

# REVIEW OF THE METHODS FOR EXTRACTION, DETECTION AND IDENTIFICATION OF SOIL MICROFLORA AND THEIR ROLE AS INDICATORS OF SOIL HEALTH

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## Abstract

Yield decline in the sugar industry might be caused by degraded soils due to the monocultivation of sugarcane. Until recently, soils were analysed primarily for their physical and chemical characteristics. Mounting evidence from sources worldwide indicates the importance of the microbial component in the health condition of soils. Soil microorganisms, including bacteria, fungi, nematodes and algae, have the potential to be important indicators of soil health. Microorganisms are responsible for the decomposition and transformation of organic matter in soils, and are also responsible for a significant number of mineral transformations. These processes affect nutrient availability, and hence soil quality and crop yield. The purpose of this paper is to review methods that can be used to measure the quantity and quality of soil microflora as indicators of soil health, and that have the potential to be used as parameters in a proposed soil health index.

*Keywords:* soil health, sugarcane, soil microorganisms, methods

## Introduction

### *Yield decline*

The loss of productive capacity of sugarcane soils was first reported by Garside *et al* in 1997. Yield decline might be caused by the degradation of soils in the sugar industry due to the long term monocropping of sugarcane with successive annual harvesting. Soil is a dynamic, living, natural body and good biological, chemical and physical properties are important to maintain a healthy soil (Doran and Safley, 2002). In 2003, a health index for soils in the South African sugar industry was proposed as part of the soil sustainability programme at the South African Sugarcane Research Institute (SASRI). In order to set up a soil health index, a minimum data set of soil health indicators has to be established. In this paper, the biological indicators of soil health will be discussed.

### *Soil microorganisms*

Soil organisms are both numerous and very diverse (Zuberer and Wollum, 2005). Many of the soil organisms are small and cannot be seen without magnification. The smallest organisms (bacteria, actinomycetes, fungi and algae) are referred to as microflora. Small soil animals are referred to as microfauna, and bigger animals such as earthworms are called macrofauna (Zuberer and Wollum, 2005). All soil organisms interact in the soil and form a soil food web (Ingham E, *The Soil Foodweb: Its importance in Ecosystem Health*. Available from: <http://www.rain.org/~sals/ingham.html> [Accessed 22 April 2004]). The numbers, biomass, activity and community structure of the members of the soil food web can be used as indicators of soil health because each of these organisms performs critical functions in the soil such as decomposition of organic compounds and release of inorganic elements (mineralisation), and the degradation of organic wastes and pollutants to carbon dioxide and

water (Zuberer and Wollum, 2005). Soil microorganisms produce extra cellular polysaccharides, glomalin and fungiform hyphae (Hartel, 2005). In this manner, soil organisms can physically bind soil particles, and they are fundamental to soil structure and soil formation (Hartel, 2005). They also form symbiotic associations with roots and act as antagonists to pathogens (Sparling, 1998). All these processes affect nutrient availability in a soil and hence soil quality.

#### *Strategies for improving soil microbial diversity*

There is a noticeable difference between a natural (virgin) soil and a soil that has been used for planting agricultural crops, this being the decline in the overall microbial biomass and microbial diversity in the latter. The reduction in diversity is usually caused by a reduction in organic matter and by a shift in plant diversity to the monocultures of modern agriculture (Villich and Sikora, 1998). The best way of improving soil microbial diversity and microbial biomass in sugarcane soils is through long term practices such as trashing, the use of green manures and intercrops and minimum tillage (Garside *et al*, 1997). All organic amendments (including the use of composts) have one thing in common: extensive and broad-spectrum activity toward the native microflora in the rhizosphere and within root tissue (You and Sivasithamparam, 1995). De Brito Alvarez *et al* (1995) suggested that composts do not stimulate the total density of rhizosphere microorganisms, but alter the number of species present, thereby causing a shift in specific groups of rhizobacteria.

Applications of single effective microorganism (EM) species or complex mixtures of unknown species and strains present in organic amendments and composts on a commercial basis have become very popular over the past few years (Villich and Sikora, 1998). Yobo *et al* (2004) reported the successful use of two *Trichoderma* species to control soil borne diseases of cucumber. Applied organisms can induce resistance in a host plant to diseases (Hasky and Sikora (1994) and may cause shifts in endemic rhizosphere community structures that will promote root health (Villich and Sikora, 1998). According to Gilbert *et al* (1993), the introduction of a single biological control agent can have an impact on the overall rhizosphere bacterial community even when the strain introduced was not part of that community. In the complex environment surrounding the rhizosphere, the success or failure of applied beneficial microorganisms is strongly influenced by biotic and abiotic factors (Villich and Sikora, 1998). The type of nutrient substrate available to some microorganisms influences their capability of producing secondary metabolites with an antibiotic activity. As the microorganisms degrade this substrate, their ability to produce the active substance may recede or terminate (Villich and Sikora, 1998).

According to Higa and Parr (1994), selection and culturing of certain Gram-positive bacteria or fungi that produce antibiotics and have a wide range of specific functions and capabilities, can develop a disease-suppressive microflora. These organisms can be grown to high populations and be applied to the soil with compost. In order to establish these microorganisms, repeated applications at regular intervals during the first growth season may be necessary, until the added microorganisms remain stable and active in the soil (Higa and Parr, 1994).

#### **Purpose of this review**

According to Pankhurst (<sup>1</sup>personal communication) high microbial biomass and activity, a fungal dominated microflora, high numbers of free-living nematodes and other micro and

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macrofauna are all indicators of a healthy system in soils in the sugar industry. Biological measurements are more sensitive to changes in land management than chemical and physical measurements. This suggests that biological indicators will be very useful in monitoring soil management practices. The purpose of this review is to identify biological indicators of soil health and to discuss the methods that are available for measuring soil microflora that can be used at SASRI as part of the introduction of a soil health index assessment.

### **Methods for measuring microflora in soils**

A number of microbiological and biochemical parameters have been suggested as indicators of soil quality, namely microbial biodiversity, N-cycling, microbial biomass (C-cycling), microbial activity, key species and bioavailability (Nielson MN and Winding A (2002) *Microorganisms as indicators of soil health*. National Environmental Research Institute Technical Report No. 388. Available from: <http://www.dmu.dk>). In the following section, methods of measuring some of these indicators will be discussed. Microbial quantification methods can be divided into three broad groups, namely microbial diversity, microbial biomass and key microbial species.

#### *Indicator 1: Microbial biodiversity*

Microbial biodiversity can be defined as the number of species present in a community (Nannipieri *et al*, 2003) and the relative contribution each species makes to the total number of organisms present (Nielson MN and Winding A (2002) *Microorganisms as indicators of soil health*. National Environmental Research Institute Technical Report No. 388. Available from: <http://www.dmu.dk>). The genetic resources present in the environment form the basis for all the actual and potential functions in the community (Nielson MN and Winding A (2002) *Microorganisms as indicators of soil health*. National Environmental Research Institute Technical Report No. 388. Available from: <http://www.dmu.dk>). More traditional methods of measuring soil microbial community structure include Phospholipid Fatty Acid Analysis (PFLA) and Substrate Utilisation Patterns (BIOLOG). PCR-based methods that can be used to examine different populations present in a community include Ribosomal Intergenic Spacer Analysis (RISA), Terminal-Restriction Fragment Length Polymorphism (T-RFLP), Denaturing Gradient Gel Electrophoresis (DGGE) and Single-Strand Conformation Polymorphism (SSCP). All these methods can be automated to process more samples and make the tests more accurate.

#### **BIOLOG:**

A number of soil microbiologists have used BIOLOG successfully to determine how specific substrate utilisation patterns differentiate between soil types, and how soil microbial communities respond to specific cropping and management practices (Bottomly, 2005). Hofman J (*Research of the soil microorganisms at the biomass-, process-, and community levels*. Available from: <http://www.recetox.chemi.muni.cz/index.php?language=en&id=139> [Accessed 21 February 2005]) found that the BIOLOG assay was a simple and quick method for routine measurement of soil microbial diversity. He *et al* (2001) found that BIOLOG will have to be modified for measuring biodiversity in acid soils. According to Nielson and Winding (2002) (*Microorganisms as indicators of soil health*. National Environmental Research Institute Technical Report No. 388. Available from: <http://www.dmu.dk>) BIOLOG is not a recommended method because of its relatively high cost and the difficulty in interpreting the data. BIOLOG is only applicable to bacteria, and it has a degree of bias towards fast-growing bacteria. The assay is also dependent on growth of cells under the

specific conditions in the micro titer plate and thereby only indicates potential functional diversity. BIOLOG cannot detect non-culturable bacteria in soil.

#### PFLA analysis:

Phospholipid fatty acids (PLFA) are stable components of the cell wall of most microorganisms (Bottomly, 2005). They are polar lipids that are specific for subgroups of microorganisms. The method gives a fingerprint of the relative PFLA composition of the resident microbial community in the soil. PFLAs are extracted from soil samples and analysed by gas chromatography (Bottomly, 2005). The technique gives estimates of both microbial community composition (Pennanen *et al*, 1996), biomass and the fungal:bacterial ratio in the soil (Zelles *et al*, 1994). The results also represent the *in situ* conditions in the soil. The method is time-consuming, and expensive apparatus and chemicals are needed to perform the tests.

#### Molecular methods:

Genetic diversity of soil microorganisms is an indicator of the genetic resource (Nielson MN and Winding A (2002) *Microorganisms as indicators of soil health*. National Environmental Research Institute Technical Report No. 388. Available from: <http://www.dmu.dk>). Genetic diversity of bacteria and fungi is most commonly studied by diversity of the 16S rDNA genes, and four PCR-based methods that are commonly used will be discussed in this paper.

#### DNA extraction from soil:

Before PCR can be done on soil samples, DNA must first be extracted from the soil. Martin-Laurent *et al*. (2001) studied the impact of three different soil DNA extraction methods on bacterial diversity and found that the phylotype abundance and the composition of the indigenous bacterial communities (using RISA) were dependent on the DNA recovery method used. For example, for clay soils, they found that the extraction protocol should include an RNA treatment. It is important to take into account the complexity of different soil types, and that there are multiple factors that may affect the performance of a DNA extraction method (Frostegard *et al*, 1999; Miller *et al*, 1999).

#### PCR methods:

- The RISA technique exploits the variability in the length of the intergenic spacer (IGS) between the small (16S) and large (23S) subunit rRNA genes on the *rrn* operon of bacteria, and by using the two internal transcribed spacers (ITS) between the 18S, the 5.8S and 28S genes of fungi (Ranjard *et al*, 2001).
- The T-RFLP method involves the amplification of the 16S/18S rDNA gene with specific primers (Liu *et al*, 1997). The primers are labelled with a fluorescent tag at the terminus, resulting in labelled PCR-products. The products are cut with several restriction enzymes, one at a time, which results in labelled fragments. PCR products are separated through electrophoresis according to their size in an agarose or polyacrylamide gel. Each band on the gel derives from a sequence with a unique restriction site and is treated as a distinct phylotype.
- The DGGE method is based on the variation in the base composition and the secondary structure of fragments of the 16S/18S rDNA molecule (Nakatsu, 2005). It allows PCR products that have the same length but not the same nucleotide sequence composition to be separated in chemical gradient gels according to the melting behaviour of the DNA (sequence variation among the different DNA molecules influences the melting behavior and therefore molecules with different sequences will stop migrating at different positions in the gel). The number and position of the fragments is an indication of the dominating bacteria/fungi in that community (Clegg and Murray, 2002).

- The SSCP method is based on the principle that, under non-denaturing conditions, single-stranded DNA molecules assume unique conformations that vary depending on their nucleotide sequence (Duthoit *et al*, 2003). The change of only one base may cause a conformational change in the DNA molecule that is sufficient to be detected on an agarose or polyacrylamide gel, or it can be assayed on an automated DNA sequencer (ARISA).

The traditional electrophoretic approach is to separate different sized PCR products by applying an electric current through an agarose or polyacrylamide gel. The high diversity of bacterial or fungal communities can be a problem when using gel electrophoresis. For a visible band on the gel, a bacterial species has to comprise 1% of the entire population (Nielson MN and Winding A (2002) *Microorganisms as indicators of soil health*. National Environmental Research Institute Technical Report No. 388. Available from: <http://www.dmu.dk>). These PCR-DGGE molecular fingerprints are complex and often difficult to interpret. More meaningful information can be obtained through numerical analysis of the banding patterns. Another limitation is the separation of relatively short (500bp) DNA fragments, which can limit the design of probes (Clegg and Murray, 2002). In order to overcome the problems with gel analysis, RISA, T-RFLP and SSCP can be automated. In the automated approach, the initial steps of DNA extraction and PCR amplification are the same as in RISA, SSCP and T-RFLP, except that PCR is conducted with a fluorescence-tagged oligonucleotide primer. The electrophoretic step is subsequently performed with an automated system, which provides laser detection of fluorescent DNA fragments. DNA is electrophoresed in a capillary tube filled with electrophoresis polymer rather than in a polyacrylamide gel. The programme output is a series of peaks (electropherogram) (Duthoit *et al*, 2003). The sizes of the peaks are estimated by comparison with fragments in the internal size standard. The GeneScan software calculates the fluorescence contained in each peak, which is proportional to the quantity of DNA in the fragment. The relative amount of each fragment in the PCR product is estimated as the ratio between the fluorescence (peak area) of the fragment of interest and the total fluorescence of all fragments in the profile (Fisher and Triplett, 1999). One of the greatest improvements of ARISA over RISA is the increased sensitivity of DNA detection, so that considerably less PCR product is needed for analysis. A problem with ARISA is that it may underestimate diversity, because unrelated microorganisms may possess spacer regions of identical length and are thus represented in the profile by a single peak. Bacterial genomes also have varying numbers of rRNA operons and these copies may exhibit length heterogeneity in the spacer region, so that in ARISA a single organism may contribute more than one peak to the community profile (Fisher and Triplett, 1999).

#### *Indicator 2: Microbial biomass (carbon cycling)*

Although the microbial biomass is defined as the living component of soil organic matter, it excludes macrofauna and plant roots (Sparling, 1998). A major activity of soil microbial organisms is the decomposition of organic matter. Soil microorganisms are generally heterotrophic and they rely on carbon energy from sources outside the microbial community (Nielson MN and Winding A (2002) *Microorganisms as indicators of soil health*. National Environmental Research Institute Technical Report No. 388. Available from: <http://www.dmu.dk>). Soil microbial biomass contributes to soil structure and soil stabilisation. Soil microbial biomass has also been recommended as an indicator of soil organic carbon (Nielson MN and Winding A (2002) *Microorganisms as indicators of soil health*. National Environmental Research Institute Technical Report No. 388. Available from: <http://www.dmu.dk>), and that is why, in this paper, microbial biomass and carbon cycling

will be discussed together as one indicator of soil health. Determination of soil microbial biomass can be done by direct methods such as microscopy, PFLAs and plating and counting, or by indirect methods that involve chloroform fumigation. Direct methods give results that are very close to the *in situ* situation in the soil. Microbial biomass is sensitive enough to measure early changes due to differences in land use and management, fertility restoration of eroded land and heavy metal contamination (He *et al*, 2001). Indicators of carbon cycling represent measurements at the ecosystem level.

#### *Direct methods of measuring microbial biomass*

##### Microscopy:

Direct counting of soil microorganisms can be done using microscopy and is the oldest method of determining microbial biomass. A known volume of a soil extraction is put onto a microscope slide, stained and counted. Several stains specific to proteins or nucleic acids have been used, namely fluorescein isothiocyanate (FITC), acridine orange (AO) and ethidium bromide. Phenol aniline blue (PAB) has been used to stain hyphae in agar films and on membrane films, whereas metabolically active hyphae have been counted after staining with fluorescent diacetate (FDA) (Nannipieri *et al*, 2003). The main disadvantages of microscopy are that it does not allow counting of specific microbial species, and some of the stains do not discriminate between living and dead organisms (Nannipieri *et al*, 2003); there can be a risk of underestimating or overestimating counts (White and Macnaughton, 1998), and it can be laborious and is affected by soil type. Variable results can be obtained because it depends strongly on the skill of the investigator performing the counts. Accuracy of counts is very low and the error large, even when counting 20 fields with adequate numbers of cells and replicate subsampling (White and Macnaughton, 1998).

##### Culture methods and plate counts:

Culturable populations of bacteria, either total populations or specific groups, have been used in a number of studies for determining soil health. A known volume of a dilution series of a soil extract is plated out onto agar-based media and the numbers of colony forming units (CFUs) are counted. However, there are problems with the use of plate counts because most types of soil microorganisms cannot be cultured. Only about 0.1-10% of organisms in the soil can be grown on agar plates (White and Macnaughton, 1998).

##### The fungal-bacterial ratio:

This ratio has been used as a microbial indicator in management studies. The ratio of fungi to bacteria is characteristic of the type of system. According to Ingham E (*The Soil Foodweb: Its importance in Ecosystem Health*. Available from: <http://www.rain.org/~sals?ingham.html> [Accessed 22 April 2004]) grasslands and agricultural soils usually have bacterial dominated biomass. Highly productive agricultural soils tend to have ratios of fungal to bacterial biomass near 1:1, or somewhat less. Forests tend to have fungal dominated food webs. The fungal-bacterial biomass ratio can be determined directly from measurements of fungal-specific and bacterial-specific PFLAs (see PFLA discussion in previous paragraphs).

#### *Indirect methods of measuring microbial biomass*

##### Soil respiration (microbial respiration):

CO<sub>2</sub> in soils is produced by roots and by microorganisms that are responsible for decomposition of organic matter (Nielson MN and Winding A (2002) *Microorganisms as indicators of soil health*. National Environmental Research Institute Technical Report No. 388. Available from: <http://www.dmu.dk>). Soil respiration can be determined either by CO<sub>2</sub>

production or O<sub>2</sub> consumption. High respiration rates are indicators of a healthy soil, if the soil is undisturbed ([www.agguide.agronomy.psu.edu](http://www.agguide.agronomy.psu.edu)). Machulla (2003) reported that soil respiration remains the best index of whole metabolic activity of soil microbial populations. It has been a traditional method since the inception of soil microbiology. Soil respiration is a robust parameter that can rapidly, and reproducibly, be determined. It allows gross comparisons of soils, and reflects soil management changes, and the impact of elevated atmospheric CO<sub>2</sub> on soil microorganisms (Machulla, 2003). However, according to Sparling (1998), soil respiration is highly variable and can show wide natural fluctuations, depending on the substrate available, moisture and temperature. Soil organisms can rapidly respond to changes in soil conditions, even after they have been inactive for a long time. Only minutes after a dry soil gets wet, an increase in respiration and mineralisation of N and C can be measured (Sparling, 1998).

Chloroform fumigation is the most commonly used indirect method to determine soil respiration, and thus indirectly microbial biomass (C). Methods used are fumigation-incubation (CFI), fumigation-extraction (CFE) and substrate-induced respiration (SIR) (Sparling, 1998). CFE measures the total microbial biomass while CFI measures the total microbial biomass under optimum environmental conditions ([www.eeescience.utoledo.edu](http://www.eeescience.utoledo.edu)). SIR measures the metabolically active portion of the microbial biomass by measuring the initial change in the soil respiration rate as a result of adding an easily decomposable substrate (e.g. glucose) (Nielson MN and Winding A (2002) *Microorganisms as indicators of soil health*. National Environmental Research Institute Technical Report No. 388. Available from: <http://www.dmu.dk>). The CFI, SIR and CFE methods, among others, were compared by He *et al* (2001) for the determination of microbial biomass. These authors found that CFI is not suitable for soil with a pH <5.0 and that SIR may underestimate microbial carbon in oxisols. Another problem encountered with soil fumigation methods was that chloroform does not kill all the microbes in the soil and thus underestimates the numbers. The SIR method is also susceptible to carbon amendments to soil that can lead to an overestimation of microbial biomass. These methods do not separate biomass contributions of fungi and bacteria. To some degree, any measurement of microbial biomass is relative – different methodologies or variations in methodologies will yield microbial biomass estimates that are not directly comparable (Fierer N (2003). Soil microbial biomass determination. Available from: <http://www.fiesta.bren.ucsb.edu/~fierer/microbilabiomassdetermination.html>. [Accessed 15 February 2005]).

#### Microbial quotient:

This can be defined as the percentage of microbial carbon in the total carbon (expressed as a percentage), which reflects the microbially 'alive' proportion of the carbon (Sparling, 1998) and is a measure of soil organic quality. The higher this ratio, the better the soil's biological fertility. Many soils worldwide range from 1-6% microbial quotient (Milton N, Braimbridge M and Murphy D (2002) *Soil quality indicators for Western Australian farmers – Part 1*. Available from: [http://www.wantfa.com.au/pub\\_pge/soils\\_alive/science4\\_Jan02.html](http://www.wantfa.com.au/pub_pge/soils_alive/science4_Jan02.html). [Accessed 15 February 2005]). The higher the percentage, the more fertile the soils. Values above 4-5% reflect optimal soil functions. Here microorganisms can easily provide a buffer against management mistakes. Healthy microbial biomass values also suggest that the chemical and physical fertility of the soil is good, since healthy soil physics and chemistry (adequate water, air, correct pH) are essential for microbial health. A low value (2%) suggests that soil attributes are poor. Soil quality problems need to be addressed through more sustainable management practices (Milton N, Braimbridge M and Murphy D (2002) *Soil quality indicators for Western Australian farmers – Part 1*. Available from: [http://www.wantfa.com.au/pub\\_pge/soils\\_alive/science4\\_Jan02.html](http://www.wantfa.com.au/pub_pge/soils_alive/science4_Jan02.html). [Accessed 15 February 2005]).

Respiratory (metabolic) quotient:

The respiratory quotient is obtained by expressing the rate of soil (microbial) respiration in terms of microbial biomass. Microbial biomass for this purpose is usually done by measuring substrate-induced respiration (SIR). Respiratory quotients are greater in soils with low microbial biomass contents (Gupta *et al*, 1994). In some cases a higher respiratory quotient suggests a stress response and poor health of a soil (Sparling, 1998). Care must be taken not to use the respiratory quotient values too literally because the quality of carbon sources can differ greatly, or soils can differ. This can make interpretation very difficult (Sparling, 1998).

### *Indicator 3: Key species*

Microbial key species in soil can be defined as organisms that fulfil important functions in the soil ecosystem, or organisms that can be detrimental to plant growth and soil health (Nielson MN and Winding A (2002) *Microorganisms as indicators of soil health*. National Environmental Research Institute Technical Report No. 388. Available from: <http://www.dmu.dk>). Microscopy, selective plating, molecular and immunological methods can be used for determination of key species in a soil. A number of criteria have to be fulfilled for key species to be useful in a monitoring programme. For example, they should be ecologically relevant, preferably abundant, and easy to enumerate and identify. Key indicator species represent measurements at the population level (Nielson MN and Winding A (2002) *Microorganisms as indicators of soil health*. National Environmental Research Institute Technical Report No. 388. Available from: <http://www.dmu.dk>). Mycorrhiza, a suppressive soil and human pathogens can be microbial indicators of soil health. In this section only mycorrhizae and suppressive soils will be discussed.

Mycorrhiza:

The abundance and diversity of mycorrhizae is determined by extraction of spores from soil samples and subsequent counting under a microscope. The determination of spore numbers is, however, poorly correlated with the actual colonisation potential of the soil. Methods for direct detection and quantification of mycorrhizae in soil samples or in roots have been developed. These include 18S rRNA, nested PCR at the species level and mycorrhizae specific PLFAs (Nielson MN and Winding A (2002) *Microorganisms as indicators of soil health*. National Environmental Research Institute Technical Report No. 388. Available from: <http://www.dmu.dk>).

Suppressive soil:

According to Hornby (1983) a suppressive soil is a soil “where disease suppression occurs when less disease is caused than would be expected in the presence of a susceptible host and a virulent pathogen under conditions normally conducive for infection.” Antagonistic microorganisms that produce antibiotics usually dominate the microflora of disease suppressive soils. These include fungi and bacteria of the genera *Penicillium*, *Trichoderma*, *Aspergillus*, *Streptomyces*, *Burkholderia*, *Pseudomonas*, *Bacillus*, *Nitrobacter*, *Nitrospora*, *Rhizobium*, *Methyococcus* sp., *Methylobacter* sp., *Nitrobacter* sp., *Nitrospira* sp., *Nitrosomonas* sp., *Azospirillum* sp., *Azotobacter* sp., *Rhizobium* sp., *Anabeaba* sp., *Thiobacillus* sp., *Agrobacterium* sp. and *Streptococcus* sp. The suppressiveness of a certain soil may thus be an indicator of plant health (Higa and Parr, 1994).

Many of the proposed soil health indicators focus on the presence of beneficial rather than the absence of detrimental organisms, although both are important. The presence of plant pathogens in soil may indicate the existence of other soil health problems, e.g. nutritional imbalance. Monoclonal antibodies, DNA probes and PCR can be used to detect beneficial as well as detrimental species that are associated with a healthy or unhealthy soil (Nielson MN

and Winding A (2002) *Microorganisms as indicators of soil health*. National Environmental Research Institute Technical Report No. 388. Available from: <http://www.dmu.dk>.

Selective isolation on agar plates:

This is the most common method for detection and quantification of specific microorganisms from environmental samples because it is easy to perform and at low cost; however, it is potentially time-consuming when a large number of growth media need to be inoculated and counted (WS Atkins Environment (2000). Review of the methodologies for the extraction, detection and identification of microorganisms in the environment, Final report. Available from: <http://www.defra.gov.uk>).

Specific PCR:

Ribosomal RNA (rRNA) genes have been used as a target for developing specific primers for the detection of a number of fungi and bacterial species (Amann *et al*, 1998). Specific primers are designed by exploiting the variability in the length of the intergenic spacer (IGS) between the small (16S) and large (23S) subunit rRNA genes on the *rrn* operon of bacteria, and by using the two internal transcribed spacers (ITS) between the 18S, the 5.8S and 28S genes of fungi (Ranjard *et al*, 2001). Specific primers can thus be developed for target species in the soil, and by using PCR to determine whether they are present in that soil or not. The problem with PCR is that it is difficult to quantify the target DNA and the tests only give indications of the presence of the microorganisms but not their activity in the soil (Dabert *et al*, 2002).

Molecular probes (dot blot and FISH):

The molecular probe approach includes Fluorescent *In Situ* Hybridisation (FISH) and dot blot. These techniques are based on the design of labelled oligonucleotides (probes) that specifically target 16S rRNA molecules of specific organisms or groups of organisms. In FISH, hybridisation is performed on fixed whole cells with fluorescently labelled oligonucleotides and the targeted cells are observed by epifluorescence microscopy or confocal laser scanning microscopy (Dabert *et al*, 2002). With dot blot, nucleic acids are extracted from the cells, spotted onto a membrane and hybridized with radioactive-labeled probes (Dabert *et al*, 2002). Because the oligonucleotide probe confers the fluorescence, it can be correlated to the ribosomal RNA of the cell and can thus give an indication of growth rate, cell activity and viability (De Long *et al*, 1989).

### **Conclusions and recommendations for future research**

Microbial indices can be sensitive measures of changing soil processes, but a major limitation to their application to assess soil health is that justifiable baseline or target values of microbial biomass and activity need to be established to define acceptable rates of change. Such values will be soil specific and will show regional differences (Sparling, 1998) because microbial biomass, activity and community structure are affected by moisture, temperature, pH, soil matrix and quantity and quality of carbon and nutrient supply. However, biological indices can be combined, modified or integrated to establish some useful tools for assessing changes in soil quality (He *et al*, 2001).

There are a wide variety of methods available for the extraction, detection and identification of microorganisms from soils and, because of this, the objective of the extraction, detection or identification process should be considered before any method is selected. Each of the methods available has a number of advantages and disadvantages, and these should also be considered. Factors to take into account when selecting a particular method should include the accuracy of the test, the cost, the ease of the operation and the time available. Using a

combination of molecular biological techniques, microbial methods and methods to determine environmental parameters will lead to an unbiased understanding of the role of microorganisms in their environment (Muyzer, 1999).

#### *Recommendations for future research*

Beneficial bacterial and fungal species must be identified and correlated with cane yield and soil health to establish the effect on indigenous microfloral communities. At SASRI, *Burkholderia* has already been identified as one of the beneficial bacterial species associated with sugarcane. Other indigenous microbial species can be identified and can then be used as applicators to enhance soil health and subsequently sugarcane yields. Implementation and standardisation of methods or techniques for measuring microbial biomass, activity and diversity must take place so that they can be used for soil health analysis. The methods must be applicable to different soil types and climates. Methods such as selective plating, PCR or dot blots can be used to identify beneficial species in soils in the sugar industry, while RISA, DGGE or SSCP analysis can be used to measure shifts in microbial populations after planting green manures, after intercropping, after the application of efficient microbes (preferably indigenous to sugarcane soils), and after changes in tillage practices. The data that are obtained by these methods can then be used to calibrate the NIR to measure whether a particular soil can be classified as healthy or poor in terms of its microbial community.

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