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***Chapter 10***

# POTENTIAL OF BIOLOGICAL NITROGEN FIXATION AS INDICATOR OF SOIL POLLUTION

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## Abstract

Soil microorganisms contribute significantly to the maintenance of soil health by controlling the decomposition of plant and animal residues and participate of biogeochemical cycling, as nitrogen cycle. In the nitrogen cycle, biological nitrogen fixation has an important contribution. Biological nitrogen fixation (BNF) is the process by which atmospheric nitrogen gas is converted into ammonia and its is subsequently available for plants. The process is carried out by bacteria of the genera *Rhizobium* where they form symbiotic associations with legume roots. The bacteria reside in nodules where they fix $N_2$ and provide the plant with nitrogen for growth. In return, the plant provides the bacteria with organic substrates. The *Rhizobium*-legume symbiosis is characterized by high host specificity and the BNF and numbers of nodules has been proposed as an indicator of soil pollution based on the organisms sensitivity to pesticides, urban and industrial wastes and heavy metals. Actually, changes in microbial populations, or specific functional groups, as nitrogen fixing bacteria and root nodule bacteria, have been used as measure of the impact of organic and inorganic pollution in soils. However, the conflicting results observed in evaluation of soil pollution, mainly by use of urban and industrial wastes, indicates the need of search others methods for use of microorganisms, especially rhizobia. New techniques for measuring the structure and

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functional diversity of rhizobial, for example DNA techniques, offer new and largely unexplored potential for using rhizobial and biological nitrogen fixation as indicator of soil pollution.

**Key words:** heavy metals, microbial activities, $N_2$ fixation, pesticides, wastes.

## Introduction

As results of increase in the industrialization and the intensity of agricultural practices, the soils of the world have become permanently contaminated with a range of potentially toxic organic and inorganic chemicals. These chemicals include those deliberately introduced into the soil to maintain and increase plant production, such as pesticides and other agrochemicals. Additionally, disposal of urban and industrial wastes, as biosolids (for example, sewage, tannery and textile sludge) and effluents have the potential to pollute soil especially with heavy metals.

Contamination of soil due the agricultural activities, as use of agrochemicals, presents varying levels of damage and risk to environment. For example, high soil fertility (often originating from high chemical fertilization) and soil contamination (from use of pesticides) may prevent the successful establishment of native plant communities that are generally poorly adapted to these conditions (Dickinson, 2003). In many situations, for example areas in process of erosion, there is a lack of significant topsoil, important to recycling of green wastes, paper pulp and sewage sludge (Fasham, 2000).

In relation of urban and industrial wastes disposal, the amounts of heavy metals accumulated in soils are dependent on the application levels, the transport of the metal from the source to the accumulation site and the retention of the metal once it has reached the soil (Alloway, 1995). Environmental pollution with heavy metals is a global issue. Living organisms are not able to prepare and adapt rapidly to a sudden and huge environmental load with different toxic substances, and therefore, the accumulation of certain elements, especially of heavy metals with mainly toxic effect, can cause undesirable changes in the biosphere. In this way, due the pronounced cumulative soil capacity for different kinds of xenobiotics, we have considered it very appropriate to examine the possibility of potential of microbiological properties as indication of soil pollution due application of pesticides and solid wastes.

## Soil Microbial as Indicators of Soil Pollution

The biological activity in soil is largely concentrated in the topsoil, the depth of which may vary from a few to 30 cm. In topsoil, the biological components occupy a tiny fraction (<0.5%) of the total soil volume and make up less than 10% of the total organic matter in soil. These biological components consist mainly of soil organisms, especially microorganisms. Soil microorganisms contribute significantly to the maintenance of soil health by controlling the decomposition of plant and animal residues, biogeochemical cycling (including biological nitrogen fixation) and the fate of agrochemicals and organic pollutants entering the soil (Pankhurst et al., 1998).

In addition to the effect on nutrient cycling, microorganisms also affect the physical properties of soil. Production of extra-cellular polysaccharides and other cellular debris by microorganisms help in maintaining soil structure, as these materials function as cementing agents that stabilize soil aggregates.

In recent years, soil microbial properties have been seen to be early and sensitive indicators of soil pollution and can be used to predict long-term trends in the soil quality (Saviozzi et al., 2002). The use of microbial properties as indicator of soil pollution is supported by criteria proposed by Brookes (1995) and include: a) general scientific validity and relevance to soil; b) is easily measured; c) can be measured accurately across a wide range of soil types and soil conditions; d) has sufficient sensitive to indicate pollution but is sufficiently robust not to give false alarms; e) is of a nature that background (control) measurements can be made so that effects of pollution can be precisely determined.

Indicators of microbial activity in soil represent measurements at the ecosystem level (e.g. processes regulating decomposition of organic residues and nutrient cycling, especially nitrogen, sulphur, and phosphorus). Measurements at the community level include bacterial DNA and protein synthesis (Pfaffl and Hageleit, 2001).

The content and diversity of mRNA molecules will give very accurate pictures of the *in situ* function and activity of the microbial community. Detection and quantification of a specific mRNA molecule can be done by reverse transcription PCR (RT-PCR), which is a very sensitive method (Pfaffl and Hageleit, 2001). Two methods have been developed to examine the diversity of 16S rDNA sequences in total DNA extracted from soil microbial communities, namely Denaturing Gradient Gel Electrophoresis (PCR-DGGE) (Muyzer et al., 1993) and Terminal Restriction Fragment Length polymorphism (T-RFLP) (Liu et al. 1997).

Soil microbial is primarily affected when environmental conditions are changed (Brookes, 1995). Soil microbial assays are used for estimation of the effect of various synthetic products or environmental pollutants (pesticides, fertilizers, sewage sludge containing heavy metals, tannery and textile sludge, etc.) on soil fertility and agricultural productivity (McGrath, 1994; Hani et al., 1996, Araújo et al., 2003; Araújo and Monteiro, 2006, Araújo et al., 2007). According to Chander and Brookes (1993), microbial parameters are useful indicators of changes in soil conditions caused by chemical pollution long before any detectable change in the soil organic C content. On the hands, for example, it might be expected that the microbial activity in soil would increase with the application of organic matter in sewage sludge. On the other hand, negative effects on the microflora by the simultaneous enrichment of inorganic and organic pollutants have been reported (Fließbach et al., 1994; McGrath, 1994; Hani et al., 1996).

In the reported international project, Filip (2002) evaluated more than 20 parameters in order to roughly characterize the composition of soil microflora (including total biomass), microorganisms participating in carbon and nitrogen cycling, and some essential soil biochemical processes as possible indicator of undisturbed and/or anthropogenically-affected soils. The author found relative sensitivity of the most reliable parameters evaluated and observed that $N_2$ fixing bacterial (rhizobia) is highly sensitivity (Table 1). This high sensitivity showed by rhizobia and the BNF suggest a great potential of use these parameters as indicator of soil pollution.

## Biological Nitrogen as Indicator of Soil Pollution

Biologically fixed $N_2$, either asymbiotic, associative or symbiotic, is considered a renewable resource, which should constitute an integral part of sustainable agro-ecosystems globally. The contribution of legume N fixation to the N-economy of any ecosystem is mediated by: the efficiency of the $N_2$ fixing system; the contribution of Biological Nitrogen Fixation (BNF) to the soil N pool; and the total amount of $N_2$ fixed that actually is recycled by human practices and animal manure into the system. Several opportunities to enhance BNF inputs are available across different agro-ecosystems and socio-economic conditions (Sims, 1990).

**Table 1. Relative sensitivity of the selected microbiological and biochemical parameters for the assessment of soil quality based on long-term soil analyses from 49 differently anthropogenically-affected European soils (Adapted of Filip, 2002).**

| Parameter | Relative sensitivity[1] |
|---|---|
| Microbial biomass | +/++ |
| Composition of microbial communities | |
| Copiotrophic bacteria (Colony forming unity) | +/++ |
| Oligotrophic bacteria | +/++ |
| Actinomycetes | ++ |
| Fungi | ++ |
| Celulose decomposer | +/++ |
| $N_2$ fixing bacteria | ++++ |
| Biochemical process-linked activities | |
| Respiration ($CO_2$ release) | +++ |
| Amonification ($NH_4$ release) | ++ |
| Nitrification | ++/+++ |
| Dehydorgenase activity | +++/++++ |
| Humification activity | ++ |

[1]Sensitivity (relative to a control soil): (+) low; (++++) maximum.

Biological Nitrogen Fixation (BNF) is the process by which atmospheric nitrogen gas ($N_2$) is converted into ammonia ($NH_3$) and its is subsequently available for plants. In agricultural settings, perhaps 80% of this biologically fixed $N_2$ comes from symbioses involving leguminous plants and bacteria of the family *Rhizobiaceae*. The family *Rhizobiaceae* currently includes six genera: *Rhizobium, Sinorhizobium, Mesorhizobium, Allorhizobium, Azorhizobium*, and *Bradyrhizobium*, which are collectively referred to as rhizobia (Vance, 1998). In recent years, however another α-proteobacterias have been showed to produce nodules in the legume (Moullin et al., 2002) *Methylobacterium* (Sy et al., 2001); *Blastobacter* (Van Berkun e Eardly, 2002), e *Devosia* (Rivas et al., 2002).

They have the ability to infect the roots of legumes and to produce nodules. The differentiated forms of rhizobia in the nodule, called bacteroids, fix atmospheric nitrogen into ammonia and export the fixed nitrogen to the host plant (Long, 1989). Symbiotic nitrogen fixation is of great importance not only in the production of leguminous crops but also in the global nitrogen cycle. Nitrogen-fixing nodules are formed as a consequence of a series of interactions between rhizobia and leguminous host plants.

Nodulation is a highly host-specific interaction in which, with few exceptions, specific rhizobia strains infect a limited range of plant hosts. Plants secrete flavonoids that are recognized by the compatible bacteria, resulting in the induction of nodulation genes. These nodulation genes encode enzymes that synthesize a specific lipochitin nodulation signal (Nod signal), which activates many of the early events in the root hair infection process (Oldroyd et al., 2005). During the infection process, the bacteria enter the plant via the root epidermis and induce the reprogramming of root cortical cell development and the formation of a nodule.

Nodulins are nodule-specific proteins synthesized in association with the symbiosis process established among leguminous and Rhizobia. These proteins play distinct by crucial functions during the biological nitrogen fixation of $N_2$ (Silveira et al., 1999). Nitrogenase is the principal nodulin involving in the BNF and catalyzes both the reduction of $N_2$ to ammonia and $H^+$ to molecular hydrogen. Additionally, phosphoenolpyruvate carboxylase-PEPC (E.C.4.1.1.31) and glutamine synthetase-GS (E.C.6.3.1.2) are particularly important. PEPC supplies carbon skeletons and as a contributory factor for ATP synthesis, both essential for GS and nitrogenase acitivity (Rodrigues et al., 1998). GS is crucial in the assimilatory process of atmospheric nitrogen, as it catalyzes the first step in the conversion of inorganic nitrogen (ammonium) into its organic form (glutamine) (Gonnet and Diaz, 2000). Some evidence indicates that there is synergism played by nitrogenase, PEPC and GS activities on $N_2$ fixation efficiency by legumes nodules. The leghemoglobin content of the nodules also influences biological nitrogen fixation rates, since leghemoglobin provides a flux of $O_2$ for rhizobial respiration and maintains $O_2$ at a concentration that does not render the nitrogenase complex inactive (Appleby, 1984).

The rhizobial-legume symbiosis is characterized by high host specificity and the BNF and numbers of nodules has been proposed as an indicator of soil pollution based on the sensitivity of rhizobial to pesticides, urban and industrial wastes and heavy metals (Chaudri et al., 1993). Biological nitrogen fixation is a biochemical process that has been suggested as important measure of the effects of soil disturbances (Viser and Parkinson, 1992). Brookes (1995) recommended the measurement of biological nitrogen fixation as an indicator of soil stress resulting from pollutants, and Wetzel and Werner (1995) reported that nodulation is an important parameter related to toxic effects of soil pollution.

The nodulation by rhizobia can be determined in soil using a simple pot test, where a diverse set of legume seeds are sowed in the test soil and number of nodules formed are determined after a specific growth period. Additionally, the BNF can be determined by nodulins activities (nitrogenase, glutamine synthetase, glutamato sinthase, glutamate dehydrogenase and leghemoglobin). The effect in the specific bacteria may be quantified by direct isolation from soil using selective growth media together with morphological and physiological characterizations. A number of molecular methods can be also applied for diversity measurements of these bacteria. On the hands, evaluation of soil pollution using *Rhizobium* by growing legumes in the test soil and determining root nodule-formation and enzyme activities are rather simple methods. The molecular methods, on the other hand, are more technically demanding and a combination of both methods will allow a screening of potential of *Rhizobium*-legume symbiosis as indicator of soil pollution.

Additionally, the use of bioluminescence-based biosensors (using lux-marked *Rhizobium*) has become widely adopted as a sensitive and rapid method for indication of soil pollution (Steinberg et al., 1995). In this way, Chaudry et al., (2000), used a luminescence based biosensor (Rhizotox-C), with the lux cassette for luminescence incorporated into it, as a

possible indicator of heavy metal pollution of soil. They demonstrate the potential for using luminescence-based biosensors in a laboratory bioassay for rapidly identifying contaminated sites.

## Pesticides on Biological Nitrogen Fixation

Modern agricultural production is often dependent of chemical pest control for superior crop yield and quality. Pesticides are synthetic organic chemicals used to control weeds in fields and lawns, and unwanted or harmful pests, such as insects and pathogenic microorganisms that feed on crops. Pesticides are divided into categories, according to the target organisms they are designed to control, as insecticides, fungicides and herbicides (e.g., insecticides control insects).

Pesticides are important components of many agricultural management systems and their effects on soil and its ability to process them should be included when evaluating soil quality. Pesticides management thresholds must be established to minimize potential non-target effects on soil biota and processes (Locke et al., 2004). Leguminous crops, not unlike other crops, are routinely subject to a wide gamut of pesticides. Manny of these commonly used pesticides, in some instances inhibit nodulation, BNF and legume growth in symbiotic association with rhizobia (Schnelle and Hensley, 1989; Hernandez et al., 1999; Fox et al., 2004; Araújo and Araújo, 2006; Schnelle and Hensley, 2006; Fox et al., 2007).

In recent published paper, Fox et al. (2007) demonstrating that pesticides can reduce the symbiotic efficiency of nitrogen fixing bacteria and the host leguminous plants. They showed that some agrochemicals, which are either applied to crops or found as contaminants in the soil, significantly disrupt symbiotic nitrogen fixation and subsequently lower plant yields. The data indicate that a one-time treatment with some natural and synthetic environmental chemicals is sufficient to significantly inhibit nodule formation and BNF. The results of the study demonstrate that one of the environmental impacts of pesticides and contaminants in the soil environment is disruption of chemical signaling between host plants and rhizobial necessary for efficient BNF and optimal plant yield. Previously, Fox et al. (2004) using in vitro reporter assays, demonstrated that ~30 different pesticides and environmental contaminants specifically disrupted crucial symbiotic signaling between flavonoid phytochemicals and NodD receptors.

Herbicides are by far the most commonly used pesticides in the world. They range from non selective to highly selective for control of specific weeds in specific crops, with different products having post emergence, preplant, and preemergence uses. These compounds which are used for weed control in legumes can have adverse effects on nodulation and nitrogen fixation. There are reports that symbiotic nitrogen fixation is decreased following application of alachlor, metribuzin, trifuralin and glyphosate to soybean (Mallik and Tesfai, 1985; Hernandez et al., 1999) and oxyfluorfen, linuron, metribuzin and oxadiazon to lentil (Sandhu et al. 1991; Sprout et al. 1992). Hernandez et al. (1999) evaluated the effect of glyphosate on the bacteroid nitrogenase activity of three *Bradyrhizobium japonicum* strains with different sensitivities to herbicide. The authors observed that the glyphosate caused an inhibition in the bacteroid nitrogenase activity that was related with the sensitivity of the nodule-forming strains (Figure 1).

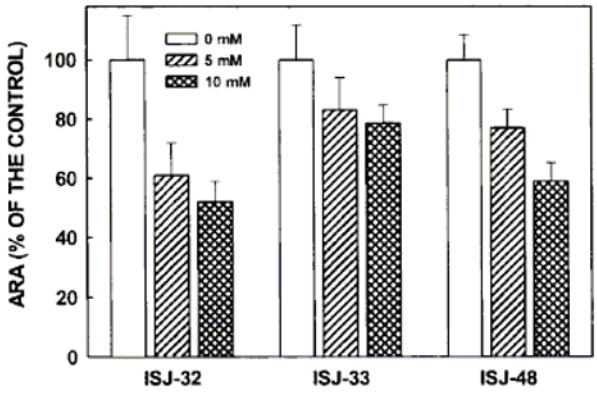


Figure 1. Inhibition of ARA (nitrogenase acitivity) in bacteroids of *B. japonicum* (strains ISJ-32, ISJ-33, and ISJ-48) extracted from plants 7 days after application of 1 mL of a solution of 0, 5, or 10 mM glyphosate (Hernandez et al., 1999).

Fungicides, used for plant pathogens control, are applied directly on seeds and consequently can be prejudicial to rhizobial survival and nodulation. Recently, the effect of fungicides on survival of rhizobial inoculated in common bean seeds was evaluated by Araújo and Araújo (2006) and the results showed that the fungicides benomyl and carboxyn inhibited the survival of rhizobial in the seeds. On the other hands, the fungicides captan, vitavax and thiram caused a decrease in the survival of rhizobial in the seeds.

## Sewage Sludge on Biological Nitrogen Fixation

Sewage sludge (often called biosolids) contains a substantial amount of nutrients such as C, N, P and trace elements which are essential major and minor plant nutrients. Thus, the addition of sewage sludge to agricultural soil improves chemical and physical characteristics and promotes plant growth. However, there is concern over the use of sewage sludge that has been contaminated with potential toxic elements, as heavy metals, from both industrial and domestic sources. Utilization of sewage sludge on agricultural land increases the concentration of heavy metals in soil. Studies suggest that leguminous crops dependent on symbiotic N fixation may be sensitive to the toxic effects of heavy metals present in sludge (Heckman et al., 1986; Reddy et al., 1983, Obbardy, 2001; Chaudhary et al., 2004).

Biological nitrogen fixation has been used to evaluate the effect of sewage sludge on soil (Reddy et al., 1983; Kinkle et al., 1987; Angle et al., 1992; Abd-Alla et al., 1999; Vieira, 2001; Vieira et al., 2004). The results are conflicting on the effects of sewage sludge on soil population of rhizobia, N fixation and yield of legumes. Reddy et al., (1983) studied the survival of *Brdyrhizobium* in soil after application of sewage sludge and found a decline in bacterial population. They conclude that this decline was the result of heavy metal toxicity. In the clover-*Rhizobium leguminosarum* association, reduced nitrogen fixation and clover yields have been associated with poorer survival of the rhizobial in contaminated soil with heavy metal (McGrath, 1994). Angel et al. (1992) attributed a rapid decline in population of *Bradyrhizobium* as to toxic concentration in soluble salts in soil after addition of sewage sludge.

On the other hands, studies on the effects of sewage sludge on symbiotic nitrogen fixation not demonstrated negative effects on nodulation (Kinkle et al., 1987; Angle et al. 1992; Ibekwe, 1997; Vieira, 2001; Vieira et al., 2004). In a study to evaluate the effect of sewage sludge application on dinitrogen symbiotic fixation in soybean, Vieira (2001) observed that the waste promoted a beneficial effect on nodulation, However, according to the same author, attention must be paid to heavy applications of sewage sludge to soils, which can negatively affect *Bradyrhizobium* and indirectly inhibit nodulation. Previously, Kinkle et al. (1987) observed an increase in the number of rhizobial in soil, which received high rates of sludge application. They concluded that the application of heavy metal-containing sewage sludge did not have a long-term detrimental effect on soil rhizobial numbers

## Industrial Wastes on Biological Nitrogen Fixation

Industrial activity generates large volumes of organic waste, as textile and tannery sludge, and release into the environment. Concerns about environmental quality have led to the introduction of alternative disposal methods such as the use as nutrient source for plants and as soil conditioners. Thus, the use of industrial organic matter in agricultural lands can be justified by the need of finding an appropriate destination for waste recycling. Textile and tannery sludge have a variable composition and normally contain high organic matter, N, P, K and micronutrients contents. Additionally, heavy metals and pathogenic microorganisms may be present in these wastes.

The use of industrial sludge in agricultural soils needs of defined action, in order to not cause damage to environment, mainly to the soil. Some studies evaluated the effects of textile (Araújo et al., 2007) and tannery (Teixeira et al., 2006; Araújo et al., 2006) sludge on nodulation and biological nitrogen fixation (BNF) in legumes.

Some studies were conduced for evaluate the effect of tannery sludge on growth and nodulation in legumes in Leucaena and Prosopis (Araújo et al., 2006) and cowpea (Teixeira et al., 2006). The tannery sludge was applied in four rates (0, 11.5, 23 and 46 ton $ha^{-1}$) and the results showed that the tannery sludge did not have a detrimental effect on nodulation in Leucaena and Prosopis. However, for cowpea, the tannery sludge, in high rates, caused an inhibition in the nodulation. The authors attributed this behavior to high content of salts presents in the waste (Table 2).

**Table 2. Nodule number of Leucaena, Prosopis and cowpea in soil plus tannery sludge and inoculation (Teixeira et al., 2006; Araújo et al., 2006).**

| Rate of tannery sludge | Nodule number (nodule per plant) | | |
|---|---|---|---|
| | **Leucaena** | **Prosopis** | **Cowpea** |
| 0 t $ha^{-1}$ | 44.7 b | 39.6 a | 53.0 a |
| 11.5 t $ha^{-1}$ | 97.0 a | 36.3 a | 43.0 a |
| 23 t $ha^{-1}$ | 75.3 a | 63.3 a | 39.3 ab |
| 46 t $ha^{-1}$ | 39.0 c | 44.3 a | 22.6 b |

In each column the means followed by the same letter do not differ statistically ($P<0.05$) from each other, according to Duncan's Test.

The effect of textile sludge on soil microbial was evaluated by Araújo & Monteiro (2006) in a laboratory experiment. The results show an inhibitory effect of the wastes on soil microbial activity and biomass. The same authors proposed the composting as an alternative method for detoxification of textile sludge. Previously, Araújo et al. (2001) and Araújo & Monteiro (2005) observed that the composting decreased or eliminated the toxicity of textile sludge. Additionally, Kaushik and Garg (2003) evaluated the composting of textile sludge using worms, and concluded that the waste could be converted into a stabilized product for agricultural use.

Thus, Araújo et al. (2007) evaluated the effect of composted textile sludge on growth, nodulation and nitrogen fixation of soybean and cowpea. The compost was applied in the soil at four rates (0, 9.5, 19 and 38 t $ha^{-1}$) and were evaluated nodulation, shoot nitrogen accumulation and nodulins activity. The results shows that nodulation and nodule glutamine synthetase (GS) activity and leghemoglobin content of the soybean and cowpea were not affected by the application of compost (Table 3).

**Table 3. Effect of different rates of composted textile sludge on nodule glutamine synthetase and leghemoglobin content of soybean and cowpea (Araújo et al., 2007).**

| Compost rates | Soybean | Cowpea | Soybean | Cowpea |
|---|---|---|---|---|
| | GS activity (µmoles of glutamyl $h^{-1}$ $g^{-1}$ nodules) | | Leghemoglobin content (mg $g^{-1}$ nodules) | |
| 0x | 101.3 a | 176.8 a | 63.4 b | 37.5 b |
| 0.5x | 126.9 a | 160.7 a | 91.6 a | 45.5 a |
| 1x | 125.7 a | 169.8 a | 78.5 ab | 40.9 ab |
| 2x | 109.2 a | 165.7 a | 74.3 ab | 38.2 ab |

In each column the means followed by the same letter do not differ statistically ($P<0.05$) from each other, according to Duncan's Test.

## Conclusion

The characteristics of soil microbial communities place these indicators as potentials in the evaluation of the soil quality and consequently the soil pollution. In early 1900s, Waksman (1927) considered several microbial criteria as indicator of soil, included numbers of microorganisms and nitrogen-fixing capacities. The potential of using different components of soil biota and its activity as biological indicators has been cited by different authors. Such indicators include soil microbial biomass, soil enzyme activity, soil micro-fauna, including bacteria, fungi, algae and plant root pathogens, soil micro-fauna (protozoa, nematodes), macro-fauna, total soil biodiversity, etc. Soil organisms have been shown to be potentially useful indicators of soil health. Actually, changes in microbial populations, or specific functional groups, as nitrogen fixing bacteria and root nodule bacteria, have been used as measure of the impact of organic and inorganic pollution in soils. With the current state of knowledge, microbial indicators are probably only useful for measuring changes in soil quality resulting from some pollution. However, the conflicting results observed in

evaluation of soil pollution, mainly by use of urban and industrial wastes, indicates the need of search others methods for use of microorganisms, especially rhizobia.

One of the major difficulties in the use of soil organisms *per se*, or of soil processes mediated by soil organisms, as indicators of soil health has been methodological - what to measure and how and when to measure it and how to interpret changes in term of soil function. Despite those difficulties there have been major advances in our understanding of the soil biota and its functioning at the community level in recent years. New techniques for measuring the structure and functional diversity of rhizobial, for example DNA techniques, offer new and largely unexplored potential for using rhizobial and biological nitrogen fixation as indicator of soil pollution.

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