THE STATE OF THE ART OF RESEARCH ON MICROBIAL ECOLOGY IN THE SOIL AND COMPOST

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1. BIOLOGICAL ACTIVITY OF THE SOIL AND THE APPLICATION OF COMPOST

Soil is a medium that is very favourable to life, so a great many living organisms have made their home in it. These organisms carry out a wide range of functions, which are responsible for the well-being of the soil. They will thus have a fundamental role in the functioning of the soil system [1].

Both vegetable and animal organisms are involved, their size varying from bacteria and other microscopic beings to the great burrowing animals and tree roots, and the different life forms present in the soil are interdependent. Green vegetables derive nutrients from the soil and carbon dioxide from the air to produce their tissues, which will serve animals as food. On the other hand, the waste products and remains of plants and animals return to the soil, where they are processed by insects, worms, fungi, bacteria and other living beings, generating humus. Thus the cycle is completed, as the nutrients and the carbon dioxide again become available to plant growth through this decomposition in the soil [2].

Decomposition of organic matter is a speciality of soil organisms as they need energy to live, together with nutritive elements, from which they synthesize their constituents. Therefore the organic fraction of residues applied to the soil such as compost has great importance as it affords a food source for a wide range of micro-organisms which, via the carbon cycle, will help to keep up a reserve of organic matter in the soil, along with its biological activity.

Among the various organisms in the soil, microbes play a fundamental part in the decomposition of organic matter. They include bacteria, fungi, actinomycetes, algae and protozoa. Bacteria are usually the most numerous, but as they are very small, the predominant part of the microbial mass is made up of fungi.

Microbial populations are very sensitive to changes in soil conditions. Any effort to stimulate the activity of micro-organisms in the soil is of vital importance when a regeneration or reactivation of the soil ecosystem is needed in cases where, for whatever reason, it has been

damaged. In this way, an increase in microbial activity in the soil will have to be the prime aim in any project for restoring or recuperating soils [1].

Moreover, the study of the role of organisms in these processes in the soil has recently been extended to include the biological control of plagues and diseases, the degradation of pollutants and the transformation of metals. More studies are also being made of microbial ecology and the interactions of micro-organisms in the soil with the environment [3], and the addition of compost of various origins to the soil and its beneficial biological effects [4, 5, 6]. The microbial population of the soil therefore has a fundamental influence on the functioning and ecology of the soil, whence its great importance. The importance of the microbial population in the biological treatments of biowaste must also be mentioned. In this study, then, we seek to offer an overview of the current state of affairs for people who may not be experts in microbiology.

2. METHODOLOGY FOR ASSESSING THE MICROBIAL COMMUNITY IN THE SOIL AND IN COMPOST

In the last few years, much importance has been given to research into everything concerning soil study, especially as regards techniques for assessing its biodiversity, the methods often being used also after the application of compost or other organic materials to the soil, as well as for the analysis and microbiological monitoring of the composting process itself.

The microbial activity of the soil has long been used as an indicator of soil quality or of the health of the soil ecosystem. Different indices of this activity have been widely included in the assessment of soil quality (carbon of microbial biomass, microbial respiration and diverse enzymatic activities such as dehydrogenase and protease as representatives of the system's activity). These parameters are useful in the monitoring of changes in soils.

Other techniques are also being used for analysing the microbial community in the soil and compost. They fall into two categories: those generating phenotypical data (analysis of lipids, substrate utilization profiles [Biolog^{TM}], and certain enzymatic activities) and those generating genotypical data (techniques of nucleic acid analyses). They give more complete information on the microbiota present than simply measuring biomass and microbial respiration.

We shall now give a brief overview of the different methods and techniques used today in the study of soil and compost ecology, ranging from traditional microbiological methods to molecular techniques.

COUNTS (Traditional Microbiology)

This name is given to quantitative determinations of the microbial biomass. Different methods exist for determining the numbers of micro-organisms, such as:

- Plate counting, either on the surface or included;
- Multiple dilution method, or the Most Probable Number Method;
- Filtration through a membrane and incubation on a solid culture medium;
- Direct microscopic counting of micro-organisms, on smears or with cameras.

INDICES OF MICROBIAL ACTIVITY

Soil Respiration

Soil respiration may be defined as the oxygen consumption or carbon dioxide release of living organisms. Basal respiration is considered a useful parameter for measuring the biological activity of the soil [14, 15]. It is found by means of the quotient of C-CO_2 given off during the respiration experiment and its duration. Several methods have been developed for determining soil respiration, both in the field and in the laboratory, in both dynamic and static systems.

Analysis of the Microbial Biomass

The microbial biomass is the main agent of decomposition of organic wastes, of the food cycle and the energy flow in the soil ecosystem. The carbon of the microbial biomass has been used as a bio-indicator of the changes undergone by organic matter in the soil as this parameter responds more quickly to changes in soil conditions than the organic matter itself. There are several ways of determining it [7, 8], such as CFI (chloroform fumigation-incubation) [9, 10], CFE (chloroform fumigation-extraction) [11, 12, 13, 14, 15] and SIR (substrate-induced respiration) [10, 11, 16, 17, 18].

CFI
This method is based on the extra flow of CO_2 produced by a soil previously fumigated with chloroform, after the chloroform has been eliminated and the soil then aerobically incubated. The additional carbon dioxide produced in comparison with a non-fumigated soil is due to the decomposition by the next colonizing population of micro-organisms killed by the fumigation. Jenkinson and Powlson [19] suggest that the measure of this CO_2 flow gives an estimate of the amount of carbon in the biomass of the soil.

CFE
Vance *et al.* [20] relate the biomass C to the parameter E_C, which is equal to the difference between the organic carbon extracted with K_2SO_4 0.5M from a fumigated soil and that extracted in the same way from the same soil without fumigation. For digestion these authors used dichromate, but organic carbon can also be determined with automatic analysers using persulphate and UV oxidation.

SIR
This method relies on the fact in the first hours after the addition of an excess of glucose to a soil sample, the soil's respiration is only limited by the quantity of micro-organisms. The respiration rate in the first few hours, considered the maximum initial respiratory response, will be proportional to the microbial biomass.

Other methods also exist for measuring microbial biomass based on the specific detection of some cellular component or metabolic product, such as the concentration of proteins or ATP.

Enzyme Activities

The biochemical reactions involved in the food cycles in soil or compost are catalysed by enzymes, which are specific to the type of reaction in which they take part, that is, they are specific for a given substrate. These enzymes may be studied indirectly through their activity.

The study of enzymes has become much more widespread owing to their role in evolution and in the degradation of organic matter. They play a basic part in the nitrogen cycle (urease and proteases), the phosphorous cycle (phosphatases) and the carbon cycle (β-glucosidases). It should be pointed out that measurements of enzyme activities are considered potential measurements, as they are carried out *in vitro* in optimal conditions, but they can be useful for the monitoring of soil characteristics after damage or use.

The measurement of dehydrogenase activity affords information on the overall metabolic activity of micro-organisms. It includes different dehydrogenase systems and it originates in biological oxidation through the dehydrogenation of different organic compounds. Dehydrogenase activity is considered to be associated with the respiratory processes of micro-organisms, and is therefore thought to be more dependent on the metabolic state and overall biological activity than any of the other enzymes present, and therefore more directly related to microbial activity.

As the activities of enzymes are substrate-specific, a group of enzymes has to be measured in order to have an idea of the general state of the nutrients or to determine indices of microbial activity. Functional microbial diversity depends on many metabolic relationships, so it would not be realistic to assume that there is a relationship between the measurement of an enzyme activity and the microbial diversity of the soil or compost. Recently kits have been used with a number of cells containing different substrates, making it possible to measure different enzyme activities simultaneously in a single sample, although it is very difficult to interpret the results obtained.

SUBSTRATE UTILIZATION PROFILES (Biolog^{TM})

Assessing use profiles of substrates with the Biolog^{TM} system is an approach to the study of functional diversity in bacterial communities, as it shows how they are potentially able to make use of a range of carbon substrates.

The Biolog^{TM} system consists of 96 cells containing 95 different carbon substrates plus a negative control with no carbon source. The extent to which these substrates will be used will depend on the structure complexity of the microbial community inoculated into the cells and their capacity to use the substrate and compete with it.

This system does not afford an overall estimate of the functional diversity of the microbial communities, just information on the members of the community that can be cultivated in the conditions existing. This method must therefore be considered only as a simple step for describing certain microbial functions, as otherwise the ecological meaning of the results obtained will be very difficult to interpret. It is also thought that the results represent potential metabolic capacities of a microbial community, but not their activity *in situ*. On the other hand, this method omits many important micro-organisms, such as fungi and actinomycetes.

PHOSPHOLIPID FATTY ACID ANALYSIS (PLFA)

Another widely used way of characterizing microbial communities in the soil and in compost [7, 14, 21, 22] is ester-linked phospholipid fatty acid analysis (PLFA). PLFAs are constituents of all cell membranes, and are compounds with no storage function, which therefore represent

a relatively constant fraction of the cell mass. There are now methods of extraction and derivation of PLFAs from live organisms (they degrade quickly after death).

Later, gas chromatography and mass spectrometry analyses are run, which make it possible to quantify a wide range of PLFAs. The result gives profiles of many PLFAs defined from the structure of each compound and the amount of it present in the sample. The total amount of PLFAs may be used as an indicator of viable microbial biomass. Some PLFAs can be used for the identification of certain taxonomic or functional groups of micro-organisms. Although it is not possible to detect each species of micro-organism, as there is an overlap of PLFA profiles, this technique can be useful for comparing the composition of the microbial community in different samples with different properties or environmental conditions.

It must, anyway, be taken into account that the lipid concentration varies with the age of the organism, its food resources, habitat, etc. Furthermore, biotic and abiotic conditions will affect the amount and type of lipids extracted, as will the clay and organic matter content of the soil.

MOLECULAR TECHNIQUES IN SOIL AND COMPOST MICROBIOLOGY

Most soil micro-organisms cannot be cultured in the laboratory. Less than 1% of the bacteria can be cultured in a pure form for later characterization. Therefore, the application of molecular techniques and methods is being developed and studied for detecting, enumerating and characterizing soil micro-organisms. A gram of soil can contain 10^{12} bacteria and probably about 5000 different microbial genomes. This great diversity makes complete knowledge of them impossible, even with the use of these techniques. So, a complete quantification of the species of bacteria and fungi in the soil is still very difficult, and the same is true for compost.

These methods are based on analysing the nucleic acids extracted from a sample, and are now the most promising techniques for describing soil or compost ecology. There are very many methods, each with its own advantages and drawbacks, but this is a relatively new field and much research still has to be done into everything concerning molecular biology as applied to these studies.

The first and possibly the most critical stage in the development of these methods is the isolation of DNA or RNA from the sample. Two basic techniques are available for obtaining DNA from soil:

- Indirect extraction, whereby micro-organisms are first separated from the rest of the soil material by differential centrifuging, with lysis straight afterwards.
- Direct extraction, where lysis of the micro-organisms is carried out and the free DNA is then extracted and purified.

The second procedure yields greater amounts of DNA, but other soil components are often extracted alongside it, including humic substances which could cause problems as they can interfere with the subsequent amplification by PCR, in DNA hybridization and digestion with restrictor enzymes. On the other hand, soils with different clay and organic matter content will respond differently to different techniques for isolating nucleic acids. Furthermore, cell lysis can affect results as, for example, large cells break up easily under sonication, while the smallest ones seem to be extremely resistant to it.

The range of molecular methods for studying soil DNA or RNA is very wide [23, 24, 25, 26, 27]. By way of example, we shall now look at some of the methods most used in soil study.

Once DNA is extracted and purified, normally Polymerase Chain Reaction (PCR) will be effected to amplify the nucleic acids extracted.

The fragments of 16SrDNA, products of PCR, can be separated according to their sequence by denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE). The methods consists in the separation of DNA fragments obtained by PCR according to the heterogeneity of their sequence, which gives rise to different denaturing properties of the chain through the gradient created in the gel. In DGGE, this gradient will be the concentration, while in TGGE it is the temperature. The model of bands obtained with gel will be an echo of the diversity of populations present in the sample. Later analyses are possible of the bands obtained with reamplification and sequencing.

Another of the most widely used methods of detecting nucleic acids is restriction fragment length polymorphism (RFLP), also known as amplified ribosomal DNA restriction analysis (ARDRA), which consists in the analysis of the fragments resulting from cutting rDNA amplified with restriction enzymes, which will be separated by electrophoresis in gel according to their size. The complexity of the band model will depend on the complexity of the original population of the sample.

When RNA is the molecule under study, reverse transcriptase will be used to transcribe a sequence of RNA, yielding complementary DNA (cDNA), which will then be amplified by PCR. Then one of the techniques described above will be applied: RFLP, DGGE or TGGE. In this case, the band patterns of the electrophoresis gel will show the metabolically active populations of a sample, which may be related to the functional diversity.

As well as these methods, hybridization techniques have also been developed for the study of the structure of the bacterial community of a sample, such as fluorescent *in situ* hybridization (FISH), where fluorescent probes are used, which hybridize with the rRNA of the micro-organisms. This technique can be used to identify key species and detect their quantity in the sample.

All these techniques have also been applied to the study of compost, especially with a view to its application to the soil [28], and to the composting process itself [29, 30, 31]. Molecular techniques certainly offer high possibilities of rapid detection, identification and characterization of populations in different environments, including soil and compost, which are probably not easy media for their application because of the difficulties in cell lysis, in the extraction and purification of DNA – because of the humic acid pollution in the soil – and also because of the great diversity of bacteria present in a sample of soil or compost.

3. THE FUTURE OF RESEARCH INTO SOIL AND COMPOST ECOLOGY

Today, in many countries, both in the European Union and outside it, field projects are underway of research into soil and compost ecology. Papers, books and reporters have been published on the subject over the past decade to show the results of the specialized research carried out in some of these countries.

However, it is still a relatively new research field and much work will therefore be necessary before we can fully understand the importance of the role of microbial biodiversity in many processes, as there are still problems for the accurate determination of the microbial community in samples of this type.

Future research will be necessary or useful on the following points:

Concepts:

For bacteria there is no appropriate and clear definition of the term "species". At present, a bacterial species is defined as the set of organisms that have a 97% similarity in sequences of its 16S rRNA, which makes the concept very debatable.

On the other hand, it would also help to have the most accurate definition possible of "soil biodiversity", possibly at different levels, given the complexity of the soil ecosystem in this regard.

Methodological development:

At present, for soil and compost micro-organisms it is not possible to make an easy and accurate determination of their number (wealth of species) or relative abundance (diversity), as most of the techniques used for determining microbial diversity have a number of drawbacks and limitations. It is therefore necessary to improve these techniques and develop others in order to have a reliable tool for detecting microbial biodiversity in soil and compost.

Trials based on genetic analysis are presumably the closest approach to microbial diversity, but more work must still be done. Until now, most molecular methods have provided taxonomic information, but functional genes have been codified more rarely. Methods are therefore needed for analysing the functional genome, to echo the wide range of functional diversity of microbial communities.

It is important to remember that genomic DNA is present in all bacteria, whether active or dormant, and also in an extracellular form protected by the adsorption of soil particles. So a genetic DNA-based analysis of the microbial community might overestimate the number of species in the sample. On the other hand, the RNA content is obviously higher in active bacteria than in dormant ones. But much still has to be done concerning the direct analysis of mRNA.

To sum up, the use of molecular techniques to assess microbial diversity in the soil still has some shortcomings, such as the difficulty of lysis of all bacteria, the presence of DNA of other organisms, the extraction of DNA originating from dead bacteria, etc. Nevertheless, there is no doubt that molecular methods offer a great opportunity for advance in the knowledge of the field of microbial ecology of the soil and compost.

Biodiversity and soil ecology:

This is a very wide field, covering many areas of knowledge and research and including the following possibilities for study:

- Relationship between structural and functional diversity. Together with the species wealth studies already mentioned, simultaneous research is also necessary into functional diversity, which will bring knowledge of functional interrelationships within a specific area, and the identification of food chains, guilds, indicator species, etc.
- The relationship between the biodiversity of the soil and the general functioning of the soil ecosystem, especially its relationship with fertility.
- The biodiversity of the soil will obviously have an effect on the "health" of the soil ecosystem, perhaps even serving as an indicator of it. In this context, the biodiversity of a soil will serve indirectly to monitor the efficacy of a remediation process (bioremediation or phytoremediation) carried out on it.
- The effects of environmental and climatic factors (drought, salinity, emission of greenhouse gases, atmospheric pollutants, pollution, waterlogging, lowering of pH, changes in the organic carbon content, etc.) in the diversity and activity of microbial communities in the soil.
- The effects of farming and forestry practices, of fertilizing soils, of the application of sludges from wastewater treatment plants, manures, composts and other organic residues on the biodiversity of the soil.
- The effect of genetically modified crops on the ecology of the soil.
- The relationship between the microbial biodiversity of the soil and its capacity for eliminating plant diseases, as the term "soil health" sometimes includes its microbiological capacity to counteract the activity of phytopathogenic organisms.
- The biodiversity of arbuscular mycorrhizal fungi (AMF), as they colonize the roots of most land plants. It would be interesting to establish a useful protocol for carrying out standardized functional AMF tests under a range of conditions. In fact, the techniques used up to now have been quite limited to the study of bacteria in the soil, thus bringing about a great ignorance of soil fungi.

In conclusion, it is obvious that soil ecology is a new field of research in which we are a long way from any far-reaching or accurate knowledge.

Soil is an extremely complex matrix, which makes it very difficult to quantify its biodiversity while more money and time are required for research and monitoring, which is probably the most serious obstruction to the advance of knowledge.

All this shows that the diversity of the soil is a subject of great interest and potential because of the number of possible applications and its contribution to a wider understanding of soil ecology, a discipline in its infancy.

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