen measurements of microbial biomass in 0.5 M K_2SO_4 soil extracts. Soil Biol Biochem 22:1023–1027

Joergensen RG, Figge RM, Kupsch L (1997) Microbial decomposition of fuel oil after compost addition to soil. Z Pflanzenernähr Bodenk 160:21–24

Joergensen RG, Mueller T (1996) The fumigation-extraction method to estimate soil microbial biomass: Calibration of the k_{EN} value. Soil Biol Biochem 28:33–37

Joergensen RG, Olfs HW (1998) The variability between different analytical procedures and laboratories for measuring soil microbial biomass C and biomass N by the fumigation extraction method. Z Pflanzenernähr Bodenk 161:51–58

Joergensen, RG, Schmaedeke F, Windhorst K, Meyer B (1994a) Biomasse und Aktivität von Mikroorganismen eines mineralölkontaminierten Bodens. In: Alef K, Fiedler H, Hutzinger O (eds) Band 6: Bodenkontamination, Bodensanierung, Bodeninformationssysteme. Eco-Informa'94, Umweltbundesamt/Wien, pp 225–236

Joergensen RG, Schmaedeke F, Windhorst K, Meyer B (1994b) Die Messung der mikrobiellen Biomasse während der Sanierung eines mit Dieselöl kontaminierten Bodens. VDLUFA-Schriftenr 38:557–560

Joergensen RG, Schmaedeke F, Windhorst K, Meyer B (1995) Biomass and activity of microorganisms in a fuel oil contaminated soil. Soil Biol Biochem 27:1137–1143

Kalembasa SJ, Jenkinson DS (1973) A comparative study of titrimetric and gravimetric methods for the determination of organic carbon in soil. J Sci Food Agric 24:1085–1090

Moore S (1968) Amino acid analysis: Aqueous dimethyl sulfoxide as solvent for the ninhydrin reaction. J Biol Chem 243:6281–6283

Moore S, Stein WH (1948) Photometric ninhydrin method for use in the chromatography of amino acids. J Biol Chem 176:367–388

Mueller T, Joergensen RG, Meyer B (1992) Estimation of soil microbial biomass C in the presence of living roots by fumigation-extraction. Soil Biol Biochem 24:179–181

Ocio JA, Brookes PC (1990) Soil microbial biomass measurements in sieved and unsieved soils. Soil Biol Biochem 22:999–1000

Plante AF, Voroney RP (1998) Decomposition of land applied oily food waste and associated changes in soil aggregate stability. J Environm Qual 27:395–402

Powlson DS, Brookes PC, Christensen BT (1987) Measurement of soil microbial biomass provides an early indication of changes in total soil organic matter due to straw incorporation. Soil Biol Biochem 19:159–164

Powlson DS, Jenkinson DS (1976) The effects of biocidal treatments on metabolism in soil. II gamma irradiation, autoclaving, air-drying and fumigation. Soil Biol Biochem 8:179–188

Vance ED, Brookes PC, Jenkinson DS (1987) An extraction method for measuring soil microbial C. Soil Biol Biochem 19:703–708

Widmer P, Brookes PC, Parry LC (1989) Microbial biomass nitrogen measurements in soils containing large amounts of inorganic nitrogen. Soil Biol Biochem 21:865–867

Wu J, Joergensen RG, Pommerening B, Chaussod R, Brookes PC (1990) Measurement of soil microbial biomass C – an automated procedure. Soil Biol Biochem 22:1167–1169

Wu J, O'Donnell AG, Syers JK (1993) Microbial growth and sulphur immobilization following the incorporation of plant residues into soil. Soil Biol Biochem 25:1567–1573

15 Determination of Adenylates and Adenylate Energy Charge

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Introduction

Objectives. The determination of adenosine-triphosphate (ATP) extracted from soil was introduced a long time ago as an estimate of the soil microbial biomass (Oades and Jenkinson 1979). After a conditioning pre-incubation, close linear relationships exist between ATP and microbial biomass C determined either by the fumigation incubation technique (Jenkinson 1988) or by the fumigation extraction method (Chapt. 14; Contin et al. 2001; Dyckmans et al. 2003). A similar close linear relationship exists also between microbial biomass C and the sum of all three adenylates AMP, ADP, and ATP (Dyckmans et al. 2003). The determination of adenylates is the quickest way of estimating microbial biomass, because 24-h incubation periods or manipulations such as substrate addition are not required as in the fumigation extraction or the substrate induced respiration methods, respectively. The measurement of adenylates by high-performance liquid chromatography (HPLC) has been repeatedly used to monitor the effects of heavy metal contamination (Chander et al. 2001) and salinization (Sardinha et al. 2003), but no information is available regarding fuel oil contaminated soil. However, enzymatic ATP has been successfully used to monitor microbial activity during fuel oil decomposition, although some quenching of the bioluminescence by fuel oil residues occurred (Wen et al. 2003).

An important index for the energetic state of the soil microbial community is the adenylate energy charge (AEC), which was defined by Atkinson and Walton (1967) as follows:

$$\left(\mathrm{ATP} + 0.5 \times \mathrm{ADP}\right) / \left(\mathrm{ATP} + \mathrm{ADP} + \mathrm{AMP}\right)$$

High AEC values (> 0.7) have frequently been described in soils (Brookes et al. 1987; Brookes 1995; Chander et al. 2001; Dyckmans et al. 2003). Low AEC values have been demonstrated under drought stress conditions (Raubuch et al. 2002), but also in Cu contaminated soils (Chander et al. 2001) and in acidic saline soils (Sardinha et al. 2003).

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Principle. Soil adenylates (AMP + ADP + ATP) are extracted with dimethylsulfoxide (DMSO) under strong alkaline conditions in combination with an ethylene-diamine-tetraacetic acid (EDTA)-containing phosphate buffer. DMSO destroys microbial cells, the phosphate buffer completely prevents the adsorption of adenylates under the strong alkaline conditions, and EDTA promotes the irreversible inactivation of ATP-converting enzymes.

Theory. ATP is rapidly destroyed outside living cells and can be used as an estimate for the soil microbial biomass assuming a constant ATP-to-microbial biomass ratio, which is fairly true in the absence of living plant roots and after a conditioning pre-incubation (Jenkinson 1988). The ATP-to-microbial biomass C ratio is affected by drought (Raubuch et al. 2002), temperature (Joergensen and Raubuch 2003), and N limitation (Joergensen and Raubuch 2002). However, the main problems in measuring ATP in soils are (1) the enzymatic breakdown of ATP after cell death and (2) adsorption of ATP to clay minerals during extraction (Martens 2001). The alkaline DMSO-EDTA-phosphate-buffer extractant solved nearly all methodological problems reported earlier (Bai et al. 1988; Martens 1992). This is especially true in combination with HPLC analysis after derivatization with chloroacetaldehyde to form the fluorescent 1-N^6-etheno-derivatives (ε-adenylates), which are highly selective for fluorometric determination (Bai et al. 1989; Dyckmans and Raubuch 1997).

Equipment

- Multipoint magnetic stirrer
- Ultrasonic bath
- Evacuation units and filters (0.45-µm cellulose nitrate membrane filters)
- Heating water bath
- Test tube stirrer
- Glassware: 100-mL glass beaker (tall form), 20-mL test tubes
- Pipettes
- HPLC equipment: automatic injector, isocratic precision pump, column oven, solvent delivery system, fluorescence detector and recording unit
- Analytical column (250 × 4.6 mm; 5 µm ODS Hypersil, Thermo Electron Corp., Waltham, MA, USA) with guard column (10 × 4.0 mm, 5 µm ODS Hypersil)

Reagents

- DMSO
- Extraction buffer: 20 mM EDTA dissolved in 10 mM $Na_3PO_4 \times 12H_2O$ containing 0.1 M KOH at pH 12
- Tris buffer: 2 mM EDTA dissolved in 10 mM ammonium acetate/20 mM Tris(hydroxymethyl)-aminomethane, adjusted to pH 7.75 with acetic acid (store at 4 °C)
- Adenylate releasing reagent: 0.05 mL benzalkonium chloride solution (ca. 50% in water, Fluka, Fluka AG, Buchs, Switzerland, purum grade) added to 49.95 mL Tris buffer (store at 4 °C)
- 0.1 M KH_2PO_4
- Chloroacetaldehyde
- TBAHS buffer: 50 mM ammonium acetate, 1 mM EDTA, 0.4 mm tetra-*n*-butylammonium hydrogen sulfate (TBAHS, LiChropur, Merck KGaA, Darmstadt, Germany)
- Mobile phase for HPLC: TBAHS buffer mixed with methanol at a ratio of 89.5 to 10.5 (v/v)
- Calibration stock solution I (100 µg/mL): 14.35 mg AMP-$Na_2 \times 6H_2O$, 11.59 mg ADP-$K_2 \times 2H_2O$, or 11.90 mg ATP-$Na_2 \times 3H_2O$; each dissolved in 100 mL extraction buffer (store at 4 °C)
- Calibration stock solution II (1 µg/mL): 1/100 dilution of stock solution I (store at 4 °C)
- Working standard solutions: a set of four standards each containing 2, 4, 6, 8 ng of AMP, ADP, ATP, respectively, prepared by mixing 100–400 µL stock solution II with 0.2 mL chloroacetaldehyde and adding 0.01 M $Na_2HPO_4 \times 2H_2O$ to give a final volume of 10 mL, heated for 3 min at 85 °C, and cooled in an ice bath (store at 4 °C for maximum 7 days)

Sample Preparation

Use moist sample equivalent to 1–5 g oven-dry soil, sieved (< 2 mm). The experimental design reflects the fact that adenylate content responds to actual conditions, is influenced by mechanical disturbance, water content, and temperature.

Procedure

1. Weigh moist soil equivalent to 1–5 g oven-dry soil into a 100-mL glass beaker (tall form).
2. Add 4 mL DMSO and stir for 2 min on a magnetic stirrer using a magnetic stirring bar.
3. Add 16 mL extraction buffer and stir again for 2 min.
4. Sonify for 2 min in an ultrasonic bath.
5. Mix an aliquot of 0.5 mL of soil suspension with 0.5 mL of adenylate releasing reagent in a 20 mL test tube, mix using a test tube stirrer, and sonify for another 5 s.
6. Pass the suspension through a membrane filter (0.45 µm) and wash the soil residue twice with 1 mL 0.1 M KH_2PO_4.
7. Add 0.2 mL chloroacetaldehyde and make up to a final volume of 5 mL by addition of 0.1 M KH_2PO_4.
8. Incubate in a water bath for 30 min at 85 °C to yield the fluorescent 1-N^6-etheno-derivatives and cool afterward in an ice bath.
9. Store at 4 °C for a maximum 7 days before HPLC measurements.
10. Adjust the column oven to 27 °C.
11. Run HPLC with the mobile phase at 2 mL/min for 3 h for equilibration of the column.
12. Use a sample loop of 200 µL.
13. Fluorometric emission is measured at 410 nm with 280 nm as excitation wavelength.
14. Clean the HPLC after measurement for 30 min at 1 mL/min with a methanol/water (50:50 v/v) solution.
15. Treat calibration standards like soil extractants to prepare calibration curves.
16. Standard solutions correspond to concentrations 2 ng, 4 ng, 6 ng, 8 ng of AMP, ADP and ATP in 200 µL, respectively.
17. There is a linear relationship in adenylate content and signal response up to 8 ng of each adenylate. The adenylates are detected on the chromatogram in the order AMP, ADP, and ATP.

Calculation

1. Identify AMP, ADP and ATP by retention time according to the retention time of the standards.
2. Calculate nanograms from areas and linear equation of standards.
3. Take dilution into account (analogous for ADP and AMP).

$$\text{ATP (ng/gsoil)} = \frac{H \times (E + SW) \times I}{A \times DM}$$

H ATP in 200 µL injection volume (ng)

E extractant (4 mL DMSO + 16 mL extraction buffer; mL)

I 25; conversion factor of the injection volume (200 µL from 5 mL)

SW total amount of water in the soil sample (mL)

A aliquot (0.5 mL)

DM total mass of dry soil sample (g)

4. Molecular masses for conversion into nmol:

   AMP = 347.2 g, ADP = 427.2 g, ATP = 507.2 g

5. Total adenylate content (nmol/g soil) = AMP + ADP + ATP
6. Adenylate Energy Charge (AEC)
   = (ATP + 0.5 ADP)/(AMP + ADP + ATP)

Notes and Points to Watch

- The mobile phase must be degassed in advance. Oxygen disturbs the measurement, especially of ATP.
- The retention time must be checked before the first measurement with a standard mixture of AMP, ADP, and ATP standards, but do not use a standard mixture of AMP, ADP and ATP for calibration. ATP contains impurities of AMP and ADP, ADP contains impurities of AMP.
- The column temperature should be constant at 27 °C. The separation of ε-AMP, ε-ADP and ε-ATP from extracted impurities is improved at 27 °C. Changing temperatures causes shifts in the retention times.

References

Atkinson DE, Walton GM (1967) Adenosine triphosphate conservation in metabolic regulation. Rat liver cleavage enzyme. J Biol Chem 242:3239–3241

Bai QY, Zelles L, Scheunert I, Korte F (1988) A simple procedure for the determination of adenosine triphosphate in soils. Chemosphere 17:2461–2470

Bai QY, Zelles L, Scheunert I, Korte F (1989) Determination of adenine nucleotides in soil by ion-paired reverse-phase high-performance liquid chromatography. J Microbiol Meth 9:345–351

Brookes PC (1995) Estimation of the adenylate energy charge in soils. In: Alef K, Nannipieri P (eds) Methods in Applied Soil Microbiology and Biochemistry. Academic Press, London, pp 204–213

Brookes PC, Newcombe AD, Jenkinson DS (1987) Adenylate energy charge measurements in soil. Soil Biol Biochem 19:211–217

Chander KC, Dyckmans J, Joergensen RG, Meyer B, Raubuch M (2001) Different sources of heavy metals and their long-term effects on soil microbial properties. Biol Fertil Soils 34:241–247

Contin M, Todd A, Brookes PC (2001) The ATP concentration in the soil microbial biomass. Soil Biol Biochem 33:701–704

Dyckmans J, Chander K, Joergensen RG, Priess J, Raubuch M, Sehy U (2003) Adenylates as an estimate of microbial biomass C in different soil groups. Soil Biol Biochem 35:1485–1491

Dyckmans J, Raubuch M (1997) A modification of a method to determine adenosine nucleotides in forest organic layers and mineral soils by ion-paired reversed-phase high-performance liquid chromatography. J Microbiol Meth 30:13–20

Jenkinson DS (1988) The determination of microbial biomass carbon and nitrogen in soil. In: Wilson JR (ed) Advances in nitrogen cycling in agricultural ecosystems. CABI, Wallingford, pp 368–386

Joergensen RG, Raubuch M (2002) Adenylate energy charge of a glucose-treated soil without adding a nitrogen source. Soil Biol Biochem 34:1317–1324

Joergensen RG, Raubuch M (2003) Adenylate in the soil microbial biomass at different temperatures. Soil Biol Biochem 35:1063–1069

Martens R (1992) A comparison of soil adenine nucleotide measurements by HPLC and enzymatic analysis. Soil Biol Biochem 24:639–645

Martens R (2001) Estimation of ATP in soil: extraction methods and calculation of extraction efficiency. Soil Biol Biochem 33:973–982

Oades JM, Jenkinson DS (1979) Adenosine triphosphate content of the soil microbial biomass. Soil Biol Biochem 11:193–199

Raubuch M, Dyckmans J, Joergensen RG, Kreutzfeldt M (2002) Relation between respiration, ATP content and adenylate energy charge (AEC) after incubation at different temperatures and after drying and rewetting. J Plant Nutr Soil Sci 165:435–440

Sardinha M, Müller T, Schmeisky H, Joergensen RG (2003) Microbial performance in a temperate floodplain soil along a salinity gradient. Appl Soil Ecol 23:237–244

Wen G, Voroney RP, McGonigle TP, Inanaga S (2003) Can ATP be measured in soils treated with industrial oily waste? J Plant Nutr Soil Sci 166:724–730

16 Determination of Aerobic N-Mineralization

Rainer Georg Joergensen

Introduction

Objectives. N mineralization is the transformation of organic N into inorganic N components (Beck 1983). It is thus an important biological process of the nitrogen cycle in ecosystems, reflecting the ability of a soil to provide available nitrogen to plants (Alef 1995). In terrestrial ecosystems, especially in arable crop production, N is often the most limiting nutrient for plant growth. The nitrogen availability to soil microorganisms often limits the decomposition of fuel oil in contaminated soils (Joergensen et al. 1995). N mineralization mainly depends on temperature, moisture, aeration, type of organic N, and pH. NH_4^+ is subject to fixation by clays. NO_3^- can be lost through denitrification and leaching (Alef 1995).

Principle. A soil is incubated aerobically after removal of plant debris for two periods. The soil is extracted with 2 M KCl before and after each of the two incubation periods. In the soil extracts, NH_4^+, NO_3^-, and, if necessary, NO_2^- are measured.

Theory. N mineralization is the catabolic use of N-containing organic components, e.g., amino acids, amino sugars, amines, and nucleic acids derived from plant and animal debris as well as from soil organic matter to meet the energy demand of the soil microbial biomass. The N mineralization process can be divided into two steps (Alef 1995): (1) the first step is ammonification, which is the breakdown of organic NH_2 groups to NH_4^+. Except for the hydrolysis of urea by extracellular urease, ammonification is carried out by proteases bound to cell membranes of all heterotrophic microorganisms in soil, i.e. more than 95% of the soil microbial community. (2) The second step is nitrification, which is carried out by heterotrophic fungi in acidic soils or obligatory aerobic chemoautotrophic bacteria [e.g., *Nitrosomonas*: $NH_4^+ \rightarrow NO_2^-$ ($G0 = -273.9$ kJ/mol) and *Nitrobacter* $NO_2^- \rightarrow NO_3^-$ ($G0 = -76.7$ kJ/mol) under neutral and slightly alkaline soil conditions. Nitrification is inhibited by fuel oil contamination in contrast to ammonification (Joergensen et al. 1995). In compacted soils,

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N mineralization is more strongly affected than CO_2 production (Nieder et al. 1993; Ahl et al. 1998).

Equipment

- 100 mL low-density, wide-neck polyethylene (PE) bottles
- Funnels
- 50-mL Erlenmeyer flasks
- Horizontal or overhead shaker
- Folded filter papers (e.g., Whatman 42 or Schleicher & Schuell 595 1/2)
- Room or incubator at 25 °C
- Steam distillation apparatus
- Burette or autotitrator

Reagents

- 2 M KCl solution
- 2% H_3BO_3 (extra pure)
- 10 µM HCl
- MgO (extra pure)
- Devarda alloy

Sample Preparation

Use field-moist, sieved (between < 2 and < 5 mm) soil at approx. 40–50% water holding capacity.

Procedure

1. Weigh 15 g field moist soil into nine PE bottles.
2. Add 5 mL of water slowly.
3. Incubate at 25 °C in the dark.
4. Remove three replicates after 0, 14, and 28 days.
5. Extract with 60 mL 2 M KCl (extractant-to-soil ratio of 4:1) for 30 min by oscillating shaking at 200 rpm (or 45 min overhead shaking at 40 rpm).

6. Filter through a folded filter paper.
7. Pipette a 30 mL aliquot into the sample flask of the distillation apparatus.
8. Add approx. 200 mg MgO rapidly to volatize NH_4^+ as NH_3 under alkaline conditions.
9. Stop the first distillation when the distillate reaches the 30 mL mark on the receiver flask (a 50 mL Erlenmeyer flask containing 5 mL 2% H_3BO_3).
10. Add approx. 200 mg Devarda alloy rapidly to reduce NO_3^- and NO_2^- to NH_4^+, which is volatilized under the alkaline conditions of the distillation flask.
11. Stop the second distillation when the distillate reaches the 30 mL mark on the receiver flask (a 50-mL Erlenmeyer flask containing 5 mL 2% H_3BO_3).
12. Titrate NH_4^+ in each of the two distillates with 10 µm HCl to pH 4.8.

Calculation

1. Calculation of extractable NH_4^+-N and NO_3^--N

$$\text{N}\,(\mu\text{g/g soil}) = \frac{(S - B) \times M \times N \times (VK + SW)}{A \times DM} \tag{16.1}$$

S HCl consumed by sample extract (µL)
B HCl consumed by blank extract (µL)
M molarity of HCl
N molecular mass of nitrogen (14)
VK volume of K_2SO_4 extractant (mL)
SW total amount of water in the soil sample (mL)
A sample aliquot (mL)
DM total mass of dry soil sample (g)

2. Calculation of net N mineralized

$$\text{N}\,[\mu\text{g N/(g soil} \times \text{day)}] = \frac{(\text{NH}_4^+\text{-N} + \text{NO}_3^-\text{-N})_{t_{d+1}} - (\text{NH}_4^+\text{-N} + \text{NO}_3^-\text{-N})_{t_d}}{n} \tag{16.2}$$

t_d sampling day before the last sampling day (day 0 or day 14)

t_{d+1} last sampling day (day 14 or day 28)

n incubation period (days)

Notes and Points to Watch

- If the N mineralization rate of the first incubation period (0–14 days) does not differ significantly from that of the second incubation period (14–28 days), the average value of both periods should be used (Beck 1983; Kandeler 1993a). If the N mineralization rate of the first incubation period is significantly lower than that of the second incubation period, e.g., due to N immobilization during the decomposition of plant residues, only the value of the second incubation period should be used. If the N mineralization rate of the first incubation period is significantly higher than that of the second incubation period, e.g., due to the increasing recalcitrance of decomposable soil organic matter, only the value of the first incubation period should be used.
- The steam distillation method is especially suitable for colored extracts (Keeney and Nelson 1982; Forster 1995).
- If a soil accumulates NO_2^- in the soil solution, a colorimetric method must be used to determine it (Keeney and Nelson 1982; Forster 1995).
- Colorimetric methods are also available for the manual determination of extractable NO_3^- (e.g., Kandeler 1993a; Forster 1995), and for automated segmented flow or flow injection, analyses are also available (e.g., Kutscha-Lissberg and Prillinger 1982).
- It is possible to estimate the NO_3^- content in soil extracts by the decrease in UV absorbance after reduction of NO_3^- (Kandeler 1993b).
- Colorimetric methods are also available for the manual determination of extractable NH_4^+ (e.g., Keeney and Nelson 1982; Kandeler 1993; Forster 1995), and for automated segmented flow or flow injection, analyses are also available.
- Contamination of chemicals, especially KCl, but also of filter paper, funnel, extraction bottles, and glassware should be avoided and regularly checked.

References

Alef K (1995) Nitrogen mineralization in soils. In: Alef K, Nannipieri P (eds) Methods in Applied Soil Microbiology and Biochemistry. Academic Press, London, pp 234–245

Ahl C, Joergensen RG, Kandeler E, Meyer B, Woehler V (1998) Microbial biomass and activity in silt and sand loams after long-term shallow tillage in central Germany. Soil Till Res 49:93–104

Beck T (1983) Die N-Mineralisation von Böden im Brutversuch. Z Pflanzenernähr Bodenk 146:243–252

Forster JC (1995) Soil nitrogen. In: Alef K, Nannipieri P (eds) Methods in Applied Soil Microbiology and Biochemistry. Academic Press, London, pp 79–87

Joergensen RG, Schmaedeke F, Windhorst K, Meyer B (1995) Biomass and activity of microorganisms in a fuel oil contaminated soil. Soil Biol Biochem 27:1137–1143

Kandeler E (1993a) Bestimmung der N-Mineralisation im aeroben Brutversuch. In Schinner F, Öhlinger R, Kandeler E, Margesin R (eds) Bodenbiologische Arbeitsmethoden, 2nd ed. Springer, Berlin, pp 158–159

Kandeler E (1993b) Bestimmung von Nitrat. In: Schinner F, Öhlinger R, Kandeler E, Margesin R (eds) Bodenbiologische Arbeitsmethoden, 2nd ed. Springer, Berlin, pp 369–371

Keeney DR, Nelson DW (1982) Nitrogen – inorganic forms. In: Page AL, Miller, RH, Keener DR (eds) Methods of Soil Analysis, Part 2. Am Soc Agron, Soil Sci Soc Am, Madison, pp 643–698

Kutscha-Lissberg P, Prillinger F (1982) Rapid determination of EUF-extractable nitrogen and boron. Plant Soil 64:63–66

Nieder R, Scheithauer U, Richter J (1993) Dynamics of nitrogen after deeper tillage in arable loess soils of West Germany. Biol Fertil Soils 16:45–51

17 Determination of Enzyme Activities in Contaminated Soil

Rosa Margesin

17.1 General Introduction

Soil biological activities are sensitive to environmental stress; each change in environmental conditions may result in a shift in the species composition of the soil microflora and modification of their metabolic rate. Soil enzyme activities are attractive as indicators for monitoring various impacts on soil because of their central role in the soil environment. Soil enzymes are the catalysts of important metabolic processes including the decomposition of organic inputs and the detoxification of xenobiotics (Schinner et al. 1996; Dick 1997).

Soil enzyme activities have been used as a biological indicator of pollution with heavy metals, pesticides, and hydrocarbons (Schinner et al. 1993; Sparling 1997; van Beelen and Doelman 1997; Margesin et al. 2000a, 2000b). A number of studies have demonstrated that soil enzymes hold potential for assessing the impact of hydrocarbons and of fertilization on soil microorganisms and are a useful tool to monitor the early stages of remediation of contaminated soil (Margesin et al. 2000a, 2000b). The usefulness of various enzyme parameters depends on the composition and concentration of the hydrocarbons, as well as on other factors such as the age of contamination and physico-chemical soil characteristics. While some enzymes activities are appropriate to monitor the most active phase of biodegradation, others are also indicative of low hydrocarbon concentrations (Margesin et al. 2000a).

A broad spectrum of soil enzyme activities should be used to evaluate the effect of contamination on the different nutrient cycles. In this Chapter, a small selection of methods for the determination of enzyme activities in contaminated soil is described. Detailed descriptions of supplementary methods are given in Schinner et al. (1996). Of course, additional information on the soil biological status should be obtained from complementary methods, such as soil microbial counts (Chap. 13), soil biomass (Chap. 14), molecular biology (Chap. 10), and fatty acid profiles (Chap. 12).

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17.2 Lipase-Esterase Activity

Introduction

Objectives. Soil lipase activity is a valuable tool to monitor the biodegradation of petroleum hydrocarbons, such as diesel oil, in freshly contaminated soil (Margesin et al. 1999, 2002a, b). The residual hydrocarbon content correlates negatively with soil lipase activity in unfertilized as well as in fertilized soil (Margesin and Schinner 2001). Generally, a correlation between soil lipase activity and other biological parameters can be found. Soil lipase activity increases with increasing initial oil loading rates, which demonstrates the induction of this enzyme activity by the contamination. This induction is attributed to the appearance of products released from hydrocarbon biodegradation, which are the substrate for hydrolases including esterases-lipases. A strong relationship between the ability of microorganisms to degrade diesel oil and their lipolytic activity was described by Mills et al. (1978) and Kato et al. (2001). No increase of lipase activity was observed during the biodegradation of polycyclic aromatic hydrocarbons (PAHs) in soil (Margesin et al. 2000a). Assaying lipase activity is also useful to monitor the biodegradation of carboxyl esters such as lipids and biodegradable polyesters in soil (Sakai et al. 2002). The described method can also be applied to determine lipase activity in non-contaminated soil and could be useful for the screening of lipase-producing soil microorganisms.

Principle. Using p-nitrophenyl butyrate (pNPB) as substrate, soil samples are incubated at 30 °C and pH 7.25 for 10 min. After cooling on ice and centrifugation, the released p-nitrophenol (pNP) is determined spectrophotometrically at 400 nm. To allow for the adsorption of pNP onto soil, a calibration curve is prepared in the presence of soil (Margesin et al. 2002).

Theory. A significant proportion of lipids, such as pesticide emulsions, oils, and lipid conjugates, enter soil in the form of triacylglycerols, the primary storage fat in plant and animal tissue. The degradation of lipids is initiated by lipases (glycerol ester hydrolases) acting on the carboxylester bonds present in acylglycerols to liberate fatty acids and glycerol. Lipases are produced by a large variety of microorganisms, plants, and animals.

The standard assays to determine the hydrolytic activity of lipase in soil are based on titration or fluorimetry. Methods that determine the fatty acids produced from tributyrin (Pokorna 1964; Hankin et al. 1982) or Tween 20 (Sakai et al. 2002) titrimetrically are easy to use, however, the disadvantages are the long incubation time (between 18 h and 3 days) and the possible adsorption of the released fatty acids onto soil colloids.

Fluorimetric methods (Pancholy and Lynd 1972; Cooper and Morgan 1981) are more sensitive and specific, but the substrate is relatively expensive. Colorimetric methods are quick and simple. Chromogenic substrates, such as p-nitrophenyl esters, are commonly used to assay microbial esterase and lipase activity (Shirai and Jackson 1982; Plou et al. 1998; Ishimoto et al. 2001; Wei et al. 2003). In soil enzymology, p-nitrophenyl derivatives are widely used as substrates for measuring phosphatases, arylsulfatase, and ß-glucosidase activity. The described method is based on the use of pNPB as substrate for the rapid, precise, and simple measurement of lipase activity in soil.

Equipment

- Centrifuge and centrifuge tubes (2,000 *g*, 2–4 °C)
- Water bath (30 °C)
- Spectrophotometer

Reagents

- Phosphate buffer: 100 mM NaH_2PO_4-NaOH, pH 7.25 (store at 4 °C)
- Substrate: 100 mM p-nitrophenyl butyrate (pNPB) diluted in 2-propanol (store aliquots at −20 °C)
- Calibration standards (p-nitrophenol, pNP)
  - Stock solution: 1 mg pNP/mL buffer (store at 4 °C)
  - Working standard solution: 100 µg pNP/mL buffer (prepare daily fresh)
  - Standards: 0, 25, 50, 75, 100, and 125 µg pNP/5 mL buffer (adjust volumes of 0–1.25 mL of the working standard solution to 5 mL with buffer)

Sample Preparation

Use field-moist, sieved (< 5 mm) soil.

Procedure

1. Weigh 0.1 g of soil into centrifuge tubes, prepare 3–4 replicates (samples).
2. Add 5 mL of buffer, and prewarm at 30 °C in a water bath for 10 min.
3. Prepare a control (3–4 replicates) without soil.
4. Add 50 µL of substrate solution to each tube.

5. Mix the contents and incubate the tubes in a water bath at 30 °C for exactly 10 min.
6. Stop the reaction by cooling the tubes for 10 min on ice.
7. Centrifuge the tubes at 2,000 *g* and 2–4 °C for 5 min.
8. Pipette the supernatants in test tubes that are held on ice.
9. Immediately afterward, measure the absorbance of the released pNP in samples and controls at 400 nm against the reagent blank. Dilute the solution with buffer when absorbance values are too high.

To prepare a calibration curve, treat calibration standards like the soil samples:

1. Weigh 0.1 g of soil into each of six centrifuge tubes.
2. Add 5 mL of standard solution containing 0 (= reagent blank), 25, 50, 75, 100, and 125 µg pNP.
3. Mix and incubate at 30 °C for exactly 10 min.
4. Proceed as described for the soil samples (6–8), and measure the absorbance of the calibration standards at 400 nm against the reagent blank.

Calculation

1. Calculate the pNP concentration from the calibration curve.
2. Subtract the control reading (hydrolysis in absence of soil) from the sample reading (hydrolysis in presence of soil) and express soil lipase activity as "µg of released pNP per gram soil dry mass over 10 min" using the following formula:

$$\text{µg pNP/(g dry soil} \times \text{10 min)} = \frac{S - C}{wm \times dm}$$

S pNP concentration of the soil sample (µg)

C pNP concentration of the control (µg)

wm Soil wet mass (0.1 g)

dm Soil dry mass (g)

Notes and Points to Watch

- To measure the chemical pNP release from the substrate, it is necessary to prepare a control without soil.

- To allow for the adsorption of pNP onto soil, the calibration curve is prepared in the presence of soil.
- It is important to use a neutral pH for this assay since the ester bond in pNPB is very labile and is fully hydrolyzed at alkaline pH. The degree of dissociation of pNP (colorless) into p-nitrophenoxide (yellow) is 0% below pH 5.0, 50% at pH 7.0 and 100% at pH 9.0. Enzyme assays based on p-nitrophenyl derivatives are usually carried out at a pH of 7.25 to 7.3 (Ishimoto et al. 2001; Wei et al. 2003).
- A reaction temperature of 30 °C is chosen to avoid a high rate of non-enzymatic substrate hydrolysis, which occurs at higher temperatures.
- Drying and freeze-thawing of soil may affect soil lipase activity, however, this effect is soil-specific.
- The presence of heavy metals in soil inhibits lipase activity. The metal sensitivity of soil lipases depends on the soil properties.
- Lipase activity determined with the described assay has been found to correlate significantly with titrimetric and fluorimetric assays.

17.3 Fluorescein Diacetate Hydrolytic Activity

Introduction

Objectives. The rate of fluorescein diacetate (FDA) hydrolysis in soil has been considered a suitable index of overall enzyme activity (Schnürer and Rosswall 1982). It is a suitable indicator to indicate the onset of biodegradation of diesel oil and of monoaromatic compounds, such as BTEX (Margesin et al. 2000a, 2003). The time course of FDA hydrolytic activity during the bioremediation of soil contaminated with diesel oil is comparable to that of lipase activity (Sect. 17.2). The biodegradation of PAHs (naphthalene, phenanthrene) in soil also results in an increase of FDA hydrolytic activity, which, however, is followed by a marked activity decrease (Margesin et al. 2000a). A short-term, reversible inhibition of FDA hydrolysis has been noted in BTEX-contaminated soil (Margesin et al. 2003) and in jet-fuel-contaminated soil (Song and Bartha 1990), being substantially higher in unfertilized than in fertilized soil. The inhibition terminated after a significant part of the contamination had disappeared, and a stimulation of the activity was observed after most of the fuel had been mineralized. Generally, a correlation between FDA hydrolytic activity and other soil biological parameters can be found.

Principle. Using FDA as substrate, soil samples are incubated at 25 °C and pH 7.6 for 2 h. The released fluorescein is extracted with acetone, and quantified photometrically at 490 nm.

Theory. FDA is hydrolyzed by a number of different enzymes, such as proteases, lipases, and esterases. The ability to hydrolyze FDA is widespread among soil organisms and has been detected among heterotrophic bacteria, fungi, algae, and protozoa. The product of this enzyme conversion is fluorescein, which can be visualized within cells by fluorescence microscopy. Fluorescein can also be quantified using fluorometry and spectrophotometry. Schnürer and Rosswall (1982) developed a simple, rapid, and sensitive spectrophometric method for the measurement of total microbial activity in soil straw litter.

Equipment

- Water bath (25 °C)
- Spectrophotometer

Reagents

- Phosphate buffer: 60 mM $NaH_2PO_4 \times H_2O/Na_2HPO_4 \times 2H_2O$, pH 7.6 (store at 4 °C)
- Substrate solution: Fluorescein diacetate (FDA; 2 mg/mL) dissolved in acetone (store aliquots at −20 °C)
- Acetone (technical grade)
- Calibration standards (fluorescein)
  - Stock solution: 1 mg fluorescein/mL acetone (store at 4 °C)
  - Working standard solution: 100 µg fluorescein/mL buffer (prepare daily fresh)
  - Standards: 0, 20, 50, 70, 100, and 150 µg fluorescein/10 mL buffer (adjust volumes of 0–1.5 mL of the working standard solution to 10 mL with buffer)

Sample Preparation

Use field-moist, sieved (< 5 mm) soil.

Procedure

1. Weigh 1 g of soil into 100 mL Erlenmeyer flasks, prepare 3 replicates (samples).

2. Add 10 mL of buffer.
3. Prepare a control (3 replicates) without soil.
4. Add 100 µL of substrate solution to each flask.
5. Mix the contents and incubate the stoppered flasks at 25 °C for 2 h.
6. Stop the reaction by adding 10 mL of acetone.
7. Filter the contents of the flasks.
8. Immediately afterwards, measure the absorbance of the released fluorescein in samples and controls at 490 nm against the reagent blank.

To prepare a calibration curve, treat calibration standards like the soil samples:

1. Weigh 1 g of soil into each of six flasks.
2. Add 10 mL of standard solution containing 0 (= reagent blank), 20, 50, 70, 100, 120, and 150 µg fluorescein.
3. Proceed as described for the soil samples (5–7), and measure the absorbance of the calibration standards at 490 nm against the reagent blank.

Calculation

3. Calculate the fluorescein concentration from the calibration curve.
4. Subtract the control reading (hydrolysis in absence of soil) from the sample reading (hydrolysis in presence of soil) and express activity as "µg of released fluorescein per gram soil dry mass over 2 h" using the following formula:

$$\text{µg Fluorescein/(g dry soil} \times 2\,\text{h)} = \frac{S - C}{wm \times dm}$$

S pNP concentration of the soil sample (µg)
C pNP concentration of the control (µg)
wm Soil wet mass (1 g)
dm Soil dry mass (g)

Notes and Points to Watch

- To measure the chemical fluorescein release from the substrate, it is necessary to prepare a control without soil.

- To allow for the adsorption of fluorescein onto soil, the calibration curve is prepared in the presence of soil. This is especially important in case of soil containing high amounts of organic matter or clay.
- Depending on the soil to be tested it might be necessary to optimize soil mass, substrate concentration, and incubation time.
- It is important to use a neutral pH for this enzyme assay since chemical hydrolysis of FDA occurs under acidic and alkaline pH conditions.
- Chemical hydrolysis of FDA occurs also at higher temperatures, therefore a reaction temperature of 25 °C is used for this enzyme assay. Store aliquots of the substrate solution at −20 °C to avoid chemical substrate hydrolysis, which occurs when the solution is stored for longer periods at 4 °C or room temperature.
- Acetone is added not only to stop the enzyme reaction, but also to solubilize cell membranes in order to facilitate the extraction of fluorescein from microbial cells.
- High amounts of heavy metals in soil may interfere with the method.

17.4 Dehydrogenase Activity

Introduction

Objectives. Soil dehydrogenase activity is a useful method to monitor the bioremediation of soil contaminated with petroleum hydrocarbons, such as diesel oil. The method has been applied to soil containing fresh (Margesin and Schinner 1997; Margesin et al. 2000a) and aged contamination (Margesin and Schinner 1999, 2001). A statistically significant positive correlation between this activity and the residual hydrocarbon content has been repeatedly observed. The substantial increase in soil dehydrogenase activity after hydrocarbon contamination reflects the adaptation and exponential growth of hydrocarbon degraders due to the availability of new carbon sources introduced by the contamination. Soil dehydrogenase activity declines with decreasing hydrocarbon content due to the loss of available compounds as a consequence of biodegradation (Margesin et al. 2000a, b). Measuring dehydrogenase activity is also a useful tool to monitor environmental contamination by anionic surfactants (Margesin and Schinner 1998), since this activity is inhibited in presence of high concentrations of anionic surfactants, such as sodium dodecyl sulfate. However, dehydrogenase activity is not a suitable parameter to monitor biodegradation of PAHs in soil (Margesin et al. 2000a).

Principle. Soil samples are mixed with [2(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride] solution (INT), and incubated for 2 h at 40 °C. The reduced iodonitrotetrazolium formazan (INTF) is extracted with dimethylformamide and ethanol, and quantified photometrically at 464 nm (von Mersi and Schinner 1991; Schinner et al. 1996).

Theory. Dehydrogenases belong to oxidoreductases and catalyze the removal from a substrate of two hydrogens that are taken up by a hydrogen acceptor or coenzyme. Nicotinamide adenine dinucleotide (NAD) is the coenzyme used by the majority of dehydrogenases, others use nicotinamide adenine dinucleotide phosphate (NADP) or flavin adenine dinucleotide (FAD). Since dehydrogenases are important components of the enzyme system of all microorganisms, soil dehydrogenase activity reflects a broad range of microbial oxidative activities, and can be taken as a measure for the intensity of microbial metabolism in soil (Schinner et al. 1996). Because of increased sensitivity and reproducibility, the substrate INT has been used by a number of authors (Trevors 1984; Griffiths 1989; von Mersi and Schinner 1991) to determine soil dehydrogenase activity. An alternative substrate is 2,3,5-triphenyltetrazolium chloride (for details, see Schinner et al. 1996).

Equipment

- Water bath or incubator (40 °C)
- Spectrophotometer

Reagents

- Buffer: 1 M Tris-HCl, pH 7.0 (store at 4 °C).
- Substrate: dissolve 500 mg of 2(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT; Serva, Heidelberg) in 2 mL of N,N-dimethylformamide by shaking vigorously and using an ultrasonic water bath. Make up to volume with distilled water in a 100 mL volumetric flask (prepare daily fresh, and store it in the dark until use).
- Extraction solution: mix 1 part of N,N-dimethylformamide with 1 part of 96% ethanol.
- Calibration standards (iodonitrotetrazolium formazan (INTF)).
  - Stock solution: 100 µg INTF/mL extraction solution (store at 4 °C)
  - Standards: 0, 100, 200, 300, and 500 µg INTF/13.5 mL extraction solution (adjust volumes of 0–5 mL of the stock solution to 13.5 mL with extraction solution)

Sample Preparation

Use field-moist, sieved (< 5 mm) soil. Additionally, prepare autoclaved soil.

Procedure

1. Weigh 1 g of soil into 100 mL Erlenmeyer flasks, prepare 3 replicates (samples).
2. Weigh 1 g of autoclaved soil into 100 mL Erlenmeyer flasks, prepare 2 replicates (controls).
3. Add 1.5 mL of buffer and 2 mL of substrate solution to both samples and controls.
4. Mix the contents and incubate the stoppered flasks at 40 °C for 2 h.
5. Add 10 mL of extraction solution to each flask. For extraction of the released INTF keep the flasks for 1 h at room temperature in the dark, and shake vigorously every 20 min.
6. Filter the contents of the flasks.
7. Immediately afterwards, measure the INTF concentration in samples, controls and calibration standards at 464 nm against the reagent blank. Dilute solutions with extraction solution when absorbance values are too high.

Calculation

1. Calculate the INTF concentration from the calibration curve.
2. Express soil dehydrogenase activity as "µg of released INTF per gram soil dry mass over 2 h" using the following formula:

$$\text{µg INTF/(g dry soil} \times 2\,\text{h)} = \frac{S - C}{wm \times dm}$$

S INTF concentration of the soil sample (µg)
C INTF concentration of the control (µg)
wm Soil wet mass (1 g)
dm Soil dry mass (g)

Notes and Points to Watch

- INT is very sensitive to light. Both incubation and filtration have to be carried out in the dark.

- The control contains autoclaved soil to estimate the chemical substrate reduction due to reactive soil components.
- Depending on the soil to be tested, it might be necessary to optimize soil mass, substrate concentration, and incubation time.
- High amounts of heavy metals (e.g., copper) in soil can interfere with the method.
- Dehydrogenase activity is significantly reduced in acidic soils.

References

Cooper AB, Morgan HW (1981) Improved fluorimetric method to assay for soil lipase activity. Soil Biol Biochem 13:307–311

Dick RP (1997) Soil enzyme activities as integrative indicators of soil health. In: Pankhurst CE, Double BM, Gupta VV (eds) Biological indicators of soil health. CAB Int, Oxon, pp 121–157

Griffiths BS (1989) Improved extraction of iodonitrotetrazolium-formazan from soil with dimethylformamide. Soil Biol Biochem 21:179–180

Hankin L, Hill DE, Stephens GR (1982) Effect of mulches on bacterial populations and enzyme activity in soil and vegetable yields. Plant & Soil 64:193–201

Ishimoto R, Sugimoto M, Kawai F (2001) Screening and characterization of trehalose-oleate hydrolyzing lipase. FEMS Microbiol Lett 195: 231–235

Kato T, Haruki M, Imanaka T, Morikawa M, Kanaya S (2001) Isolation and characterization of psychrotrophic bacteria from oil-reservoir water and oil sands. Appl Microbiol Biotechnol 55:794–800

Margesin R, Feller G, Hämmerle M, Stegner U, Schinner F (2002) A colorimetric method for the determination of lipase activity in soil. Biotechnol Lett 24:27–33

Margesin R, Schinner F (1997) Bioremediation of diesel-oil-contaminated alpine soils at low temperatures. Appl Microbiol Biotechnol 47:462–468

Margesin R, Schinner F (1998) Biodegradation of the anionic surfactant sodium dodecyl sulfate at low temperatures. Int Biodet Biodegradation 41:139–143

Margesin R, Schinner F (1999) A feasibility study for the in situ remediation of a former tank farm. World J Microbiol Biotechnol 15:615–622

Margesin R, Schinner F (2001) Bioremediation (natural attenuation and biostimulation) of diesel-oil-contaminated soil in an alpine glacier skiing area. Appl Environ Microbiol 67:3127–3133

Margesin R, Walder G, Schinner F (2000a) The impact of hydrocarbon remediation (diesel oil and polycyclic aromatic hydrocarbons) on enzyme activities and microbial properties of soil. Acta Biotechnol 20:313–333

Margesin R, Walder G, Schinner F (2003) Bioremediation assessment of a BTEX-contaminated soil. Acta Biotechnol 23:29–36

Margesin R, Zimmerbauer A, Schinner F (1999) Soil lipase activity – a useful indicator of oil biodegradation. Biotechnol Tech 13:859–863

Margesin R, Zimmerbauer A, Schinner F (2000b) Monitoring of bioremediation by soil biological activities. Chemosphere 40:339–346

Mills AL, Breuil C, Colwell RR (1978) Enumeration of petroleum-degrading marine and estuarine microorganisms by the most probable number method. Can J Microbiol 24:552–557

Pancholy SK, Lynd JQ (1972) Quantitative fluorescence analysis of soil lipase activity. Soil Biol Biochem 4: 257–259

Plou FJ, Ferrer M, Nuero OM, Calvo MV, Alcalde M, Reyes F, Ballesteros A (1998) Analysis of Tween 80 as an esterase/lipase substrate for lipolytic activity. Biotechnol Tech 12:183–186

Pokorna V (1964) Method of determining the lipolytic activity of upland and lowland peats and muds. Soviet Soil Sci 1:85–87

Sakai Y, Hayatsu M, Hayano K (2002) Use of Tween 20 as a substrate for assay of lipase activity in soils. Soil Sci Plant Nutr 48:729–734

Schinner F, Bayer H, Mitterer M (1993) The influence of herbicides on microbial activity in soil materials. Austrian J Agric Res 34:22–30

Schinner F, Öhlinger R, Kandeler E, Margesin R (eds; 1996) Methods in Soil Biology. Springer, Berlin Heidelberg New York

Schnürer J, Rosswall T (1982) Fluorescein diacetate hydrolysis as a measure of total microbial activity in soil and litter. Appl Environ Microbiol 43:1256–1261

Shirai K, Jackson RL (1982) Lipoprotein lipase-catalyzed hydrolysis of p-nitrophenyl butyrate. J Biol Chem 257:1253–1258

Song HG, Bartha R (1990) Effects of jet fuel on the microbial community of soil. Appl Environ Microbiol 56:646–651

Sparling GP (1997) Soil microbial biomass, activity and nutrient cycling as indicators of soil health. In: Pankhurst CE, Double BM, Gupta VV (eds) Biological indicators of soil health. CAB Int, Oxon, pp 97–119

Trevors JT (1984) Dehydrogenase activity in soil: A comparison between the INT and TTC assay. Soil Biol Biochem 16:673–674

van Beelen P, Doelman P (1997) Significance and application of microbial toxicity tests in assessing ecotoxicological risks of contaminants in soil and sediment. Chemosphere 34:455–499

Von Mersi, Schinner F (1991) An improved and accurate method for determining the dehydrogenase activity of soils with iodonitrotetrazolium chloride. Biol Fertil Soils 11:216–220

Wei YL, Kurihara T, Suzuki T, Esaki N (2003) A novel esterase from a psychrotrophic bacterium, *Acinetobacter* sp. strain no. 6, that belongs to the amidase signature family. J Mol Cat B-Enzym 23:357–365

18 Assessment of Ecotoxicity of Contaminated Soil Using Bioassays

Adolf Eisentraeger, Kerstin Hund-Rinke, Joerg Roembke

18.1 General Introduction: Strategy

Bioassays provide important information for the assessment of pollutant effects of chemicals or environmental samples. In contrast to chemical analyses, they also detect effects of multiple contaminants and metabolites. Standardized bioassays can be used for the path-related, toxicological characterization of soils and soil materials, taking into account possible transfer of pollutants to the groundwater and potential effects on soil microorganisms, earthworms, and plants. A large number of bioassays have been applied for the characterization of contaminated soil or soil materials (Spurgeon et al. 2002). Most of them have been developed for the testing of chemicals and then have been adapted for testing of contaminated soil samples. In the first case, uncontaminated soil is spiked with chemicals in defined concentrations and dose-response relationships are obtained and evaluated further. While this is a straightforward and often standardized approach, testing and assessing contaminated soils is more difficult, since many soil samples are contaminated with different kinds of known and unknown chemicals that can not be quantified comprehensively. In addition, the soils have different properties (e.g., pH, texture), which themselves can affect organisms. Whereas the uncontaminated soil sample can be used as reference sample for the testing of chemicals, it is very difficult to select uncontaminated reference samples for contaminated soils. Therefore, bioassays can not be transferred easily to the testing of contaminated soils and the evaluation of test results is completely different.

The results of bioassays using soil are affected by mobilization, bioavailability, and pathways of transfer of contaminants. The latter ones can be varied by the kind of organisms and the test design chosen. In addition, by using a battery of different test systems the effects of contaminants can be assessed as a whole (i.e., whether they are known or not), thus covering

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potential synergistic or antagonistic effects. Therefore, bioassays are useful tools for complementing chemical analysis.

Several joint research projects were carried out in the recent years in order to optimize bioassays with respect to sample treatment, test performance, and evaluation of data (Hund-Rinke et al. 2002a, 2002b). After performing a round-robin test and a laboratory intercomparison test, a testing strategy was proposed by researchers involved in these projects (Dechema 2001; Eisentraeger et al. 2004). The strategy allows a cost efficient assessment of soil samples in addition to chemical analysis in a stepwise approach using a maximum of nine standardized bioassays. Detailed advice both on sample treatment and on the interpretation of test data is given. Depending on the results, recommendations are given whether remediated soils can be incorporated at unsealed or sealed sites and whether they can be used as upper or lower soils. The proposed strategy can be expected to contribute to the discussion on the standardization of soil testing and to promote the incorporation of biological test methods into soil protection legislation.

As mentioned above, nine different bioassays are applied in a stepwise approach. The four pathways from soil to (ground)water, to soil microflora, to soil fauna and higher plants are assessed using several test systems.

The water-extractable ecotoxicological potential of soils and soil materials is examined to assess whether undesirable effects in the groundwater or surface water might occur (i.e., after toxic substances have been set free) by using two aquatic ecotoxicity tests with bacteria and algae (Table 18.1). Since only the potential is of significance in this context, the selected test systems are not considered to be ecologically relevant.

Genotoxic substances in contaminated soils may be hazardous both for soil organisms and human beings. The latter may be exposed via the path soil – groundwater – since it is one of the major sources for drinking water. Therefore, the water extractable genotoxic potential is assessed by testing water extracts using a test of genotoxicity known as the umu test (because of its dependence on *umuC* gene induction) according to ISO 13829 (2000; Table 18.1). The *Salmonella*/microsome test (Ames test) according to DIN 38415 T4 (1999) should be carried out additionally, but only if the umu test is negative and there are strong hints from chemical analysis or site history that mutagenic compounds are present (Table 18.1). It should be noted that the approach presented here is a screening method to identify substances that can cause gene mutations; it cannot be used to identify clastogenic substances.

Several bioassays are employed to ascertain different aspects of the habitat function of soils (Table 18.2): Microorganisms are chosen for these that differ in trophic levels, exposition, and habitat (e.g., bulk soil, air-filled soil pores; water film of soil pores). Effects on the soil microflora are quantified via respiration and ammonium oxidation activity. The combined earth-

Table 18.1. Biological test systems for the ecotoxicological assessment of the water-extractable ecotoxic and genotoxic potential of contaminated soil or soil materials. (Eisentraeger et al. 2004)

Test system	Standard	Comments/modifications
Water quality – determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* luminescent bacteria test – parts 1–3	ISO 11348 (1998)	–
Water quality – fresh water algal growth inhibition test with *Scenedesmus subspicatus* and *Selenastrum capricornutum*	ISO 8692 (1989)	Alternatively testing on microplates possible (Eisentraeger et al. 2003; Rila et al. 2003)
Water quality – determination of the genotoxicity of water and waste water using the umu test	ISO 13829 (2000)	–
Water quality – determination of the genotoxicity of water and waste water using the Salmonella/ microsome test (Ames test)	DIN 38415 T4 (1999)	–

worm mortality/reproduction test and the Collembola reproduction test are used to assess effects on soil fauna. Further, the emergence and growth of *Brassica rapa* and *Avena sativa* are used to assess a soil's capacity to function as a habitat for higher plants.

18.2 Sample Preparation

Introduction

Objectives and Principles. Suitable sample preparation is a prerequisite for obtaining reliable results (ISO 15799 2003). The preparation of soil samples includes transport, sieving, determination of maximum water-holding capacity, and water content, as well as adjustment of water content and storage.

Theory. Tests should be performed as soon as possible after sampling. The period of storage should be minimized, at least for soils containing degrad-

Table 18.2. Overview of relevant soil organisms and respective test systems considered in the test battery for the assessment of the habitat function of soils; the selection represents different habitat and exposure routes. (Eisentraeger et al. 2004)

Organism	Test system	Habitat	Exposition	Test parameter	Reference
Soil microflora	Soil quality – determination of the activity of soil microflora using respiration curves	Water film of soil pores	Soil pore water	Basal respiration, substrate induced respiration	ISO 17155 (2002)
Soil microflora	Ammonium oxidation – a rapid method to test potential nitrification in soil	Water film of soil pores	Soil pore water	Nitrification	ISO15685 (2004)
Collembola: *Folsomia candida*	Effects of pollutants on Collembola (*Folsomia candida*) – method for the determination of effects on reproduction	Air filled soil pores	Food	Reproduction	ISO 11267 (1999)
Earthworms: *Eisenia fetida*	Effects of pollutants on earthworms – combined Earthworm mortality/reproduction test with *Eisenia fetida*	Bulk soil	Soil pore water, food	Reproduction	ISO 11268–1 (1993) ISO 11268–2 (1998) (combination of the two ISO guidelines)
Plants: *Brassica rapa* (monocotyledon) *Avena sativa* (dicotyledon)	Soil-quality – determination of the effects of pollutants on soil flora – part 2: effects of chemicals on the emergence and growth of higher plants	Bulk soil	Soil pore water	Germination, growth	ISO 11269–2 (1995) (this guideline is currently under re-consideration)

able contaminants. Soil sampling may cause an alteration of the soil conditions, especially of the redox potential. This may result in a degradation of the contaminants and lead to an inaccurate evaluation of toxic potential. Due to altered environmental conditions, for example, it was found at a munition site that the TNT concentration after excavation quickly decreased by a factor of about 10.

Microbial activity may diminish in storage, leading to erroneous results with respect to microbial respiration and nitrification rates (see below). The extent and time period of decrease varies for different soils. If no degradation is expected, the maximum storage period for uncontaminated soil samples should not exceed 3 months at 4 °C in the dark. If soil samples have to be stored for a longer period, −20 °C may be best. Certain aspects of these procedures, however, remain controversial, especially regarding potential ammonium oxidation activity (Sect. 18.5.2).

Samples should be stored in a way that changes in the soil water content are minimized. The vessels used should not influence the composition of the samples. For soil samples contaminated with organic pollutants, stainless steel, aluminum, or glass vessels should be used. Container materials of lower quality may be used for large amounts of soil, in which case it must be assured that the ratio of soil mass to vessel mass is appropriate. The decrease of contaminant concentrations (e.g., by sorption to the wall of the vessel) also must be negligible. Suitable containers for samples containing inorganic contaminants (heavy metals) usually made of plastic and or other materials free of heavy metals.

A draft version of a sequential approach to estimate the storage capacity of soil samples contaminated with volatile organic compounds (VOC) was set up by Rila and Eisentraeger (2003). This approach is based on the quantification of the VOCs, on the one hand, and of the microbial respiratory activity, on the other, under the assumption that toxic characteristics of samples with a high microbial activity and a high VOC concentration are altered during storage.

The procedure of sample preparation is summarized in Fig. 18.1. According to ISO/DIS 21268 (2004), for the tests with soil eluate, and for those using terrestrial test organisms other than microorganisms, the soil samples are sieved to $\leq$ 4 mm. For microbial tests the soil fraction $\leq$ 2 mm is needed. The microbial tests are performed using the indigenous soil microflora, whereas the other tests are performed with introduced organisms. These latter tests usually require huge amounts of soil. Especially for highly silty and loamy soils, sieving of large soil volumes to smaller soil fractions may be difficult with an acceptable expenditure of work, as the holes of the sieves may plug up within several minutes. This makes frequent cleaning necessary. Therefore, depending on the organisms introduced, it was decided to apply different procedures.

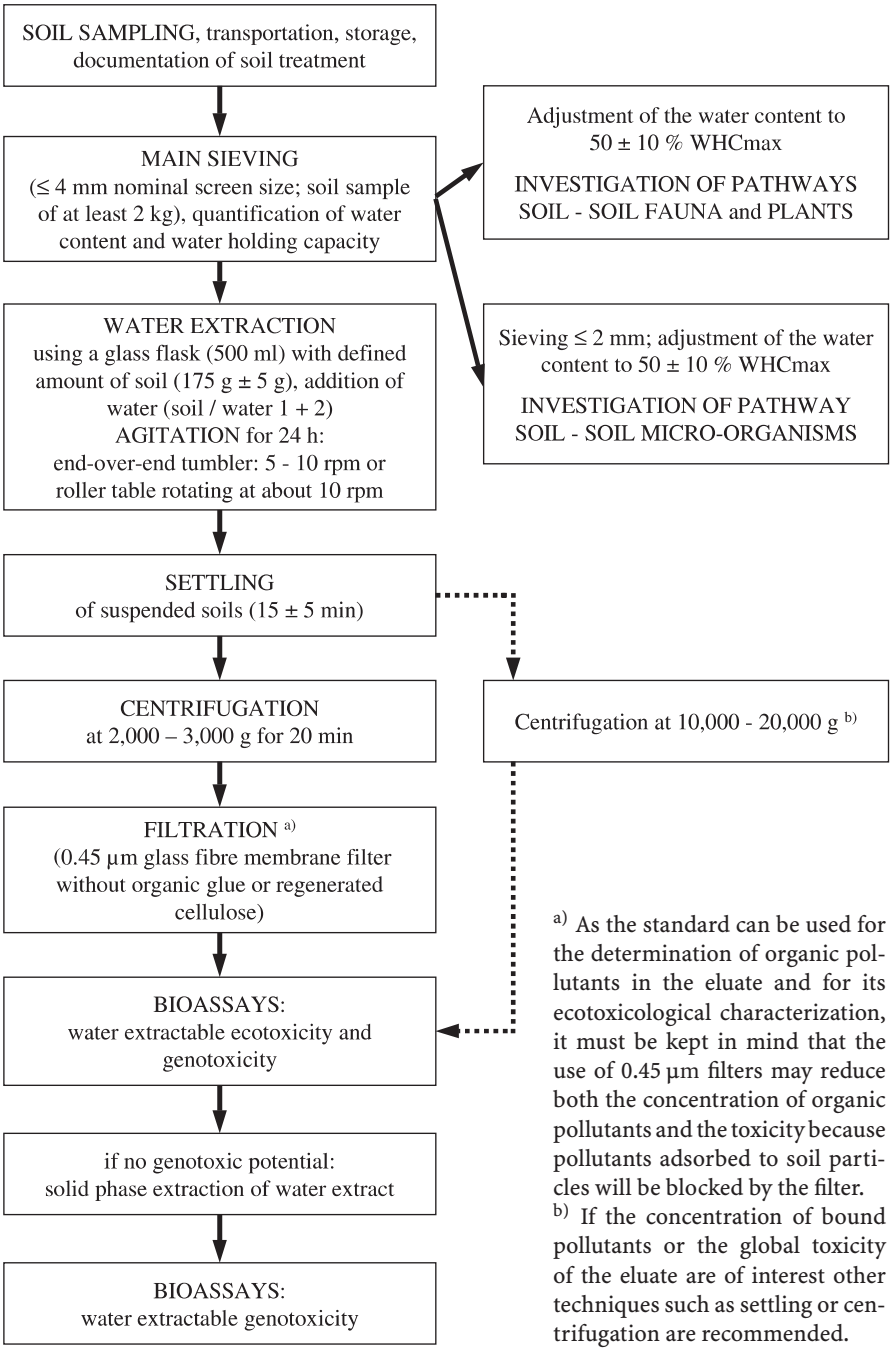

Fig. 18.1. Procedure proposed for the preparation of soil samples for ecotoxicological testing (modified according to Pfeifer et al. 2000; Dechema 2001; Rila and Eisentraeger 2003; Eisentraeger et al. 2004, ISO/DIS 21268–1 2004)

Sample Preparation for Investigation of the Pathways: Soil to-Soil Organisms, and Soil to Plants. It has to be considered that biological determinations require optimal water content. The water content also has an influence on the oxygen supply; water saturation, for example, results in a limited oxygen supply. As the demands of organisms regarding humidity and oxygen supply differ, the optimal water content depends on the planned investigations. For microbiological assessments a water content of 50 ± 10% of the maximum water holding capacity (WHC_{max}) is recommended. Soils with this water content provide the microorganisms living in the soil pore water with sufficient water as well as oxygen. Similar conditions are recommended for collembola that live in air-filled soil pores. For tests with earthworms the appropriate water content is higher, since their surface is rather sensitive to desiccation. Due to their large size, the thin water film in soil pores, sufficient for microorganisms, is insufficient for them. Furthermore, the earthworm species used in ecotoxicological tests is a compost worm, adapted to higher humidity.

The soil has to be dried if the water content is too high for the planned investigations or for sieving. During the drying process local complete drying should be avoided. This is essential for microbial investigations, since complete drying causes a reduction of the microbial population. Moreover, the aggregates formed in silty and loamy soils will be difficult to destroy. Localized drying can be prevented by turning the soil periodically. In addition, structural changes of the soil due to drying can cause problems in plant tests since roots cannot permeate hard soil aggregates. In such cases, the soil samples must be re-wetted carefully by hand in order to reach a moisture suitable for plants, i.e., automatic moistening via wicks (Sect. 18.5.5) will not be sufficient.

Water Extraction for Ecotoxicological and Genotoxicological Testing. In order to make a pragmatic estimation of the fraction of contaminants that might migrate to the groundwater, soil samples are extracted with water in a simple batch assay. By choosing a dry soil-to-water ratio of 1:2 it is guaranteed, on the one hand, that enough water sample is available for biotesting and, on the other, that concentrations of the water-extractable contaminants remain high. Meanwhile, this approach is accepted, has been successfully validated in a ring test, and its standardization is in progress (ISO/DIS 21268–1 2004).

Preparation of Solid-Phase Extracts from the Water Extracts for Genotoxicological Testing. As stated earlier, groundwater contaminated with genotoxic substances can be hazardous. The water-extractable genotoxic potential is assessed in order to roughly estimate whether genotoxic compounds might be mobilized by water. In the first step of the procedure, the same water extract is tested as is used for the assessment of the water-extractable eco-

toxicological potential. If there is a genotoxic effect in the umu test, with or without metabolic activation, a high risk of transfer of genotoxic substances from soil to groundwater exists. If there is no genotoxic effect, the water extract should be concentrated by a (low) factor of 15 using Serdolit PAD-1 resin (Boehringer Ingelheim Pharma GmbH & Co. KG, Ingelheim, Germany) as described below.

Equipment and Reagents

- 2 mm sieve (in exceptional cases 4 mm, see Sect. 18.1)
- Cylinders of metal, glass, or plastic (diameter 5–8 cm), sealed at one end with a finely meshed fabric for determining water-holding capacity
- Sand bath fitted out at the bottom with a discharge valve, filled with fine sand (grain size 0.1–0.7 mm); to about 10 cm, then saturated with water before starting the test by closing the valve while letting in the water and opening it afterward (so that the surplus water can run off), and the sand then covered with a moist fabric
- Analytical balance
- Heating apparatus for determining water content or drying cabinet and exsiccator
- Shakers for water extraction
- Centrifuge
- Glass microfiber filters
- Pentane, acetone, dimethylsulfoxide (DMSO), methanol, dichloromethane, conc. HCl, conc. NaOH (of analytical grade)
- Resin (e.g., Serdolit PAD-1 resin; Boehringer Ingelheim, No. 42442)

Sample Preparation

The preparation of the soil consists of the following steps:

- Sieving
- Water extraction for aquatic test systems
- Solid phase extraction of the water extract for aquatic genotoxicity test systems
- Determination of the WHC_{max} (Chap. 2)
- Determination of the water content of the sieved soil (Chap. 2)
- Adjustment of the water content to a specific percentage of WHC_{max} (Chap. 2)

Procedure

In the guideline ISO 10381–6 (1993) collection, handling, and storage of soil for the assessment of aerobic microbial processes in the laboratory is described. For testing contaminated soils it has to be considered that some contaminants may interact with vessel material (see Sect. 18.1). Moreover, alteration of the redox potential during storage should be minimized for anaerobic soils for which only investigation by aquatic ecotoxicological and genotoxicological tests is relevant.

Sieving (According to ISO 10381–6 1993)

If the soil is too wet for sieving, it should be spread out, where possible in a gentle air stream, to facilitate uniform drying. The soil should be finger crumbled and turned over frequently to avoid excessive surface drying. Normally this procedure should be performed at ambient temperature. The soil should not be dried more than necessary to facilitate sieving.

Water Extraction (According to ISO/DIS 21268–2 2004)

The soil samples are extracted by a ratio of 1 part soil dry mass to 2 parts of water with a minimum amount of 100 g soil dry mass. The water content in the soil has to be considered. The samples are shaken intensively to simulate worst-case conditions for 24 h and then centrifuged. The supernatant is filtered with a glass microfiber filter and stored at 4 °C in Duran (Schott AG, Mainz) glass bottles in the dark. The pH of the elutriates is adjusted to 7 ± 1 with conc. HCl or NaOH. Ecotoxicological and genotoxicological testing should be performed within 8 days.

Preparation of Solid-Phase Extracts from the Water Extracts for Genotoxicological Testing

The solid-phase extraction of the water extract is performed with Serdolit PAD-1 resin, an ethylstyrene-DVB-copolymer with a particle size of 0.3–1.0 mm and a pore diameter of ca. 25 nm with a specific surface of ca. 250 m^2/g. The PAD-1 beads are pretreated by rinsing for 2 h in warm 10% (v/v) HCl, Millipore water, 10% (v/v) NaOH, and Millipore water successively followed by 8 h Soxhlet extraction with pentane/acetone in a ratio of 1:2. The beads are dried at a temperature of 110 °C. Shortly before solid phase extraction 10 g PAD-1 beads are preconditioned by shaking them with 25 mL methanol.

The water extract should be concentrated by a factor of 15 by mixing 375 mL with 10 g Serdolit PAD-1 beads. This suspension is placed on an overhead shaker for 2.5 h. The beads are removed from the water extract and dried under nitrogen atmosphere in a Baker-spe-10 system (J.T. Baker,

Phillipsburg, New Jersey, USA). The dried beads are then extracted with a mixture of 9 parts dichloromethane and 1 part methanol. One mL of DMSO is added to the solvent, which is then evaporated under nitrogen atmosphere to a final volume of 1 mL. The concentrated sample is stored for less than 8 days at 4 °C. The sample is adjusted with distilled water to a volume of 25 mL for the genotoxicity tests. The final DMSO concentration is 4%. Therefore, the concentration factor for the water soil extract is 15.

Notes and Points to Watch

- As already mentioned in Sect. 18.1, localized drying of the soil has to be avoided.
- The soil should be processed as soon as possible after sampling. Any delays due to transportation should be minimized.
- Microbial tests: if storage is unavoidable, this should not exceed 3 months, unless evidence of continued microbial activity is provided. Even at low temperatures the active soil microflora decreases with increasing storage time; the rate of decrease depends on the composition of the soil and the microflora.
- Soil fauna tests and tests using higher plants: there are no specific recommendations for soil storage with respect to soil fauna and higher plants in ISO standards. Therefore it is recommended to store the soil samples under the same conditions as for testing of microbes and microbial processes.
- Aquatic tests: for testing the leaching potential, water extracts for aquatic tests should be prepared immediately after sieving. If the tests cannot be performed within 8 days (storage of the extracts at 4 ± 2 °C in the dark), extracts should be stored at −20 °C.
- An ISO guidance paper on the long and short term storage of soil samples is in process.

18.3 Water-Extractable Ecotoxicity

18.3.1 *Vibrio fischeri* Luminescence-Inhibition Assay

Introduction

Objectives. This test is an acute toxicity test with the marine luminescent bacterium *Vibrio fischeri* NRRL B-11177 (formerly known as *Photo-*

bacterium phosphoreum). It is standardized for the determination of the inhibitory effect of water samples in the ISO guideline 11348 parts 1-3 (1998). In the strategy presented here, it is used to determine whether toxic substances are present in the aqueous soil extracts.

Principle. The test system measures the light output of the luminescent bacteria after they have been challenged by a sample and compares it to the light output of a blank control sample. The difference in light output (between the sample and the control) is attributed to the effect of the sample on the organisms. The test is based on the fact that the light output of the bacteria is reduced when it is introduced to toxic chemicals.

Theory. *V. fischeri* emits a part of its metabolic energy as blue-green light (490 nm). Biochemically luminescence is a byway of the respiratory chain. Reduction equivalents are separated and transmitted to a special acceptor (flavin mononucleotide, FMN; Engebrecht et al. 1983). During the oxidation of substrates by dehydrogenase hydrogen is transferred to nicotinamide adenine dinucleotide (NAD). The reduced NAD ($NADH_2$) transfers the hydrogen normally to the electron transport chain. To get bacterial luminescence, a part of the $NADH_2$ is used to build reduced flavin mononucleotide ($FMNH_2$). $FMNH_2$ builds a complex with luciferase which involves the oxidation of a long-chain aliphatic aldehyde, developing an excited energy state. The complex decomposes and emits a photon. The oxidation products FMN and the long chain fatty acid are reduced in the next reaction cycle by $NADPH_2$.

$$FMNH_2 + RCHO + O_2 \rightarrow \text{Luciferase} \rightarrow FMN + RCOOH + H_2O + h\nu$$

This luminescence is inhibited in the presence of hazardous substances. Since it is dependent on reduction equivalents, the luminescence inhibitory test is a physiological test belonging to the electron-transport-chain-activity group.

Procedure

Equipment, reagents, sample preparation, procedure, and calculations are described in detail in ISO 11348 (1998).

18.3.2 *Desmodesmus subspicatus* Growth-Inhibition Assay

Introduction

Objectives. This fresh water algal growth inhibition assay is performed according to the standard ISO 8692 (1989). It is applicable both for the

characterization of chemicals and aquatic environmental samples. While the standard allows the testing with two strains (*Desmodesmus subspicatus*, formerly *Scenedesmus subspicatus*, and *Selenastrum capricornutum*), the strategy for soil characterization presented here has been set up and validated using the strain *D. subspicatus*. The algal growth inhibition test complements the acute bacterial luminescence test with *V. fischeri*.

Principle. The growth of *D. subspicatus* in batch cultivation in a defined medium over 72 ± 2 h is quantified both in the presence and the absence of a sample. The cell density is measured at least every 24 h using direct methods like cell counting or indirect methods correlating with the direct methods, such as in vivo chlorophyll fluorescence measurement. The inhibition is measured as a reduction in growth rate.

Theory. *D. subspicatus* is a fresh water algae that can be easily cultivated under defined conditions at 23 ± 2 °C with a light intensity in the range of 35×10^{18} to 70×10^{18} photons/m^2/s. Since it is based on growth inhibition, all specific or nonspecific toxic effects relevant to reproduction of these algae are assessed with this test system.

Procedure

Equipment, reagents, sample preparation, procedure, and calculations are described in detail in ISO 8692 (1989).

18.4 Water-Extractable Genotoxicity

18.4.1 The umu Test

Introduction

Objectives. The umu test is a short-term genotoxicity assay carried out on microplates within less than 8 h. It is standardized for the examination of water and waste water (ISO 13829 2000). The water-extractable potential of soil samples is assessed by testing the water extract and (if the water extract is not genotoxic) the 15-fold concentrated water extract. The results give hints as to whether genotoxic substances might migrate to the groundwater. The umu test was chosen since it is widely applied for the examination of aquatic environmental samples and since both costs and time needed are reasonable. The procedure has been optimized and validated by characterizing large numbers of contaminated and uncontaminated soil samples (Ehrlichmann et al. 2000; Rila et al. 2002; Rila and Eisentraeger 2003).

Principle. The bioassay is performed with the genetically engineered bacterium *Salmonella choleraesuis subsp. choleraesuis* TA1535/pSK1002 (formerly *Salmonella typhimurium*). This strain is exposed to different concentrations of the samples. Different kinds of genotoxic substances can be detected using this test since the strain responds with different types of genotoxic lesions, depending on the toxin.

Theory. The test is based on the capability of genotoxic agents to induce the *umuC* gene which is a part of the SOS repair system in response to genotoxic substances. The *umuC* gene is fused with the *lacZ* gene for β-galactosidase activity. The β-galactosidase converts ONPG (*o*-nitrophenol-β-D-galactopyranoside) to galactose, and the yellow substance *o*-nitrophenol is quantified photometrically at 420 ± 20 nm. The tests are preformed both with and without metabolic activation by S9-mixture (liver enzymes). Cytotoxic characteristics of the samples are quantified photometrically in parallel.

Procedure

Equipment, reagents, sample preparation, procedure, and calculations are described in detail in ISO 8692 (1989).

18.4.2 Salmonella/Microsome Assay (Ames Test)

Introduction

Objectives. The Salmonella/microsome assay (Ames test) is a bacterial mutagenicity assay that is standardized according to DIN 38415 T4 (1999) for the determination of the genotoxic potential of water and waste water (Ames et al. 1975). In the strategy presented here, it is recommended if the umu test is negative and if there are strong hints from chemical analysis or site history that mutagenic compounds are present. Thus it complements the umu test in some cases.

This method includes sterile filtration of the aquatic sample prior to the test. Due to this filtration, solid particles will be separated from the test sample. It may be possible that genotoxic substances are adsorbed by these particles. If so, they will not be detected.

Principle. The bacterial strains *Salmonella typhimurium* TA 100 and TA 98 should be used. The possible mutagenic activity of the sample is detected by comparing, for the bacterial strain and its activation condition, the number of mutant colonies on plates treated with the negative control and on plates treated with undiluted and diluted test samples.

The bacteria will be exposed under defined conditions to various doses of the test sample and incubated for 48–72 h at 37 ± 1 °C. Under this exposure, genotoxic agents contained in water or waste water may be able to induce mutations in one or both marker genes (hisG46 for TA 100 and hisD3052 for TA 98) in correlation with the dosage. Such induction of mutations will cause a dose-related increase of the numbers of mutant colonies of one or both strains to a biologically relevant degree above that in the control.

Theory. Bacteria that are not able to synthesize histidine are exposed to mutagenous substances inducing a reversion to the wild type growing in the absence of histidine. Histidine auxotrophy is caused by different mutations in the histidine operon: *S. typhimurium* TA 98 contains the frameshift mutation hisD3052 reverting to histidine independency by addition or loss of base pairs. *S. typhimurium* TA 100 bears the base pair substitution hisG46 which can be reverted via base pair substitutions (transition or transversion).

The tester strains are deep rough enabling larger molecules also to penetrate the bacterial cell wall and produce mutations (rfa mutation). The excision repair system is disabled (*Δ*uvrB), increasing the sensitivity by reducing the capability to repair DNA damage. Furthermore, they contain the plasmid pKM101 coding for an ampicillin resistance.

Procedure

Equipment, reagents, sample preparation, procedure, and calculations are described in detail in DIN 38415 T4 (1999). An ISO standard is in preparation.

18.5 Habitat Function: Soil/Microorganisms, Soil/Soil Fauna, Soil/Higher Plants

18.5.1 Respiration Curve Test

Introduction

Objectives. The determination of respiration curves provides information on the microbial biomass in soils and its activity. The method is suitable for monitoring soil quality and evaluating the ecotoxicological potential of soils. It can be used for soils sampled in the field or during remediation processes. The method is also suitable for soils that are contaminated experimentally either in the field or in the laboratory (chemical testing).

Principle. The CO_2 production or O_2 consumption (respiration rate) from unamended soils as well as the decomposition of an easily biodegradable substrate (glucose + ammonium + phosphate) is monitored regularly (e.g., every hour). From the CO_2-production or O_2-consumption data the different microbial parameters, such as basal respiration, substrate-induced respiration, lag time, are calculated.

Theory. Basal respiration and substrate-induced respiration (SIR) are widely used physiological methods for the characterization of soil microbial activity and biomass. Basal respiration gives information on the actual state of microbial activity in the soil. After addition of an easily biodegradable carbon source respiration activity increases. At the time of substrate addition the activity can be described by

$$\mathrm{SIR} = r + K$$

where r is the initial respiration rate of growing microorganisms.

In the course of an incubation period the respiration rate increases and can be described by

$$\mathrm{d}p/\mathrm{d}t = re\mu t + K$$

This equation is based on the assumption that the increase of the respiration rate $\mathrm{d}p/\mathrm{d}t$ after substrate addition in the SIR method represents the sum of the respiration rates of growing ($re\mu t$) and non-growing (K) microorganisms (Stenström et al. 1998).

The microbial respiration activity is affected by several parameters. Water content, temperature (Blagodatskaya et al. 1996), the quality of the soil organic matter (Wander 2004), as well as contaminants (e.g., Blagodatskaya and Anan'eva 1996; Kandeler et al. 1996) show an influence.

Procedure

Sample preparation, equipment, reagents, procedure, and calculations are described in detail in ISO 17155 (2002). A prerequisite is equipment that allows the determination of CO_2 release or O_2 uptake at short time intervals. Basal respiration is measured first. The respiration rates should be measured until constant rates are obtained. After measuring the basal respiration, a defined substrate mixture containing glucose, potassium dihydrogen phosphate, and diammonium sulfate is added. The mixture is made up of: 80 g glucose, 13 g KH_2PO_4, and 2 g $(NH_4)_2SO_4$. In testing, 0.2 g mixture is used per gram of soil in which at least 1 g organic matter is found in 100 g soil dry mass. The measurement of CO_2 evolution or O_2 consumption has to be continued until the respiration curve reaches its peak and the respiration rates are declining.

The ecotoxicological potential of soils is described by several parameters:

- Respiratory activation quotient: basal respiration rate divided by substrate-induced respiration rate ($Q_R = R_B/R_S$)
- Lag time (t_{lag}): the time from addition of a growth substrate until exponential growth starts, – a reflection of the vitality of the growing organisms
- Time to the peak maximum ($t_{peakmax}$): the time from addition of growth substrate to the maximum respiration rate – another reflection of the vitality of the growing organisms

According to the guideline, $Q_R > 0.3$, $t_{lag} > 20\,h$, and $t_{peakmax} > 50\,h$ indicate polluted materials.

Notes and Points to Watch

- Increased respiratory activation quotients may occur for two reasons. On one hand, they are an indicator of bioavailable carbon sources. These may be of biological origin, as for example compost, or biodegradable organic contaminants (e.g., mineral oil, anthracene oil, phenanthrene) that have the same effect (Hund and Schenk 1994). Sufficient amounts of biodegradable carbon sources always result in increased respiration activities when a sufficient amount of further nutrients (e.g., nitrogen, phosphate) is available. On the other hand increased Q_Rs may be an indicator of contaminants that are not biodegradable, e.g., heavy metals (Nordgren et al. 1988). Up to now, it is not known how to distinguish which parameters are responsible for a stress-induced respiration causing increased quotients.
- It has to be considered for the assessment that increased values indicate amended/contaminated soils, whereas not all contaminated soils show higher values. Accordingly, it cannot be concluded that the habitat function of a soil is intact when the respiration values are in a normal range.
- In the literature, the derivation of a metabolic quotient (basal respiration divided by microbial biomass) as an indicator for an ecosystem is described (Insam and Domsch 1988; Anderson and Domsch 1990). In soils with a recent input of easily biodegradable substrates, mainly r-strategists occur. They usually respire more CO_2 per unit degradable C than k-strategists, which prevail in soils that have not received fresh organic matter and have evolved a more complex detritus food web (Insam 1990). Since the substrate-induced respiration can be used to calculate the microbial biomass, it could be concluded that the metabolic quotient

and the respiration activation quotient are comparable. In this context it should be noted that the estimation of the microbial biomass by Anderson and Domsch (1978) is based on a linear regression between SIR and the microbial biomass according to the fumigation-incubation method. The conversion factor was elaborated on the basis of a range of soils. However, in other soils the population may differ from the originally investigated soils (e.g., forest soils vs. contaminated soils) and different conversion factors may be necessary (Hintze et al. 1994). One should, therefore, avoid calculating the microbial biomass of soils on the basis of the substrate-induced respiration for which the conversion factor is unknown.

18.5.2 Ammonium Oxidation Test

Introduction

Objectives. This test is a rapid procedure for determining the potential rate of ammonium oxidation in soils. The method is suitable for all soils containing a population of nitrifying organisms. It can be used as a rapid screening test for monitoring the quality of soils and wastes, and it is suitable for testing the effects of cultivation methods, chemical substances, and pollution in soils.

Principle. Ammonium oxidation, the first step in autotrophic nitrification in soil, is used to assess the potential activity of microbial nitrifying populations. Autotrophic ammonium-oxidizing bacteria are exposed to ammonium sulfate in a soil slurry. Oxidation of the nitrite formed by nitrite-oxidizing bacteria in the slurry is inhibited by the addition of sodium chlorate. The subsequent accumulation of nitrite is measured over a 6-h incubation period and is taken as an estimate of the potential activity of ammonium oxidizing bacteria. For the assessment of soil quality the nitrification activity in a test soil, in a control soil, and in a mixture of both soils is determined.

Theory. In soils with pH > 5.5 nitrification is performed by chemoautotrophic nitrifiers (Focht and Verstraete 1977). The procedure consists of two steps. Ammonium is oxidized to nitrite by one group of nitrifiers, while nitrite is oxidized to nitrate by a second group. Since nitrite is oxidized as it is produced, the rate at which ammonium is oxidized is equal to that at which nitrite plus nitrate accumulate. To avoid the application of two methods – one for the determination of nitrite and one for determining nitrate – a procedure was developed to completely and specifically block the oxidation of nitrite. With this method it is possible to get information on the

nitrification process by using only one analytical method, since the rate at which nitrite alone accumulates equals the rate of ammonium oxidation. In soils with a high background of nitrate this method is much more sensitive, since nitrite normally is undetectable at the beginning of the incubation. A prerequisite for a correct measurement is (1) that the inhibitor does not inhibit ammonium oxidation, and (2) that the inhibitor completely blocks nitrite oxidation. Chlorate has proved to be an appropriate inhibitor. At suitable concentrations an inhibition of ammonium oxidation seems to be negligible. Although, in some cases, the inhibition of nitrite oxidation can be incomplete, this does not seem to be a real problem. It is negligible when V_{max} for nitrite oxidation is lower than the rate of ammonium oxidation. It might be a problem, if V_{max} is larger. Since chlorate mainly influence the K_m of the reaction, the initial rate of the reaction is the best estimate of the ammonium oxidation rate. Leakage will be lowest at low nitrite concentrations (Belser and Mays 1980).

The results present a potential activity, since several test parameters are different from natural conditions: Ammonium is added in surplus, aeration is probably more intensive by shaking in the laboratory than under field conditions, and the incubation temperature of 25 °C usually far exceeds real soil conditions.

Several methods exist to get information on nitrification in soil. Some of these are characterized by incubation periods of several weeks (e.g., ISO 14238 1997). For soil assessments the determination of the ammonium oxidation activity was selected since this procedure has several advantages, especially for investigation of contaminated soils and for soil remediation procedures. These applications frequently require results within a short period of time, as they contribute to decisions whether a soil has to be remediated, whether a remediation has to be continued, or whether the habitat function of the soil (at least with respect to microorganisms) is intact so that the soil can leave the remediation plant. This is important in avoiding unneeded and expensive retention of soil in the remediation plants. As the potential ammonium oxidation method yields results in a short period of time, and furthermore is suitable for soils with high nitrate contents (during bioremediation nitrogen has to be added to achieve degradation of contaminants), this method was selected for the ecotoxicological soil assessment.

Procedure

Sample preparation, equipment, reagents, procedure, and calculations are described in detail in ISO 15685 (2004). For soil assessments three different test designs are applied:

1. Test soil
2. Reference soil (uncontaminated soil with a nitrification activity of about 200–800 ng N/g dry mass of soil/h)
3. Mixture of test soil and reference soil (1:1 with regard to soil dry mass)

The soils are adjusted to 60% of WHC_{max} and incubated for 2 days at 20 °C. The mixture is prepared immediately before testing. The mixture and the two soils are incubated again for 1 day at 20 °C, after which the nitrification activity is determined. Soil samples are mixed with test medium containing phosphate, sodium chlorate and diammonium sulfate. The slurries are incubated for 6 h at 25 ± 2 °C on an orbital shaking incubator (about 175 rpm). 2-mL samples are taken after 2 and 6 h, and the nitrite content is determined. The mentioned time interval is a recommendation.

Calculation

The rate of ammonium oxidation (ng NO_2^--N /g dry mass of soil/h) is calculated from the difference of NO_2^--N concentrations at the different measuring times. The following formula is applied for the assessment of the test soil:

$$M_m + SD_m < 0.9 \times (M_C - M_P)/2 \quad (18.1)$$

M_m mean ammonium oxidation activity in soil mixture

SD_m standard deviation of ammonium activity in replicate test vessels with soil mixture

M_C mean ammonium oxidation activity in control soil

M_P mean ammonium oxidation activity in polluted soil

The polluted soil is considered to be toxic if the mixture has an ammonium oxidation activity significantly slower than 90% of the calculated mean activity of the two single soils.

Notes and Points to Watch

- The suitability of storing soil samples at −20 °C is discussed controversially. The investigation of 12 soils differing in their physico-chemical properties has revealed that storage at −20 °C for 13 months does not affect the nitrifiers in annually frozen soils in any decisive way (Stenberg et al. 1998). As the procedure, however, does not seem to be suitable for every soil, in the guideline ISO 15685 (2004) storage at −20 °C is not generally recommended. The different results found in the literature on the effects of freezing as a storage method can be explained in a number

of ways: The populations in soils annually subjected to several freeze and thaw cycles seem to be adapted and more resistant to freezing than the microflora in soils where freeze and thaw cycles are not a regular occurrence. Furthermore, the growth status of the microorganisms at the time of sampling may play a role. Active cells seem to be more sensitive to freezing and thawing than less active cells. Therefore, samples collected shortly after managing processes such as fertilizing or tilling may show cell depletion. Furthermore, the selected procedure of freezing and thawing may influence the results. Slow rates of temperature change seem to result in greater microbial losses. Storage in small portions and rapid temperature flux may be preferable (Stenberg et al. 1998). In conclusion, soils should only be stored if the effect is known and acceptable.

18.5.3 Combined Earthworm Mortality/Reproduction Test

Introduction

Objectives. The determination of the survival and the reproductive success of earthworms as representatives of soil macrofauna provide information on these saprophagous soft-bodied invertebrates that in many soils play an important role as ecosystem engineers. The method is suitable for monitoring soil quality and the evaluation of the ecotoxicological potential of soils. It can be used for soils sampled in the field or during remediation processes. Furthermore the method is suitable for soils that are contaminated experimentally in the field or in the laboratory (e.g., chemical testing, in particular pesticide testing).

Principle. Adult earthworms are either exposed to potentially contaminated soil samples or to a range of concentrations of a test substance mixed in an artificial or natural control soil. The mortality and the biomass of the adult worms are measured after 28 days. The effect on the reproduction is determined by counting the number of juveniles hatched from the cocoons after an additional period of 4 weeks. Based on these measurements, the ecotoxicological potential of the test soil is determined.

Theory. Earthworms are important members of the soil community due to their ability to change or create their habitat through various activities, thus correctly considered to be "ecosystem engineers" (Lavelle et al. 1997):

- Penetrating the soil and building burrows, as well as increasing pore space
- Transporting soil and organic matter by casting

- Comminuting organic material (including cattle feces in meadows) as a first step in its breakdown
- Providing nutrients to plants (e.g., by concentrating them in burrow linings or by increasing the availability of nutrients like phosphorus)
- Relocating seeds in the soil profile
- Changing the diversity and improving the activity of the microbial community by selective feeding and providing feces rich in nutrients

Finally, earthworms are closely exposed to all contaminants occurring in the soil solution but also – by feeding – to all chemicals adsorbed to soil particles.

These activities thus finally lead to an improved soil structure, i.e. to stabilization of soil aggregates, to increase in water infiltration (partly by higher water-holding capacity; Urbanek and Dolezak 1992; Edwards and Shipitalo 1998), often to the formation of a humic layer close to the soil surface (mainly in forest ecosystems; Doube and Brown 1998), and to an increased yield in orchards or grassland (Blakemore 1997). The activities described above are performed by various earthworm species to a very different extent. Still, large, deep-burrowing worms like *Lumbricus terrestris* are involved in several of these activities, especially concerning soil structure and organic matter breakdown (Swift et al. 1979). In the light of this knowledge, it is difficult to understand why the main earthworm species used in tests are the two closely related compost worms *Eisenia fetida* or *Eisenia andrei*. Ecologically, these species are less important than the deep-burrowing worms (Løkke and van Gestel 1998). On the other hand, from a practical point of view the compost worms are more suitable than any other lumbricid species because they reproduce very quickly and easily in the laboratory, and mass cultures can be obtained. In addition, the sensitivity of these species is in the same general order of magnitude as other earthworm species. In most cases the differences between species are, depending on the chemical or contaminant mixture tested, not larger than by a factor of 10 (Roembke 1997; Jones and Hart 1998).

Concerning the test endpoints, the determination of mortality covers strong acute effects. However, from an ecological point of view such effects are clearly less important than long-term, chronic effects usually occurring at relatively low and thus more realistic concentrations (see "Notes and Points to Watch"). For this reason, reproduction is the test variable of highest relevance.

Procedure

Equipment, reagents, sample preparation, procedure, and calculation of the test results are described in detail in the ISO guidelines 11268–1 (1993)

and 11268–2 (1998). In deviation from these guidelines in which the acute and chronic endpoints are determined in individual test runs, it is recommended to use a combined test method for the assessment of contaminated soils. For the assessment of single chemicals, separate tests should still be used in order to be in agreement with legal requirements concerning the risk assessment of chemicals (e.g., the EU guideline describing the registration of pesticides; European Union 1991).

Ten adult earthworms of the species *E. fetida* or *E. andrei* per test vessel are exposed to a series of mixtures of the potentially contaminated test soil and an uncontaminated control or reference soil at 20 ± 2 °C for 4 weeks. If the mortality in the contaminated test soil is higher than 20%, the test is stopped. Otherwise, at the end of this period, the adult worms are removed from the vessels and the surviving animals are counted and weighed. Afterwards, the test soil remains in the same vessels for another 4 weeks. After 56 days the juveniles are extracted from test and control soils and counted. For the endpoint reproduction the data of the test soil vessels are compared with those from the controls. An inhibition of reproduction of 50% compared to the control is indicative of a contaminated soil sample. A soil that causes mortality higher than 20% is also classified as contaminated.

Notes and Points to Watch

- As already mentioned, the acute test endpoint mortality is ecologically not relevant due to the following reasons: Lumbricid worms die slowly and only at high concentrations of soil contaminants. In real field situations (with the exception of relatively small areas like mining deposits) the concentrations of chemicals are low but these substances, in particular metals, are often persistent. Such effects are much better determined by using chronic sensitive endpoints like reproduction. Ecologically, in many populations of earthworms any impact more strongly affects the reproductive rate than it does mortality rate. A short-term decrease in the number of individuals is easier to compensate than a long-term reduction in the number of juveniles. For this reason, the assessment of the biological quality of soil should be based on the chronic endpoint reproduction.

18.5.4 Collembola Reproduction Test

Introduction

Objectives. The determination of the survival and the reproductive success of collembolans as representatives of soil mesofauna provides information

on these saprophagous hard-bodied invertebrates, an important part of the soil food web in many soils. The method is suitable for monitoring soil quality and evaluating the ecotoxicological potential of soils. It can be used for soils sampled in the field or during remediation processes. Furthermore, the method is suitable for soils contaminated experimentally in the field or in the laboratory (e.g., chemical testing, in particular pesticide testing).

Principle. Juvenile collembolans are either exposed to potentially contaminated soil samples or to a range of concentrations of the test substance mixed in artificial soil. The mortality of the adult springtails as well as the reproduction (= number of juveniles) are measured at the end of the exposure period of 28 days. Based on these measurements, the ecotoxicological potential of the test soil is determined.

Theory. The species *Folsomia candida* (Collembola) is tested as a representative of hard-bodied soil invertebrates, in particular arthropods (Achazi et al. 2000). These organisms, mainly consisting of springtails (Collembola) and mites (Acari), are among the most numerous invertebrates in a wide range of soil types, especially of the Northern hemisphere. Due to their high numbers they are an important part of the soil food web (Weigmann 1993). In addition, the springtails control by their feeding activity the population cycles of microorganisms, which in turn are extremely important as mineralizers of organic matter (Swift et al. 1979). To a lesser extent, springtails can also influence the numbers of nematodes (Hopkin 1997). Finally, they are exposed to contaminants via pore water and air space.

The species *F. candida* is distributed worldwide (mainly by anthropogenic activities). It prefers soils with an elevated content of organic matter but is not only a compost inhabitant (e.g., it occurs in comparatively low numbers in agricultural soils; Petersen 1994; Hopkin 1997). Its use is criticized for the same reasons discussed for compost worms. However, the response is similar: *F. candida* is easily cultured and its sensitivity, as far as known, is not considerably different from other collembolans (Achazi et al. 2000). As in the case of earthworms, the endpoint reproduction is ecologically highly important (see Sect. 18.5.5).

Procedure

Equipment, reagents, sample preparation, procedure, and calculation of the test results are described in detail in the ISO guideline 11267 (1999). Ten juvenile springtails of the species *F. candida* per test vessel are exposed to a potentially contaminated soil sample or a series of mixtures between the test soil and an uncontaminated control or reference soil (plus a control) at $20 \pm 2\,^{\circ}C$ for 4 weeks. At the end of this period, the collembolans are removed

from the vessels and the surviving animals are counted (juveniles and adults separately) by using photographs or an automatic image processing system. For the endpoint reproduction, the data from the test soil vessels are compared with the controls. An inhibition of reproduction of 50% compared to the control is indicative of a contaminated soil sample.

Notes and Points to Watch

- The common test species *F. candida* is difficult to distinguish from other species of the same genus, in particular *F. fimetaria* (Wiles and Krogh 1998). This species has also been proposed for ecotoxicological testing, but it reproduces sexually and is, as such, more difficult to handle. Due to such practical problems and since it is not known whether the two species are equally sensitive to chemicals, any mixing of them must be carefully avoided. In cases of doubt a taxonomist specialized in collembolans should be consulted.

18.5.5 Plant Growth Test

Introduction

Objectives. The determination of the emergence and growth of different plant species allows assessment of the quality of a certain soil as a habitat for terrestrial primary producers (i.e., in terms of nutrient cycling, the basis of the whole ecosystem). The method is suitable for monitoring soil quality and evaluating the ecotoxicological potential of soils. It can be used for soils sampled in the field or during remediation processes. Furthermore, the method is suitable for soils that are contaminated experimentally in the field or in the laboratory (chemical testing, in particular pesticide testing).

Principle. This phytotoxicity test is based on the emergence and early growth response of a variety of terrestrial plant species to potentially contaminated soil. Seeds of selected species of plants are planted in pots containing the test soil and in control pots. They are kept under growing conditions for the chosen plants and the emergence and mass of the test plants are compared against those of control plants.

Theory. The importance of plants as the basis of ecosystem performance, but also for the production of food and forage, cannot be overestimated (Riepert et al. 2000). In 1984, plants were added to the list of terrestrial test species by the OECD. These selected species still represent agricultural plants, while wild herbs, trees, etc., are usually not tested (Boutin et al. 1995).

For the testing of chemicals, often two exposure pathways are distinguished: airborne via aboveground plant parts (e.g., after the spraying of pesticides) or via soil mixtures. Obviously, in the case of contaminated soil only the latter test version is used.

Concerning the measurement endpoints, the fresh biomass of the aboveground parts has been selected due to the practicability of evaluating it and its high sensitivity. However, one must be aware that this selection has been done for an acute test with a duration of 14 days. Further research will clarify whether long-lasting chronic tests (e.g., using the endpoint reproduction) will be more sensitive (ISO 22030 2004).

Procedure

Equipment, reagents, sample preparation, procedure, and calculation of the test results are described in detail in the ISO guideline 11269–2 (1995). In supplementing the guideline the test was changed in two ways:

1. In addition to the pure test soils, mixtures of the potentially contaminated soils with a suitable control or reference soil are made in a ratio of 50:50.
2. While the ISO lists 15 potential test species, it is recommended to use only the monocotyledonous species *Avena sativa* (oat) and one of the two named dicotyledonous species, either *Brassica rapa* (turnip) or *Lepidum sativum* (cress), for soil quality assessment. Each treatment is tested in four replicates (10 seeds per replicate (= test vessel)). Watering is done by using a semi-automated wick method (Stalder and Pestemer 1980). After emergence, the seedlings are thinned to a final number of five per vessel. Fourteen days later the aboveground parts of the plants (fresh mass) are harvested and weighed.

Evaluation

The evaluation is done according to the following formula (Winkel and Wilke 2000):

$$M_g + SD_{Mg} < 0.9 \times M_b \tag{18.2}$$

M_g Biomass measured in the vessels with the 50:50 mixture of test and control soil

SD_{Mg} Standard deviation of the 50:50 mixture between test and control soil

$0.9 \times M_b$ The calculated mean between the test and the control soil (biomass$_{\text{test soil}}$ + biomass$_{\text{control soil}}$) divided by 2 minus a tolerance value of 10%.

A soil is classified as toxic if the biomass measured in the vessels with the 50:50 mixture of test and control soil is > 10% lower than the mean biomass determined in the test and control soils.

Notes and Points to Watch

- In addition to storage problems already mentioned in the context of other terrestrial tests, it must be pointed out that in the case of plant testing the amount of plant-available nitrogen is very important for the growth of the test organisms, including the controls. If the plants grow badly in the controls it is difficult to identify effects occurring in the test vessels with test soils. For this reason, Riepert and Felgentreu (2000) recommended to avoid the use of fresh field soils because they don't contain enough available nitrogen due to high microbial activity. In order to solve this general problem fertilizer could be added to the water reservoirs used in the plant tests. Since all plants (both in the test as well as in the control vessels) are on the same nutrient level any effect caused by nitrogen availability would be eliminated. However, one must be cautious since some soils might be already so rich in nutrients that over-fertilization could occur.
- Another problem in testing potentially contaminated soils with plants is the fact that structural properties of the soil can affect the plants too. If the habitat function of the soil has to be assessed in general, the distinction between chemical and physical properties is not necessary. However, there are many field soils which are not suitable for the growth of crop species (e.g., acid soils). In order to avoid false positive results, the ecological requirements of the common test species (oat, turnip) are currently being studied (Jessen-Hesse et al. 2003). These data will allow the determination of which soils can be tested with the current test species and which cannot.

18.5.6 Test Performance for the Derivation of Threshold Values

Introduction

Objectives. The described terrestrial ecotoxicological tests are also suitable for the derivation of threshold values to protect the habitat function of

soils for soil organisms. The protection of this soil function is required in the German Soil Protection Act (BBodSchG 1998). The threshold values indicate the contamination pathway soil to soil organisms.

Principle. Soils are contaminated experimentally and the biological effect is investigated (chemical testing). Several concentrations are tested and the ecotoxicological potential is determined. Based on these measurements LC_{50} (lethal concentration) or EC_{50} (effective concentration) values for the different endpoints are calculated, using appropriate statistical methods.

Theory. Ecotoxicological tests provide information on the toxicity of priority contaminants. For the derivation of trigger values it has to be kept in mind that only a limited number of species and organisms have been tested. To protect the "whole" ecosystem, extrapolation methods have to be applied. Depending on the amount of available data the extrapolation method DIBAEX (distribution based extrapolation; Wagner and Løkke 1991) or FAME (factorial application method; European Union 1996) may be suitable. For the derivation of trigger values concerning the pathway soil to soil organisms, this procedure was successfully applied in Germany (Wilke et al. 2001; Wilke et al. 2004). In Germany trigger values are those which, if exceeded, indicate a harmful soil change or site contamination. If such cases occur, investigations of the site have to be performed. Since they indicate a potential effect, EC_{50} and LC_{50} values instead of NOEC (no-observed-effect concentration) or LOEC (lowest-observed-effect concentration) values are applied for the derivation.

Procedure

Chemical testing is described in detail in the different guidelines (mainly from OECD) mentioned in the pertinent sections.

Notes and Points to Watch

- Control soils have to be selected carefully (ISO 15799 2004). For the derivation of trigger values, natural soils are recommended, but at least a sandy soil with low sorption capacity should be used. For higher environmental relevance, loamy and silty soils should be employed. If artificial soil is used and if the test chemical has a high $\log K_{ow}$ value (octanol-water partitioning coefficient; e.g., > 2; European Plant Protection Organization 2003) this test substrate should contain only 5% peat instead of 10% in order to test a more field-relevant situation concerning the bioavailability of the test substance.

18.6 Combined Performance of Bioassays and Assessment of the Results

18.6.1 Water-Extractable Ecotoxic Potential

The procedure proposed here, and based on only two bioassays, is a qualitative one that offers a way to quickly obtain results and keep costs down (Fig. 18.2). Dilution values are defined to indicate ecotoxicological potential if exceeded. The water extracts should be diluted using a factor of 2, and lowest ineffective dilution values (LID) should be assessed. The LID is defined as the lowest dilution with less than 20% inhibition in the luminescence algae test. For the qualitative evaluation it is not necessary to determine EC values by data transformation from dose response curves. Of course, it might be useful to determine EC values if toxic potentials of soil samples (e.g., from the same site during remediation) have to be compared.

The *V. fischeri* luminescence inhibition assay (ISO 11348 1998; Sect. 18.3.1) should be performed at first. If the LID value exceeds 8, a risk of pollutant leakage exists and it is recommended that the remediated soils should not be incorporated at unsealed sites. If the luminescence inhibition assay is negative, the 72 h algae growth inhibition assay (ISO 8692

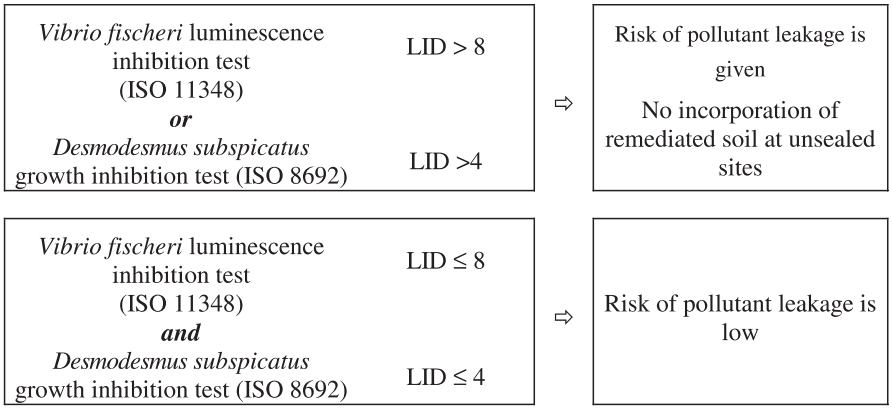

Fig. 18.2. Procedure proposed for the assessment of the water extractable ecotoxicological potential of soils and soil materials. The assessment based on LID values is allowed if (1) a dose response relationship is obtained, or (2) nearly 100% inhibition is obtained in several tested dilutions, or (3) no significant inhibition is obtained in the dilutions up to the threshold value. (Maxam et al. 2000; Pfeifer et al. 2000; Dechema 2001; Rila and Eisentraeger 2003; Eisentraeger et al. 2004)

1989; Sect. 18.6.2) should be performed additionally. The risk of pollutant leakage is low if this LID value is ≤ 4 (or the LID value of the luminescence inhibition assay is ≤ 8). These threshold values are derived from the experiences gained during the earlier-mentioned research projects (Rila and Eisentraeger 2003) and from the results of a ring test (Hund-Rinke et al. 2002a, b). Low inhibitions are obtained with uncontaminated soil samples such as the natural standard soils LUFA 2.1, 2.2 and 2.3 (landwirtschaftliche Untersuchungs- und Forschungsanstalt, Speyer, Germany). This "background toxicity" might be caused by humic substances.

Ecotoxic effects of a wide range of water-extractable contaminants can be detected by using these two test systems. In a round robin test eight contaminated soils were investigated using four aquatic test systems (luminescence and growth test with *V. fischeri*, tests with algae and daphnids). It was shown that daphnids are mostly less sensitive than the tests with algae and the luminescence test with *V. fischeri*. The daphnids test was more sensitive, however, for soils contaminated with heavy metals (Hund-Rinke et al. 2002c). As heavy metals are routinely measured by chemical analyses, it was decided to exclude the test with daphnids from the base set of aquatic ecotoxicological test systems for soil assessment. The approach presented here is cost effective: No range-finding test has to be carried out and the algae growth inhibition test can be performed in microplates, so long as the validity criteria of ISO 8692 are fulfilled (Eisentraeger et al. 2003). If other or further testing is regarded as necessary, ecological relevance and practicability should be considered.

18.6.2 Water-Extractable Genotoxicity

Cost effectiveness and speed are also major aspects of the assessment scheme for water-extractable genotoxic potential. It should thus be noted that this is a screening method that cannot be used to identify clastogenic substances, but is able to roughly estimate whether genotoxic compounds can be mobilized by water. The procedure is mainly based on the assessment of the genotoxic potential of water extracts using the umu test according to ISO 13829 (2000; Sect. 18.4.1). The umu test can be performed in less than a day with and without metabolic activation. The Salmonella/microsome test (Ames test) according to DIN 38415 T4 (Sect. 18.4.2) should be carried out additionally if the umu test is negative and if there are strong hints from chemical analysis or site history that mutagenic compounds are present.

In the first step of the procedure (Fig. 18.3) the same water extract is tested as used for the assessment of the water-extractable ecotoxicological potential. If there is a genotoxic effect in the umu test, with or without

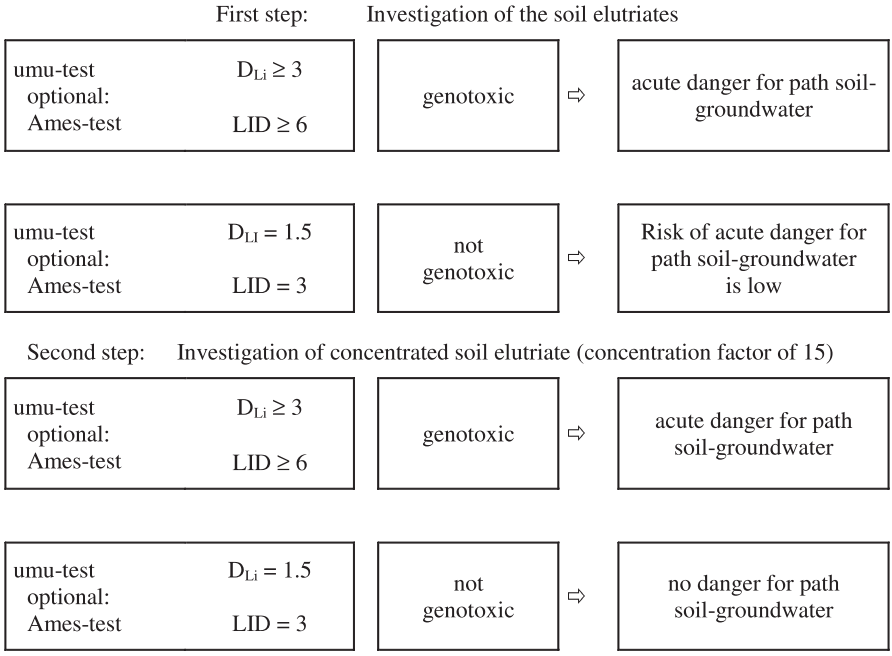

Fig. 18.3. Assessment of the water-extractable genotoxic potential of soils and soil materials using the umu test according to ISO 13829. The Salmonella/microsome test (Ames test) according to DIN 38415 T4 should be carried out if the umu test is negative and there are strong hints from chemical analysis or site history that mutagenic compounds are present. (Eisentraeger et al. 2000; Dechema 2001; Eisentraeger et al. 2001; Rila and Eisentraeger 2003; Eisentraeger et al. 2004; modified according to Ehrlichmann et al. 2000)

metabolic activation, a high risk of transfer of genotoxic substances from soil to the ground water exists. If there is no genotoxic effect, the water extract should be concentrated by a (low) factor of 15 using Serdolit PAD-1 resin. During the ring test mentioned above (Hund-Rinke et al. 2002a) the water extracts were concentrated by a factor of 30, as performed by Ehrlichmann et al. (2000). The factor was reduced to 15 on the basis of results obtained during this test and further studies (Rila and Eisentraeger 2003), since several obviously uncontaminated soil samples (e.g., LUFA 2.1 and LUFA 2.2) tested positive after 30-fold concentration.

18.6.3 Assessment of the Habitat Function

Criteria for the combined assessment of the pathways from soil to soil microorganisms, fauna, and higher plants were elaborated (Fig. 18.4). The

Path soil / microorganisms			Path soil / soil fauna			Path soil / higher plants	
Respiration curve test (ISO 17155)	$Q_r > 0.3$ If $0.2 < Q_r \leq 0.3$ (see legend)		Combined earthworm mortality/reproduction test (ISO 11268-1,-2)	>20% Mortality or reproduction <50% of control		Plant growth test (ISO 11269-2)	biomass reduction > 30% or significant differences between test and control or $BM_m + SD_{BMm} < 0.9 \times BM_{calc}$
Ammonium oxidation test (ISO 15685)	$A_m + SD_{Am}$: $< 0.9 \times A_{calc}$		Collembola reproduction test (ISO 11267)	Reproduction <50% of control			

Overall assessment for evaluation of contaminated and reuse of remediated soil and soil materials

none of the tests positive	low risk of toxic effects
one test positive	case-by-case decision
at least two tests positive	risk of toxic effects given (toxicity identification is recommended)

Q_r Respiratory activation quotient (basal respiration/SIR; if 0.2–0.3, a soil is considered to be toxic if additionally lag phase > 20 h or $t_{peakmax}$ > 50 h).

A_m Activity in the mixture;

A_{calc} calculated mean activity of the test and control soils $(A_{test\ soil} + A_{control\ soil}) \times 2^{-1}$;

SD_{Am} standard deviation of activity in the mixture;

BM_m biomass in the mixture;

BM_{calc} calculated mean biomass of the test and control soils $(BM_{test\ soil} + BM_{control\ soil}) \times 2^{-1}$;

Fig. 18.4. Assessment of habitat function of contaminated/remediated soils and soil materials using ecotoxicological test systems with respect to incorporation in upper soils. A soil sample is considered to be toxic for a certain test organism if the specific toxicity criterion (a) is (are) fulfilled. (Dechema 2001; Hund-Rinke et al. 2002b; Eisentraeger et al. 2004).

test design was approved in a round robin test. For special requirements it is possible to complement the basic test set by further tests. Test results are interpreted using different strategies selected, depending on the test system employed.

Soil Microflora – Respiration Activity. The respiration activity is assessed by the ratio basal respiration:SIR, and by considering the O_2 uptake or CO_2 production over time.

Soil Microflora – Ammonium Oxidation Activity. The nitrification activity of the test soil is assessed by comparison with a control soil and a 1:1 mixture of test soil and control soil. If the nitrification activity in the mixture is below 90% of the mean value of the activity in the test and control soil, the habitat function is assessed as "disturbed" for this criterion.

Soil Fauna. Regarding soil fauna, a minimal habitat function is demanded. The assessment is based on the comparison between the mortality rate and the reproduction in the test soil and in the control. The habitat function is considered disturbed if the mortality rate surpasses 20% and the reproduction rate falls below 50% compared to the control.

Soil Flora. To evaluate potential effects on the soil flora two test strategies have been elaborated. For both strategies a control soil is needed. The first strategy directly compares the biomass production in the test soil and in the control soil. A second possibility is to compare the biomass production in (1) the test soil, (2) a control soil, and (3) a 1:1 mixture of the test and control soils. A biomass determined to be less than 70% in the test soil as compared to the control or less than 90% in comparison to the mean value of the mixed test and control soils is regarded as insufficient and the sample is assessed "toxic".

Preferably, a control soil from the site should have the same physicochemical soil properties as the contaminated soil but no contamination. However, in many cases such a soil is not available and it is then recommended to use a sandy soil (e.g., LUFA standard soil 2.2) to avoid a high sorption of contaminants (for more details see ISO 15799 2003). In cases where the geographical or pedological typicality of the selected soil is important, approaches like the EURO Soil concept (Kuhnt and Muntau 1992) or the German Refesol proposal can help to find appropriate control soils.

The terrestrial tests were selected to give information on the habitat function of the soil. If the habitat function of a soil is reduced, this may result from anthropogenic contaminants (e.g., heavy metals, PAHs, TNT), a high salt content caused by the addition of large amounts of organic material (e.g., compost), or a low pH. Therefore, expert knowledge is needed to decide whether a test is suitable for a specific soil or soil material.

Moreover, results indicating a toxic potential have to be critically examined with respect to further decisions regarding the use of the test material. If there seems to be a need to replace a test or to perform further tests, ecological relevance and practicability should be considered. Under certain circumstances, field monitoring approaches at the assessment site may be appropriate (Roembke and Notenboom 2002).

18.6.4 Overall Assessment – Combined Strategy

In Fig. 18.5, a stepwise procedure for the combined evaluation of remediated soil samples is given as an example for the cost effective application of these bioassays.

1. In the first step it is determined by chemical analyses whether threshold values for single contaminants are exceeded; these values are laid down in national laws, decrees, or guidance papers (e.g., in Germany: BBodSchV 1999). If a threshold value is exceeded, different possibilities exist. Risk-reduction measures to decrease the contaminant levels may be necessary, and/or the further use of this soil is restricted, because the remediation goal for this soil has not been reached. It should be evident that those soils which are clearly contaminated, where threshold values are exceeded, do not have to be tested biologically at all. For the other soils, in which such thresholds have not been exceeded, the water-extractable ecotoxicological and genotoxic potential is tested. If the threshold values of at least one bioassay are exceeded, the source of the toxicity should be identified and appropriate measures taken. If the test results do not indicate a risk for groundwater or surface water, the remediated soil can, for example, be incorporated as subsoil.
2. If, depending on the envisaged use of the soil, the habitat function of the soil has to be assessed, terrestrial ecotoxicological tests have to be performed in a second step. Again, if the assessment criteria are exceeded, the source of the toxicity should be identified and appropriate measures taken. If the values are not exceeded, the habitat function is substantiated and the remediated soil can be used as topsoil.

In the overview thus far presented it has been shown that ecotoxicological test systems are available for the assessment of the retention function and for the habitat function of soils. In addition, the results of these tests can be evaluated to determine whether the soil might cause a risk to the environment. Finally, it should be noted that it may be necessary to modify

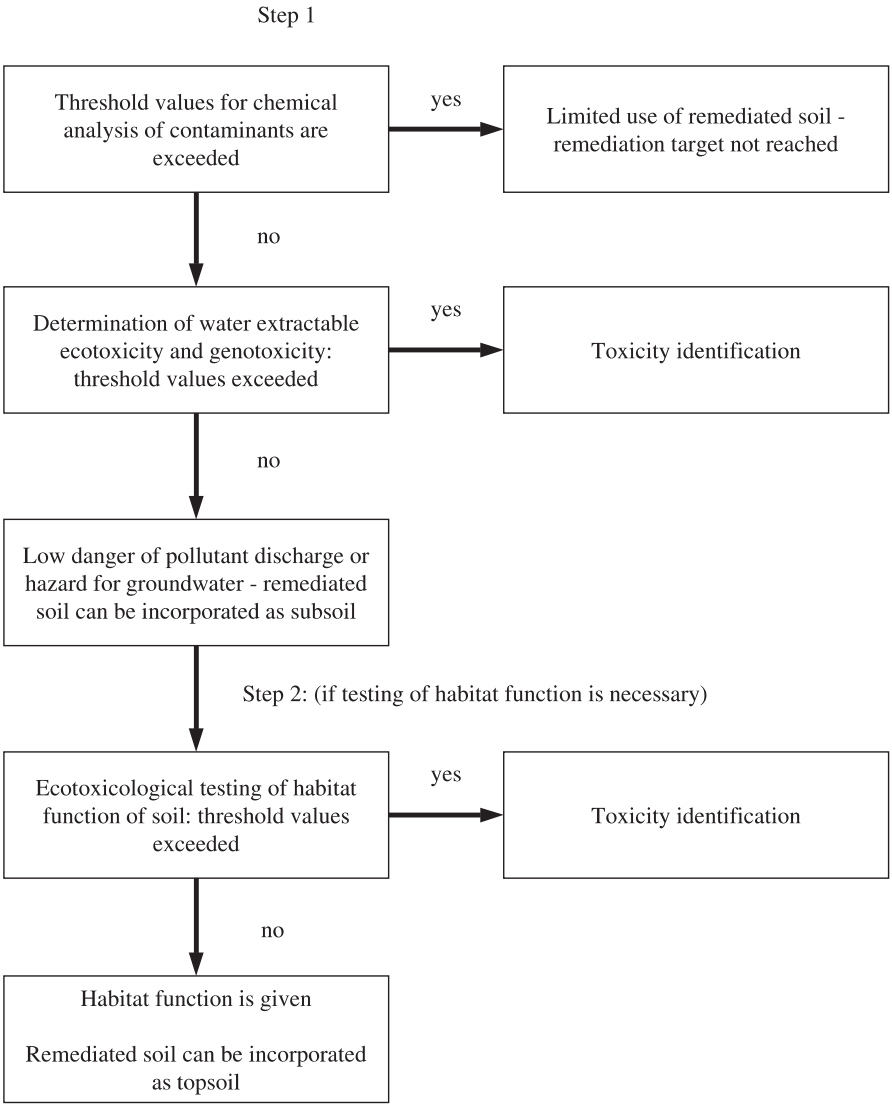

Fig. 18.5. Stepwise procedure for the examination of soils or soil materials using the test systems of Tables 18.1–18.2 and Figs. 18.2–18.4 for remediated soil. (Dechema 2001; Eisentraeger et al. 2004)

the stepwise procedure presented here in specific cases. These modifications might depend on the kind of sample to be tested, the kind of site, the kind of contamination, the overall aim of the investigation (precautionary, complementary to remediation, on-site analytics), and of course on the money available.

References

Achazi R, Roembke J, Riepert F (2000) Collembolen als Testorganismen. In: Heiden S, Erb R, Dott W, Eisentraeger A (eds) Toxikologische Beurteilung von Böden. Leistungsfähigkeit biologischer Testverfahren. Spektrum Akademischer Verlag, Heidelberg, pp 83–103

Ames BN, McCann J, Yamasaki E (1975) Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test. Mutation Res 31:347–364

Anderson JPE, Domsch KH (1978) A physiological method for the quantitative measurement of microbial biomass in soils. Soil Biol Biochem 10:215–221

Anderson T-H, Domsch KH (1990) Metabolic process and soil microbial biomass. Soil Biol Biochem 22:251–255

BBodSchG (Bundes-Bodenschutzgesetz) (1998) Gesetz zum Schutz vor schädlichen Bodenveränderungen und zur Sanierung von Altlasten. Bundesgesetzblatt I, p 502 vom 17. März 1998

BBodSchV (Bundes-Bodenschutzverordnung) (1999) Bundes-Bodenschutz- und Altlastenverordnung. Verordnung zur Durchführung des Bundes-Bodenschutzgesetzes. BGBl I, 36, pp 1554– 582 vom 16.06.1999

Belser LW, Mays EL (1980) Specific inhibition of nitrite oxidation by chlorate and its use in assessing nitrification in soils and sediments. Appl Environ Microbiol 39:505–510

Blagodatskaya EV, Anan'eva ND (1996) Assessment of the resistance of soil microbial communities to pollutants. Eurasian Soil Sci 29:1251–1255

Blagodatskaya EV, Anan'eva ND, Myakshina TN (1996) Description of a soil microbe community in terms of metabolic quotient. Eurasian Soil Sci 28:86–95

Blakemore RJ (1997) Agronomic potential of earthworms in brigalow soils of south-east Queensland. Soil Biol Biochem 29:603–608

Boutin C, Freemark KE, Keddy CJ (1995) Overview and rationale for developing regulatory guidelines for nontarget plant testing with chemical pesticides. Environ Toxicol Chem 14:1465–1475

DECHEMA (2001) Methods for toxicological/ecotoxicological Assessment of Soil. 7th Report of the interdisciplinary DECHEMA committee "Environmental Biotechnology - Soil". DECHEMA Deutsche Gesellschaft fuer Chemisches Apparatewesen, Chemische Technik und Biotechnologie eV, Frankfurt am Main, Germany

DIN 38415 T4 (1998) Deutsche Einheitsverfahren zur Wasser-, Abwasser- und Schlammuntersuchung – Suborganismische Testverfahren (Gruppe T) - Teil 4: Bestimmung des erbgutveraendernden Potentials mit dem Salmonella-Mikrosomen-Test (Ames-Test) (T 4). VCH, Weinheim, Germany

Doube BM, Brown GC (1998) Life in a complex community: Functional interactions between earthworms, organic matter, microorganisms and plants. In: Edwards CA (ed) Earthworm ecology. CRC Press, Boca Raton, pp 179–212

Edwards WM, Shipitalo MJ (1998) Consequences of earthworms in agricultural soils: Aggregation and porosity. In: Edwards CA (ed) Earthworm ecology. CRC Press, Boca Raton, pp 147–161

Ehrlichmann H, Dott W, Eisentraeger A (2000) Assessment of the water-extractable genotoxic potential of soil samples from contaminated sites. Ecotoxicol Environ Safety 46:73–80

Eisentraeger A, Dott W, Klein J, Hahn S (2003) Comparative studies on algal toxicity testing using fluorometric microplate and Erlenmeyer flask growth inhibition assays. Ecotoxicol Environ Safety 54:346–354

Eisentraeger A, Pfeifer F, Dott W (2000): Erfassung des waeßrig extrahierbaren genotoxischen Potentials kontaminierter Boeden. In: Heiden S, Erb R, Dott W, Eisentraeger A

(Hrsg.): Toxikologische Beurteilung von Boeden – Leistungsfaehigkeit biologischer Testverfahren. Spektrum Akademischer Verlag, Heidelberg, Berlin, pp 131–146
Eisentraeger A, Rila J-P, Ehrlichmann H, Dott W (2001): Erfassung des waeßrig extrahierbaren genotoxischen Potentials von Boeden und Bodenmaterialien mit optimierten bakteriellen Genotoxizitaetstests. In: Neumann-Hensel H, Ahlf W, Wachendoerfer V (eds) Nachweis von Umweltchemikalien – Auswerte- und Interpretationsmethoden fuer Toxizitaetsdaten aus einer oekotoxikologischen Testkombination. (Initiativen zum Umweltschutz Band 29). Erich Schmidt Verlag, Berlin, pp 47–60
Eisentraeger A, Rila J-P, Hund-Rinke K, Roembke J (2004) Proposal of a testing strategy and assessment criteria for the ecotoxicological assessment of soil or soil materials. J Soil Sediment 4:123–128
Engebrecht J, Nealson KH, Silverman M (1983) Bacterial bioluminescence: Isolation and genetic analysis of functions from *Vibrio fischeri*. Cell 32:773–781
European Plant Protection Organization (2003) EPPO Standards. Environmental Risk Assessment scheme for plant protection products. Bull OEPP/EPPO 33:195–208
European Union (1991) Council directive concerning the placing of plant protection products on the market No. 91/414/EEC. Brussels, Belgium
European Union (1996) Technical guidance documents in support of Directive 93/67/EEC on risk assessment of new notified substances and Regulation (EC) No. 1488/94 on risk assessment of existing substances. Brussels, Belgium
Focht DD, Verstraete W (1977) Biochemical ecology of nitrification and denitrification. Adv Microbiol Ecol 1:135–214
Hintze TH, Gehlen P, Schröder D (1994) Are microbial biomass estimations equally valid with arable soils and forest soils? Soil Biol Biochem 26:1207–1211
Hopkin SP (1997) Biology of the springtails (Insecta: Collembola). Oxford Univ Press, Oxford, pp 1–330
Hund K, Schenk B (1994) The microbial respiration quotient as indicator for bioremediation processes. Chemosphere 28:477–490
Hund-Rinke K, Koerdel W, Hennecke D, Eisentraeger A, Heiden S (2002a) Bioassays for the ecotoxicological and genotoxicological assessment of contaminated soils (results of a round robin test). Part I Assessment of a possible groundwater contamination: Ecotoxicological and genotoxicological tests with aqueous soil extracts. J Soil Sediment 1:43–50
Hund-Rinke K, Koerdel W, Hennecke D, Achazi R, Warnecke D, Wilke B-M, Winkel B, Heiden S (2002b) Bioassays for the ecotoxicological and genotoxicological assessment of contaminated soils (results of a round robin test). Part II Assessment of the habitat function of soils. J Soil Sediment 2:83–90
Hund-Rinke K, Koerdel W, Heiden S, Erb R (eds; 2002c) Oekotoxikologische Testbatterien – Ergebnisse eines DBU-gefoerderten Ringtests. Erich Schmidt Verlag, Berlin,
Insam H (1990) Are the soil microbial biomass and basal respiration governed by the climatic regime? Soil Biol Biochem 22:525–532
Insam H, Domsch KH (1988) Relationship between soil organic carbon and microbial biomass on chronosequences of reclamation sites. Microb Ecol 15:177–188
ISO 10381–6 (1993) Soil quality – Sampling – Part 6: Guidance on the collection, handling and storage of soil for the assessment of aerobic microbial processes in the laboratory
ISO 11267 (1999) Soil quality – Inhibition of reproduction of collembola (*Folsomia candida*) by soil pollutants
ISO 11268–1 (1993) Soil quality – Effects of pollutants on earthworms (*Eisenia fetida*) – Part 1: Determination of acute toxicity using artificial soil substrate
ISO 11268–2 (1998) Soil quality – Effects of pollutants on earthworms (*Eisenia fetida*) – Part 2: Determination of effects on reproduction

ISO 11269–2 (1995) Soil quality – Determination of the effects of pollutants on soil flora – Part 2: Effects of chemicals on the emergence and growth of higher plants
ISO 11348 parts 1–3 (1998) Water quality – Determination of the inhibitory effect of water samples on the light emission of *Vibrio fischeri* (Luminescent bacteria test)
ISO 11465 (1993) Soil quality – Determination of dry matter and water content on a mass basis – gravimetric method
ISO 13829 (2000) Water quality – Determination of the genotoxicity of water and waste water using the umu-test
ISO 14238 (1997) Soil quality – Biological methods – Determination of nitrogen mineralization and nitrification in soils and the influence of chemicals on these processes
ISO 15685 (2004) Soil quality – Determination of potential nitrification and inhibition of nitrification – Rapid test by ammonium oxidation
ISO 15799 (2003) Soil quality – Guidance on the Ecotoxicological characterization of soils and soil materials
ISO 17155 (2002) Soil quality – determination of the activity of the soil microflora using respiration curves
ISO 22030 (2004) Final Draft: Soil quality – Biological methods – Chronic toxicity in higher plants
ISO 8692 (1989) Water quality – Fresh water algal growth inhibition test with *Scenedesmus subspicatus* and *Selenastrum capricornutum*
ISO/DIS 21268–1 (2004) Soil quality – Leaching procedures for subsequent chemical and ecotoxicological testing – Part 1: Batch test using a liquid to solid ration of 2 l to 1 kg
ISO/DIS 21268–2 (2004) Soil quality – Leaching procedures for subsequent chemical and ecotoxicological testing – Part 2: Batch test using a liquid to solid ration of 10 l/kg dry matter
Jessen-Hesse V, Terytze K, Roembke J, Jänsch S, Eisentraeger A, Filzek P, Grabert E, Hüttner S, Hund-Rinke K, Jander JP, Neumann-Hensel H (2003) Preparation and performance of ecotoxicological test methods under practical conditions – a project overview and first results. Mitt Deut Bodenkundl Ges 102:773–774
Jones A, Hart ADM (1998) Comparison of laboratory toxicity tests for pesticides with field effects on earthworm populations: a review. In: Sheppard SC, Bembridge JD, Holmstrup M, Posthuma L (eds) Advances in earthworm ecotoxicology. SETAC Press, Pensacola, pp 247–267
Kandeler E, Kampichler C, Horak O (1996) Influence of heavy metals on the functional diversity of soil microbial communities. Biol Fertil Soils 23:299–306
Kuhnt G, Muntau H (1992) EURO-Soils: Identification, collection, treatment, characterization. Joint Research Center, European Commission, Ispra
Lavelle P, Bignell D, Lepage M (1997) Soil function in a changing world: The role of invertebrate ecosystem engineers. Eur J Soil Biol 33:159–193
Løkke H, Gestel CAM (1998) Handbook of soil invertebrate toxicity tests. Wiley & Sons, Chichester
Nordgren A, Baath E, Söderström B (1988) Evaluation of soil respiration characteristics to assess heavy metal effects on soil microorganisms using glutamic acid as a substrate. Soil Biol Biochem 220:949–954
OECD (Organisation for Economic Co-operation and Development) (1984) Guidelines for testing chemicals No. 208: Terrestrial plants, growth test
Petersen H (1994) A review of collembolan ecology in ecosystem content. Acta Zool Fennica 195:11–118
Pfeifer F, Haake F, Koerdel W, Eisentraeger A (2000) Untersuchung der Rueckhaltefunktion von Boeden mit aquatischen Testsystemen. In: Heiden S, Erb R, Dott W, Eisen-

traeger A (eds) Toxikologische Beurteilung von Boeden – Leistungsfaehigkeit biologischer Testverfahren. Spektrum Akademischer Verlag, Heidelberg, pp 1–18
Riepert F, Felgentreu D (2000) Relevance of the soil storage to biomass development, N-mineralization and microbial activity using the higher plant growth test. Appl Soil Ecol 20:57–68
Riepert F, Wilke B-M, Kalsch W, Winkel B (2000) Pflanzen als Testorganismen zur Charakterisierung der Lebensraumfunktion des Bodens als Pflanzensstandort. In: Heiden S, Erb R, Dott W, Eisentraeger A (eds) Toxikologische Beurteilung von Böden. Leistungsfähigkeit biologischer Testverfahren. Spektrum Akademischer Verlag, Heidelberg, pp 19–42
Rila J-P, Eisentraeger A (2003) Application of bioassays for risk characterization and remediation control of soils polluted with nitroaromatics and PAHs. Water Air Soil Poll 148:223–242
Rila J-P, Hund-Rinke K, Pfeifer F, Dott W, Eisentraeger A (2003) Validation of microplate bioassays for the assessment of contaminated and remediated sites (laboratory-intercomparison study). J Soil Sediments 3:273–283
Rila J-P, Maxam G, Eisentraeger A (2002) Kap. IV: Genotoxizitätstests. In: Hund-Rinke K, Koerdel W, Heiden S, Erb R (eds) Ökotoxikologische Testbatterien – Ergebnisse eines DBU-geförderten Ringtests. Erich Schmidt Verlag, Berlin, pp 131–156
Roembke J (1997) Taxonomische Probleme in der terrestrischen Ökotoxikologie (Laborebene). Abh Ber Naturkundemus Görlitz 69:33–44
Roembke J, Notenboom J (2002) Ecotoxicological approaches in the field. In: Sunahara G, Renoux AY, Thellen C, Gaudet CL, Pilon A (eds) Environmental analysis of contaminated sites. Wiley, Chichester, pp 181–195
Spurgeon, D, Svendsen C, Hankard P, Weeks J, Kille P, Fishwick S (2002) Review of sublethal ecotoxicological tests for measuring harm in terrestrial ecosystems. Environment Agency, Bristol
Stalder L, Pestemer W (1980) Availability to plants of herbicide residues in soil. Part I: A rapid method for estimating potentially available residues of herbicides. Weed Res 20:341–347
Stenberg B, Johannson M, Pell M, Sjödahl-Svensson K, Stenström J, Torstensson L (1998) Microbial biomass and activities in soil as affected by frozen and cold storage. Soil Biol Biochem 30:393–402
Stenström J, Stenberg B, Johannson M (1998) Kinetics of substrate-induced respiration (SIR). Theory. Ambio 27:35–39
Swift MJ, Heal OW, Anderson JM (1979) Decomposition in terrestrial ecosystems. Studies in ecology, vol 5. Blackwell Sci Publ, pp 1–372
Urbanek J, Dolezal F (1992) Review of some case studies on the abundance and on the hydraulic efficiency of earthworm channels in Czechoslovak soils, with reference to the subsurface pipe drainage. Soil Biol Biochem 24:1563–1572
Wagner C, Løkke H (1991) Estimation of ecotoxicological protection levels from NOEC toxicity data. Water Res 25:1237–1242
Wander M (2004) Soil organic matter fractions and their relevance to soil function. In: Magdoff F, Weil R (eds) Advances in agroecology. CRC-Press, Boca Raton, pp 67–102
Weigmann G (1993) Zur Bedeutung von Bodenarthropoden für die Funktion und die Kennzeichnung von von Ökosystemen. Mitt Deut Ges Allg Angew Entomol 8:479–490
Wiles JA, Krogh PH (1998) Tests with the collembolans *Isotoma viridis, Folsomia candida* and *Folsomia fimetaria*. In: Løkke H, van Gestel CAM (eds) Handbook of soil invertebrate toxicity tests. Wiley, Chichester, pp 131–156
Wilke B-M, Hund-Rinke K, Pieper S, Roembke J, Marschner A (2004) Entwicklung von Prüfwertempfehlungen für ausgewählte Schadstoffe zum Schutz des Bodens als Lebensraum für Bodenorganismen. UWSF – Z Umweltchem Ökotox 16:145–214

Wilke B-M, Pieper S, Roembke J. (2001) Ableitung von Prüfwerten für den Wirkungspfad Boden – Bodenorganismus. Bodenschutz 3:93–100
Winkel B, Wilke B-M (2000) Abschlußbericht des BMBF-Verbundvorhabens: Biologische Verfahren zur Bodensanierung, Teilverbund 4.1: Praxiserprobung biologischer Testverfahren

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