Section 3.
Guidance document on methods for detection of micro-organisms introduced into the environment: Bacteria

1. Introduction

1.1. Aim and scope

The aim of this Guidance Document is to provide information on the "state-of-the-art" detection methods available for micro-organisms released into the environment. The document is meant to offer guidance to regulators and applicants on how to interpret and evaluate data from scientific studies. It is therefore not an exhaustive list of all detection methods presently available, but a document that discusses the merits and pitfalls of a number of the most current, frequently used methods. However, this can also be helpful for similar discussions on alternative, or newly developed methods.

Although this document focuses on the detection of prokaryotes (bacteria), many of the techniques described are also used or developed for detection of fungi and other eukaryotic micro-organisms. However, detection of micro-organisms belonging to these latter groups may be complicated by specific features (for example, multi-cellular structure, sexual reproduction) and so fall outside the scope of this document.

In fact, the scope of this document is limited to situations where the introduced bacteria have previously been characterised in laboratory studies.

The document is meant to assist in the risk assessment of genetically modified micro-organisms (GMMs or GEMs, genetically engineered micro-organisms) introduced into the environment, or indeed, any strain of bacteria or any other micro-organism that is amenable to the methods and techniques described.

1.2. General background

Due to their metabolic versatility, micro-organisms are present everywhere in the earth's biosphere, playing many vital functions in environmental metabolism, for example, mineralization of organic matter, nitrification, nitrogen fixation, and so on. Because of their versatility, micro-organisms can be used in various geochemical processes, for example, bioremediation and mineral leaching. In addition, many beneficial micro-organisms are being explored for agronomic use in crop protection.

In the future, there may be possibilities to exploit the beneficial properties of micro-organisms in the environment. Genetically engineered strains could be constructed that combine useful traits derived from different sources. However, environmental applications of GMMs have raised concerns about possible hazards to the environment such as that the introduced strains may upset natural balances.

The deliberate introduction of micro-organisms into the environment, and of GMMs, in particular, requires risk assessments based on detailed information about the properties and ecological fate of the GMM in question. These assessments are based on assumptions derived from data from previous studies on the survival of the same or similar organisms in the same or similar environmental conditions.

Following the actual introduction of micro-organisms into the environment, monitoring the fate of these organisms is usually done, both to evaluate the validity of the assumptions made in the risk assessment, and to collect additional data to refine future risk assessments.

An adequate risk assessment requires data on the GMM about survival, dispersal, activity and possible interactions with the indigenous microflora. Recent advances have provided new molecular tools and techniques that can be applied in the risk assessment of GMMs. Both traditional and modern techniques provide researchers with a large variety of methods for marking and detection of micro-organisms in environmental studies, in general, and in risk assessment studies, in particular. Proper review of these studies requires extensive knowledge of the merits and limitations of the various detection methods.

In general, the methods rely on the detection of the heterologous gene(s), or other DNA sequences, or on detection of intrinsic properties of the micro-organism. The specificity of the detection technique depends on the uniqueness of the properties of the particular strain in question. The properties may have been introduced by genetic modification, or they may be the result of traditional mutagenesis and selection; therefore, no specific emphasis will be put on the aspects of genetic modification.

This document does not deal specifically with gene transfer in the environment, but the process is a complicating factor when interpreting the results of detection techniques. The issue will be discussed in this context.

Detection of micro-organisms is different from identification, though both may make use of the same techniques. Identification involves the characterisation of previously unknown strains, whereas detection serves the purpose of monitoring the presence and activity of previously characterised strains.

1.3. Outline of the document

Section 2 presents a discussion of general issues of the detection of micro-organisms. It tries to put the questions around detection methods into perspective, as an aid to the risk assessor.

Section 3 presents detailed discussions of these issues, against the general background of section 2, for individual methods and markers commonly used for detection. The detailed information in section three is cross-referenced to the corresponding paragraphs in section 2 and vice versa.

The reliability of experimental data of environmental studies is dependent on the robustness of the experimental methods. The methods should be properly validated; application of the methods should be subject to quality assurance procedures. Section 4 presents a discussion on these issues.

Section 5 presents information on the importance of quality control and quality assurance of methods used to detect micro-organisms in the environment. Quality control can help to eliminate inter-laboratory variability in test results, allowing a comparison of data from different studies.

In Section 6, examples are presented that show how the interpretation of data on survival of bacteria in the environment is dependent on the detection method that has been used, and how the environmental conditions can affect the results.

1.4. Sources used for the present study

The discussion in section three will focus on the nature and quality of data obtained through different detection methods. Peer-reviewed articles will be used as a source of information, as well as published textbooks. However, it should be noted that, generally, not all of these issues are addressed concomittantly in peer-reviewed literature. Data will be scrutinised as to their quality and relevance.

Special attention will be given to important parameters such as specificity[1], sensitivity[2], reproducibility[3] and repeatability[4]. Literature included in this document does not solely involve field studies, but also studies of microcosm and mesocosm experiments. The latter studies are only included if they contain results that allow extrapolation to environmental situations.

2. Detection of micro-organisms in the environment: Overview of physiology and methods

2.1. Introduction

This section presents an overview of the characteristics of methods for the detection of bacteria introduced into the environment. Section Three presents more detailed information.

The detection of a particular organism in a particular environment requires:

- presence of at least one unique trait or unique nucleotide sequence in the strain that is suitably stable under the physiological conditions set by the environment, and that allows for discrimination of the organism from, in principle, all other organisms present in the sample; and

- a robust detection method that allows a (semi-) quantitative assessment of the trait.

A bacterial strain that is considered for introduction into the environment for some particular use will have been characterised in terms of its general physiology, as well as the specific traits that are needed for the purpose.

This means that a thorough knowledge is available on the growth characteristics and requirements of the strain, as well as its physiological behaviour under laboratory conditions, and possibly concomittantly under micro or mesocosm conditions. At the same time, the strain must have been characterised for traits that can be used for environmental detection.

The traits used for detection may be indigenous to the strain, or they may be acquired from any form of mutation or exchange of genetic information or deliberate genetic modification. As the specificity and sensitivity of the detection depends on the presence of the same trait in the receiving environment before the introduction, a survey must have been made before the introduction of the bacteria, to assess the presence of micro-organisms with the same trait.

Detection can have different goals. It may aim at detection of the number of 'live' viable, bacteria present, or the detection of some specific environmental activity of the bacteria. Here mainly techniques for the enumeration of bacteria will be discussed. But as many of these techniques depend on specific metabolic activity of the bacteria, the problems of assessing such activity will also be covered.

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1 Specificity is defined in general as the capacity to specifically recognize the target organism, distinguishing it from similar non-target organisms. Specificity of a diagnostic test is the probability not to detect a target organism (negative response) in non-infected or non-contaminated test material.

2 Sensitivity is defined in general as the capacity to record small variations in concentration of a target organism in the test material. Sensitivity of a diagnostic test is the probability of detecting a target organism (positive response) in an infected or contaminated test material.

3 Reproducibility is defined as the difference between two single test results with the same method on identical samples under different conditions (*e.g.* different laboratories, different operators, different equipment).

4 Repeatability is defined as the difference between two single test results with the same method on identical samples under the same conditions.

After introduction into the environment, the strain will no longer be under controlled conditions. This may affect its physiology in many ways, changing expression patterns, and possibly even diminishing our ability to isolate it from the environment.

The interpretation of detection results, therefore, requires some understanding of the interplay between the organism, its physiological state, the trait, and the detection method.

This section presents an overview of factors that influence this interplay, as a background for the more detailed description of specific traits and methods in the following sections.

2.2. Physiological and other states of micro-organisms introduced into the environment

The physiological and other states (*e.g.* lysed cells, cell debris) of micro-organisms introduced into the environment determine what techniques can be applied for detection (see Table 1). In general, readily culturable cells introduced into the environment can enter into different states, relevant to detection: viable/culturable; viable but not culturable (due to different mechanisms); ghosts/lysed cells; and cell debris.

2.2.1. Overview of states

Viable/culturable – 'Viability' indicates the ability of an organism to grow; culturability is the ability to grow in a certain growth medium under defined conditions of atmosphere and temperature. For all detection methods that require growth, viability of the organism under the conditions of culture is essential. Optimal culturing conditions in the lab may not be the optimal conditions for retrieval of viable organisms from the environment. Only those organisms will be observed that can adapt from the environmental conditions to culture conditions in the laboratory. Adaptation may occur only after a certain lag time; the number of viable organisms observed may therefore increase with the length of the observation period.

Viable but not culturable (VBNC) - It is now recognised that a large proportion of micro-organisms in the environment are in a physiological state where they may be viable or metabolically active without cell growth. This not only applies to indigenous organisms for which suitable growth conditions have not (yet) been established, but also to micro-organisms that have been cultured in the lab and subsequently intoduced into the environment. The latter may lose their culturability, requiring special media to recover them from environmental conditions. If this is not recognised, the detection method will overlook these bacteria.

Viable, metabolically active organisms will not manifest their complete capabilities all of the time. Conditions in the environment will influence the expression pattern of many genes. Moreover, it has recently been realised that the expression pattern may also be influenced by the complex interactions with other organisms in the environment. Active organisms in the environment may therefore 'look' very different from the same organisms under laboratory conditions.

Ghosts/lysed cells - Immediately after cell death some cells may still possess an intact cell wall. Eventually, the cell wall will lose its integrity (or loss of integrity results in cell death), resulting in empty cell envelopes without cytoplasm. These 'ghosts' will be recognised by methods that rely on markers that are present in or on the cell envelope. The markers that are present on the ghost depends on the physiological activity of the cell before death, and the possible instability of the marker, for example, due to degradation after cell death.

Cell debris and constituents - After lysis of the cell, complex biological molecules may remain present and recognisable by detection techniques for some time. Proteins, RNA and DNA are generally not stable in the environment, but marker molecules bound to soil particles may escape degradation, and

remain biologically intact and available for detection. Some proteins (for example, bacterial Bt pro-toxins and the crystal proteins of baculoviruses) are especially stable in the environment.

In general, the environmental stability of macromolecular compounds is not well understood, and it should be kept in mind that on the one hand, ghosts may lose their distinctive markers, while on the other hand, cell debris may remain intact for some time after cell death.

Enzymes may remain active in ghosts and cell debris. However, enzymes that require cofactors for activity will not be active for long, as the biosynthesis of cofactors like ATP and NAD(H)P require metabolic activity.

Table 1. Methods which can be used to detect micro-organisms which might be present in the environment in various states

	Detection Method				
State	Cultivation based	*gfp* marked	Immuno-based	RNA-based	DNA-based
Viable/culturable	+	+	+	+	+
VBNC	-	+	+	+	+
Ghost/Lysed Cell	-	-	+	-	-
Cell Debris	-	-	-	-	+

+ indicates suitable for detection, - indicates not suitable for detection

2.3. Detection methods

This section deals with the general characteristics of detection methods and the requirements which pose on the physiology and traits of the bacteria that are to be detected.

2.3.1. Methods based on bacterial growth under laboratory conditions

2.3.1.1. Direct plating

Methods typically rely on growth of the organism from a single cell to a visible colony on solid medium. The time required to form a visible colony depends on the growth rate of the micro-organism, which is influenced by intrinsic factors (*e.g.* physiological status) and external parameters (for example, temperature, availability of nutrients). Usually colonies are observed by the naked eye, but by the use of a microscope the visibility of (small) colonies can be enhanced.

Type of information - Numbers of organisms can be assessed by counting the number of colonies derived from a known volume of a known dilution of a sample.

If a selective medium can be employed that only allows for growth of the organism, detection can be straightforward by looking at the number of colonies. If other organisms can also grow, there must be a possibility to recognise the desired organism against this background. In that case, the conditions of growth must allow for 'election' of the organism, based on some specific reaction with the culture medium (for example, substrate conversion due to β-galactosidase activity).

As a spin off, the direct plating methods for single colonies of the organisms can result in pure cultures that can be used for further characterisation.

Physiological requirements - The method only assesses cells that are culturable, and remain so under the conditions of sampling, plating and growth (culture medium, temperature) in the laboratory.

Requirement for marker traits - Depending on the number of other organisms present in the samples, capable of growth under the chosen conditions, it will be necessary to have a selective or, at least, an elective trait in the organism.

Technical requirements - Special techniques may be required to detach organisms from particles in the sample, which is one of the critical steps for reliability of the method.

Speed, sensitivity, reliability - Time required depends on the growth rate of the organism under the conditions of plating.

Sensitivity is typically in the order of 10^2 bacteria per gram soil; growth and recognition of bacteria at low dilutions of the sample may be hindered by a large background of other organisms that also grow under the conditions of plating. Even if plating is carried out under selective conditions (for example, in the presence of antibiotics) unexpected background growth may occur.

The reliability of the method is limited by the impossibility of retrieving all organisms that are viable in the environment as culturable organisms under laboratory conditions. This is not always recognised in environmental studies, which usually assume that plating methods retrieve all viable organisms present in the environment. The first requirement, suitable growth conditions for the bacterial strain in the laboratory are known from the previous characterisation of the strain.

The conditions during sampling and the switch to growth conditions in the laboratory may put extensive physiological stress on the bacteria. Even the sudden dilution of cells from environmental conditions to a situation where they are single and separated from other cells may impose stress on the cells. For some bacterial species this phenomenon has been shown to be related to the loss of signal molecules that play a role in quorum sensing. The term 'quorum sensing' is used to describe the phenomenon whereby the accumulation of signalling molecules enable a single cell to sense the number of bacteria (cell density). In the natural environment, there are many different bacteria living together which use various classes of signalling molecules. As they employ different “languages” they cannot necessarily communicate with all other bacteria. Quorum sensing enables bacteria to co-ordinate their behaviour. As environmental conditions often change rapidly, bacteria need to respond quickly in order to survive. These responses include adaptation to availability of nutrients, defence against other micro-organisms which may compete for the same nutrients and the avoidance of toxic compounds potentially dangerous to the bacteria.

2.3.1.2. Most probable number methods

Most probable number methods are based on detecting the presence of a particular organism in dilutions of a sample. Samples are diluted to concentrations at which there is a high probability that a relatively large number of samples do not contain the organism. The most probable number of bacteria in the original sample can be inferred by statistical methods, from the number of samples in a dilution that contain zero organisms.

In practice, the organisms in the diluted sample are allowed to grow, and the presence of the organism that is to be detected is assayed by some biological effect that is specific to the organism, for example, an enzyme reaction.

Type of information - Similar to direct plating. The method does not, however, yield single, pure colonies for further study and characterisation (still, these could be obtained by sub-culturing from the samples after growth).

Physiological requirements - Similar to direct plating.

Requirements for marker traits - This method requires growth and a unique biological activity of the strain, by which it can be assayed.

Technical requirements - This method requires a homogeneous distribution of the bacteria, and therefore, relies on the detachment of organisms from particles in the sample. Special technical facilities may be

required in particular systems, to assay the biological effect (*e.g.* production of a specific metabolite) by which the presence of the organism is recognised.

Speed, sensitivity and reliability - Dependent on the biological effect, and the way this is measured, the method may require more extensive growth than direct plating methods, and will therefore take longer.

The sensitivity is comparable to direct plating techniques; this is, however, dependent on the sensitivity of the measurement of the biological effect by which the presence of the organism is recognised.

The reliability depends, in the first place, on the soundness of the assessment of the biological effect. The reliability further depends on the experimental approach chosen for the determination of the most probable number, *i.e*. depends on the dilution steps and the number of samples analysed per step. It is generally felt that most probable number determinations are less reliable than results from direct counting.

2.3.2. Detection by assessment of marker gene products

These methods rely on the determination of the product of a marker gene, which is specific to the detected organism.

The gene product may be detected based on its enzyme activity, or on the basis of its physico-chemical properties, for example, a green fluorescent protein that can be detected by its fluorescence when illuminated by light of a suitable wavelength.

Type of information - From the data one can calculate, in principle, the number of protein molecules present in the sample. If it can be assumed the organisms are homogeneous as to their content of the protein, the method is applicable for the assay of relative numbers of bacteria.

In situ detection of the presence of the protein is possible, in principle. It should be kept in mind that data obtained by these methods only show the presence of an intact protein, not necessarily of an active organism.

Physiological requirements - The marker gene must be (or have been) actively transcribed and translated. If the protein needs cofactors for its activity, these must be available for *in situ* measurement. Gene expression can be influenced by environmental conditions. It should be kept in mind that gene expression may be under the control of either constitutive or inducible promoters. Also more global regulation processes may also affect the level of gene expression. It cannot be assumed a priori that gene expression will be the same, for instance, in culturable cells and viable but not culturable cells. The time span during which the gene product can be assayed after its production varies with the stability of the protein under environmental conditions. Gene products that are inserted into the cell envelope may be assayed on ghosts. The presence of proteins that are stable enough to be detectable in cell debris may cause a background noise which interferes with the detection of cells.

Requirements for marker traits - The gene of interest must be present in a stable form in the genome; gene expression must be stable under environmental conditions.

Technical requirements - Extraction of enzymes should be performed under conditions where loss of enzyme activity due to inactivation is minimised. Assay of enzyme activity *in vitro* is usually straightforward.

Assessment of protein activity *in situ* may require specialised equipment. Cofactors or specific substrates may have to be made available during the assay; if the proteins are assayed in whole cells, cell envelopes may have to be made permeable for these substances.

Speed, sensitivity and reliability - The assessments can be done very fast, or even in real time. Preparation of samples may however be time consuming.

The sensitivity strongly depends on the marker gene product and the detection method used.

Reliability is dependent on the reliability of expression and stability of the gene products.

2.3.3. DNA and RNA hybridisation methods

These methods usually rely on the assessment of DNA sequences of a marker gene (although intrinsic markers present in entire genomic DNA may be used as well), that is specific for the detected organism, or of RNA transcribed from the sequence. The provisos mentioned for detection by assessment of marker gene products apply in a similar way to this section.

2.3.3.1. Direct detection by hybridisation

Detection methods are based on hybridisation of probes, marked or tagged for detection, to a specific target sequence. The target sequence is extracted from the sample and immobilised for the hybridisation reaction. Hybridisation may also be performed *in situ*.

Detection of the hybridised probes may be performed on the basis of fluorescence or a chromogenic enzyme reaction, or on radioactive labelling.

Assays of this type usually allow qualitative conclusions: the sequence is either present or absent. Quantitative results may be obtained through the use of extensive controls which are run along with the assay.

Type of information - The presence or absence of the DNA or RNA molecules is determined, but with the proper controls the quantity of target molecules can be calculated. In principle, the number of organisms present in the sample can be calculated if the copy number (for DNA) or the number of molecules per cell (for RNA) is known.

It should be kept in mind that if data obtained by these methods detects the presence of a target DNA sequence, this does not necessarily reflect the presence of an active organism. However, the presence of RNA can serve as an indicator of active cells.

As RNA is usually less stable than DNA, methods based on RNA targets rely on active transcription of the sequence. The regulatory status of the cell will influence the abundance of RNA in a similar way as it influences abundance of proteins.

Physiological requirements - The results indicate the presence in a sample of a sequence, irrespective of whether the sequence is derived from a viable or active organism, or from inactivated organisms or cell debris (lysed cells and ghosts have the status of cell debris in this discussion).

Requirements for marker traits - Marker traits should be unique for the strain in question. The most frequently used intrinsic marker genes, that is, genes coding for ribosomal RNA (rRNA), do not necessarily meet this condition, as they are species specific rather than strain specific. These sequences can only be used if the abundance of strains of the same species in the receiving environment is low. On the other hand, as the concentration of rRNA is very high, at least in active cells, and as rRNA carries species-specific sequences, rRNA is the target of choice in many environmental studies.

Technical requirements - Preparation of probes, if done by oligonucleotide synthesis, requires specialised equipment and sufficient know-how.

DNA and RNA samples for hybridisation do not have to meet very stringent quality criteria. Extensive fragmentation of the material should be prevented, especially when the sequences are separated by gel electrophoresis before hybridisation. Fragments that are heterologous in size will form smears in electrophoresis, which are less easily visualised by hybridisation methods.

Speed, sensitivity and reliability - These methods are relatively time consuming, because of the sample preparation, and because of the time needed for hybridisation and visualisation.

Sensitivity is usually low and is dependent on the number of target sequences present in the cell. If the copy number is high, as is the case for rRNA for instance, the sensitivity for the detection of cells can be quite high. The speed of the procedure and the sensitivity can be enhanced by use of special equipment. In-situ background noise may be a problem with some environmental samples due to autofluorescence. This requires the proper selection fluorophore for different samples

Reliability depends on the specificity of the hybridisation. This is very much influenced by the stringency of the conditions for hybridisation.

Quantitative reliability is low; usually in order of magnitude of the amount of target sequence can be determined.

2.3.3.2. Detection after amplification of DNA or RNA sequences

These methods allow for a much more sensitive assay of specific DNA or RNA sequences by the use of an amplification step by means of a polymerase chain reaction (PCR).

The PCR may be carried out qualitatively, in which case only the presence or absence of a sequence can be tested. Quantitative PCR methods have been developed, and allow for quantitative assays. The method is, however, much more sensitive, as in principle one target molecule can be detected.

Type of information - Similar to direct hybridisation methods (2.3.3.1), but the assay is more sensitive.

Physiological requirements - Similar to direct hybridisation methods (2.3.3.1).

Requirements for marker traits - Similar to direct hybridisation methods (2.3.3.1), but in this case the chosen sequence should additionally contain short sequences on both 3' and 5' side, which can be used as unique primers for the PCR reaction.

Technical requirements - Sample preparation should yield DNA or RNA preparations that do not contain inhibitors of the PCR reaction. This turns out to be difficult and time consuming, especially for preparations from soil samples. A thorough check for the quality of the preparation should always be carried out. Presently various commercial soil DNA extraction kits are available, which claim to extract high quality DNA. Although these are quick and easy to use, they may not yield a satisfying DNA preparation for all soil types.

PCR requires equipment that is no longer very specialised; quantitative PCR, however, still requires special equipment and specific know-how.

Speed, sensitivity and reliability - Speed and reliability are similar to direct hybridisation methods (2.3.3.1), except in the case of real-time PCR, which can be very fast. To obtain reliable results a dedicated laboratory is required, to prevent false positive results. Sensitivity can be very high; as little as one or a few DNA or RNA molecules as the lower detection limit.

Table 2 Overview of considerations on technical requirements, speed, sensitivity and reliability for detection methods addressed in Section 2.3

	Techincal requirements	Speed	Sensitivity	Reliability
Direct plating	Detachment of organisms from particles in sample	Dependent on growth characteristics of detected organisms	Typically 10^2 bacteria per gr soil	Limited by retrieval of organisms in a state viable under laboratory conditions
MPN methods	Detachment of organisms from particles in sample. Recognizable trait.	May require more extensive growth than direct plating	Dependent on sensitivity of measurement of biological effect; comparable to direct plating	Dependent on reliability of assessment of biological effect, and on statistical validity of experimental design
Detection of gene products	Extraction of enzyme without loss of activity	Typically fast (within hours) or real time	High	Dependent on reliabilty of expression and stability of the gene product
DNA/RNA hybridization, direct	Prepration of probes requires equipment and know-how	Time consuming due to sample preparation and time required for hybridization	Dependent on specificity of probes and stringency of hybridization conditions	Dependent on specificity of probes and stringency of hybridization conditions; quantitative reliability is low
DNA/RNA hybridization after amplification	Samples should be free of PCR inhibitors	Time consuming due to sample preparation and time required for hybridization	Very high, up to a few DNA or RNA molecules	Dependent on experimental conditions to avoid false positives

3. Detection of micro-organisms in the environment: Details of methods

The aim of this section is to provide a more detailed description of the most commonly used techniques to enumerate micro-organisms in environmental samples.

It is by no means the aim of the authors to treat and describe all methods that could be used.

3.1. Traits of the organism which can be used for specific detection

Detection and enumeration of specific micro-organisms is of prime importance for the monitoring of the fate of micro-organisms introduced into the environment (Smit *et al*., 1992). One of the major challenges is the specific detection of a certain micro-organism in all of its physiological states within the tremendously diverse natural microbial community. In order to be able to detect only the species of interest, it is essential that the organism possess at least one trait that can be used to distinguish it from all other micro-organisms. Such traits can be intrinsic, that is, a property that the micro-organism possesses naturally, or it can be introduced by selection, for example, spontaneous antibiotic resistance; alternatively the marker genes may be introduced into the micro-organism through genetic modification.

The nature and properties of the marker gene are very important and determine which detection method(s) should preferentially be used, how sensitive the methods will be and in what way the measured data should be interpreted (Akkermans *et al*., 1998). A marker can either be selective such as antibiotic resistance genes or elective. An elective marker allows an organism to be recognized specifically among other, non-target organisms. Some elective markers can also be used for direct detection and enumeration of cells without the need for cultivation, for instance, by directly measuring the signal, by microscopy or by flow cytometry. Some marker systems can be used to detect metabolically active cells. Although such data are highly informative, this approach might be less suited for enumeration in samples where the signal may be low as a result of starvation.

Currently, quite a range of different elective markers is being used such as *lux*, *luc*, *xyl*E, *gus*, *lac*Z, *gfp* and *celB*. There are several prerequisites for the successful use of these markers: 1) they should be present in a stable condition, in the micro-organism; 2) the marker should be expressed at a detectable level; 3) there should not be a high background in the samples which are studied; and 4) there should be a known relationship between the signal produced and the number of cells, in cases where the marker is to be used for enumeration. In order to facilitate the choice of a marker system and the interpretation of the obtained data, it can be of great help to distinguish different classes of markers, which exhibit similar properties. In this document, five classes of marker genes are described:

1) markers which encode antibiotic or heavy metal resistance and which can only be used in a cultivation based detection method;

2) markers which encode compounds which can be detected directly and which can be used without cultivation;

3) markers encoding enzymes which mediate an enzymatic reaction, requiring the addition of a substrate and which can be used for either direct detection or in a cultivation based approach;

4) markers encoding enzymes which mediate energy dependent reactions and which can be used for both direct detection and detection after cultivation; and

5) specific DNA sequences which can be detected without cultivation.

The use of these markers and the choice of the detection method depend on the purpose of the study. The experimental system and the scientific questions will ultimately determine which markers and which detection methods are most suitable.

Markers of class 1 can only be used in culture based detection methods. Markers of class 2, for example, the genes coding for stable *gfp,* are very suitable for direct enumeration of cells in the environment, while markers of class 3 are generally used as confirmation after cultivation, although they are occasionally used for direct detection. Markers of class 4 are more suited for direct detection of cell activity in the environment to distinguish between active and non-active cells. Class 5 markers allow direct detection and do not depend on expression of the DNA sequence. Finally, it should be noted that most markers could also be used in combination with a number of other, less obvious detection methods, *e.g.* direct detection of an antibiotic resistance gene by PCR amplification.

3.1.1. Markers which confer resistance

Traditionally micro-organisms in a certain environment are detected *via* plating or MPN techniques. For these methods, it is essential that the organisms of interest harbor certain selective traits, or at least, traits that enable them to be recognized. For instance, when certain bacteria in soil lacking any known selective trait are plated onto a non-specific medium, they will form colonies along with a large number of colonies from other micro-organisms. When such organisms harbor an elective marker they can be recognized based on color, size or some other characteristic. However, since many other bacteria are able to grow on these plates, the detection limit will still be between 10^5 and 10^6 cells per gram of soil

because colonies of the bacterium of interest will be completely overgrown when lower dilutions are plated. Therefore, it is almost inevitable to use a selective marker as well to counter select other micro-organisms. Markers such as antibiotic resistance genes and heavy metal resistance genes have been used to selectively cultivate the micro-organism of interest from the environment.

3.1.1.1. Antibiotic resistance

The majority of the selective markers used for the specific detection of bacteria introduced into the environment have been antibiotic resistances (Smit *et al*., 1996b). Important prerequisites for the use of antibiotic resistance as selective marker are: 1) the micro-organism has to be culturable; 2) the resistance should be stable; 3) the environment should be investigated for the presence of high numbers of resistant micro-organisms which will produce background growth; and 4) the antibiotic resistance should be expressed. However, there is a world wide trend to limit the environmental introduction of these genes. The use of these genes as markers for environmental use should therefore not be promoted.

There are two methods to mark bacteria with antibiotic resistance. Cells can be cultured and plated onto antibiotic containing medium to select for spontaneous resistant mutants, or an antibiotic resistance gene can be introduced into the bacterium by traditional genetic exchange or by genetic modification. The use of spontaneous mutants resistant against rifampicin has been successful in many environmental studies (Liang *et al*., 1982; van Elsas *et al*., 1986; Turco *et al*., 1986; Compeau *et al*., 1988; Glandorf *et al*., 1992; Nijhuis *et al*., 1993). The number of antibiotic resistant bacteria that can be detected in soil depends on the type of antibiotic and the type of soil. A certain percentage of the micro-organisms in the environment are naturally resistant to certain antibiotics which results in a background level of CFU's on antibiotic containing plates. Generally, a combination of more than one antibiotic resistance will result in a lower background of resistant colonies of the natural microflora.

3.1.1.2. Heavy metal resistance

Another class of selective markers is resistance to heavy metals. Although the natural background of heavy metal resistance or tolerance in soil seems higher than that of antibiotic resistance, heavy metals have the advantage that they do not interfere with therapeutic use (Mergeay, 1995). Researchers from the lab of Mergeay have developed a heavy metal gene cassette consisting of genes encoding for resistance against cobalt, zinc and cadmium, which allows a very specific detection. Factors that can affect the results when using heavy metal resistance are the choice of the medium, since its components should not form complexes with the metals, and the level of expression in the micro-organism.

3.1.2 Markers which code for compounds which can be detected directly

3.1.2.1. Green fluorescent protein

The green fluorescent protein (*gfp*) gene was originally obtained from the jellyfish *Aequoria victoria* and has been expressed in prokaryotic and eukaryotic cells (Chalfie *et al*., 1994). *Gfp* is a protein that emits green light at 508 nm when excited with blue light of 396 nm. *Gfp* fluorescence is independent of the energy charge of the cell. The product of the wild type gene is very stable and there is no need to add a substrate (Chalfie *et al*., 1994). In an experiment with *gfp* marked pseudomonads in phosphate buffer Cassidy *et al*. (2000) showed that fluorescence was almost constant up to 48 days. The use of *gfp* as marker is particularly suited to monitor cell number by detecting individual cells without cultivation. Detection of marked cells can be achieved by epifluorescence microscopy, laser confocal microscopy, flow cytometry and spectrofluorometry (Tombolini and Jansson, 1998). It is possible to study the location of the cells marked by *gfp* on plant roots by studying samples with confocal laser scanning

microscopy (Normander *et al*., 1999; Bloemberg *et al*., 2000). Because of these advantages *gfp* is chosen as a marker instead of *lacZ* and Xyl*E*.

Recent developments can provide even more information on cell physiology by fusing the *gfp* gene to specific promoters which respond to specific environmental conditions in combination with labeled 16S DNA probes (Moller *et al*., 1998). Using different derivatives of the green fluorescent protein, namely enhanced cyan (*ecfp*), enhanced green (*egfp*), enhanced yellow (*eyfp*) and the red fluorescent protein (*rfp*), it is also now possible to simultaneously visualize different populations of micro-organisms. Bloemberg *et al*. (2000) could visualize and distinguish *ecfp*, *egfp* and *rfp* marked *P. fluorescens* cells in the rhizosphere of tomato plants by triple imaging using a confocal laser scanning microscope with negligible cross talk.

The versatility of the *gfp* system is further enhanced by the possibility of using both stable and unstable *gfp* as reporter genes. Especially unstable *gfp* reporter genes are suitable to measure specific physiological reactions to conditions in the environment since they will remain intact only for a short period (Jansson, 2000). Currently, most research is not focussed on simply detecting the introduced micro-organism; the aim is to investigate bacterial activity in relation to the conditions in the environment. For this purpose reporter genes can be combined with the appropriate promoters, such as those which respond to amino acid, carbon, phosphate or nitrogen starvation (Jansson, 2000).

3.1.3. Markers which mediate enzymatic reactions which require a substrate

3.1.3.1. LacZY[5]

LacZY is an elective marker with a selective component, which can be used in microbial ecology studies. The *lacZ* gene codes for ß-galactosidase which can cleave the chromogenic substrate 5-bromo-4-chloro-3-indolyl-ß-D-galactosidase (X-gal) and change the appearance of the colonies when present in the plates. The selective part consists of the *lacY* gene, which codes for lactose permease, which enables the cell to take up lactose. This is particularly useful for pseudomonads since they are unable to utilize lactose as a carbon source. However, lacZY by itself is not selective enough to prevent the naturally occurring microflora from growing on the plates. Therefore, the *lacZY* marker cassette has been used in combination with one or more antibiotic resistances (Hofte *et al*., 1990; Kluepfel, 1993; Drahos *et al*., 1992; Bailey *et al*., 1995; Nairn and Chanway, 1999). The use of *lacZY* as a marker in microbial ecology has proved very useful in studies on dispersion and survival of bacteria. However, it is mainly used in cultivation based detection systems and it is less suited to be used in direct detection procedures. *LacZ* has also been used in experiments in which plant-bacteria interactions were studied, however its use seems limited because of high background in rhizosphere bacteria (Lambrecht *et al*., 2000). To date *lacZY* is gradually being replaced by the more versatile *lux* or *gfp* marker systems.

3.1.3.2. XylE

The marker gene *xylE* codes for catechol-2,3-dioxygenase (C230), which can cleave catechol. Catechol can be sprayed onto plates with colonies of bacteria marked with *xylE*. In this reaction a yellow substance, 2-hydroxymuconic semialdehyde, is formed which can be seen in colonies on plates. As with *lacZY*, *xylE* is an elective marker, which is mainly used in conjunction with antibiotic resistance, and a cultivation based detection method. There are a number of studies in which *xylE* has been used as a marker to facilitate detection of the introduced cells (De Leij *et al*., 1998; Morgan *et al*., 1989; Winstanley *et al*., 1991; Wipat *et al*., 1991). Currently, *xylE* is not often used as a marker to study bacteria introduced into the environment.

[5] Lactose Fermentation is an example of a metabolic trait that is often used for wild type strains in MPN.

3.1.4. Markers which mediate enzymatic reactions which require energy and substrate addition

3.1.4.1. Bioluminescence

There are currently two bioluminescence genes used as markers, *lux* from the bacteria *Vibrio fisheri* and *luc* from the eukaryotic firefly *Photinus pyralis*. The production of light by *lux* or *luc* is mediated *via* the enzyme luciferase and is energy dependent. For luminometry, both *lux*AB and *luc* marked strains require the addition of a substrate. For *lux*AB, it is necessary to add n-tetradecyl aldehyde, dodecanal or decanal and for *luc* one has to add luciferin. The firefly luciferase is very efficient and has a high yield. Application of *luc*, however, is limited by the fact that the substrate luciferin is quite expensive and it can not permeate through bacterial cell walls. Cells must be permeabilized before the measurement can take place. The *luc* system is therefore recommended for use only when high sensitivity is required (Prosser *et al*., 1996). The whole *lux* operon consists of *lux*R, I, C, D, A, B and E. *Lux* A and B are the structural genes for the luciferase enzyme and *lux* C, D and E are involved in the synthesis of the aldehyde. Bioluminescence is regulated by *lux*I and R. In most cases bacteria are marked using the *lux*AB only and the aldehyde, which is freely permeable, is added to the cells. An important advantage is that light can be measured in real time, sometimes even without extraction or cultivation (Prosser, 1994; Yolcubal *et al*., 2000). Light output seems to be correlated with the amount of ATP in the cells and is therefore an indicator of the energy charge (Maechler *et al*., 1998). A very specific property of light is that it can not accumulate in the cells as a result of former activity, so light output gives an instant impression of the energy status of the cell. When the promoter is down regulated or when the amount of ATP is low, production stops, and the signal disappears. Currently, *lux* and *gfp* are the markers, which are employed most frequently in studies on bacterial activity and survival in the environment. In Table 3 a summary is given of advantages and disadvantages of *gfp* and *lux* in combination with various detection methods.

Table 3 Advantages and disadvantages of *lux* and *gfp* in combination with several detection methods

Marker	Detection Method	Advantage (+) or Disadvantage (-)
Lux	MPN cultivation or plating and bioluminescence measurements	+ suited for enumeration + sensitive; strong signal - combination needed with resistance - detects only culturable cells
	Bioluminescence in extract	- not suited for enumeration + suited to measure activity - not sensitive + detects culturable and non-culturables
Gfp	MPN or plating and detection of fluorescence	+ suited for enumeration + sensitive - combination needed with resistance - detects only culturable cells
	Flow cytometry	+ suited for enumeration + sensitive + detects culturable and non-culturables
	Microscopy of cells fixed on filter or slides	+ suited for enumeration - not sensitive + detects culturable and non-culturables
	In situ confocal laser scanning microscopy	- not suited for enumeration + suited to study localization + detects culturable and non-culturables

There are numerous intrinsic traits which can be used for the selective detection of a certain micro-organism (Tas and Lindstom, 2000). Some examples will be given. Bacilli form spores which will

survive heat treatments of 80ºC for a certain period; such a treatment will kill most other bacteria and can be used prior to plating to specifically detect bacilli. Researchers can also make use of outer membrane properties of a micro-organism to develop specific fluorescence labeled poly or monoclonal antibodies. A very elegant example was developed by Raaijmakers *et al.* (1998), who showed that incorporation in the medium of the siderophore pseudobactin 358 allowed the detection of a specific *Pseudomonas* strain. This specific *Pseudomonas* strain is the only bacterium that can take up iron complexed to this siderophore and it is therefore the only strain able to grow on this medium. Since intrinsic traits, which could be used for detection, can be very diverse and used in combination with a great variety of methods they will not be discussed in this document. However, the utilisation of intrinsic markers or of the added functional genes as markers for detection may make the introduction of extra genes into a GMO redundant.

Direct measurement of bioluminescence can be achieved by: 1) visual detection; 2) photographic or X-ray film; 3) charge coupled device (CCD) camera; 4) optical fibre systems; 5) scintillation counter; and 6) luminometry (Prosser *et al*., 1996). In most samples it is necessary to extract the cells first before accurate measurements can be done.3.1.5 Miscellaneous intrinsic markers

Table 4: Most commonly used marker genes in microbial ecology studies

Marker Gene	Detectable Phenotype	Detection Method	Experimental Sensitivity	Back-ground	Pitfalls and Limitations
Antibiotic Resistance (Km^r, Tet^r)	Growth on antibiotic containing media	Plating or MPN	High	Low	Environmental stress can reduce expression and cultivation
Heavy Metal Resistance (Mer^r)	Growth on heavy metal containing media	Plating or MPN	High	Low	Environmental stress can reduce expression and cultivation
Luminescence (*lux*, *luc*)	Light output	Luminometry	Medium	No	Signal determined by activity in extract*1 Stress might reduce signal
		Fibre optic detection	*2		
Chromogenic (LacZ, Xy1E)	Production of a colored product	Plating and Screening	Medium	No	Reduced culturability and expression
		Product Measurement	Medium	Low	Limited sensitivity
Green fluorescent protein (*gfp*)	Fluorescence in blue light	*In situ* microscopy	Medium	Low	Particles producing background Also signal from less active cells
		Facs sort analysis	High	Low	Optimal calibration is required

*1 Reaction (*luc* or *lux*AB only) may require substrate addition; *2 Insufficient data

3.2. Detection methods based on bacterial growth

3.2.1. Direct plating

3.2.1.1. Type of information

Plating techniques are widely used to enumerate micro-organisms introduced into the environment. The main advantage of the plating technique is that the colonies can be counted and that the strain of interest can subsequently be isolated and studied in more detail using any typing method available. For enumerating introduced strains, plating techniques generally have a relatively low detection limit, they are sensitive and easily performed and are, therefore, the usual method of choice for detecting introduced cells.

3.2.1.2. Physiological requirements

Survival of introduced bacteria is determined by the characteristics of the strain and by the environmental conditions to which the cells are exposed, and although an introduced strain is supposed to be well characterized and the re-isolation medium used is optimized, part of the introduced population might shift into a viable but non-culturable state as a result of the stressful conditions in the environment (Wilson and Lindow, 1994; Heijnen *et al*., 1993, Mahaffee and Kloepper, 1997, Warner and Oliver, 1998). Once introduced into the environment bacterial cells can revert to various physiological states. Cells can be: 1) culturable; 2) viable but non-culturable (VBNC); 3) dead, but still intact; or 4) lysed. The choice of markers, used in combination with specific detection methods, determines which of these various physiological states can be detected, and thus has important implications for interpretation of the results for risk assessment purposes (Table 1 and 4). There are several studies which show that introduced cells can revert to a non-culturable state resulting in lower plate count while cells remain detectable by other methods. Wendt-Potthoff *et al*. (1994) compared PCR mediated detection with plating methods for the detection of a recombinant *Pseudomonas amyloliquefaciens* in the phylosphere and found that while the strain was no longer detectable using plate counts, the genetic marker could still be detected by PCR. Troxler *et al.* (1997) observed a progressive decrease in plate counts from 8 to 2 log CFU/g soil of a *Pseudomonas* strain introduced into soil in the field while the number of cells detected by immunofluorescence was several log units higher. This shift to VBNC cells occurred especially in the surface horizon, where the culturable cell numbers declined to less than 2% of the number of viable cells. Mahaffee and Kloepper (1997) determined CFU counts of *Pseudomonas fluorescens* introduced into soil using a rifampicin containing medium, using plates which were screened for bioluminescent colonies and by immunofluorescent colony staining. Both the selective and elective media showed significantly lower CFU counts, which suggested that the selection in combination with environmental stress reduced culturability of the introduced cells.

Binnerup *et al*. (1993) have observed the formation of microcolonies when they tried to detect *Pseudomonas fluorescens* cells that had been introduced in soil. These microcolonies could be seen by epifluorescence imaging and were formed from target cells that ceased multiplying after a limited number of cell divisions. Whether or not these microcolonies are included in the plate count can make a considerable difference.

3.2.1.3. Requirement for marker traits

To enable the specific detection of an introduced micro-organism one can use a general or a specific medium in combination with a selectable trait of the organism (Van Elsas *et al*., 1986; Compeau *et al*., 1988), or with a genetic marker such as antibiotic or heavy metal resistance and *lacZ* or *lux* (Hofte *et al*., 1990; Kluepfel, 1993). It is also possible to use a combination of growth medium with specific fluorescent antibodies (Van Vuurde, 1990). The most important prerequisite is that the micro-organism

can be detected with a minimal background of other micro-organisms. The traits or markers which are selected for should be stable and expressed in the micro-organism (See 3.1). Chabot *et al*. (1996) studied survival and root colonization of rifampicin resistant mutants of *Rhizobium leguminosarum*, *Enterobacter sp*. and *Pseudomonas sp*. marked by inserted kanamycin resistance (*npt*II) and *lux* genes *via* plate counts. Counts were confirmed by measuring light emission from the colonies. The combination of both antibiotic resistances and the additional elective *lux* marker proved to be sufficient to reduce the background of non-target bacteria.

Huertas *et al.* (1998) used both antibiotic resistance and toluene as the sole carbon source to select for the introduced bacteria to assess the survival of several toluene degrading pseudomonads. This resulted in a detection limit of 100 CFU/g soil.

De Leij *et al*. (1998) studied survival of a *Pseudomonas* strain marked *via* insertion of the *aph-I* gene (kanamycin resistance), the *xylE* gene and the *lacZY* genes. Such a triple marked strain appeared to have a reduced environmental fitness since this strain survived less well than the wild type strain. In combination with antibiotic resistances such as nalidixic acid, rifampicin and kanamycin, the *lacZY* marker was shown to be very sensitive with detection limits ranging from 100 CFU to 25 CFU per gram of soil. Winstanley *et al*. (1991) was able to monitor *Pseudomonas* strains in lake water marked with *xylE* without using antibiotics, whereas Morgan *et al.* (1989) studied survival of *xylE* marked pseudomonads by selective plating on streptomycin and ampicillin containing medium and by using an ELISA-based detection method for the *xylE*-enzyme.

3.2.1.4. Technical requirements

In order to enumerate bacteria in soil, the cells have to be dislodged from soil particles and from each other. This can be accomplished by various methods which are usually based on suspending the soil in a buffer followed by shaking and diluting the sample. Buffers which are commonly used are sodium pyrophosphate (0.1% $Na_4P_2O_7$) or $MgCl_2$ (0.1 M). Soil is usually added to, for instance, Erlenmeyer flasks in a ratio of 1:10 (w/w) and sterile gravel is added to improve dispersal of the cells. Samples are subsequently shaken at, for example, 200 rpm for 10 minutes. Smaller amounts of soil can be added to 50 ml tubes with gravel and vortexed for one minute. Alternatively soil samples in buffer can be added to a stomacher. Studies in which these methods are compared for efficiency and reliability are lacking.

Most micro-organisms which are introduced into soil are, in origin, soil micro-organisms. Usually a specific medium is required to culture them. A wide variety of media are available for enumeration of the total number of culturable bacteria or specific groups of bacteria in soil. The optimal medium and culture temperature has to be determined for each individual species. Generally, a low nutrient medium should be used to culture micro-organisms from environmental samples, since cells introduced into the environment generally experience oligotrophic conditions.

The fungal inhibiting antibiotic cycloheximide is always included in the medium to prevent fungal growth.

3.2.1.5. Speed, sensitivity and reliability

If a low detection limit is required then a detection technique based on cultivation using selective markers such as antibiotic resistances can be combined with an elective marker such as *lux* to rule out any background (Cassidy at el., 2000). However, such an approach will fail to detect non-culturable cells or cells which have lost resistance. Most molecular markers can also be used in a hybridization or PCR based detection set-up which will allow detection of non-culturable cells. The major drawback of such an approach is that DNA from inactive and dead cells will also be detected (Table 1).

The sensitivity of the plate count method is determined by the antibiotic(s) used to select for the introduced bacterium and the percentage of the community that is naturally resistant to this antibiotic.

To obtain optimal sensitivity Liang *et al.* (1982) used three antibiotic resistances that lowered the detection limit to 25 CFU per gram of soil. In practice antibiotic resistance is combined with other markers. To enhance sensitivity multiple antibiotic resistance markers can be used simultaneously (Liang *et al*., 1982). Although the use of resistance has been quite successful, there are several reports of problems. Some rifampicin resistant mutants have been shown to have a reduced competitive ability and a diminished nodulation competitiveness (Compeau *et al*., 1986). It is important to investigate the occurrence of possible negative effects of the marker(s) on the environmental fitness of the micro-organisms before they are actually used.

The speed of the method mainly depends on the time of incubation which the micro-organisms require to form a visible colony. While the cell extraction and plating itself is relatively fast, the incubation period can vary between 2 to 14 days or longer.

The reliability of the plate count method is high and can only be negatively affected by differences in marker expression and culturability of the micro-organism.

3.2.2. Most Probable Number culturing

3.2.2.1. Type of information

MPN (Most Probable Number) is an alternative to plating, which can be applied for enumerating viable cells by culturing. The technique uses serial dilutions of the original sample in liquid culture medium and relies on the principle that only a single cell is needed to produce a population of new cells. Growth is detectable by changes in properties of the medium, *e.g.* a color change. After the incubation period each culture is scored either negative or positive, the latter indicating the presence of at least one cell, able to grow in the medium. From the scores of the different dilutions the most probable number of culturable organisms in the original sample can be calculated by statistical methods (Cochran, 1950; Gerhardt *et al*., 1981; Alexander, 1982). Being based on the growth of cells, the MPN culturing technique shares many of the advantages and disadvantages of direct plating methods.

3.2.2.2. Physiological requirements

Being a culture-based technique, MPN only detects cells that will multiply in the liquid medium. Most studies in which both MPN culturing and direct plating were used, have shown a similar result for both methods (*e.g.* Line *et al*., 2001; Massa *et al*., 2001). However, MPN culturing will not detect those target cells that would form microcolonies when plated on a solid medium (Binnerup *et al*., 1993). Cassidy *et al*. (2000) have compared both methods for detection of *gfp*-marked *Pseudomonas fluorescens* cells in soil. They found that the counts from MPN culturing were significantly lower than those from direct plating, due to the presence of microcolonies on the solid medium.

3.2.2.3. Requirement for marker traits

For MPN culturing, both selective and elective markers can be used, but as with direct plating, the best results can be obtained by a combination of both. If the organism of interest only contains an elective marker it may be overgrown by other organisms present in the sample, resulting in a false negative result.

3.2.2.4. Technical requirements

The result of MPN culturing strongly depends on the success of the procedure to extract the target cells from the environmental samples. Inefficient extraction and clumping of cells will lead to an underestimate of the number of culturable target cells present.

3.2.2.5. Speed, sensitivity and reliability

Being based on growth, the speed of an MPN assay is determined by the ability of the organism to grow in the liquid medium and to visibly change the characteristics of this medium. Especially for slow growing cells, such as auxotrophs, this may result in a very lengthy assay. Ekelund *et al*. (1999) have shown that it is possible to automate the reading of an MPN experiment by using a multi-well microtitre plate format for the incubation of the cultures. They could enumerate phagotrophic protist (protozoa) from soil in a medium containing *lux*-labeled bacteria as the growth medium. Wells were scored positive if the light emission had decreased more than 30% compared to a non-inoculated control.

In general, the MPN culturing technique mostly yields results similar to plate counts, but lower numbers than found by methods that also detect non-culturable cells.

3.3. Detection by assessment of marker gene products

There are quite a number of different methods to detect the various marker gene products from introduced bacteria (see Table 4). The methods which are commonly used, such as luminometry, flow cytometry, fluorescent microscopy and confocal laser scanning microscopy, will be discussed.

3.3.1. Enumeration of micro-organisms by luminometry

Bacteria marked with *lux* or *luc* constructs (See 3.1.4.1) can be enumerated in crude extracts by measuring the amount of light which is produced. In this chapter bioluminescence measurements after a cultivation step are excluded, since that method should be regarded as cultivation based rather than direct luminometry. A possible alternative for luminometry might be *in situ* fibre optic detection (Yolcubal, *et al*., 2000), which will not be discussed separately.

3.3.1.2. Type of information

The method will detect the amount of light that is produced. In order to calculate the number of cells, one has to know the relationship between cell number and light output. Since this relationship is dependent on the energy status of the cell, it is difficult to relate a given signal to cell numbers. Actually luminometry of *lux* marked cells gives information on the energy charge of the cells and should be combined with an enumeration method.

Luminometry of bacteria marked with *lux* or *luc* genes is also very suitable to investigate various conditions in the environment when used in combination with certain promoter sequences that respond to environmental stimuli (Prosser *et al*., 1996; Jansson, 2000).

3.3.1.2. Physiological requirements

The measurement is limited both by the level of expression of the *lux* or *luc* genes by the bacteria and by the number of bacteria in the sample. On the other hand the amount of light produced is a direct measurement of the *in situ* activity of the cells and will yield information on the effect of the environmental conditions on gene expression (Meikle *et al*., 1994).

3.3.1.3. Requirement for marker traits

There are quite a number of different *lux* based marker cassettes which have been used in environmental studies. The original *lux* pathway, which consists of *lux*CDABE with its original promoter, is not useful to conduct environmental studies (De Weger *et al*., 1991). Most work is done using the *lux*AB construct with a constitutive promoter. However, it is necessary to add n-decanal and to provide O_2 (Kragelund *et al*., 1997, Meikle *et al*., 1994) and the amount of light production depends on the metabolic activity of the cell. On the other hand *lux* constructs can be combined with specific

promoters which respond to environmental conditions and can be used a biosensor (Kragelund *et al*. 1997, Hestbjerg-Hansen *et al*., 2001).

3.3.1.4. Technical requirements

A disadvantage of the method can be quenching of light by soil particles which will result in a lower value. This can be overcome by separating the cells from the soil particles. Cells can be separated from soil using a Nycodenz density gradient centrifugation procedure (Unge *et al*., 1999; Elväng *et al*., 2001; Unge and Jansson, 2001). However, it is not known what the effect of this procedure is on the physiology of the bacteria and what percentage of the cells is lost.

3.3.1.5. Speed, sensitivity and reliability

Luminometry on crude or purified environmental samples which contain the luminescent bacteria is relatively straightforward and fast. Sensitivity lies between 10^3 and 10^4 cells, which is adequate in most cases. Reliability depends on the environmental conditions and physiology of the introduced cells. Care should be taken when interpreting results from studies in literature which use *lux* since quite a number of studies employ a culture based approach in their detection scheme (Errampalli *et al*., 1998; Tresse *et al*., 1998; Cassidy *et al*., 2000). In such a scheme, the cells are first cultured in an MPN approach and then light emission is measured. These studies do not take the advantage of the possibility to directly detect the marker, and when these studies claim that the luminometry data give similar results to plate counts this is not surprising. A better approach to fully use the advantages of the marker system is direct detection of the product (Unge *et al*., 1999; Yolcubal *et al*., 2000; Elväng *et al*., 2001).

3.3.2. Enumeration of micro-organism by fluorescent microscopy or confocal laser scanning microscopy

Fluorescent microscopy is a sensitive technique to detect marked or labeled cells. Although microcopy is not particularly suited for cell enumeration the method has been successfully applied for this purpose (Putland and Rivkin, 1999; Unge and Jansson, 2001) The strength of the method is in its use to study the *in situ* localization of cells (Bloemberg *et al*., 2000; Unge and Jansson, 2001).

3.3.3. Enumeration of micro-organisms by flow cytometry

Flow cytometry is a relatively new technique for the enumeration of specific bacterial cells in environmental samples. The technique is very promising and seems optimally suited for reliable and fast enumeration of cells. The current disadvantage is the isolation procedure required to separate cells from soil particles of other debris. Currently, work is in progress to optimize such procedures (Ziglio *et al*., 2002).

3.3.3.1. Type of information

Enumeration without cultivation of micro-organisms in environmental samples can be achieved by flow cytometry (Unge *et al*., 1999; Elväng *et al*., 2001). Flow cytometry is commonly used for the analysis of microbial communities in freshwater and marine samples (Rice *et al*., 1997; Chen *et al*., 2001; Lopez-Amoros *et al*., 1995; Marie *et al*., 1996). Soil samples are more complicated because of the presence of fluorescent particles. To eliminate these, the cells have to be separated from the soil which can be achieved by using a Nycodenz density gradient (Unge *et al*., 1999; Elväng *et al*., 2001). The flow cytometer can detect and enumerate cells with a specific fluorescent signal and with a specific shape or size.

3.3.3.2. Physiological requirements

In order to stain cells with a fluorescent dye there are no clear physiological requirements other than that the cell wall and membrane are permeable to the compound. However, *gfp* marked cells should be metabolically active enough to produce amounts of *gfp* that can be detected. On the other hand, once the *gfp* is produced it is generally quite stable. If, however, its metabolic activity in the environment is the subject of the study, *lux* or *luc* in combination with luminometry is a better choice.

Currently, alternatives for such studies have been developed by using marker genes encoding unstable *gfp* mutants. In reporter gene studies the promoter will start expression of the marker once a specific condition in the environment is met. In this situation, it is important that the product formed is not stable, which means that the signal will disappear quickly once the conditions change.

3.3.3.3. Requirements for marker traits

While in some specific cases cells might be detected which produce an autofluorescent signal, in most cases, the micro-organisms can only be detected if they are stained with a fluorescent dye. One can choose a dye which specifically binds to DNA such as DAPI, Hoechst33342, SYBR Green and SYBR Gold, YOYO or YOPRO, PicoGreen. However, these dyes will stain all micro-organisms and can thus not be used to specifically detect introduced ones. The flow cytometer can also detect cells which are specifically stained by *in situ* hybridization probes (FISH) or which have a marker gene inserted into the genome such as *gfp*. Since the excitation peak of natural *gfp* does not match the standard 488nm laser of the flow cytometer, Tombolini *et al.* (1997) used a red shifted mutant gene which displayed a high and stable fluorescence signal. The *gfp* gene was expressed by a constitutive *psb*A promoter from *Amaranthus hybridus* which resulted in a stable and evenly distributed signal in all *Pseudomonas fluorescens* cells. During the growth phase fluorescent intensity varied. The fluorescence intensity decreased to 30% in exponential phase while it increased again in stationary until the intensity per cell was the same as that of the inoculum (Tombolini *et al*., 1997)

The choice of a specific detection method in combination with a certain marker gene and promoter sequence depends on the aim of the experiment. If researchers want to study the survival of a micro-organism, it is best to use a constitutively expressed, stable *gfp* marker gene which is indicative for the presence of a bacterium and suited to determine cell number by FacsSort analysis.

3.3.3.4. Technical requirements

The main disadvantage of flow cytometers is that they are relatively expensive. Less expensive types are limited by the fact that they will have a laser which can only work at one wavelength, which is usually 488 nm. This limits the use to dyes that can only be excited at this wavelength.

The apparatus is designed in such a way that the micro-organisms are forced to go through a capillary with a fluorescence detection device one by one at high speed. While passing the detector the signal is recorded and, after the run, the data can be visualized in a 2-D plot. In this plot the fluorescence is set against the forward light scatter, which is determined by the shape and size of the cell. It is important to distinguish the cells of interest from other things such as particles or cell debris with autofluorenscence. It is recommended, therefore, to analyze positive controls and blank samples. By analyzing blanks (that is, samples without the cells of interest) one can identify the background. By analyzing dilutions of cultures of the organism of interest one can validate if the region in the plot which gates the cells can be separated from the background. Moreover, by analyzing the dilutions researchers can validate the enumeration of the cells.

3.3.3.5. Speed, sensitivity and reliability

The use of flow cytometry for the enumeration of cells introduced into the environment is fast and reliable if the right procedures and controls are used.

3.3.4. Enumeration by enzymatic measurements

A specific *LacZ* construct has been used as reporter to monitor the starvation and stress response of a *Pseudomonas* strain in soil by an extraction procedure. In this experiment cells were lysed and a ß-galactosidase assay was performed on the crude lysate (Van Overbeek *et al*., 1997). In this study ß-galactosidase activity was measured by chemiluminescence as a measure of metabolic activity (Van Overbeek *et al*., 1997). In a later study, the activity of the introduced *Pseudomonas* could even be determined up to 21 days in a wheat rhizosphere. A prerequisite for such an approach is that the introduced micro-organisms survive well and have a sufficiently high level of expression to allow detection.

3.4. Enumeration of micro-organisms by DNA or RNA analysis

DNA or RNA based detection and enumeration techniques for micro-organisms introduced or present in environmental samples are very important in modern microbial ecology studies. These methods are not hampered by cultivation problems, poor expression of markers or changes in physiology of the bacteria and even enable researchers to detect sequences from formerly unknown groups of micro-organisms. Currently, there are quite a number of different methods for the extraction and purification of nucleic acids from the environment (Holben *et al*., 1988; Smalla *et al*., 1993; Van Elsas *et al*., 2000). Briefly, two different approaches can be discerned, one is based on extraction of microbial cells prior to lysis and the other is based on direct lysis of the cells in the sample. The disadvantage of the methods which are based on cell extraction is the impossibility to recover all cells of the microbial community. The advantage is that once the cells are obtained it is relatively easy to extract and purify their RNA or DNA. Methods based on direct cell lysis in the sample are better suited to cover the whole community. Nevertheless, the co-extraction of contaminants from the samples could give problems in the purification. In order to obtain efficient cell lysis, mechanical based methods such as using a bead beater or ribolyser have been shown to give high yields of DNA (Smalla *et al*., 1993; Van Elsas *et al*., 2000; Borneman *et al*., 1996). Currently, there is a kit on the market (Fast DNA Spin Kit for soil, Bio101) which is specifically for the isolation and purification of DNA from soil which should be used in combination with a ribolyser

3.4.1. Enumeration of micro-organisms by hybridization

Direct hybridization of a probe to DNA or RNA extracted from the environment has been used in the past to enumerate bacteria. However, the use of this approach is limited since it has a high detection limit and the data which are generated give only an indication of cell numbers. Therefore simple hybridization assays, dot blots or Southern blots are not particularly suited for the enumeration of micro-organisms. However, current developments in microarray techniques, which are essentially miniaturized hybridization assays, might yield very powerful tools for future microbial ecology studies.

The most important application of currently used techniques based on hybridization is fluorescence *in situ* hybridization (FISH). This technique is based on hybridizing specific oligonucleotides with a fluorescent label to intact whole cells which can be subsequently visualized using a microscope (Moter and Gobel, 2000). Using this technique, cells of a specific taxonomic group for which the oligonucleotide was designed, can be counted. It is very powerful technique, and the selection of the oligonucleotide is of the utmost importance. The much used general prokaryotic oligo EUB 388 has recently been demonstrated not to cover the whole microbial kingdom (Manz *et al*., 1992; Daims *et al*., 1999). This method is very valuable for studying micro-organisms in the environment (Amman *et al*., 1991;

Ramsing *et al*., 1996; Ludwig *et al*., 1997; Felske *et al*., 1998b). However, it is not used to enumerate introduced cells.

3.4.2. Enumeration of micro-organisms by PCR amplification

3.4.2.1. Type of information

DNA or RNA based methods which do not rely on cultivation, might be better suited to enumerate bacteria which are difficult to culture or which can enter a non-culturable state (Van Elsas *et al.*, 2000). PCR techniques for the detection and enumeration of introduced cells seem to offer good possibilities in studying dynamics of micro-organisms. Since the PCR reaction involves an exponential increase of the target molecule, it can not easily be used for quantification. For this purpose three different PCR strategies have been developed: MPN-PCR (Sykes *et al*., 1992), real-time PCR (Blok *et al*., 1997) and competitive PCR (Gilliland *et al*., 1990).

MPN-PCR is based on a normal PCR amplification of serial dilutions of a DNA extract from a sample. The result of each PCR assay is scored as either positive or negative. Using the same statistics as in other MPN procedures the number of target molecules (reflecting the number of cells) in the initial sample can then be calculated.

The advantage of MPN-PCR is that it is relatively easy to perform and that it does not require expensive equipment. A drawback is that the method is very labour intensive and requires a large number of PCR reactions per sample. Moreover, the suitability of MPN-PCR for the quantification of micro-organisms has been questioned (Hermansson and Lindgren, 2001).

In real-time PCR, the accumulation of the PCR product is monitored during amplification, this is in contrast to normal PCR where the amplicons are only detected at the plateau phase of the reaction. This enables monitoring of the product during the exponential phase of the reaction. This exponential phase is usually limited to a few cycles where the amplification curve is log-linear. This part of the curve can be used to accurately determine the original concentration of the target. The advantages are: 1) an accurate quantification; 2) an increased dynamic range and a low detection limit; 3) no post-PCR manipulation and thus a reduced risk of cross contamination; and 4) a quick, reproducible and less labour intensive procedure. A disadvantage is that the efficiency of the PCR reaction in the standard samples can be different from the efficiencies in the environmental samples, which may contain PCR inhibitory substances. Currently, there are various methods to overcome this problem (Hristova *et al*., 2001; Widada *et al*., 2001). Another practical drawback is that real-time PCR requires expensive equipment and reagents. Presently, several different systems for real-time PCR are commercially available such as those which are fast and flexible and use capillaries such as the "LightCycler" (Roche) and the Rotorgene (Corbett research) and the high throughput machines based on 96 or 384 well plate formats such as the ABI Prism Sequence Detection Systems the "iCycler" (Biorad), the MX4000 Multiplex Quantitative PCR system (Stratagene) and the DNA Engine Opticon (MJ Research).

Competitive PCR is based on the simultaneous amplification of the target and a competitor DNA in a single tube. The competitor molecule differs in size, yielding a slightly smaller or larger PCR product than the target. The PCR products are subsequently separated on an agarose gel and by a comparison of the intensities of both bands the number of copies in the original sample can be calculated (Johnsen *et al*., 1999). The advantage of the procedure is that it does not require expensive equipment and that the competitor DNA is amplified in the same reaction mixture as the target. A disadvantage is that the amount of product is compared in the plateau phase of the PCR reaction, which is less accurate than a measurement in the exponential phase.

Several studies describe the use of quantitative PCR for the enumeration of introduced cells in the environment (Rosado *et al*., 1996; Halier-Soulier *et al*., 1996; Moller and Jansson, 1997; Farelly *et al*., 1995; Felske *et al*., 1998a).

Rosado *et al*. (1996) used MPN-PCR for the enumeration of introduced *Paenibacillus azotofixans* in soil. Results between plate counts and MPN-PCR correlated well; however, after prolonged dry conditions plate counts decreased rapidly while MPN-PCR counts decreased little which eventually lead to a difference of 4 log units which could be indicative of either the presence of non-culturable cells or naked DNA. Van Elsas *et al*. (1997) compared immunofluorescence counts with MPN-PCR of *Mycobacterium chlorophenolicum* in soil. Although cell numbers correlated relatively well, there were 10 fold differences in numbers between IF and PCR counts.

Similarly, Hallier-Soulier *et al*. (1996) did not find a clear correlation between the number of colony forming units (CFU) and competitive PCR. Both Lee *et al*. (1996) and Lechner and Conrad (1997) found a good correlation between competitive PCR results and colony counts from soil. However, the presence of dead cells with their DNA still intact could lead to false enumeration results.

3.4.2.2. Physiological requirements

To enumerate cells by quantitative PCR of DNA, cell physiology does not play a role. However, critics have pointed out that these methods will also detect naked DNA from dead and lysed cells. There is only limited and circumstantial evidence that naked DNA is of minor importance. Rosado *et al.* (1996) found that while introduced cells could be easily quantified by MPN-PCR, similar concentrations of naked DNA added to soil were no longer detectable within three hours after introduction. Coolen and Overmann (1998) detected ancient DNA in lake sediment layers up to 9000 years old; however, they also showed that 99% of the DNA was degraded and only small fragments were present. Therefore, amplifying a specific rRNA sequence by competitive or real-time RT-PCR could be more promising (Felske *et al*., 1998a), since RNA has a short half life and will be degraded rapidly when the organism dies. Moreover, the rRNA content of a cell represents the activity of organism and not just its presence, which might be more important from an ecological point of view.

More basic studies are needed to fully investigate the extent to which the presence of naked DNA or dead cells can influence the PCR mediated detection results of an introduced strain.

3.4.2.3. Requirement for marker traits

The markers or genes which are amplified should be specific for the micro-organism which is to be detected and should allow the development of specific primers for the amplification of a DNA sequence of approximately 1.0 kb in length. For real-time PCR the amplification of DNA sequences smaller than 0.5 kb is recommended.

3.4.2.4. Technical requirements

A robust procedure for the extraction of DNA from soil is a key to efficient PCR detection and quantification. Ideally, all micro-organisms are lysed and the isolated DNA is of high quality, that is, pure and not degraded. The extraction procedure should yield high quality DNA/RNA to ensure optimal PCR amplification. Care should be taken to check the occurrence of reduced amplification efficiency due to inhibitory compounds present in the extract.

A common problem with PCR amplification of DNA extracted from environmental samples is the occurrence of inhibitory substances (Van Elsas *et al*., 1997). Van Elsas *et al*. (1997) found that DNA extracted from different soils required different purification steps. A clay soil from the Netherlands did not require any further purification while an organic rich soil from Finland required three additional purification steps to remove inhibitory substances. Chandler and Brockman (1996) observed PCR

inhibition at most 10^{-1} dilutions in a MPN assay aimed at quantifying the presence of a number of biodegradative genes.

For bacteria it is relatively easy to correlate cell number with quantitative PCR amplification; however, for hyphal fungi it is not straightforward.

When detecting a specific micro-organism with PCR, it is of the utmost importance that the primers are specific and will not amplify other DNAs. Therefore, evidence should be given which proves that the primers will amplify only the target. To increase the specificity one can use a hot start technique or touch-down PCR protocol, that is, the gradual decrease in annealing temperature during amplification.

3.4.2.5. Speed, sensitivity and reliability

Although PCR seems to be an ideal detection method there is little evidence that DNA which is amplified from environmental samples is actually representing living micro-organism. Amann *et al.* (1996) provided evidence that PCR amplified cloned 16S rRNA sequences indeed represented the diversity present in their activated sludge sample. The authors used specific oligonucleotide probes for each clone and could detect all micro-organisms by fluorescent microscopy.

The sensitivity of real-time PCR is higher than normal PCR. Cullen *et al*. (2001) showed that the detection of *Helminthosporum solani* by real-time PCR was as sensitive as a nested PCR in a conventional set-up, since both were able to detect 1.5 spore/g of soil. Generally real-time PCR amplification is more sensitive than normal PCR; on the other hand it can be optimized with higher precision. Mygind *et al*. (2001) were able to detect the equivalent of two copies of the genome of *C. pneumoniae* in their samples. The reproducibility was found to be good, by repeating their assays on the standard curve 10 times. The coefficient of variance was found to range from 1.4% to 3.9% (Mygind *et al*., 2001). Only at the lowest concentration (one copy per µl) 3 out of 10 assays were negative. They also found a good correlation between traditional immunohistochemical analysis and the real-time PCR assays.

A lot of parameters can influence the amplification efficiency in real-time PCR. Wilhelm *et al*. (2000) observed with particular samples and primers, differences in amplification efficiencies which were dependent on the place of the capillary in the rotor. These problems could largely be overcome if the samples were completely denatured before starting the amplification. The dynamic range of real-time PCR is high. Böhm *et al*. (1999) were able to detect the arbuscular mycorrhiza fungus *G. mossae* in quantities ranging from 10^{-8} to 10^{-2} µg DNA per ml.

3.5. Determining cell numbers by immunofluorescence techniques

3.5.1. Type of information

Immunofluorescent techniques can also be used to study introduced cells. Using immunological methods, samples are incubated with a specific antibody to which a fluorescent label is attached and cells can be counted using a microscope. Heijnen *et al*. (1988) studied survival of introduced *R. leguminosarum* using a polyclonal antibody for immunofluorescence microscopic counts and found that cell numbers were higher as from day 15 than plate counts. On day 60 cell numbers determined with IF were half a Log unit higher than those obtained by plate counts, suggesting that part of the introduced population had become refractory to cultivation.

Leeman *et al*. (1995) determined the survival of *P. fluorescens* introduced in the radish rhizosphere in a commercial greenhouse using the immunofluorescence colony staining method of Van Vuurde and Roozen (1990). The disadvantage of this method is that it requires a cultivation step; however, the advantage is that the organisms do not need to be marked. Wiehe *et al*. (1996) used strain specific polyclonal antibodies and a chemiluminescence immunoassay to determine colonization of *Pseudomonas*

on roots of *Lupinus albus* and *Pisum sativum.* The use of fluorescently labeled 16S rDNA probes for the microscopic detection of bacteria in environmental samples is not particularly suited for the detection and enumeration of introduced cells. Problems such as high background signals, autofluorescence of soil particles and relative high detection limits have hampered wide scale use. Immunological techniques are gradually being replaced by the use of specific marker genes that can be detected without cultivation by direct measurement of the protein.

4. Sampling introduced micro-organisms, experimental design and sampling practice

4.1. Introduction

Representative sampling of soils is crucial for assessing the survival and distribution of soil micro-organisms. Soils may be very heterogeneous, depending on intrinsic factors, but also on usage, for example, for various agricultural practices. Depending on the purpose of the study, the sampling strategy should take into account the level of precision (defined as the accuracy with which the real mean value of the parameter being assayed is determined) of the data needed in relation to the commonly observed variability. A thorough treatment of sampling methods and strategies is given by Van Elsas (2002). Over the last few years the European Committee for Standardization (CEN) has produced a number of standards which contain useful guidelines for the design of sampling and monitoring strategies of genetically modified micro-organisms, introduced into the environment (for example, EN-12685: "Biotechnology; Modified organisms for application in the environment; Guidance for the monitoring stategies for deliberate releases of genetically modified micro-organisms, including viruses." and EN-12686: "Biotechnology; Modified organisms for application in the environment; Guidance for the sampling strategies for deliberate releases of genetically modified micro-organisms, including viruses.").

4.2. Experimental design

The design of an experiment and the sampling strategies chosen must be clear, in order to yield data that can be analysed by adequate statistical methods (Green, 1979; Totsche 1995). Various experimental designs have been developed to achieve this. In general, the field site to be tested is divided into blocks, according to a pattern (for example, completely randomised, randomised complete block, randomised incomplete block, latin square, or split plot designs) that serves the specific goals of the experiment (Anon, 1992; Totsche, 1995). Sampling of the soil may be done using various strategies, serving different purposes. It must be clear which strategy has been followed, and for what rationale.

Examples of sampling strategies are:

Judgement samples: non-random samples, taken for specific purposes, for example, isolation of organisms, but not suitable for statistical analysis of soil composition;

Simple random samples: samples are collected randomly over the site to be studied, *e.g.* according to a grid pattern, selecting a random sampling site within each section of the grid. Data from simple random samples can be treated statistically, and are appropriate for purposes such as the characterisation of fields by mean parameter values, variation, and spatial distribution;

Stratified random samples: this sample strategy takes into account the different (*e.g.* physical) properties of a plot. The plot is divided according to these properties, and random samples are taken from each subplot;

Systematic sampling: samples are taken in a non-random fashion, of an entire area. They are useful for systematic characterisation of the spatial variability of a parameter across a whole field or area; and

Composite samples: samples are obtained by bulking and mixing individual samples. In this way the variability of individual samples is reduced. This reduces the variance between samples, but composite

samples can only be compared if they are similarly constructed and if there are no statistical interactions between the sampling units.

4.3. Sampling strategies

From the data of detection assays on a limited number of soil samples, conclusions have to be drawn on the population of micro-organisms present in the entire field site. The statistical methods that can be used to derive an adequate description of the total population from the sample populations depend on the population distribution of the tested micro-organism in the field site. The most commonly used mathematical description of a population, the normal distribution, can only be applied to populations that are randomly distributed. However, populations often are non-randomly distributed over fields. Mathematical methods, for example, log transformation, are available for transformation of data from non-random populations to a form that can be analysed as a normal distribution (Isaacs and Srivastava 1989; McIntosh, 1990; Pielou, 1983, McSpadden and Lilley, 1997).

In practice, the distribution of the population under study often will be unknown. In such cases the most likely spatial distribution of the whole population can be approximated from a limited number of samples, but this approach represents just a first rough attempt at characterising the actual distribution. As it is often assumed that the variations in populations of micro-organisms over a field result in log-normal distributions, a log-normal distribution is commonly taken as most likely. However, this assumption can be challenged. Microbial activity and diversity will be influenced by a number of factors of the field site, for example, its history, topography, type of soil, degree of homogeneity, type and variability of vegetation and slope, and presence of water streams. Field history is important, as management or disturbance will certainly impact microbial activity and diversity. For instance, previous use of a fungicide may leave residues that can impact microbial populations in soil. Cropping history is also important for soil microflora and processes such as nitrogen fixation. Knowledge of the site's topography and surroundings is a key to understanding other possible influences such as *via* water movement along slopes.

The deviation of the sample mean from the actual population mean is dependent on the number of samples analysed. This deviation can be determined by statistical methods (McIntosh, 1990; Wollum, 1994; Lamé and Defize, 1993; McSpadden and Lilley, 1997). Using these statistical methods one can calculate the minimal sample number needed to determine the population size in a field site to a given accuracy, with a given confidence interval.

ISO norm 10381-4-1992 (International Organization for Standardization, Geneva, Switzerland[6]) provides general rules for sampling soil, for example, for microbiological analyses. According to this norm, fields with homogeneous utilization of up to 2 ha (most agricultural practice) are well-sampled with one composite sample composed of 15 subsamples per replicate to yield an average whole field estimate. Homogeneous fields of 2 to 5 ha require two such composite samples, fields of 5 to 10 ha require three, fields of 10 to 20 ha five, and so on.

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[6] The International Organization for Standardization (ISO, Geneva Switzerland) has developed standards for adequate soil sampling, which have been described in a series of ISO norms (ISO/CD 10 381-1-1992: Soil quality - Sampling - Part 1: Guidance on the design of sampling programmes; ISO/CD 10 381-2-1992: Soil quality - Sampling - Part 2: Guidance on sampling techniques; ISO/CD 10 381-3-1992: Soil quality - Sampling - Part 3: Guidance of safety; ISO/CD 10 381-4-1992: Soil quality - Sampling - Part 4: Guidance on the procedure for the investigation of natural and cultivated sites; and ISO/DIS 10 381-6-1992: Soil quality - Sampling - Part 6: Guidance on the collection, handling and storage of soil for the assessment of aerobic microbial processes in the laboratory). These norms can be obtained *via* ISO.

4.4. Soil sampling in practice

The results of soil sampling and analysis will be very much dependent on the practical conditions during these processes. Applicants should provide a detailed and well rationalised description of the approaches and techniques chosen, taking into account the following aspects.

Sample size, sampling apparatus: Sample size will depend on statistical considerations, the purpose of the experiment, and the practical requirements of the assays to be performed. Small (up to 100 g), medium (100 g to several kg) or large sample sizes (over several kg) may be required. Most microbiological, biochemical and soil chemical assays will require small (up to 100 g) to medium (100 g to several kg) size samples. Small to medium size samples can be obtained for each soil horizon by using presterilised tools (hand auger, sample corer, spade, shovel or trowel). Rhizosphere soil and rhizoplane (surface of plant roots) samples are obtained by carefully excavating plants from soil with a sterile shovel or trowel. Roots and other plant parts should be left intact as much as possible so as to avoid introducing sampling artefacts. Sampling depth is defined by the type of soil and the experimental requirements. As examples, the plough layer (0-25 cm deep) is commonly sampled in agricultural soils, whereas in grassland, soils from the most densely rooted layer (0-10 cm) are taken.

Conditions during transport and storage: Changes in humidity and temperature as well as exposure to direct sunlight may influence the number of viable organisms that can be retrieved from samples. Long storage periods should be evaded if possible; however, samples may have to be stored for very long periods, *e.g.* for comparison with samples taken later in time.

Sample processing: Information of the spatial distribution of the organisms in the sample will be lost during sample processing unless special precautions are taken, *e.g.* for *in situ* determinations, or for obtaining specific samples of the rhizosphere. Samples should not be exposed to excessive temperatures or conditions that will cause desiccation. Excessively moist samples may be dried, but not to less than 30% of the water holding capacity. If samples are processed according to a 'logical' pattern, this may result in changes during processing that may be interpreted as influences of other, *e.g.* geographical, parameters.

Recovery of the bacterial fraction from a sample: In order to retrieve the bacterial fraction from a sample, the soil aggregates should be dispersed in a suitable liquid medium, which allows for dislodgement of the micro-organisms from the soil particles, and the bacterial fraction should be purified.

Dispersion and dislodgement: Dispersion is brought about by mechanical means such as shaking, blending, ultrasonic treatment (at energy levels that do not disintegrate bacteria) (Ramsey, 1984; Bakken, 1985; Faegri *et al*., 1977; MacDonald, 1986). Dislodgement may be helped by addition of detergents, *e.g.* sodium deoxycholate and the use of ion exchange resins (Hopkins and O'Donnell, 1992; Jacobsen and Rasmussen, 1992; MacDonald, 1986). The applicant may be able to report on their own experience with specific methods; *e.g.* Van Elsas (2002) reports that sodium pyrophosphate is a good soil dispersing agent that allows for recoveries of total and specific bacteria of the same order of magnitude as estimated in soil based on microscopic cell counts.

Separation and purification of the bacterial fraction; This is usually brought about by low speed centrifugation, which removes soil particles and most fungal hyphae, yielding cleared supernatant containing bacteria. The recovery of bacterial cells depends on the efficiency of their previous dislodgement from the soil, which is in general dependent on the type of soil: separation from a sandy soil is easier than from a clay type soil. The bacterial fraction may be further purified by density gradient centrifugation. This is however laborious, and may lead to loss of micro-organisms. It should be kept in mind that recovery of micro-organisms from soil samples is always only partial. It has been estimated that only up to 30% of microscopically detectable bacteria are often recovered from soil by established methods (Steffan *et al*., 1988). This recovery rate may be acceptable if coupled with sensitive analytical methods such as selective plating for viable counts of specific culturable organisms. However, it may be

inadequate for immunofluorescence or DNA-based methods used for monitoring populations because of the enhanced limit of detection. An exception is PCR (Briglia *et al*., 1996) which by its nature can overcome the reduction in sensitivity; however, the presence of PCR inhibitors in humus rich soils is notorious and this should be taken into account.

5. Validation and quality control

Quality control and assurance is an important part of the analysis and procedures in certain microbiological laboratories. For instance, for laboratories which monitor microbiological quality of food, drinking water and recreational water, there are national and international guidelines and standards to work with. Most of these laboratories also have a quality assurance system which involves both technical assessment of the equipment which is used and the documentation of the samples, procedures and data. The extra costs and working hours are insignificant compared to the severe economic and social implications which could be caused by the measures which have to be taken as result of incorrect test results (Lightfoot and Maier, 1998). Most of the research in microbial ecology is generally performed by high-tech experimental R&D laboratories and most of the methods used are highly experimental. These laboratories usually do not work with a quality assurance system and standard procedures. Most laboratories which perform microbial ecology research use "in house" developed protocols, specific microbial strains and study specific environments. There are no standard procedures for sampling and plating of soil bacteria, or for the isolation and purification of DNA from soil. This makes it impossible to compare studies in terms of reproducibility and to distinguish intra- and inter-laboratory variation from actual ecological effects. While there is quite some knowledge on variation in bacterial enumeration using plate counts in food and water analysis, there are hardly any data on the reproducibility of molecular detection methods.

Detection of micro-organisms in complex samples in different laboratories can yield very different results, due to known differences in materials and procedures. However, in collaborative studies it was found that even when uniform samples are examined, using a standardized laboratory protocol, repeatability may show considerable intra-laboratory variability, while reproducibility may show considerable inter-laboratory variability (for example, Mooijman *et al*., 1992). This is an important finding to bear in mind when results from literature are compared which have been obtained in different laboratories, on different samples and with different methods. Collaborative studies to gather information on the variability of results concerning detection of micro-organisms in microbial ecology are rare. In the fields of food and medical microbiology these studies are more generally available and they indicate that the variability of results can be considerable. For example, a multicenter comparison trial was carried out to examine the detection rate of *Chlamidia pneumoniae* in atherosclerotic lesions by PCR (Apfalter *et al*., 2001). It showed that there was no consistent inter-laboratory pattern of positive results and no correlation between the detection rates and the sensitivity of the assay used. There is no reason to assume that the detection results of micro-organisms in ecological studies are not similarly variable.

Another very important aspect of quality control is to check the identity of the strain which is to be introduced (Smalla *et al*., 2002). DNA based methods for identification such as sequencing of the rDNA gene, or rep-, BOX-, or ERIC-PCR are recommended methods (Smalla *et al*., 2002). The strain should not be repeatedly sub-cultured in the laboratory since this might change its genotype and/or its phenotype and affect its environmental fitness (Lenski, 1991). The number of generations that the strain is cultured should be limited and a large number of stocks should be frozen at -80ºC. Especially in the case of large scale introductions, when extensive culturing of bacteria is required to obtain a sufficient quantity of inoculant, contaminants could easily take over.

6. Examples of the use of different detection methods for studies of the fate and survival of bacteria introduced into the environment

6.1. Environmental conditions affecting the detection of introduced bacteria

The interpretation of data on the quantification of bacterial survival in the environment is not straightfoward. Environmental conditions can and will affect the physiological state of the bacteria, which, as has been discussed, may have strong implications for the outcome of a detection technique used. When different detection techniques are applied to identical samples, the results may differ to an extent that they seem incompatible, untill the influence of the physiological state of the bacteria in the sample is sufficiently taken into account. The relevance of data on bacterial survival in the environment, provided in scientific literature or in an application, to answer a specific question should be judged on a case-by-case basis, taking into account these factors. The examples in this section are meant to illustrate how the interpretation of results depends on the method that was used.

Untill recently information on the survival of micro-organisms in ecosystems was obtained mainly by the use of culture based detection and enumeration methods. These methods have indicated that several bacterial genera, such as *Pseudomonas, Rhizobium, Agrobacterium, Azospirillum, Bacillus, Azotobacter, Xanthomona*s and *Erwinia,* have adapted to growth in the rhizosphere. Knowledge of the interaction between micro-organism and evironment is important since introduction of these micro-organisms into bulk soil will not be successful as they will not survive very well. In soil, the presence of plant roots was shown to be the major factor for survival of *Azospirillum* cells (Bashan et. al., 1995). Similarly *Bacillus megaterium* was shown to increase in number in soybean rhizosphere from Log 6.28 one week after inoculation to Log 7.21 four weeks after inoculation (Liu and Sinclair, 1993). This is typical behavior of bacteria adapted to the rhizosphere. While all reports on the survival of pseudomonads in soil demonstrated that their numbers decline fairly rapidly (Table 5), *P. fluorescens* has been shown to increase in number and survive very well when its host plant was continuously grown in microcosms in monthly cycles (Raaijmakers and Weller, 1998). In the rhizosphere, there could be a continouos succession of different species or specific genotypes, types adapted to a certain growth phase of the roots (Duineveld and van Veen, 1999: Semenov *et al*., 1999). This was shown to occur on leaf surfaces by Rainey *et al*. (1994) and Ellis *et al*. (1999).

6.2 Bacterial characteristics and physiology affecting their survival

Generally bacteria introduced into the environment are subject to stress because of the transition from a pure culture in the laboratory to a harsh oligothrophic environment, which may affect the bacterial physiology and thus the number of cells that can be detected with a given method. This should be kept in mind when evaluating the results of survival studies, obtained with culture methods. The humidity (or matric potential) of the soil is an important parameter for the survival of introduced bacteria (Heijnen *et al*., 1993). The survival of *P. azotofixans* in very dry soil as determined by MPN-PCR was 4 Log units higher than plate count values whereas under normal conditions both methods yielded similar data (Rosado *et al*., 1996). This indicates that cells might enter a non-culturable state when experiencing dry conditions. Rattray *et al*. (1992) concluded from their data that both the activity and viable cell counts were negatively influenced by matric potential stress.

To investigate if the physiological conditions of the cells influence their survival, Masher *et al*. (2000) studied the fate of *P. fluorescens* CHAO in soil, which was incubated prior to inoculation under various stress conditions, by IF counts and plating. IF counts and plate counts were similar up to 12 days in soil with cells which had been incubated in minimal medium prior to inoculation. Differences between total cell numbers and viable counts started to occur at day 26 and at day 54 total counts were one Log unit higher than viable counts. This difference was much more pronounced when the cells had been subjected to oxygen and redox stress prior to inoculation. The amount of viable but non-culturable (VNBC) cells,

assessed by Kogure's cells elongation test (Kogure *et al*., 1979), appeared to be intermediate, which suggests that the total cell counts was made up of culturable, viable and dead cells. *P. fluorescens* CHAO appeared to be tolerant to moderate levels of NaCl concentrations at which other strains such as *P. aeruginosa* and *E. coli* were already affected (Masher *et al*., 2000). Tolerance to high NaCl concentrations is suggested to be an important property for rhizosphere bacteria (Miller and Wood, 1996). These results suggest that biotic factors and the culture conditions before the introduction can affect survival. The change from culturable cells to a viable but non-culturable state did not appear to represent a successful adaptive response to adverse environmental conditions (Masher *et al*., 2000). The relation between the non-culturable state and physiological adaptation to the conditions in soil remains unclear. While studying survival of *E. coli* in freshwater systems Dan *et al*. (1997) found a large discrepancy between plate counts and direct counts. The apparent non-culturable cells were shown to be viable since glucose uptake activity was not impaired. Arana *et al*. (1997) could also detect viable but non-culturable transconjugants in river water. Strains of *Xenorhabdus nematophilus* and *Photorhabdus luminescens* genetically marked with kanamycin resistance and *Xyl*E introduced into river water decreased to undetectable levels after 6 days (Morgan *et al*., 1997). However, in sterile water, evidence was found that the strains remained viable but had become non-culturable and had thus escaped detection by plating. England *et al*. (1995) used both plating techniques and PCR mediated detection methods to study the fate of genetically modified *Pseudomonas aureofaciens* introduced into soil. Results suggested the occurrence of non-culturable cells or the persistence of naked chromosomal DNA in the samples. Kluepfel (1993) was able to detect *lacZY* marked pseudomonads released in the field three months after they became undetectable by plate counts. This suggested the presence of non-culturable genetically modified bacteria, since the extraction method used involved isolation of intact cells first. The meaning of the presence of viable but non-culturable cells, dead cells, or naked DNA for DNA based detection techniques remains largely unsolved and will definitely require further study.

The characteristics and physiology of the bacteria also play a key role for their survival. Based on their physiology, bacterial species can roughly be divided into two ecological groups, r-strategists and K strategists. K-strategists have characteristics which make them better adapted to survive in oligotrophic environments and r-strategists thrive better in nutrient rich environments. The fact that the characteristics of the receiving ecosystem and the characteristics of the introduced strain play a key role for survival was shown by Thompson *et al*. (1990) who compared the fate of an *Arthrobacter* and a *Flavobacterium* species. Both strains were introduced at a level of about Log 7 per gram of soil and while the *Flavobactrium* decreased rapidly in number to below the detection limit in less than 20 days *Arthrobacter* decreased to a level Log 5 at day 50 and remained at that level until the end of the experiment at day 100. The *Flavobacterium* survived much better in the rhizosphere of wheat in which it could be detected up to 50 days. Soil is an oligotrophic environment and a major abiotic factor influencing bacterial survival is nutrient limitation, *Arthrobacter* is apparently adapted to such an environment while *Flavobacterium* is not. Respiration measurements on sterile soil microcosms to which both strains had been added showed that the *Arthrobacter* reduced its respiration rate to a lower level than the *Flavobacterium* (Thompson *et al.*, 1990). Differences in physiology of the strains are supposedly responsible for the different survival characteristics. This is strongly supported by the fact that *Flavobacterium* showed much better survival when it was starved prior to inoculation. It should be noted that all experiments were analyzed by plating techniques, so viable but non-culturable cells could have remained undetected. Van Elsas *et al*. (1986) studied the survival of two different bacterial species *P. fluorescens* and *B. subtilus* in two soils of different texture. *P. fluorescens* decreased more slowly in silt loam than in loamy sand, while *B. subtilus* decreased much more rapidly in both soils until it reached a level at which it survived as spores. Kim *et al*. (1997) demonstrated that cell numbers of an introduced *Bacillus* strain remained relatively stable in wheat rhizosphere while the number of *P. fluorescens* cells gradually declined in numbers. *B. megaterium* was shown to survive for two years after introduction into the field for biocontrol purposes (Liu and Sinclair, 1993).

Table 5: Decline rates of introduced cells calculated as a decrease of cell numbers in Log per week and mean decline rates of different bacterial divisions.

Taxon/Species	Decline Rate	Ecosystem	Detection Method[2]	Reference
Proteobacteria				
Alpha subdivision (x=0.11)[1]				
Rhizobium leguminosarum	0.21	Soil	Cult	Heijnen *et al*., 1988
Rhizobium leguminsosarum	0.15	Soil	IF	Heijnen *et al*., 1988
R. leguminosarum RSM2004	<0.01	Soil*	Cult.	Hirsch, 1996
Azospirillum brasilense	-0.1	Rhiz.	Cult	Bashan *et al*., 1995
Azospirillum brasilense	0.46	Rhiz.	Cult.	Bashan *et al*., 1995
Bradyrhizobium japonicum	<0.01	Soil	IF	Brunel *et al*., 1988
Sinorhizobium meliloti	0.07	Soil*	*Luc*	Schwieger *et al*., 2000
Gamma Subdivision (x=0.35)				
Pseudomonas stutzeri	0.26	Soil	Cult. + Cat.	Byzov *et al*., 1996
P. stutzeri	0.22	Soil	Cult. + Cat.	Byzov *et al*., 1996
P. putida	0.42	Soil	Cult.+ Tol.	Huertas *et al*., 1998
P. putida WCS358	0.40	Rhiz.*	Cult.	Glandorf *et al*., 2001
Pseudomonas fluorescens	0.20	Rhiz.	Cult.	Frey-Klett *et al*., 1997
P. fluorescens	1.2	Soil	Cult.	Kozdroj, 1997
P. fluorescens R2f	0.18	Rhiz.*	Cult.	Wernars *et al*., 1996
P. fluorescens Q2-87	-0.06	Rhiz.	Cult.	Raaijmakers and Weller, 1999
P. fluorescens CHAO	0.26	Soil	IF	Masher *et al*., 2000
P. fluorescens CHAO	0.39	Soil	Cult.	Masher *et al*., 2000
CFB Group				
Flavobacterium sp.	2.45	Soil	Cult.	Thompson *et al*., 1990
Firmicutes (x=0.05)				
Paenibacillus azotofixans	-0.2	Rhiz.	MPN-PCR	Rosado *et al*., 1996
P. azotofixans	0.5	Soil	MPN-PCR	Rosado *et al*., 1996
Bacillus megaterium	-0.3	Rhiz.	Cult.	Liu and Sinclair, 1993
Bacillus thuringiensis	0.12	Soil	Cult.	Byzov *et al*., 1996
Arthrobacter globiformis	0.14	Soil	Cult.	Thompson *et al*., 1990

* Data obtained from field experiment; [1] x = mean decline rate for each division, [2] Cult. = cultivation based detection method; *Luc* = *Luc* used as marker for confirmation; Cat. = 2,3 di-oxygenase gene for degradation of catechol was used as marker; Tol. = Toluene degradation was used as marker; IF = immunofluorescent counts; MPN-PCR = quantification by PCR

There are several reports of bacteria which can survive very well for months or even years in certain soils (See Table 5). *Rhizobium Bradyrhizobium* and *Sinorhizobium* have been reported to survive in soil for years sometimes even without the presence of their specific host (Hirsch, 1996; Diatlof, 1977; Brunel *et al*., 1988; Schwieger *et al*., 2000). *Rhizobium* was shown to be able to form nodules when its host plant was planted again after several years (Hirsch, 1996). This shows that not only the ability of the strains to form associations with plant roots affects their survival but also the characteristics of the strain allow them to survive in bulk soil for years. Although *Rhizobium* species have been shown to survive extremely well in soil, both fast and slow growing species with different survival characteristics have been observed (Marshall, 1964). Fast growing *Rhizobium* species were found to be more susceptible to desiccation than the slower growing *Bradyrhizobium* (Marshall, 1964). *Rhizobium* species are sensitive to pH since they are generally not found in soils with pH 5 and lower (Lowendorf et. al., 1981). Competition between introduced and indigenous *Rhizobium* species is also reported to affect inoculant survival. In a study by Vlassak *et al*. (1996) introduced *Rhizobium tropici* which has superior nitrogen fixing capabilities was followed in the field. During successive bean crops an increase in number was

found in the second year after introduction. However, in the third year, only small number of *R. tropici* could be recovered and mainly indigenous *R. etli* and *R. leguminosarum* were found. *Rhizobium* inoculants are difficult to establish when they have to compete with the indigenous strains which are generally much less effective in nitrogen fixation (Thies *et al*., 1991).

6.3. Survival of genetically modified bacteria

Survival of genetically modified bacteria is generally similar to that of non-modified bacteria. Before considering the introduction of an engineered strain, it is important to know in which category the organism fits and to determine which methods will be used to gather data for risk assessment of GMM's. Besides the conditions in the receiving ecosystem, the fate of an introduced GMM is determined by its environmental fitness. The environmental fitness might be impaired as a result of the genetic modification. In a number of experiments in which wild-type and GMM are compared, the GMM's survived less well than their non-modified parent strains (Brockman *et al*, 1991; Bromfield and Jones, 1979; De Leij *et al*., 1998; Van Elsas *et al*., 1991; Wang *et al*., 1991). However, there are also quite a number of studies in which no difference in survival between GMM and parent strain could be detected (Bailey *et al*., 1995; Kline *et al*., 1988; Orvos *et al*., 1990; Wernars *et al*., 1996; Glandorf *et al*., 2001). Only in studies with artificial growth conditions did GMM's survive better then the wild-type strain (Biel and Hartl, 1983, Edlin *et al*., 1984). It is generally assumed that the inserted genes and their expression pose an extra metabolic burden for the strains, which could reduce their environmental fitness (Lenski *et al*., 1991). Results from a study of De Leij *et al*. (1998) showed that the presence of a number of constitutively expressed marker genes in a GMM had a negative effect on its survival in competition with the wild type strain. The site of insertion into the chromosome did not affect survival. The evidence suggested that it was purely the metabolic load which was responsible for the decreased fitness since the study also indicated that this effect did not occur under nutrient rich conditions. An increase in metabolic load resulting from the expression of heterologous genes which do not give the organism a selective advantage resulted in a decrease of fitness. In such experiments, the experimental design is also important. It seems that most studies in which the GMM competed less well with its parent were based on mixed inoculations (Van Elsas *et al*., 1991; De Leij *et al*., 1998). In this way, there was direct competition between the parent and the GMM while studies in which no differences were found, the strains were introduced separately. In a study of van Elsas *et al*. (1991), a reduced survival of a GMM in comparison to the parent strain could only be detected in mixed inoculation experiments.

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