

Cranfield University
Faculty of Medicine and Biosciences
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**Microbial volatile fingerprints: potential use for
soil/water diagnostics and correlation with
traditional microbial parameters**

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**Microbial volatile fingerprints: potential use for
soil/water diagnostics and correlation with
traditional microbial parameters**

Supervisor:

Prof. N. Magan

This degree is submitted in partial fulfillment of the requirements for the Degree of
Doctor of Philosophy.

*This thesis is dedicated to my family,
1809 kilometres away, and yet always so close...*

ABSTRACT

This project used an electronic nose (E-nose) system composed of an array of 14 non-specific conducting polymer sensors for soil and water diagnostics, based on qualitative microbial volatile production patterns. It tested the feasibility of using soil microbial volatile fingerprints for detecting and monitoring changes in microbial activity in three soils, as a response to key environmental factors such as temperature (16, 25, 37°C), water potential (-0.7, -2.8 MPa), and nutrient (glucose and wheat straw) inputs. It also investigated their potential use for atrazine detection when applied to soil at usual field application rates (2.5 ppm) as well as for monitoring its bioremediation using the white-rot fungus *Trametes versicolor* (R26), for up to 24 weeks. Furthermore, statistical correlations were investigated between soil volatile profiles and traditional microbial parameters for characterising microbial communities and their metabolic activities such as respiration, dehydrogenase (DHA) and laccase (LAC) activities, bacterial and fungal colony counts and fungal community structure under different soil conditions. Finally, this study explored the potential of microbial volatile production patterns for monitoring the activity and differentiation of two *Streptomyces* species (*S. aureofaciens* A253 and *S. griseus* A26) in potable water and in soil, as well as the production of geosmin in both environments.

Data in this research has demonstrated that the production of volatile organic compounds (VOC) in soil is likely to arise from microbial metabolism. The E-nose was able to detect variations in the patterns of volatile production from soil according to treatments, functioning as indicators of shifts in microbial activity and community structure. The potential for discrimination between soil types in relation to environmental factors and nutrient addition has been demonstrated for the first time using principle component analysis (PCA). Significant ($p < 0.05$) correlations were also found between soil volatile patterns (through PC1) and traditional soil microbial parameters. The close relationship ($r > 0.80$) between PC1 and soil respiration was particularly relevant, since it indicates that microbial volatile fingerprints, similarly to respiration, respond quickly to changes in soil conditions. The sensor array was also able to detect *Streptomyces* activity and differentiation as well as discriminate

between bacterial species at different concentrations in potable water and in soil. Using this approach, the presence of geosmin was detected in water at 0.5 ppb (below its human odour threshold detection, OTD) and in soil at 100 ppb (OTD not established).

This study has, therefore, demonstrated that an E-nose can be employed as a rapid, sensitive, reproducible and non-invasive tool for characterising changes in soil environmental conditions, as well as for monitoring key soil processes such as organic matter decomposition and atrazine degradation. It also suggests that this approach can complement, and perhaps replace, some of these methods for a quick and routine evaluation of the impact of environmental factors on soil microbial communities. Furthermore, this study showed that an E-nose can also be employed for assessing *Streptomyces* activity and detecting geosmin production at an early stage in water and soil.

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List of Acronyms and Abbreviations

ABTS	2,2-azino-bis-ethylbenthiazaline-6-sulphonic acid
ANN	Artificial neural network
ANOVA	Analysis of variance
ATP	Adenosine triphosphate
BTEX	Group of compounds which include benzene, toluene, ethylbenzene, xylene
CFU	Colony forming units
DF	Discriminant factor
DFA	Discriminant function analysis
DHA	Dehydrogenase activity
E-nose	Electronic nose
GC	Gas chromatography
GC/MS	Gas chromatography/mass spectrometry
HPLC	High pressure liquid chromatography
INF	Iodonitrotetrazolium formazan
INT	2(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride
LAC	Laccase
LDF	Litter-decomposing fungi
MnP	Manganese-peroxidase
MEA	Malt extract agar
NA	Nutrient agar
OTD	Odour threshold detection
PAH	Polycyclic aromatic hydrocarbons
PARC	Pattern recognition
PC	Principle component
PCA	Principle component analysis
PCB	Polychlorinated biphenyls
PCP	Pentachlorophenol
RO	Reverse osmosis

SE	Standard error
SIR	Substrate-induced respiration
SOM	Soil organic matter
TPF	Triphenyl formazan
TTC	2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazolium chloride
VOC	Volatile organic compound
r	Correlation coefficient
p	Significance level

List of Units

MPa	Mega Pascal
%	Percentage
µm	Micrometres
µg	Micrograms
kg C ha⁻¹	Kilograms of carbon <i>per</i> hectare
m g⁻¹	Metres <i>per</i> gram
°C	Degrees centigrade
ppm	Parts <i>per</i> million
ppb	Parts <i>per</i> billion
mm	Milimetres
CFU	Colony forming units
Log CFU g⁻¹	Logarithm of colony forming units <i>per</i> gram
µg CO₂ g⁻¹ soil h⁻¹	Micrograms of carbon dioxide <i>per</i> gram of soil <i>per</i> hour
µg INF g⁻¹ soil 2h⁻¹	Micrograms of INF <i>per</i> gram of soil <i>per</i> two hours
U g⁻¹ soil	Units of enzyme <i>per</i> gram of soil

(one enzyme activity unit U was defined as the amount of enzyme required for producing a 0.001 increase in the optical density of the reaction mixture *per* minute)

CHAPTER I

Literature Review and Research Aims

CHAPTER I

LITERATURE REVIEW AND RESEARCH AIMS

1.1 INTRODUCTION

The major components of soil are mineral and organic matter, water and air (Brady and Weil, 2002). However, soil is a complex and dynamic habitat (Harris and Steer, 1997). Its physical, chemical and biological characteristics are determined by the relative proportion of those components, markedly influenced by environmental factors and biotic activities over time (Punkhurst *et al.*, 1997; Bloem *et al.*, 2006). Soil characteristics, in turn, determine those of natural vegetation, as well as mesofauna and microbial community structure and activity (Stengel and Gelin, 2003).

Organic matter degradation (i.e. nutrient cycling) is considered the most important function of soil organisms: soil fauna is valuable for mixing the litter; and microorganisms, mainly bacteria and fungi, degrade and release simple and complex nutrients, which are then available for plant growth (Schinner *et al.*, 1996; Bloem *et al.*, 2006). The role of soil and the influence of its properties on crop production, animal health, water quality and on the recycling of compounds ranging from biological remains and wastes to xenobiotics (such as pesticides) has been recognised (Bridges, 1998; Doran, 2002; Stengel and Gerin, 2003; Crawford *et al.*, 2005). This increasing awareness of the importance of soil has led to scientific discussions on defining and evaluating soil quality. The most widely accepted definition for soil quality is that of Karlen *et al.* (1997), stating that “it is the capacity of a specific type of soil to function, within natural or managed ecosystem boundaries to sustain plant and animal health and productivity, maintain or enhance water and air quality, and support human health and habitation”.

This concept implies that soil quality can change over time due to environmental and human impacts, either towards an increase or a decrease in quality (Doran, 2002). Soil management and land-use (e.g. the application of pesticides and herbicides) are the

main factors responsible for abrupt and deep decreases in both soil and water quality (Doran, 2002). Unfortunately, soil contamination with pesticides and herbicides is very frequent. Bioremediation of contaminated soil using white rot fungi may offer a cost-effective treatment, with minimal impact on soil physical properties and on the quality of the environment (Kearney and Roberts, 1998; Margesin *et al.*, 2000; Tornberg *et al.*, 2003).

Environmental imbalances related to soil temperature and water potential may also be responsible for changes in soil, and consequently, water quality. An example is the development of geosmin-derived off-odours and off-flavours in crops, vegetables, fish, sea-food and potable water when soil temperature increases (Buttery and Garibaldi, 1976; Wnorowski, 1992; Wood *et al.*, 2001). In this case, a quick detection of geosmin-producing agents at an early stage of differentiation may be essential for controlling these occurrences.

It is, therefore, important to investigate the way in which environmental factors and potential soil contaminants influence soil functioning. Traditionally, this is done by measuring a pre-established set of physical, chemical and biological soil parameters. Among these, the characteristics of soil microbial communities, i.e. community size, activity and community structure are key parameters, since they respond quicker than any other soil component to changes in soil status (Bloem *et al.*, 2006). These have been widely used as sensitive and reliable indicators of changes in soil environmental conditions and land-use, as well as for comparisons between soil types and the way they respond to those changes. The impact of the presence of pesticides in soil as well as the progress of soil bioremediation strategies have also been evaluated using the same set of microbial indicators (Nannipieri *et al.*, 2003). From this point of view, the development of technologies such as the Electronic Nose (E-nose), which can provide an early, quick and reliable characterisation of microbial activity and the potential for detection and monitoring of chemical and biological contaminants, may be of major importance. Furthermore, exploring possible correlations between the E-nose and traditional microbial parameter responses to soil conditions may open doors on the use of this technology in Soil Science and Agriculture.

1.2 SOIL AS A MICROHABITAT

1.2.1 Organic matter: source and sink of soil nutrients

According to Carter (2001) there is no consensus yet on the definition of soil organic matter. According to a review by Bloem *et al.* (2006) on the subject, soil organic matter (SOM) is usually considered to comprise two main pools: the “living” (e.g. roots and microbial populations) and the “non-living” (nutrients of different ages and origins) pool. For the purpose of this study, microorganisms will be treated independently of SOM for simplification.

The decomposition of organic matter provides energy for microbial growth and carbon for synthesis of new cell material (Schinner *et al.*, 1996). Carbon is its main constituent, typically accounting for around 58% of its total weight. Besides carbon, it also includes oxygen, hydrogen, nitrogen, phosphorus, sulphur, proteins, carbohydrates, lignins, enzymes and nucleic acids (Schinner *et al.*, 1996; Miller and Donahue, 1995). Plant residues are usually considered the largest contribution to organic matter in soil, containing both labile and more recalcitrant compounds. The labile substances are generally soluble and easy to degrade such as sugars and amino-acids; the more recalcitrant are typically natural polymers such as cellulose and lignin. Nevertheless, soil is generally poor in nutrients and energy sources (estimated to be only about 5% of its solid material), when compared with the conditions used for microbial growth under laboratory conditions (Ashman and Puri, 2002; Nannipieri, 2003). Microorganisms are thus constantly competing for nutrient sources (Bridges, 1998), mainly carbohydrates, nitrogen, phosphorus and ions such as iron (Fe^{3+}), calcium (Ca^{2+}) (Miller and Donahue, 1995; Stengel and Gerin, 2003).

Nutrients are not homogeneously distributed throughout the bulk of the soil but rather heterogeneously dispersed, creating discrete microhabitats or “hot spots” (Stotzky, 1997; Nannipieri *et al.*, 2003) where the microorganisms live and interact. Chemical, physical and biological characteristics of these zones of high biological activity, are thus different from one microhabitat to the next, and also vary with time and as

influenced by environmental factors (Nannipieri *et al.*, 2003). In addition, the characteristics of organic matter in any soil are in a state of flux influenced by climate, vegetation and agricultural practices (Nannipieri *et al.*, 2003; Stengel and Gelin, 2003).

Apart from being an excellent nutrient supply to plants and microorganisms, organic matter makes the soil less compact, therefore providing better aeration and water movement, as well as protecting it from erosion. This is particularly important in hot and dry environments, where by shading the soil, rapid moisture loss and excessive temperature increases are prevented (Miller and Donahue, 1995).

1.2.2 Soil type

In this work, the concept of soil type refers mainly to properties such as texture, pH and the type and amount of organic matter it contains. Soil texture is usually considered a static property of soil, relating to the distribution of particle sizes, i.e. the proportion of sand, silt and clay, and consequently to the distribution of pore size between them (Ashman and Puri, 2002). The pores between particles of solid material are just as important to the nature of the soil as are the solids themselves (Miller and Donahue, 1995), since it is in the pore space that air and water circulate, roots grow and most of the microbial population live.

For example, gaps between sand particles tend to be large favouring aeration but also rapid water loss. In contrast, pores between clay particles tend to be small, favouring water retention (Ashman and Puri, 2002) but decreased aeration (Miller and Donahue, 1995). In this way, soil texture influences most soil processes. In a simplified explanation, it not only determines its moisture and aeration characteristics (Brady and Weil, 2002) but ultimately its ability to store and supply organic matter for plant and microbial use. For instance, clays due to their large sorption area as well as their electrostatic nature, have a greater tendency to adsorb and retain organic matter into stable aggregates (Ashman and Puri, 2002).

On the other hand, most chemical and biological reactions are dependent on the levels of H^+ and OH^- ions in soil, being markedly influenced by soil pH (Miller and Donahue, 1995). pH alone can explain the ionic character of most compounds present in the organic matter and consequently, their solubility and availability for plants and microbial use (Ashman and Puri, 2002). In a similar way, it influences the solubility of soil contaminants such as pesticides (Brady and Weil, 2002).

The properties described above are influenced by other factors such as climate, in turn determine soil natural vegetation (and therefore the type of organic matter it will contribute with) and ultimately the characteristics of its native microbial community (Stengel and Gelin, 2003).

1.2.3 Water availability and the concept of soil water potential

Most of the nutrients required by plants and microorganisms, as well as oxygen, are found dissolved in the soil solution. The availability of water in soil is a key factor influencing microbial growth and activity and therefore major soil processes such as degradation of organic matter and potential soil contaminants (Brady and Weil, 2002; Bloem *et al.*, 2006).

In soil, water is held as films on particle surfaces and within soil pores (Stengel and Gelin, 2003) through hydrogen bonding (Miller and Donahue, 1995). Therefore, the higher the clay or organic matter contents of a soil, the larger the amount of water it can store, but also the more tightly it is bond to the large surface area (Brady and Weil, 2002). However, if the water is held too tightly it becomes unavailable to plants and microorganisms, as they can only extract water at certain tensions. “Tension” here refers to the measure of suction required in order to extract water from the soil (Miller and Donahue, 1995; Ashman and Puri, 2002).

Very often, studies in the literature express soil water content in terms of percentage moisture (% MC), which is the ratio between dry and wet soil weight (Equation 1.1).

$$\text{M.C.} = [(W_{\text{wet}} - W_{\text{dry}}) / W_{\text{dry}}] * 100 \quad (\text{Equation 1.1})$$

W_{wet} is the weight of the wet soil and W_{dry} is the weight of the dry soil.

However, for the reason mentioned above, soil moisture content (or that of any given substrate) is not always a good indicator of the amount of water that is actually available for plant and microbial use (Magan, 1997; Ashman and Puri, 2002). In early studies, Orchard and Cook (1983) have recognised that expressing soil water in terms of gravimetric moisture content is not meaningful, particularly when microbiological studies are concerned. These authors, supported by others, have therefore chosen to instead use the concept of soil water potential (Ψ), which provides a measure of the force with which the water is held in soil (Orchard and Cook, 1983) and more reliably describe the water availability for microbial growth and activity.

In other words, Ψ (in Pascals, Pa) is the difference in energy level between water molecules in soil and that of free water molecules in a pool of pure water at the reference state of temperature, pressure and height (Ψ of pure water is zero). It is given by the sum of the pressure potential (or turgor pressure) (Ψ_p), solute or osmotic potential (Ψ_s) and matric potential (Ψ_m) per unit of volume (Equation 1.2):

$$\Psi = \Psi_p + \Psi_s + \Psi_m \quad (\text{Equation 1.2})$$

Ψ_p is the potential of water in soil as affected by external pressure (in biology, it represents the turgor potential of the cell contents against the cell wall); Ψ_s is the potential of water in soil as affected by the presence of solutes (Salisbury, 1992; Magan, 1997); Ψ_m is the potential of water in soil as affected by the presence of a solid matrix (it is a measure of the tendency for the matrix to adsorb water molecules) (Salisbury, 1992). Water potential is therefore a negative value (Miller and Donahue, 1995); the more tightly the water is held, the drier is the soil and the more negative is its water potential value.

Although soil microbial responses to changes in soil moisture content has been described widely in the literature, only in very few studies their response to changes

in soil water potential has been explored (Wilson and Griffin, 1974). This is of course important in any soil type of any region, but perhaps most important in Mediterranean and Mediterranean-like regions, where soils are very often subject to stress related to low water availability, aggravated by high temperatures and short supply of readily available organic matter (Garcia *et al.*, 1994; Ros *et al.*, 2003). According to Miller and Donahue (1995), soil available water for plants and microorganisms ranges between -0.03 (field capacity) and -1.5 MPa (wilting point).

The relationship between total soil water content and water potential is best described by the so called soil-water characteristic curve (soil-water curves for the soil types in this study are given in Chapter II, Sub-section 2.2). When the soil is saturated with water (e.g. after heavy rainfall), soil water potential is zero. As the soils dries out, its water potential decreases as large pores drain and the water gets progressively confined to smaller pores. Since soil properties such as texture determine the pore size distribution, each soil type has its own soil-water characteristic curve (Ashman and Puri, 2002).

1.3 ASSESSING AND CHARACTERISING SOIL MICROBIAL COMMUNITIES

Microbial communities inhabiting the soil environment are large and diverse. Because of the close relationship between microbial activity and soil functioning and the fact that microorganisms are sensitive and respond quickly to changes in soil conditions, explains why the characteristics of microbial populations have been widely used as a tool for monitoring soil quality (Nannipieri *et al.*, 1990; Brookes, 1995). Three main aspects of microbial populations are usually considered for their relationship with soil functioning: population size, activity and diversity. A wide range of methods have been used to determine such features of soil microbial communities. It is not within the scope of this work to refer to them all, but rather discuss only those relevant to this study. The remaining methods have already been extensively described elsewhere (e.g. Schinner *et al.*, 1996; Harris and Steer, 2003; Nannipieri *et al.*, 2003; Bloem *et al.*, 2006).

Characterising soil microbial populations may have several applications, such as investigating the impact of environmental factors (Zelles *et al.*, 1991), nutrient addition (Sparling *et al.*, 1981; Meli *et al.*, 2003) or soil properties on microbial populations (Yao *et al.*, 2000; Wang *et al.*, 2003), monitoring soil health (Gil-Sotres *et al.*, 2005) and bioremediation of soil contaminants (e.g. Margesin *et al.*, 2000; Fragoiro, 2004). It is worth mentioning however, that whatever the application, one should not depend on the information provided by solely one parameter, but rather use them in combination in order to obtain the wider picture (Nannipieri *et al.*, 2003). Usually in most studies, a combination of microbial counts (or biomass), soil respiration and enzyme activities (mainly dehydrogenase) are employed.

1.3.1 Microbial biomass and population size

Microbial biomass has been defined as the living component of SOM, which includes all living organisms smaller than $5\text{-}10\ \mu\text{m}^3$. This study focuses on two main groups of soil microorganisms: bacteria and fungi. Bacteria are usually smaller than $2\ \mu\text{m}$ in diameter, with population densities of about 10^9 cells g^{-1} in an agricultural soil and a biomass of $50\text{-}500\ \text{Kg C ha}^{-1}$; fungal hyphae have usual diameters ranging from $2\text{-}10\ \mu\text{m}$, easily reaching total lengths of $10\text{-}1000\ \text{m g}^{-1}$ soil and a biomass of $1\text{-}500\ \text{Kg C ha}^{-1}$ (Bloem *et al.*, 2006). Actinomycetes, particularly from the genus *Streptomyces*, are also well-adapted and highly competitive soil inhabitants (Wood *et al.*, 2001) and will receive focus in Chapter IV. They are mainly known for their ability to degrade the more complex and recalcitrant fraction of soil organic matter and to produce a wide range of antibiotics (e.g. streptomycin) and geosmin-like compounds (Wood *et al.*, 2001).

The use of both traditional (electron microscopy with staining procedures) and more advanced (gene and antibody-based) techniques have shown that 80-90% of soil microorganisms live attached to solid surfaces, such as mineral particles and organic matter (Hattori, 1973; Forster, 1994; Assmus *et al.*, 1997; Bakken, 1997). The

remaining 10-20% has been found to live in water films surrounding solid particles and inside soil aggregates (Stotzky, 1997).

Although microbial biomass represent less than 5% of soil weight, it is critical for soil functioning (Harris and Steer, 1997; Dalal, 1998). It has been estimated that soil microorganisms mediate 80-90% of all soil processes (Bollag and Stotzky, 1990; Ruggiero *et al.*, 1996; Nannipieri *et al.*, 2003) through the production of enzymes. Among other roles, microorganisms are both a source and a sink for nutrients and key in organic matter decomposition and nutrient cycling, nitrogen fixation (Bloem *et al.*, 2006), and degradation and bioaccumulation of xenobiotic compounds (e.g. pesticides, synthetic polymers and heavy metals; Nannipieri *et al.*, 2002).

Information on the size of viable microbial populations is usually a good indicator of soil status (Harris and Steer, 2003) and may be gathered using a wide range of methods. It is important however, to note that in the literature some of these techniques provide an estimation of microbial population size, whereas others give an estimation of microbial biomass. The difference between both types of information is the fact that biomass is usually expressed in units of carbon (or other essential cell components) (Bloem *et al.*, 2006). In this study, microbial population size is given in terms of colony counts, rather than biomass.

Plate counting is the most widespread culture-dependent method, allowing the isolation, identification and count of viable microorganisms (Schinner *et al.*, 1996; Bloem *et al.*, 2006). The plate counting method involves homogenising a known amount of soil suspended in sterile water, followed by serial dilution procedures. Aliquots of soil suspension are then spread onto solid agar in sterile Petri dishes containing nutritional media and incubated for an appropriate period of time (Schinner *et al.*, 1996). Discrete colonies of microorganisms can then be counted and identified (if that is required) and numbers can be multiplied by the dilution factor in order to obtain an estimate of the number of viable cells in the original soil sample (Harris and Steer, 2003). There are, however, two main disadvantages associated with this method: one is related to an inadequate homogenisation of the soil suspension; and the other is that it allows estimating only the culturable portion of soil microbial

population (Nannipieri *et al.*, 2003). Both these factors may result in an underestimation of microbial counts (Harris and Steer, 2003; Bloem *et al.*, 2006).

Despite these drawbacks, the effects of soil pollutants, particularly pesticides, on soil microbial population size have conventionally been assessed using the Plate Count method. There are some evidences that the presence of soil contaminants, such as atrazine, may influence microbial population size, although this relationship is very often contradictory (e.g. Ghani *et al.*, 1996). Very recently, Ros *et al.* (2006) used it in order to demonstrate that atrazine addition into soil at a wide range of concentrations always resulted on an increase in bacterial counts in a semi-arid soil. Earlier, Marchand *et al.* (2002) had also used this method when comparing between atrazine biodegradation in bulk and maize rhizosphere soils.

1.3.2 Microbial activity

Microbial activity is the general term used to indicate the vast range of activities carried out by soil microorganisms (Landi *et al.*, 2000). It is markedly influenced by changes in environmental factors and soil conditions (Bloem *et al.*, 2006). Generally, microbial activity (and growth) is optimal when soils are near field capacity (c.a. 0.03–0.1 MPa), with near neutral soil pH (6–7) and soil temperatures between 20–30°C (Miller and Donahue, 1995).

Since microbial activity involves a complex list of microbial processes, it cannot be evaluated by measuring a single parameter (Nannipieri *et al.*, 1990). This means that there is the need for making a careful pre-selection of the microbial parameters to be assessed, given that it is not feasible to measure a large number of parameters each time (Landi *et al.*, 2000). The decision on which set of parameters should be assessed is dependent on the objective of one's study. Soil respiration and enzymatic activities are the most widely measured microbial indicators for a diverse number of applications, since they relate to the whole (active) soil microbial community (Bloem *et al.*, 2006). Any of these parameters can be estimated in the absence (actual activity) or presence (potential activity) of added substrates.

i) Soil respiration

In a general sense, soil respiration reflects the turnover (oxidation) of organic matter under aerobic soil conditions, which results in CO₂ production (Harris and Steer, 2003; Nannipieri *et al.*, 2003). However, CO₂ is also a product of processes such as fermentation under anaerobic soil conditions and for that reason, its evolution from soil can be considered representative of the whole microbial metabolic activity (Bloem *et al.*, 2006). Frequently, its rate has been assessed for determining the impact of soil physical properties, environmental factors, presence of xenobiotics, or agricultural practices on soil microbial activity (Landi *et al.*, 2000; Harris and Steer, 2003). Soil water content, oxygen and nutrient availability are some of the main factors influencing soil respiration (Nannipieri *et al.*, 1990, 2002, 2003).

Soil respiration can be determined using simple, fast, accurate and cost-effective methods such as laboratory based- gas chromatography (GC) (Bloem *et al.*, 2006). Nowadays, due to the development of mobile GC systems, it can also be measured under non-disturbed field conditions, although in this case, it provides a measure of total biological activity (microbial populations, mesofauna and plant roots) (Harris and Steer, 2003). In either case, respiration can generally be measured using two approaches: static, in which CO₂ is concentrated within a closed incubation system containing the soil and then measured; or dynamic, where CO₂-free air is continuously flowing through the incubation system and analysed for CO₂ quantification at the outlet (Schinner *et al.*, 1996; Bloem *et al.*, 2006).

CO₂ evolution from soil under laboratory conditions is often assessed either in the absence (basal respiration) or in the presence (potential respiration) of nutritional supplements, according to the objective of the experiment. Basal respiration originates from the turnover of native organic matter and its rate reflects both the amount and quality of such, and the ability of microbial populations to use it under given environmental conditions (Bloem *et al.*, 2006). Potential respiration originates from the degradation of introduced nutrients and provides an estimate of the status of microbial activity when nutrients are not the limiting factor. In this study, potential respiration is referred to as substrate-induced respiration (SIR), based on the concept

by Schinner *et al.* (1996) and Bloem *et al.* (2006). It is therefore, a different approach to the original concept of short-term SIR, based on which Anderson and Domsh (1973, 1975) developed their method for estimating microbial biomass.

ii) Soil enzymatic activity

Soil functional diversity comprises a wide range of metabolic activities, which are in turn catalysed by many different enzymes. In the impossibility of measuring all enzymatic activities, a representative set has to be chosen, according to the objectives of the study. It is generally agreed that the activities of those enzymes involved in the degradation of organic matter are considered representative of the overall microbial activity (Trasar-Cepeda *et al.*, 2000).

The biological dehydrogenation (oxidation) of organic matter under aerobic conditions is ultimately linked to the respiratory chain and the synthesis of adenosine triphosphate (ATP) (Trevors, 1982; von Mersi and Schinner, 1991) and is catalysed by dehydrogenases (Harris and Steer, 2003; Nannipieri *et al.*, 2002, 2003). These are a diverse range of intracellular enzymes which function within the cytoplasm of microbial, animal and plant viable cells (Burns, 1982; Nannipieri *et al.*, 2003). Similarly to any other intracellular enzyme group, these are thought to be short lived following cell lysis, since they require certain specific environmental conditions (e.g. redox potential) and co-factors in order to remain active (Burns, 1982). Since dehydrogenases are associated with the oxidative activity of viable cells only, they have been used as an indicator of the overall soil microbial activity (Garcia *et al.*, 1994), very often in combination with respiration analyses.

Dehydrogenase assays are based on the use of synthetic electron acceptors, such as 2-p-iodophenyl-3-p-nitrophenyl-5-phenyltetrazolium chloride (TTC) and 2(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT), although they are less effective than O₂. Under both aerobic and anaerobic conditions, TTC and INT are reduced to triphenyl formazan (TPF) and iodonitrotetrazolium formazan (INF) respectively, which are coloured compounds and can then be extracted from soil and estimated spectrophotometrically (Nannipieri *et al.*, 1990; Harris and Steer, 2003). The use of TTC has been reported to carry some disadvantages. It has been suggested

that this is an inefficient hydrogen acceptor and that it is only reduced when all other acceptor species have been exhausted (von Mersi and Schinner, 1991; Nannipieri *et al.*, 2002; Harris and Steer, 2003). The INT, however, is recognised by many, including von Mersi and Schinner (1991) and Nannipieri *et al.* (2002), as a more effective and reliable hydrogen acceptor.

Dehydrogenase activity (DHA) has been frequently assessed as a valuable parameter for evaluating the impact of environmental factors and the presence of soil contaminants on soil health. For example, it has been found to be a useful indicator of microbial activity in Mediterranean arid soils (Garcia *et al.*, 1994). Also, Margesin *et al.* (2000) are among the authors who determined dehydrogenase INT reduction as a tool for monitoring bioremediation processes in oil-contaminated soil. Similarly, Trasar-Cepeda *et al.* (2000) employed several enzyme activities, among which dehydrogenase, for investigating limitations of soil enzymes as indicators of soil pollution. According to the latter study, dehydrogenase was the only enzyme exhibiting consistent responses to soil contaminants, although there are suggestions that its activity may vary depending on the type and concentration of the pollutant, as well as soil type.

Many soil microorganisms are also dependent on the activity of their extracellular enzymes (such as laccase, LAC) to supply them with nutrients (Harris and Steer, 2003). These enzymes are secreted by viable cells during growth and their roles include, for example, hydrolysis of high molecular weight or insoluble compounds such as lignin and the inactivation of exogenous toxic substances (Burns, 1982). They are often immobilised (forming stable complexes) on soil organic matter and clay particles and can remain active after the death of the cell even under rigorous environmental conditions (Burns, 1982; Nannipieri *et al.*, 1990, 2002). This means that they are not always related to viable cells (Nannipieri *et al.* 2002). LAC will receive specific focus in Chapter III.

Enzymatic assays are generally rapid, simple, accurate and sensitive towards changes in soil conditions or environmental factors, as long as an appropriate substrate concentration, temperature and pH are used. On the other hand, large numbers of

samples can be analysed using small amounts of soil (Nannipieri *et al.*, 1990, 2002). However, they have associated drawbacks. One problem concerning enzymatic assays in general, is that they measure potential activities rather than actual activities, since they are performed at optimal conditions of pH, temperature and substrate concentration, as well as often using synthetic substrates instead of natural ones (Burns, 1982; Nannipieri *et al.*, 1990). In addition, they do not allow differentiating between activities of enzymes from viable cells and those found free or immobilised in soil (Nannipieri *et al.*, 2003). These are the main reasons why enzymatic activity-based techniques should not be employed alone but in combination with other methods such as respiration or population size.

1.3.3 Microbial diversity and community structure

For many authors, microbial diversity is a broad term, referring to the number and relative abundance of different microbial species that can be found in soil, as well as to the structure of the microbial community (Nannipieri *et al.*, 2002, 2003). Community structure refers to the type as well as relative amount and contribution of particular species or functional groups within the community (Nannipieri *et al.*, 2002; Harris and Steer, 2003; Bloem *et al.*, 2006).

One hypothesis for explaining the large diversity of topsoil microorganisms is based on the presence of a great variety of nutritional sources (Tiedje *et al.*, 2001). The mineralization of organic matter is carried out by a largely diversified microbial community involving a wide range of simultaneous metabolic processes (Nannipieri *et al.*, 2002). Nevertheless, determining the rates of microbial processes does not provide any information regarding which microbial species are behind those same processes (O'Donnel *et al.*, 2001). This, plus the practical difficulty in assessing soil microbial diversity means that the link between species diversity and soil functioning remains poorly understood (Nannipieri *et al.*, 2002, 2003). Yet, microbial diversity is seen by many as a good indicator of soil health (Gil-Sotres *et al.*, 2005; Bloem *et al.*, 2006).

Loreau *et al.* (2001) explored the relationship between microbial diversity and soil health in terms of plant production. Results indicated that productivity increased with increasing biodiversity until a certain point, after which any further increase in diversity resulted in a decrease in productivity. On the other hand, Degens *et al.* (2001) investigated this relationship in terms of soil resistance, i.e. the inherent capacity of the system to withstand disturbance (McNaughton, 1994) and resilience, i.e. the property of the system to recover following disturbance (McNaughton, 1994) when subjected by stress imposed by a decline in pH and Cu contamination. Although data suggested that both resistance and resilience were correlated to microbial diversity, other factors related to soil properties may have also been responsible for such a result, as suggested by the authors.

In recent years, there has been evidence that most soil microorganisms are functionally redundant, and that this redundancy is as vital for soil functioning as the number of species *per se* living in it (Nannipieri *et al.*, 2003). This hypothesis may explain divergent results obtained by different studies. It is supported by the fact that, for example, no correlation has been found between microbial diversity and key soil processes such as organic matter decomposition. Furthermore, a targeted reduction of some microbial species was also shown not to influence that same overall process, since other species appeared to be able to fulfil that function (Andren *et al.*, 1995; Brookes, 1995; Giller *et al.*, 1998; Griffiths *et al.*, 2000, 2001b).

Up to now, assessing microbial diversity has been commonly done using counting methods, such as the Plate Count technique (Nannipieri *et al.*, 2003; Bloem *et al.*, 2006). By using different incubation parameters such as the type of nutritional media, temperature, water potential and incubation period, it is possible to increase the amount of culturable microorganisms. Specific functional groups of microorganisms have already been successfully identified in this way (Nannipieri *et al.*, 2003; Bloem *et al.*, 2006). However, culture-dependent approaches have become less commonly used when applied to soil microbial diversity analyses for the reasons described previously (Sub-section 1.3.1).

Recently, a new range of culture-independent techniques emerged, such as those based on molecular biology, and are now increasingly popular as they allow characterising “unculturable” microorganisms, as well as assessing community structure. All these methods involve DNA extraction from soil followed by a series of purification steps, meaning that they are time-consuming, expensive and requiring specialised personnel throughout the analysis and interpretation of results (Harris and Steer, 2003; Nannipieri *et al.*, 2003).

1.4 GASES AND VOLATILES IN SOIL ATMOSPHERE

There is strong evidence that most of the gases and volatile organic compounds (VOCs) in the soil atmosphere, which were found to vary widely in type and relative concentrations, are likely to be produced by microbial metabolic activity (Stotzky and Schenck, 1976; Bossio and Scow, 1995; Wheatley *et al.*, 1996; Sheppard and Loyd, 2002). Soil fungi, bacteria and actinomycetes produce a wide variety of VOCs including alcohols, aliphatic and aromatic hydrocarbons, aldehydes, amines, esters, methylated halogens, terpenoids and volatile fatty acids (Stotzky and Schenck, 1976; Linton and Wright, 1993; Stahl and Parkin, 1996; Schöller *et al.*, 2002).

Although many VOCs in the soil atmosphere seem to not have any particular role, there is evidence that some may be important factors influencing bacterial and fungal activity in soil or in mediating specific microbial interactions. For example, several volatiles such as alcohols, aldehydes and hydrocarbons constitute the main readily-available source of nutrients for soil microorganisms, thereby stimulating their growth and activity (Stotzky and Schenck, 1976; Linton and Wright, 1993). Two other studies have shown that ammonia and terpenoid compounds, individually and in combination with other volatiles, were shown to stimulate some microbial groups, while inhibiting others (Ko and Hora, 1972; Amaral and Knowles, 1998). Examples of volatiles that inhibit spore germination of a variety of fungi such as ethylene, ammonia, allyl alcohol and terpenes have also been widely described and reviewed in the literature (e.g. Stotzky and Schenck, 1976; Linton and Wright, 1993; Mackie and Wheatley, 1999; Chuankun *et al.*, 2004). Mackie and Wheatley (1999) have also

demonstrated that VOCs produced by some bacteria can influence both fungal mycelial growth rate and enzyme activity. However, the effect of each volatile on microorganisms seems to be species-specific. Furthermore, many authors agree that the production of VOCs may also be linked to microbial competition strategies, survival and perhaps communication within the soil system (e.g. Stotzky and Schenck, 1976; Linton and Wright, 1993; Wheatley *et al.*, 1996).

A relevant study by Stahl and Parkin (1996) investigated whether soils inhabited by different microbial communities produced different types and concentrations of VOCs. Adding selective inhibitors and nutritional substrates into soil they found that the greatest amount of VOCs was produced in soil dominated by actinomycete and bacterial populations, when compared to that dominated by fungi, in which terpenes were the most common volatiles. Other studies corroborated these results by demonstrating that volatile profiles in the atmosphere of any given soil were not only dependent on the composition and structure of native microbial communities but also, on soil environmental conditions such as temperature, moisture, nutrients and aeration status (Stotzky and Schenck, 1976; Fiddaman and Rossal, 1993; Stahl and Parkin, 1996; Wheatley *et al.*, 1996, 1997). VOC production in soil is therefore likely to arise from microbial metabolism and therefore, be determined by the same environmental and soil type-related factors that influence microbial activity (Stotzky and Schenck, 1976).

Geosmin (trans-1,10-dimethyl-trans-9-decalol) (Figure 1.1; page 19) is a tertiary alcohol produced during secondary metabolism of certain actinomycetes, mainly from the genus *Streptomyces*, and is considered to be the main contributor to the earthy/musty odour of soil (Lechevalier, 1965; Young *et al.*, 1996). Geosmin can be found dissolved in the soil solution, often finding its way into crops and vegetables, (in particular root vegetables) being responsible for off-flavours and odours in food products (Buttery and Garibaldi, 1976). It is also commonly found in potable water systems and aquaculture-raised fish and sea-food, which makes it a major source of consumer complaints all over the world (Wnorowski, 1992). This is because, although streptomycetes are soil-borne organisms, they can occasionally be washed from soil into water courses and reservoirs, where they remain active as long as the conditions

are favourable (Wood *et al.*, 2001). Geosmin will receive particular focus in Chapter IV.

Very few studies have focused on the profiles of VOCs and other gases in soil atmosphere. For example, Wheatley *et al.* (1996) analysed the headspace of silty-clay loam soil at 50% water holding capacity using GC/MS. They have identified 35 volatile organic compounds (27 in aerobic and 13 in anaerobic soil), with the predominant groups being sulphur compounds (75%), aromatics (15%), ketones (4%), followed by alcohols/ aldehydes and some unidentified volatile organic compounds. Their relative concentrations changed when nitrogen sources were added to soil, and the types of volatiles identified also varied when incubation conditions became more anaerobic (Wheatley *et al.*, 1996). Yet, there is still very little information regarding the impact of key factors such as temperature, water potential, nutrients and even pesticides on soil microbial volatile production patterns.

Similarly, Sheppard and Loyd (2002) analysed soil depth profiles of gases such as O₂, CO₂, Ar, CH₄, N₂ and NO_xs using membrane-inlet-mass spectrometry (MIMS). They demonstrated that the concentration of each of the gases was not only associated with soil depth but also varied within the same horizon (with horizons referring to the different soil layers parallel to the surface, which differ from each other in terms of physical, chemical and biological properties) and with soil moisture content. For example, concentrations of O₂ and N₂ were found to decrease linearly with increasing soil depth, in opposition to CO₂ and CH₄ gradients, due to the transition from an aerobic environment to an anaerobic one. They also showed that production and emission of these gases from soils are enhanced by the application of amendments such as sewage sludge or lime. Elevated CO₂ and CH₄ levels were obtained independently of the amendment used.

Both these studies demonstrate that potential exists for qualitative soil volatile fingerprint analyses using sensor arrays to be used for characterising and perhaps monitoring soil microbial activity, as well as determining the factors influencing it. Up to now, soil volatile fingerprints have not been used as one of the microbial parameters for assessing soil microbial communities.

Much of what is known about soil volatiles being produced by microbial activity has come from *in vitro* studies (Linton and Wright, 1993). Generally, GC and GC/MS are traditionally used to identify and quantify soil volatiles but although they are effective, reliable and low cost, they can be time consuming, particularly if many replicates are necessary (Nagle *et al.*, 1998). In the case of geosmin, in particular, GC or GC/MS are adequate when the volatile is present at high concentrations, but it involves large sample volumes and intensive sample concentration procedures (Watson *et al.*, 2000) for detecting concentrations near human odour threshold detection (OTD) levels using this method. Human sensory panels have also been traditionally used for geosmin detection and odour evaluation, but there are generally marked differences in the responses between individuals to specific compounds, and from the same individual in different days (Young *et al.*, 1996). The potential use of an E-nose system for early detecting geosmin in soil and water at concentrations near its OTD may be of great relevance.

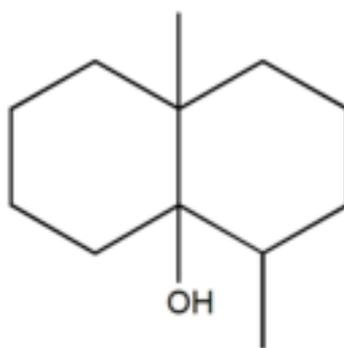


Figure 1.1 Molecular structure of geosmin.

1.5 ELECTRONIC NOSE (E-NOSE) AND VOLATILE FINGERPRINT ANALYSIS

1.5.1 E-nose and the ‘sense of smell’

In recent years, a greater understanding of the process of mammalian olfaction has inspired the development of E-nose technology and increased the interest in E-nose based research (for an historical perspective see Nagle *et al.*, (1998), Harper (2001) and Turner and Magan (2004). Firstly, a brief and simplified overview of the mechanism involved in the mammalian sense of smell will provide a clearer insight on the principle of ‘sensing technology’.

The mammalian olfaction system consists of three essential elements: (a) an array of olfactory neurones located at the roof of the nasal cavity; (b) the olfactory bulb based just above it; and (c) the brain (Craven *et al.*, 1996; Nagle *et al.*, 1998). When odorant molecules are inhaled through the nostrils and enter the nasal cavity, they become in contact with the array of olfactory neurones. Each neurone contains specialised receptor proteins bound to its cell membranes, which interact with the odorant molecules generating a series of nerve impulses. These electrical signals feed into the olfactory bulb where they are pre-processed in order to reduce noise and simplify the neurone output, converting them into the form of a signature. Finally, the information is sent into the brain, where the learning and classifying processes involve associative memories between neurone response signatures and a given smell. The number of different membrane-bound receptor proteins is estimated to be between 100 and 1000, with overlapping sensitivities and selectivities (Craven *et al.*, 1996; Gibson *et al.*, 1997; Nagle *et al.*, 1998). Although each neurone appears to express only one type of protein, the number of neurones within the array is large (ca. 100 million) and therefore, it responds to a wide range of different odorant molecules without being specific towards any particular molecule (Craven *et al.*, 1996). As a result, our sense of smell is able to identify, recognise and discriminate a wide range of volatile compounds (or odours) with high sensitivity and accuracy, even when present at parts *per* trillion (ppt) levels (Breer, 1997; Craven *et al.*, 1996)

By using arrays of non-specific sensors as “artificial receptors” it has been possible to mimic the mammalian sense of smell (Gibson *et al.*, 1997). Any electronic nose device comprises three functionally equivalent elements: (a) a sensor array located within a reaction chamber; (b) a data pre-processing unit; and (c) the pattern recognition (PARC) engine involved in odour classification and pattern recognition. A sampling conditioning unit delivers the volatiles from the sample headspace into the reaction chamber housing the sensor array (Nagle *et al.*, 1998). Generally, the basic principle of sensing technology is based on the measurement of the variation of mass, heat, magnetic, electric or optical properties of the sensor active material due to the interaction between that and volatile compounds (Nagle *et al.*, 1998; Ivnitski, 1999; Guadarrama *et al.*, 2000; Tothill, 2001). The electronic signal then undergoes a pre-processing stage where sensor drift and noise can be reduced, thus simplifying the data set and converting it into a form suitable for input to the PARC engine (Craven *et al.*, 1996). At this stage, multivariate statistical analyses or artificial neural network (ANN) can be employed for classifying samples, based on the pattern of the overall response generated by the array (Turner and Magan, 2004).

E-nose technology may therefore provide a rapid way of characterising the headspace of a given sample, independently of its complexity (such as that of a volatile mixture). However, it should not be considered an analytical technique in the common sense, since it does not involve an exact identification and quantification of the components in a volatile mixture (Craven *et al.*, 1996; Gibson *et al.*, 1997; Harper, 2001).

1.5.2 Sensor Technology

Sensors within the array can comprise many different active materials, such as conducting-polymers (CP) and metal-oxides (MO) (referred to as conductivity sensors). This study focuses on conducting-polymer based sensors but the properties of the other available materials as well as the type of signal generated and operating conditions can be found elsewhere (e.g. Breer, 1997; Haugen and Vaal, 1998; Nagle *et al.*, 1998; Fenner and Stuetz, 1999; Tothill, 2001; Turner and Magan, 2004). But irrespectively of the active material, there are some features that sensors should fulfil

in order to provide sensitive and accurate classifications. These include: high sensitivity, low selectivity, high stability (robustness) and reproducibility, short recovery time, easy calibration and the potential for portability for *in-situ* applications (Ivnitsky, 1999).

Conducting-polymer based E-nose systems fulfil those criteria and are among those more widely used (Nagle *et al.*, 1998; Guadarrama *et al.*, 2000; Barisci *et al.*, 2002; Wanekaya *et al.*, 2005). Their active material is commonly polyaniline, polypyrrole or polythiophene with different functional groups attached to the polymer backbone. A common way of fabricating this type of sensors employs two electrodes separated by a gap of 10-20 μm integrated into a substrate (which can be alumina, silicon, glass or plastic) (Figure 1.2; page 23). Polymers are usually produced by electropolymerisation of the corresponding monomers directly onto the sensor surface (Gibson *et al.*, 1997). Once exposed to volatiles and as these interact with the sensor polymer backbone (through an ionic or covalent bonding), a change in the transfer of electrons (conductivity) occurs along the polymer chain. By varying the voltage change rate applied, the variety of monomers and doping ions used and the thickness of the polymer film, a wide range of sensor characteristics can be obtained (Gibson *et al.*, 1997; Nagle *et al.*, 1998; Haugen and Kvaal, 1998). Those will in turn determine the affinity of the sensor towards each volatile, as well as the magnitude of sensor response (Nagle *et al.*, 1998).

When comparing to conventional chemical sensors/detectors, which normally employ specific receptors for the compound of interest, conducting polymer sensors are not specific to any one particular volatile. Instead, they have low selectivity and thus, respond to a wide range of volatile compounds (Craven *et al.*, 1996; Wanekaya *et al.*, 2005). This feature of the sensor array is important since the structure of the volatile molecule is a determinant for detecting and classifying the odour. On the other hand, the use of an array of sensors with broad and overlapping sensitivities to different groups of volatiles is key for enabling a unique and characteristic volatile profile (or fingerprint) of each sample/ treatment to be obtained (Breer, 1997; Nagle *et al.*, 1998; Turner and Magan, 2004; Kurup *et al.*, 2006). This is the key feature allowing them to be employed in the most diverse range of applications (Gibson *et al.*, 1997).

Conducting polymer sensors respond to both odorous and odourless volatiles and have the highest sensitivity towards high and low weight hydrophilic (polar) compounds (Craven *et al.*, 1996; Guadarrama *et al.*, 2000; Swann *et al.*, 2000; Wanekaya *et al.*, 2005). Groups such as alcohols, ketones, fatty acids, esters, amines, as well as those containing sulphur produce a particularly strong sensor responses, whereas fully oxidised volatile species (e.g. CO₂, NO₂ and H₂O) lead to lower responses (Tothill, 2001). Other important characteristics of this type of sensors include the fact that their limit of detection is within the 0.01-100 ppm concentration range for most of the volatile compounds (but can also detect at ppb levels) (Haugen and Kvaal, 1998; Nagle *et al.*, 1998) and the fact that they operate at ambient temperatures (Craven *et al.*, 1996). The relatively easy production method (at ambient temperature), the good reproducibility achieved and the potential for miniaturisation (and portability) are also important advantages of this type of sensors (Haugen and Kvaal, 1998; Harper, 2001). Drawbacks are mainly related to the high degree of sensor drift over time and high susceptibility to humidity (Haugen and Kvaal, 1998; Nagle *et al.*, 1998; Harper *et al.*, 2001).

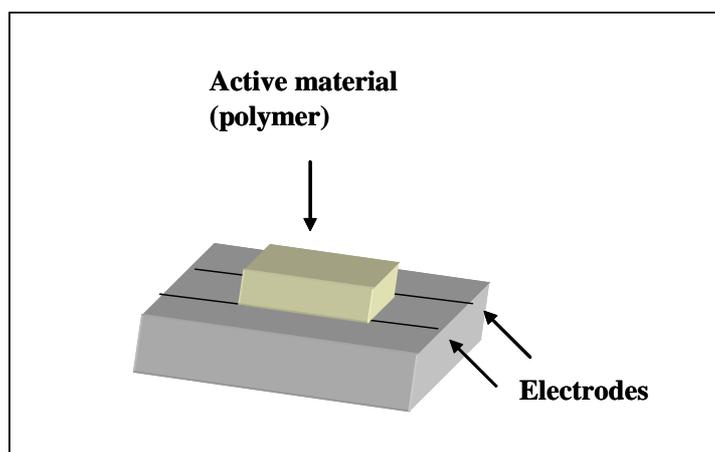


Figure 1.2 Schematic presentation of a conducting-polymer sensor (adapted from Nagle *et al.*, 1998).

1.5.3 Headspace sampling methods

There are two main odour sampling methods: static headspace analysis (SHA) and flow injection analysis (FIA). SHA is simple and cost-effective, and therefore, the most commonly used. The sample to be analysed is left inside a closed container for a period of time prior to sampling, in order to ensure an adequate accumulation (saturation) and distribution of volatiles in the headspace above the sample. Volatiles are then quickly delivered to the sensor array and almost immediately removed from it, which allows a quick recovery of the baseline and reduction of the overall sampling time (Craven *et al.*, 1996; Guadarrama *et al.*, 2000). As a result of the saturation, the magnitude of sensor response is high (Guadarrama *et al.*, 2000).

On the other hand, the FIA method is usually automated and employs a carrier gas (e.g. clean air) constantly being pumped through the sensor chamber. A sample of the headspace to be analysed is injected into the carrier gas and delivered to the sensor array in a much more diluted form. The ratio of carrier gas: headspace volatiles can be accurately controlled. Nevertheless, due to dilution, the magnitude of sensor response to volatiles is much lower when compared against that obtained using the SHA technique (Craven *et al.*, 1996).

1.5.4 Data processing and multivariate statistics

For each of the sensors comprising the array, a typical response over time includes data corresponding to different sensor parameters (i.e. sampling steps) and can be represented by a curve (Figure 1.3; page 26): (I) baseline; (II) absorption of the volatiles to the polymer (highest rate of change in resistance); (III) saturation or equilibrium (steady-state) between the volatiles and the polymer, which produces the maximal response (divergence); (IV) desorption of the volatiles from the polymer (highest negative rate of change in resistance); and (Δy) area under the response curve (directly proportional to volatile concentration) (Pavlou *et al.*, 2000; Vanneste and Geise, 2003). This generates large data sets which require rapid, effective and accurate analyses in order to obtain useful results. Multivariate statistical techniques,

such as principal component analyses (PCA) and discriminant function analyses (DFA) are widely employed for data processing and interpreting sensor response.

PCA is an exploratory (unsupervised or “untrained”) technique, which allows identification of relationships between samples/ treatments, highlighting their similarities and differences. This is done based solely on the variance within the data, meaning that no previous information regarding the samples is required (e.g. grouping of replicates). In order to achieve that, PCA reduces the number of variables that need to be considered (i.e. n sensors) to a smaller number of indices (usually two) called principal components (PCs), which together are representative of the whole data set (i.e. explain most of the variance within the data). In this two-dimensional space, similarities and/or differences between samples can be interpreted more easily without losing much of the overall information (Craven *et al.*, 1996; Dewettinck *et al.*, 2000; Goodner, 2001). Usually, over 80% of the overall variance is contained within the first two principal components (PCs 1 and 2), which according to Gardner *et al.* (2000) is a typical result.

Contrary to PCA, DFA is a supervised technique. It can be used either to discriminate between different groups of samples or to assign “unknown” samples into the appropriate group (Turner and Magan, 2004). The latest is usually the main purpose when using this technique. Typically, DFA uses a certain number (“ n ”) of characteristics (or variables) within the data set to derive “ $n-1$ ” discriminant factors (DF) which together explain the overall information within the data. The chosen characteristics are those differing the most between different groups of samples, while differing the least within the same group (Siripatrawan *et al.*, 2006). This is done using previous information regarding sample classification, meaning that previous grouping of samples is required. Although “ $n-1$ ” DF are derived, the first two factors generally express the highest eigenvalues and are thus considered to be representative of the whole data set. Eigenvalues are indicative of the relative importance of the DF in determining the group separation (Siripatrawan *et al.*, 2006).

However, prior to pattern recognition analyses using any of the methods described above, data corresponding to the different sensor parameters is usually normalised. Normalisation ensures that sensor output is independent (or less dependent) on

volatile concentration, so that only qualitative information is taken into account (Craven *et al.*, 1996). In certain E-nose applications, however, information on volatile intensity may be also required and then, pattern recognition analyses are performed on un-normalised data (Craven *et al.*, 1996).

Figure 1.3 Typical sensor response curve over time. (I) baseline; (II) absorption of the volatiles to the polymer; (III) saturation or equilibrium (steady-state) between the volatiles and the polymer (divergence); (IV) desorption of the volatiles from the polymer; and (Δy) area under the response curve (adapted from Vanneste and Geise, 2003).

1.5.5 E-nose main applications

i) Food Industry

The most common application of E-nose systems is in the Food Industry, where deterioration of quality is often associated with microbial or chemical spoilage, and the development of off-odours and flavours (Guadarrama *et al.*, 2000; Magan and Evans, 2001; Taurino *et al.*, 2003). Traditionally, changes in odour and flavour in food products (as well as drinking water) have been assessed by highly trained sensory panels and using headspace GC, GC/MS or high pressure liquid chromatography (HPLC) (Haugen and Kvaal, 1998; Guadarrama *et al.*, 2001). However, these methods are time consuming and costly, particularly when used for

routine analysis purposes (Guadarrama *et al.*, 2000, 2001). Further, the use of sensory panels for odour and flavour evaluation is regarded as having high inherent variability (MacRae and Falahee, 1995). Within the framework of Hazard Analysis Critical Control Point (HACCP) for food safety, real-time analysis is required as part of the quality control schemes (Turner and Magan, 2004). Also, a method which could provide a rapid, objective, automated and non-destructive way of characterising odours from the raw material, throughout the processing and to the final product would contribute greatly at this level (Haugen and Kvaal, 1998; Harper, 2001). E-nose technology fulfils these requirements, ensures that reproducible results can be achieved and can complement traditional methods for quality control purposes (Harper, 2001). Examples of relevant and successful applications of sensor arrays in the food industry are listed in Table 1.1 (page 32).

E-noses for evaluation of quality have been employed in a wide range of food products. For example, early fungal activity and toxin contamination in grain and bakery products have been successfully detected using this approach (Magan and Evans, 2000; Keshri *et al.*, 2002; Olsson, 2002; Needham *et al.*, 2005). E-nose potential application in the dairy industry has also been receiving particular interest. Examples of successful applications in this field have been the detection and differentiation between bacteria and yeast spoilage in milk (Magan *et al.*, 2001), as well as for quality assessment of several varieties of cheese (e.g. Trihaas and Nielsen, 2002). Other economically important food products which quality has been evaluated using this approach include meat and derivatives, fish, wine, coffee, olive and frying oil (Gardner *et al.*, 1992; Haugen and Kvaal, 1998; Guadarrama, 2000, 2001; Taurino *et al.*, 2003; Innawong *et al.*, 2004; Santos *et al.*, 2004). Promising results have also been achieved in the field of Post-harvest, where for instance, a conducting polymer sensor array has been recently employed for evaluating the quality of onion bulbs as affected by soil type, and soil nitrogen and sulphur (Abbey *et al.*, 2003).

ii) Medical Diagnostics

There is strong evidence that certain diseases, whether they are microbial-derived or not, are associated with characteristic smells. This means that volatile profiles emanating from biological samples of patients with the disorder can be used as

biomarkers for its diagnoses (Turner and Magan, 2004). A summary of key volatiles associated with different common disorders, which have been detected by traditional methods such as GC and GC/MS can be found in Turner and Magan (2004).

For instance, the diagnosis of a bacterial infection takes at least 24-48 h when using traditional microscopy and culture-dependent methods, but in the case of a fungal infection it can take up to a week (Turner and Magan, 2004). New methods for microbial detection based on molecular biology have recently been developed, but they can become too complicated and expensive, particularly for routine analysis purposes (Pavlou *et al.*, 2004). An efficient treatment, and perhaps prevention of the infection, may be dependent on the gathering of rapid results and a quick diagnosis (Gardner *et al.*, 2000; Turner and Magan, 2004). For this reason, E-nose technology has been the subject of growing interest and attention for its potential for rapid screening of biological samples and for diagnosing illness based on microbial volatile patterns. The fast achievement of results, along with the fact that it is a non-invasive technique offer advantages over traditional methods, particularly when real-time and near-patient diagnostics are required (Gardner *et al.*, 2000). A summary of the most relevant applications of sensor array for detecting and monitoring infection-causing agents as well as non-infectious diseases (both in culture and in patients) is provided in Table 1.1 (page 32).

So far, a significant amount of work has been reported on the detection and discrimination between infectious microorganisms in culture based on their volatile profiles, as reviewed by Turner and Magan (2004). For example, studies *in vitro* and *in vivo* have shown that E-nose technology was able to discriminate between different species of aerobic and anaerobic infectious bacteria, both in pure culture and in a mixture, based on their volatile profiles (Gibson *et al.*, 1997; Gardner *et al.*, 2000; Pavlou *et al.*, 2000, 2002a, 2002b). Among these reports, it is worth referring to the successful application of a polymer-based sensor array for the *in-vitro* and *in-vivo* diagnosis of urinary tract infections and tuberculosis on samples of patients carrying the disorder (Pavlou *et al.*, 2002a, 2002b, 2004). It has also been accurate in detecting and discriminating between different bacterial species responsible for causing eye infections, as well as in monitoring urinary tract infections and bacteria-derived

human vaginosis and diagnosing clinical ketosis in dairy cattle (Gardner *et al.*, 2000; Persaud *et al.*, 2006).

Additionally, this technology has also proved able to detect non-infectious diseases, such as diabetes, stomach disorders and some types of cancer with the same level of sensitivity (Wang *et al.*, 1997; Gardner *et al.*, 2000; Pavlou *et al.*, 2000; Di Natale *et al.*, 2003; Yu *et al.*, 2005). For example, diabetes is responsible for the smell of acetone on the patient breath, whereas a characteristic smell mainly due to aliphatic alkanes and aromatic compounds (such as benzene derivatives) has been associated to lung cancer (Wang *et al.*, 1997; Di Natale *et al.*, 2003; Yu *et al.*, 2005). Recently, it has also been reported the application of a conducting polymer sensor array for monitoring haemodialysis in patients with kidney disorders (Fend *et al.*, 2004).

iii) Environmental Diagnostics

In recent years, environmental monitoring has become an area of growing interest for E-nose manufacturers, due to the increasing pressure, from both consumers and legislation in demanding quality water, food and air (Tohill, 2001; Bourgeois *et al.*, 2003). The continuous development and improvement of more sensitive and versatile sensor-based E-nose systems has led to a growing list of environmental applications in sectors such as chemical, paper, soil and agriculture and waste processing (landfill sites and wastewater treatment plants) (Bourgeois *et al.*, 2003). Due to their simplicity, versatility, rapidity, as well as portability potential and reliability, they may provide a suitable solution for rapid detection and monitoring of a wide range of biological and chemical environmental contaminants.

The quick identification of trace amounts of VOCs causing off-odours and tastes is important in many sectors, but mostly when quality assessment of potable water is concerned. Flavour and odour occurrences in potable water are a common and major source of consumer complaints world-wide with extremely high associated costs (MacRae and Falahee, 1995; Hogben *et al.*, 2004). VOCs responsible for taste and odour episodes are usually identified using a variety of analytical and sensory techniques such as GC/MS. Although these provide an accurate chemical characterisation of water, they have limited application for detecting low contaminant

concentrations (at the ng l⁻¹ level) and for continuous monitoring purposes (Watson *et al.*, 2000; Hogben *et al.*, 2004).

Detection of microbial contaminants in water, at an early stage of differentiation as well as monitoring their growth is key for preventing tastes and odour occurrences. Gardner *et al.* (2000) were successful in monitoring the growth of cyanobacteria in potable water over a period of 40 days and for discriminating between different cyanobacteria strains, using a metal-oxide sensor array. Another interesting study by Hogben *et al.* (2004) tested the E-nose potential for laboratory and field-based continuous monitoring of potable water, to which pollutants (among which geosmin) had been gradually added over a period of time. A temperature controlled chamber for on-line headspace generation and volatile delivery to the sensor array, was used for conditioning the system, minimising the impact of environmental fluctuations on the sensor response. These reports have shown that this technology can provide a tool for potable water monitoring purposes.

Generally, environmental odour assessment has been subject to growing interest in diverse areas. Odour emission can affect quality of life in its vicinities and has been associated with psychological stress and symptoms such as insomnia and loss of appetite (Gostelow *et al.* 2001). For this purpose, the use of non-specific sensor arrays may be of a particular interest, since they can provide an objective characterisation of the odour, as it is perceived by humans (Bourgeois *et al.*, 2003). For instance, Figueiredo and Stentiford (2002) employed a conducting-polymer sensor array for remote monitoring of bad odours at a waste composting plant, where most frequent odours are due to sulphur and nitrogen-derived compounds, as well as volatile fatty acids (Figueiredo and Stentiford, 2002). Similarly, both a conducting-polymer and metal-oxide sensor arrays have also been tested for characterising and discriminating effluents and for *in situ* monitoring of odours at wastewater treatment plants (Stuetz *et al.*, 1998; Nake *et al.*, 2005).

Although most VOCs (such as geosmin) are probably not a threat to humans and wildlife, the range of others potentially hazardous for both is enormous. Few pertinent studies have focused on evaluating petrol quality which is an increasingly important aspect, particularly since adulteration (by adding low cost solvents e.g. kerosene) will

lead to greater pollutant emissions (Lauf and Hoffheins, 1991; Sobański *et al.*, 2006). Other groups of organic vapours have also been detected and monitored using this technology. For example, Barisci *et al.* (2002) developed an array of 8 conducting polymer sensors for identifying and differentiating between benzene, toluene, ethyl benzene and xylene (a group of compounds referred to as BTEX). Beside BTEX vapours, methanol, ethanol, propanol, butanol, acetone, cyclohexane and methane have all been successfully identified at ppm concentration levels, when alone and as part of a multicomponent mixture, using sensor arrays (mainly conducting polymers and metal-oxides) (Faglia *et al.*, 1997; Llobet *et al.*, 1997; Szczurek *et al.*, 1999; Furlong and Stuart, 2000; Lee *et al.*, 2000; Negri *et al.*, 2001; Zee and Judy, 2001; Wanekaya *et al.*, 2005).

Very recently, a small number studies have documented the use of sensor- based E-noses for detection of chemical pollutants in soil and water. Arnold *et al.* (2006) used a metal-oxide sensor based micro-nose (KAMINA) integrated into a soil gas percussion probe system for on-site detection and differentiation between 11 typical volatile soil pollutants (such as chlorinated, aromatic and aliphatic hydrocarbons) in contaminated soil. Authors were able to discriminate between different pollutants and predict their concentration in soil based on concentration *vs.* signal calibration curves. Kurup *et al.* (2006), on the other hand, described the use of an array of metal oxide sensors for rapid on- and off-site screening of gasoline contaminated sites at different concentrations. A conducting polymer sensor array had previously been used for detecting and discriminating between trace levels of microbiological and heavy metal contaminants in water (Canhoto and Magan, 2003, 2004). Authors were also able to differentiate between contaminant microbial species (*Escherichia coli*, *Enterobacter aerogenes* and *Pseudomonas aeruginosa*) at 10^2 colony forming units (CFU) ml^{-1} . Allergenic pollen in the ambient air has also been identified and differentiated according to the plant species from which it had been originated, using a metal oxide sensor array (Kalman *et al.*, 1997). Authors defend that this E-nose-based detection method for pollen would help preventing and controlling pollen induced allergic crisis at an early stage (Kalman *et al.*, 1997). Furthermore, the early *in vitro* detection of fungal colonisation in different library paper types has also recently been reported, using a conducting polymer sensor array (Canhoto *et al.*, 2004).

Table 1.1 Examples of relevant applications of sensor arrays in the food industry and post-harvest, medical and environmental diagnostics.

Main fields of application	Examples of applications	References
Food Industry	i) Evaluating quality of dairy, post-harvest and other products (e.g. meat and derivatives, fish, wine, coffee, olive and frying oil).	Gardner <i>et al.</i> (1992); Haugen and Kvaal (1998); Guadarrama (2000, 2001); Magan <i>et al.</i> (2001); Taurino <i>et al.</i> (2003); Innawong <i>et al.</i> (2004); Trihaas and Nielsen (2002); Santos <i>et al.</i> (2004).
	ii) Detecting early fungal activity and toxin production in grain and bakery products.	Magan and Evans (2000); Keshri <i>et al.</i> (2002); Olsson (2002); Needham <i>et al.</i> (2005).
Medical Diagnostics	i) Detecting and discriminating between infectious microorganisms in culture	Gibson <i>et al.</i> (1997); Gardner <i>et al.</i> (2000) Pavlou <i>et al.</i> (2000, 2002a, 2002b).
	ii) Diagnosis of infections (e.g. tuberculosis, vaginosis, those of the urinary tract and eyes) in patients and clinical ketosis in cattle.	Gardner <i>et al.</i> (2000); Pavlou <i>et al.</i> (2002a, 2002b, 2004); Persaud <i>et al.</i> (2006).
	iii) Detecting of non-infectious diseases (e.g. diabetes, stomach disorders and some types of cancer) in patients.	Wang <i>et al.</i> (1997); Gardner <i>et al.</i> (2000); Pavlou <i>et al.</i> (2000); Di Natale <i>et al.</i> (2003); Yu <i>et al.</i> (2005).
	iv) Monitoring haemodialysis in patients	Fend <i>et al.</i> (2004).
Environmental Diagnostics	i) Early detection and differentiation between off-odour and flavour compounds (e.g. geosmin) and producing agents (e.g. <i>Streptomyces</i> and	Gardner <i>et al.</i> (2000); Bastos and Magan (2006).

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- cyanobacteria) in potable water
- ii)* Remote assessment of odours from composting plants
- iii)* Detection of hydrocarbons and heavy metals in soil and potable water
- iv)* Characterisation of effluents from wastewater treatment plants
- v)* Evaluation of air quality; detection of organic vapours (e.g. methanol, ethanol, propanol, butanol, acetone, cyclohexane and methane), BTEX compounds (benzene, toluene, ethyl benzene and xylene) and allergenic pollen
- vi)* Quality assessment of petrol
- vii)* Detection of fungal colonisation in library paper
- Figueiredo and Stentiford (2002).
- Canhoto (2004); Canhoto and Magan (2003, 2004); Arnold *et al.* (2006).
- Stuetz *et al.* (1998); Nake *et al.* (2005).
- Faglia *et al.*, 1997; Llobet *et al.* (1997); Szczurek *et al.* (1999); Furlong and Stuart (2000); Lee *et al.* (2000); Negri *et al.* (2001); Zee and Judy (2001); Barisci *et al.* (2002); Wanekaya *et al.* (2005).
- Lauf and Hoffheins (1991); Sobański *et al.* (2006).
- Canhoto *et al.* (2004).
-

1.6 PESTICIDES AND SOIL CONTAMINATION: HEALTH AND ENVIRONMENTAL CONCERNS

Pesticide structures are developed as analogues in order to inhibit biological reactions (Gavrilescu, 2005). Considering the widespread use of pesticides in soil, their fate is of main concern, as they pose a major threat to our health as well as to the quality of our soil, air and surface and ground water resources (Hägglom, 1992). It was not until recently that it was found that contaminants in the soil can easily and rapidly find their way into living organisms, as well as to the atmosphere (volatile contaminants through diffusion) and water resources (through leaching) (Ashman and Puri, 2002; Šašek *et al.*, 2003).

Inadequate management practices and on-farm pesticide handling is considered one of the major sources of pesticide environmental contamination. Apart from the application for pest-management (non-point source contamination), pesticide wastes, spills and rinsates from spraying and storage equipment are often added to soil as a common and convenient disposal method (point source contamination) (Kearney and Roberts, 1998; Kuo and Regan, 1999; Ashman and Puri, 2002). In addition to agricultural applications, many pesticides are commonly used for control and maintenance of urban facilities in homes, road sides and recreational areas. Also in these cases, pesticide residues very often end up being added to soil and ultimately reaching groundwater when no mitigation practices are in force (Kearney and Roberts, 1998).

Herbicides are the leading pesticide group, accounting for nearly 50% of all pesticides used in developed countries (Gadd, 2001). Among these are the triazines (e.g. atrazine and simazine), halogenated nitrogen derivatives, produced in vast quantities over the last 50 years. Atrazine will be the herbicide receiving particular attention in this work.

Herbicides and organic amendments are usually applied together in agriculture (Ghani *et al.*, 1996). The addition of nutrients frequently alters the degradation rate of the herbicide, depending on the nature of the nutrient (Abdelhafid *et al.*, 2000). Although these are added in order to increase microbial activity, they may cause repression of

the herbicide metabolism, thus increasing its persistency in soil. A similar situation may occur in soils with high organic matter content (Houot *et al.*, 1998).

When the concentration of a pesticide or its metabolites/by-products in soil is considered too high, remediation is necessary for minimising health and environmental hazards (Kearney and Roberts, 1998). Remediation is a general term including any physical, chemical or biological process aiming to reduce pesticide concentration in soil or water. Gavrilesco (2005) described in detail the most commonly used methods for pesticide remediation in soil. In the case of triazine herbicides, although they can be degraded abiotically, its ultimate breakdown is mainly the result of microbial action (Hägglom, 1992). There is therefore, considerable interest in applying microbiological clean-up of soil and water contaminated with these chemicals. However, this process is slow and herbicides, as well as their metabolites or by-products, are often recalcitrant in the environment.

For any pesticide, the threshold concentration above which remediation is necessary is referred to as “remediation trigger level”. However, for many pesticides as well as for many other xenobiotics, the threshold concentration has not yet been established. There is also a need for establishing a target concentration when remediation is achieved. Generally, the target concentration is assumed to be in the 1 ppm (mg l^{-1}) range, but in practice it can vary from site to site (Kearney and Roberts, 1998).

Very often, urban applications of pesticides are done at excessively high concentration, resulting in pesticide waste with prolonged persistence. When applied at usual agricultural rates which can be between 1-4.5 kg ha^{-1} , pesticide degradation in soil may be around 99% over the course of a growing season (Kearney and Roberts, 1998). However, Kearney and Roberts (1998) suggested that even at these concentrations top-soil residues have been found to last for several years, ranging between 0.5 to 2.5 ppm. Unfortunately, even when present in soil at the ppb level ($\mu\text{g l}^{-1}$), many pesticides can often migrate by leaching and reach groundwater (Muszkat *et al.*, 1993).

1.7 BIOREMEDIATION AND BIOAUGMENTATION OF PESTICIDES IN SOIL

1.7.1 Pesticide metabolism

Microbial metabolism has been regarded as the most important mechanism of pesticide degradation in soils as well as in a wide range of habitats (Armstrong *et al.*, 1967; Gravilescu, 2005), and is the basis for bioremediation and bioaugmentation strategies. Therefore, conditions that favour microbial growth and activity in soil, i.e. temperature, moisture, nutrient status, pH and aeration will also generally promote metabolic degradation of pesticides (Gavrilescu, 2005).

As suggested by Gadd (2001) among others, the composition and size of soil microbial populations, as well as metabolic activity status are the determining factors as to whether biodegradation is feasible as a remediation option. Biodegradation refers to the metabolic ability of microorganisms to transform organic contaminants into less harmful compounds (Margesin and Schinner, 2001). According to Margesin *et al.* (2000), bioremediation techniques aim to accelerate the naturally occurring biodegradation process by optimising the conditions for it to occur.

In many contaminated areas, even though suitable microbial populations may be available for biodegradation of a given contaminant, environmental conditions may limit or even inhibit this process (Margesin *et al.*, 2000). In those cases, biostimulation of the degrading potential of native microbial populations and/or the addition of selected degrading microorganisms in contaminated soil (bioaugmentation) have been effective at enhancing pesticide metabolism (Häggbloom, 1992).

Biostimulation typically involves the addition of limiting nutrients (e.g. carbon and nitrogen sources, O₂), acid or bases for pH optimisation and water or specific substrates to stimulate specific enzymes (Pointing, 2001). It is an effective bioremediation strategy (Margesin and Schinner, 2001), although it may have poor reproducibility and be dependent on the characteristics of microbial populations

(Gadd, 2001). Alternatively, bioaugmentation is an attractive option. The major advantage of bioaugmentation is probably the possibility of choosing introduced specie(s) according to what is intended and the conditions of the medium, conferring a certain degree of control upon the process. In this case, the success of bioremediation is mainly dependent on the competition/proliferation capability of the introduced specie(s) (which are very often poor), and in the bioavailability of the pesticide (Kearney and Roberts, 1998). Bioavailability here refers to the acquisition and subsequent transformation/ degradation of the compound (Gadd, 2001) and is closely related to its chemical properties, as well as to a wide range of soil physical and chemical parameters (Gavrilescu, 2005).

Complete biodegradation will ultimately result in the mineralisation of the pesticide to CO₂ and water (Hägglom, 1992). In addition to mineralization (which implies the use of the pesticide as growth substrate), microorganisms can also co-metabolise pesticides, i.e. transform them into metabolites while growing and obtaining energy from other substrates found in soil (Kearney and Roberts, 1998; Gadd, 2001). Many reactions are involved, very often in simultaneous, in co-metabolic reactions of pesticides such as oxidation-reduction, de-halogenation, ring-cleavage and hydrolysis (Gadd, 2001). This transformation can lead to complete detoxification, to the production of metabolites that may be prone to further attack by other microbial groups or to the production of more toxic metabolites (Roberts and Kearney, 1995). Triazines among other halogenated aromatic compounds can often be co-metabolised into more toxic metabolites (Gadd, 2001). For example, microbial enzyme-mediated methylation reactions usually increase herbicide lipophilicity and thus, the potential for bioaccumulation in the food chain (Hägglom, 1992). Generally, biodegradation and co-metabolism happen in combination in soil.

1.7.2 Atrazine and its biodegradation in soil

Atrazine [(2-chloro-4-(ethylamino)-6-(isopropylamino)-s-triazine)] is a chlorinated aromatic herbicide heavily used for controlling a wide variety of weeds mainly in corn, sorghum, maize and sugarcane worldwide (Ghani *et al.*, 1996; Houot *et al.*,

1998; Ralebitso *et al.*, 2002) (Figure 1.3; page 39). It is also used as a non-selective herbicide for vegetation control in urban and recreational areas (Gadd, 2001).

Atrazine and related triazines are moderately persistent in soil (Pointing, 2001). Although atrazine can also be degraded abiotically (e.g. photolysis) to some extent its degradation in soil is mostly biological (Kaufman and Kearney, 1970; Kaufman and Blake, 1973; Levanon, 1993), with a major contribution by fungal populations (Levanon, 1993). It is generally accepted that factors influencing soil microbial growth and activity in soil (soil type, environmental factors), in combination with the physico-chemical features of the pesticide, will determine its biodegradation by both native and introduced microorganisms (Roberts and Kearney, 1995).

Among intrinsic properties, structural and chemical characteristics largely determine the susceptibility of any pesticide to suffer transformations, and thus its degradability in soil (Gravilescu, 2005). A wide variety of soil aerobic and anaerobic microorganisms are known to partially or completely degrade atrazine when present at low concentrations (Houot *et al.*, 1998; Paszczyński and Crawford, 2000). Among these are some species of bacteria (e.g. genera *Pseudomonas*, *Bacillus*, *Agrobacterium*, *Rhodococcus* and *Klebsiella*), actinomycetes (genus *Streptomyces*) and fungi (e.g. *Penicillium*, *Fusarium*, *Aspergillus*, *Rhizopus*, *Trichoderma*, and several white-rot species) (e.g. Kaufman and Kearney, 1970; Kaufman and Blake, 1973; Levanon, 1993; Paszczyński and Crawford, 2000). What these microorganisms have in common is the ability to utilize the triazine ring as a source of carbon, nitrogen or energy, only varying in the degradation pathway followed (Houot *et al.*, 1998; Paszczyński and Crawford, 2000).

For example, *Klebsiella* and *Pseudomonas* were found in early studies to be able to use the amino groups and ring nitrogen for biomass production (Cook and Hutter, 1981), although evidence suggests that carbon in the atrazine molecule was generally not used by bacteria (Levanon, 1993). On the other hand, two earlier studies have revealed that soil fungi, in particular *Fusarium* and *Aspergillus*, were able to cleave carbon-carbon (C-C), carbon-chloride (C-Cl) and carbon-amine (C-NH₂) bonds in atrazine and use the molecule as sole carbon and nitrogen source (Kaufman and

Kearney, 1970; Kaufman and Blake, 1973). Nevertheless, it is likely that atrazine mineralization in soil is performed simultaneously by all suitable degraders through different simultaneous pathways (Gadd, 2001). This may explain why atrazine, among most herbicides, has not always been shown to be degraded by pure cultures of microorganisms, suggesting that different enzymatic groups may be required for full degradation.

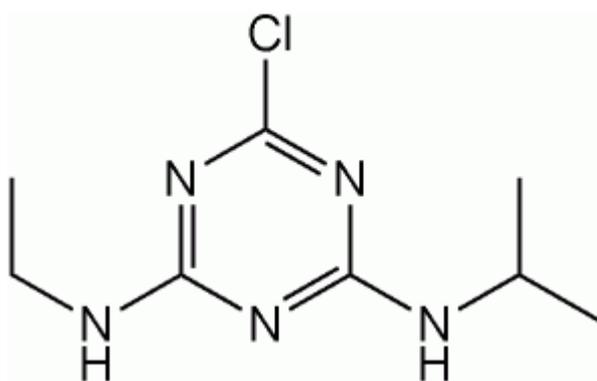


Figure 1.4 Molecular structure of atrazine (Paszczyński and Crawford, 2000).

1.7.3 White-rot fungi: evidence of enzyme-mediated degradation of xenobiotics

The application of fungi for the cleanup of contaminated soil looks promising since the mid-1980s when the white-rot fungi *Phanerochaete chrysosporium* showed to be able to metabolise a number of different organic environmental contaminants (Bumpus *et al.*, 1985; Tien and Kirk, 1988). Later, the same ability was verified for other white-rot fungi, among which *Trametes versicolor* and *Pleurotus ostreatus* are the most popular (Gadd, 2001). White-rot fungi are the most widely studied and understood ligninolytic fungi (e.g. Häggblom, 1992; Šašek *et al.*, 2003; Fragoeiro, 2004; Fragoeiro and Magan, 2005; Baldrian and Šnadjr, 2006). In nature, these fungi colonise and degrade lignocellulosic materials (normally tree wood) and are responsible for causing the white-rot of wood (Šašek *et al.*, 2003).

Lignin is a three-dimensional naturally occurring polymer present in woody plants, and one of the most structurally complex and therefore, recalcitrant to microbial degradation (Gadd, 2001; Paszczynski and Crawford, 2000; Šašek *et al.*, 2003). The ability of white-rot fungi for mineralising lignin is generally attributed to the secretion of extracellular ligninolytic enzymes, mostly LAC, lignin-peroxidase (LiP) and manganese-peroxidase (MnP) (e.g. Gadd, 2001; Šašek *et al.*, 2003; Baldrian and Šnadjr, 2006). It is accepted that both peroxidases catalyse the oxidation of (endogenously produced) low-molecular weight mediators using H₂O₂ as the oxidant. These powerful mediators then oxidise the lignin molecule leaving it partially modified and open to further attack by other enzymes, including LAC (Gadd, 2001; Pointing, 2001).

A main feature of LAC is the fact of being highly non-specific with regard to their substrate (Pointing, 2001). This, adding to the fact that all xenobiotics share at least one of the numerous sub-structures (i.e. combination of functional groups) present in the lignin molecule (Gadd, 2001) explain the ability of white-rot fungi for tolerating as well degrading such a wide range of environmental organic pollutants, even when present at high concentrations (Kearney and Roberts, 1998; Gadd, 2001; Baldrian and Šnadjr, 2006). It has been shown that both LAC and peroxidases co-metabolise these compounds with lignin through similar oxidative mechanisms (Hägglom, 1992; Paszczynski and Crawford, 2000; Gadd, 2001), yet with no net energy gain. In fact, the oxidation of lignin is performed in order for these fungi to have access to wood polysaccharides, their main energy source and which other microorganisms do not have access to (Pointing, 2001). This implies that the presence of ligninocellulosic substrates is necessary for pesticide degradation to occur (Baldrian and Šnadjr, 2006).

Although LAC activity is also involved in lignin degradation, it can also be associated with growth (Schmidt *et al.*, 2005) or as an indication of specific interactions with other microorganisms, particularly in *T. versicolor* (Baldrian, 2004). Determining the activity of this enzyme in soil inoculated with the white-rot species thus provides an indication of its colonising ability as well as a tool for monitoring the success of bioremediation of numerous soil contaminants, including triazine pesticides (Fragoero and Magan, 2005). Other applications of LAC activity include a study by

Schmidt *et al.* (2005) who studied the impact of fungal inoculum properties on *T. versicolor* growth and activity in soil. In another study, Novotný (2004) determined LAC activity for demonstrating the correlation between its production and the degradation rate of polycyclic aromatic hydrocarbons (PAHs) by several strains of white-rot fungi in both liquid culture and soil.

Paszczynski and Crawford (2000), Gadd (2001) and Šašek *et al.* (2003) reviewed a series of applications of white-rot fungi, among them *T. versicolor*, for environmental remediation of pesticides, disinfectants (e.g. pentachlorophenol, PCP) synthetic dyes, benzene derivatives (e.g. petrol and diesel), PAHs, explosives (trinitrotoluene, TNT, derivatives) and industrial solvents (e.g. polychlorinated biphenyls, PCBs). There are also descriptions of the use of these fungi in biotransformation of coal, on the treatment of effluents from paper and olive-processing plants and on the degradation of synthetic polymers (e.g. plastics) and other materials (e.g. nylon). All these applications were found to be related to the production of laccases, Mn-peroxidase or (less frequently) lignin-peroxidases, alone or in combination, which has been corroborated by many other authors (e.g. Novotný, 2004).

However, most of the studies done so far involving the application of white-rot fungi for bioremediation of environmental contaminants were performed using liquid culture media (Ryan and Bumpus, 1989) very often in bioreactors (Novotný, 2004), in sterile soil (e.g. Lamar, 1993) or soil extract broth (Fragoieiro and Magan, 2005). In non-sterile soil, where other factors influence pesticide degradation apart from the fungus degrading capabilities, our knowledge is more limited (Šašek *et al.*, 2003). Very recently, Fragoieiro (2004) reported the successful application of three white-rot species, among which *T. versicolor*, for bioremediation of simazine (alone and in a mixture of pesticides) in non-sterile sandy loam soil under low water potential conditions.

1.7.4 Main factors influencing soil bioremediation by white-rot fungi

Generally, most of the factors influencing microbial activity as well as pesticide availability in soil are likely to influence pesticide bioremediation by white-rot fungi. Key requirements for soil bioremediation to occur have been found to be 25-85% water holding capacity; over 0.2 mg l⁻¹ of dissolved O₂ (corresponds to 10% air-filled pore space); C:N:P = 120:10:1 (molar ratio); pH in the range of 5.5-8.5; and temperature between 15-35°C, as reviewed by Gavrilescu (2005). In addition to soil type and environmental factors, the characteristics of the fungal inoculum have also been shown to be key factors (Schmidt *et al.*, 2005).

Characteristics of fungal inoculum

In many studies, a rapid decline in the growth and activity of the introduced species was observed in contaminated non-sterile soil. One of the most common reasons for this is the inability of the fungus to compete with native highly adapted microbial communities (Hägglom, 1992).

The survival and activity of introduced microbial species for environmental clean-up is therefore one of the most important factors to take into consideration. When inoculating contaminated soil with these fungi, high lignin-containing amendments such as wheat straw or sawdust have been used as both carriers for the fungi as well as nutritional substrates (Šašek *et al.*, 2003). Studies have shown that when fungi were allowed to grow on these ligninolytic substrates prior to addition into soil, both growth and enzyme production in soil were enhanced, therefore improving the competition capabilities of the introduced fungi (Bumpus, 1993; Levanon, 1993). Further, adding into soil nutrients of a ligninolytic nature, which are not easily degraded by indigenous soil microorganisms, also puts the white-rot fungi in an advantageous position. Additionally, the use of mycelium-impregnated substrates as carriers may also be useful for allowing better inoculum distribution in the soil (Gadd, 2001). It is important to notice however, that according to several studies, the parameters influencing white-rot fungi colonisation in soil, may differ between fungal species.

Soil properties and environmental factors

In most of the applications, biodegradation of soil pollutants is generally dependent on the performance of aerobic microorganisms, and therefore aeration for an efficient gas exchange is required for the process to take place (Hägglom, 1992). The same is true when using white-rot fungi since they are obligate aerobes. On the other hand, there is evidence that molecular oxygen may act directly on the oxidation of the lignin molecule, and therefore may have a role in enhancing the breakdown of xenobiotic molecules. For applications in liquid culture, aeration is usually not a problem and gentle agitation of the medium has proven to be enough (Gadd, 2001). However, in soil, adequate aeration can be difficult and is often closely related to soil properties, depth and water content (Gavrilescu, 2005).

Like most fungi, white-rot species usually show optimal growth at relatively low pH. Since fungal growth on carbon substrates results in the production of organic acids, it may result in acidification of the medium, in this case the soil. Even if initial soil pH is between 5.5 and 7 (i.e. usual) the white-rot fungi can reduce it through metabolism to 4.0-5.5, at which degradation of most xenobiotics has shown to be promoted (Gadd, 2001). This implies firstly that extracellular activity (and enzyme stability) is optimum within that range, and secondly that xenobiotic degradation is not immediate but only starts after pH has reached that optimal level. On the other hand, at pH 3 *T. versicolor* was found to stop growing and exhibiting extracellular activity (Libra *et al.*, 2002). The pH range for a bioremediation process probably reflects a compromise between that optimal for the ligninolytic enzymes and that at which the mycelium can grow effectively, which can be easily controlled using buffer solutions.

The majority of white-rot species are mesophiles in their temperature requirements (Okeke *et al.*, 1996) and they can also tolerate a wide range of moisture contents (Gadd, 2001). Both parameters combined with an adequate C:N ration have shown to deeply influence soil colonisation by white-rot fungi (e.g. Okeke *et al.*, 1996). There is however, very little information on their optimum temperature and water potential ranges, especially for xenobiotic degradation. Furthermore, those ranges probably vary according to the white-rot species. According to Kearney and Roberts (1998), the optimum temperature for bioremediation should be balanced between that for enzyme

activity (degradation rate) and stability. The bioavailability of the pesticide in soil affected by organic matter and clay minerals is another important factor influencing its biodegradation by white-rot fungi (Šašek *et al.*, 2003).

1.8 RESEARCH AIMS AND OBJECTIVES

Soil health and consequently its capability for functioning, can change over time due to both environmental and human impacts (Doran, 2002). It is therefore important to early detect and assess changes in soil status, in order to determine the direction of that change. Soil microbial community size, activity and diversity have been traditionally used as indicators of changing soil conditions, due to the tight relationship between the status of microbial community and soil functioning, as well as the fact that microorganisms respond quicker than any other soil component to changes in the soil environment (Bloem *et al.*, 2006).

The development of technologies such as the E-nose, which can provide an early, quick and reliable characterisation of microbial activity, may be of major importance for soil diagnostics. Nevertheless, the potential of the E-nose for such applications has not been researched yet. Innovatively, this work has focused on investigating the potential use of qualitative soil volatile fingerprints for complementing traditional methods for assessing and characterising soil microbial populations as influenced by soil type and changes in soil conditions. Also, correlations were assessed under different soil treatments between volatile fingerprints and the more traditional microbial parameters such as respiration, dehydrogenase activity, microbial population size and community structure.

Therefore, the research aims and objectives of this work were focused on testing the E-nose potential for:

1. Assessing changes in microbial activity as influenced by soil characteristics and environmental conditions (temperature, water potential, nutrient and herbicide inputs);

2. Detecting the presence of atrazine at usual field application rate and monitoring its bioremediation using a white-rot fungus (*T. versicolor*);
3. Investigating relationships between soil volatile fingerprints and traditional methods for determining soil microbial activity, population size, and fungal community structure under the treatment conditions;
4. Detecting and monitoring *Streptomyces* activity and geosmin production in soil and potable water.

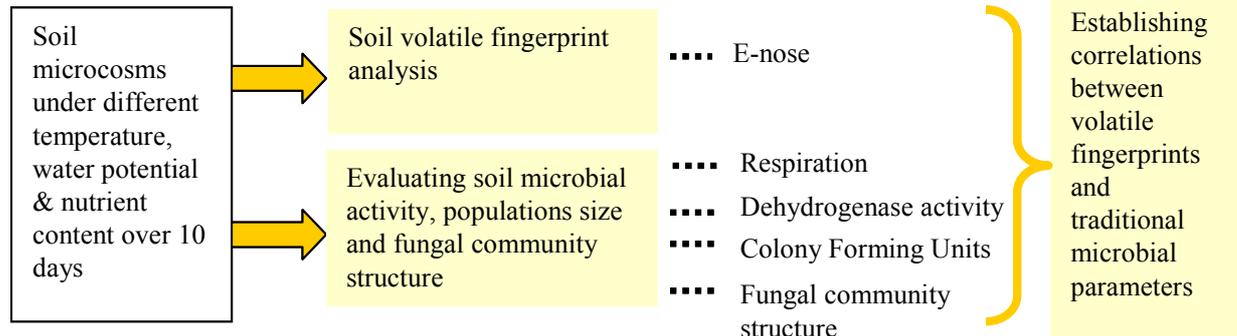
The three soils used in this study differ in their characteristics, which reflect differences in their environment: a sandy loam (Bedfordshire, UK), a calcareous clay soil (Algarve, South Portugal) and a volcanic ash (original material from Mauna Kea volcano, Hawaii), commonly known as Martian simulant soil JSC- MARS 1. The use of three different soils allowed testing whether volatile fingerprint analysis can be applied independently of soil physico-chemical and biological characteristics.

Throughout this research, particular attention was given to the influence of soil water availability on microbial activity, in particular the way it influences key soil processes such as organic matter and pesticide degradation, by both native and introduced microorganisms. In this research, the water potentials used were -0.7 and -2.8 MPa having the interval of soil available water for plants and microorganisms (-0.03 to -1.5 MPa) has reference.

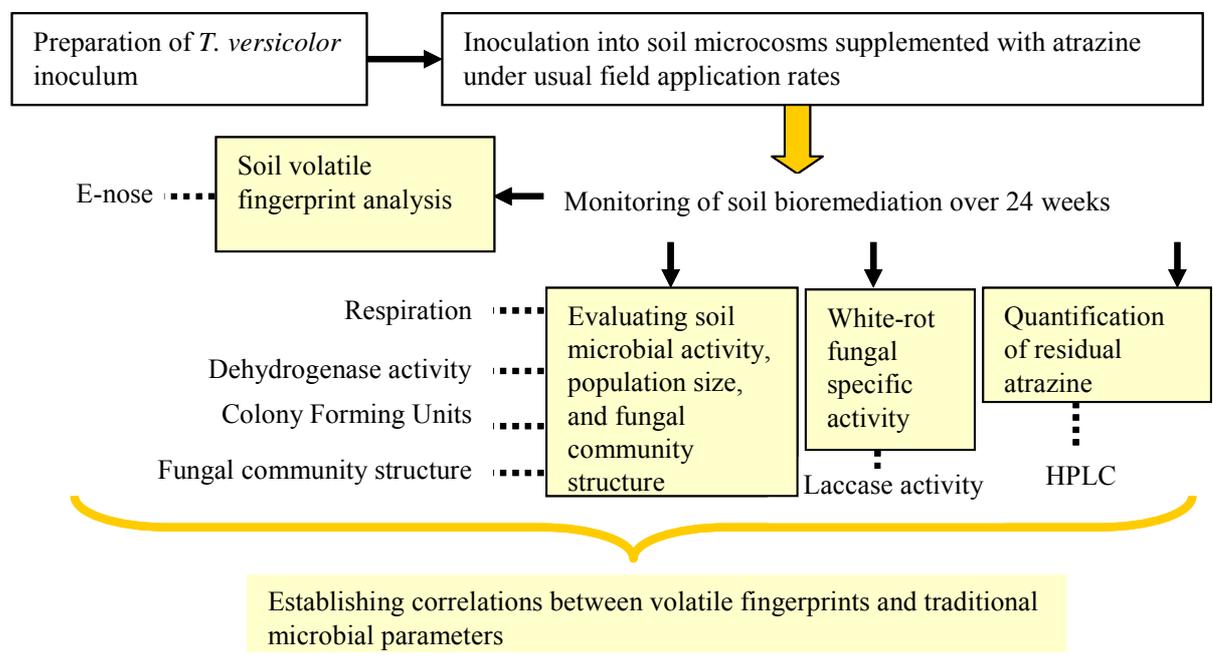
1.8.1 Thesis flowchart

Figure 1.5 shows the several experimental steps involved in this work.

CHAPTER II Evaluating the E-nose potential for differentiating between soil types and conditions



CHAPTER III Using volatile fingerprint analysis as a monitoring tool for atrazine bioremediation in soil by white-rot fungus



CHAPTER IV Volatile fingerprints: potential for early detection of *Streptomyces* activity and geosmin production in soil and potable water

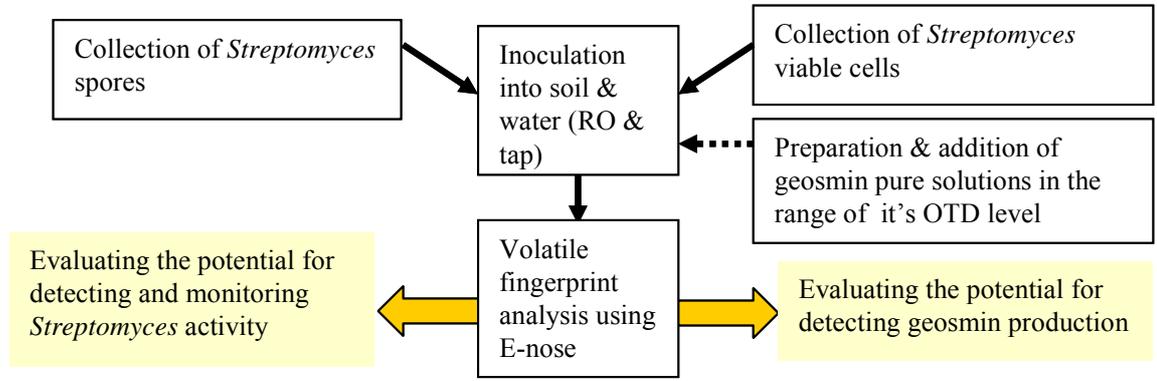


Figure 1.5 Thesis flowchart.

CHAPTER II

Evaluating the Potential of the E-nose for Differentiating between Soil Types and Conditions

CHAPTER II

EVALUATING THE POTENTIAL OF THE E-NOSE FOR DIFFERENTIATING BETWEEN SOIL TYPES AND CONDITIONS

2.1 INTRODUCTION AND OBJECTIVES

Until now, the potential of soil volatile fingerprints to be employed for characterising soil microbial communities as influenced by external factors has not been investigated. All traditional methods generally include the assessment of microbial respiration and enzymatic activities, as well as community size and structure. In this study, microbial activity was determined and characterised using both qualitative soil microbial volatile fingerprints, and traditional soil respiration analyses and DHA, as these can be considered representative of the overall soil heterotrophic activity. Microbial population size and fungal biodiversity were also estimated using colony forming units. All indicators employed in this Chapter were assessed in three soil types under different soil environmental conditions both in the absence and in the presence of added nutrients for up to 10 days. The potential use of soil volatile fingerprints in combination with traditional microbial parameters provides an overall picture of the status of native microbial communities in the three soils under the treatment environmental conditions. This information is also relevant for use as reference in subsequent Chapters.

The work in this chapter, therefore, had the following main objectives:

- (1) Investigating the potential use of qualitative volatile fingerprints as a sensitive indicator of soil microbial activity and the way it correlates with traditional microbial parameters, as influenced by soil conditions;
- (2) Studying the short-term impact of soil temperature, water potential and the addition of soluble (glucose) and recalcitrant (wheat straw) nutrients on native

- microbial activity, volatile production patterns, community size and fungal biodiversity;
- (3) Testing the potential of the same parameters as reliable methods for characterising soil microbial communities, as well as the way these parameters correlate with each other, under low soil water potential conditions;
 - (4) Comparing the responses of microbial communities in clay soil, volcanic ash and sandy loam, as influenced by those environmental factors.

2.2 MATERIAL AND METHODS

2.2.1 Main properties of soils

Physical and chemical properties of soil are key factors determining the characteristics of soil microbial populations. Table 2.1 (page 49) summarizes the main properties of each soil and those of the site of collection. Soil properties were assessed by the National Soil Resources Institute, NSRI (Cranfield University, Silsoe, UK), Direcção Regional de Agricultura do Algarve, DRAALG (Algarve, Portugal) and NASA Johnson Space Centre (USA) for each soil respectively.

The sandy loam soil (arable top-soil, 0-20 cm) was collected from an arable field plot in Silsoe, Bedfordshire, and was provided by the NSRI. The calcareous clay soil (arable top-soil, 0-20 cm) was collected from a field plot in Lagoa, inland region of Algarve, South Portugal. Occasional dry plant debris, larger stones and marine shells were manually removed at the time of collection. Both soils were collected from non-vegetated areas for which there is no recent history of soil management (nutrient or pesticide addition) previous to collection (Da Silva, pers. communication).

The martian simulant soil's original material was hand-collected at 1.850 m.a.s.l. and 40-60 cm depth from the south flank on Mauna Kea volcano, Hawaii. It was then sieved < 1 mm and dried at approximately 80°C before being sealed in plastic moisture-proof containers for shipment and storage (Allen *et al.* 1997, 1998). For the purpose of this work this soil was treated as top-soil and it was assumed that it had no nutrient inputs or pesticide applications previous to use. The sample used in this study was provided by Dr. David Cullen (Cranfield Health, Cranfield University, Silsoe).

Table 2.1 Main properties of each soil and collection site. Particle size ranges for sand, silt and clay are between 62 μm -2 mm, 4-62 μm and 1-4 μm respectively.

	Sandy loam	Clay soil	Volcanic ash
SOC content (mg) ^a	23.0	12.1	1.8
Water content (mg) ^a	690	353	282
Sand content (mg) ^a	578	320	841
Clay content (mg) ^a	181	470	13.0
Silt content (mg) ^a	241	210	146
pH	6.0	6.8	5.9
Collection site	Bedfordshire, UK	Algarve, Portugal	Mauna Kea, Hawaii
Precipitation on site (mm) ^b	560	400	300
Temperature ($^{\circ}\text{C}$) on site ^b	12	17	15

^a Values are g^{-1} oven dry soil.

^b Precipitation and temperature on site are average annual values.

2.2.2 Soil preparation, conditioning and storage

The three soils were sieved (<2 mm), which was intended to facilitate mixing and homogenisation, and thus allowing for identical sub-samples to be prepared. Soils were then air-dried at 20 $^{\circ}\text{C}$ for 5 days prior to use. In order to allow microbial activity to stabilise after these changes in environmental conditions and also by the sampling and sieving processes, soils were pre-incubated for one week at 20 $^{\circ}\text{C}$ before any analyses were performed, as recommended by Schinner *et al.* (1996) and Bloem *et al.* (2006). While not in use, soils were kept in sterile plastic bags at 4 $^{\circ}\text{C}$ in the dark, as recommended by Wollum (1994) and Nielsen and Winding (2002).

Firstly, moisture calibration curves were developed for each soil type. Then, the impact of soil water potential and temperature on soil microbial communities and volatile production patterns was assessed using un-amended soil under different environmental conditions. Later, nutritional amendments were added into soil under those same

environmental conditions in order to study the effect of nutrient addition, type and concentration on soil microbial communities.

Soil moisture calibration curves

Initially, moisture calibration curves were developed for each soil type, to enable accurate modifications to soil water potential to be made. Moisture adsorption curves were obtained by adding different volumes of RO water (ranging from 50 to 150 μl) to triplicate 5 g samples of air-dried soil. Soil samples were homogenised and left equilibrating for 24 h at 4°C before the water potential was determined using a Aqualab-Dewpoint Potentiometer WP4. Figure 2.1 shows the relationship between the volume of water added to soil ($\mu\text{l g}^{-1}$) and its water potential (MPa) for each soil type, at 20°C temperature. The increase in the amount of soil available water means that microbial populations need to spend less energy in order to extract it for use. Soil–water adsorption characteristic curves between soil moisture content (% w w⁻¹) and water potential (MPa) are shown in Figure 2.2. Soil structure explains differences between soil-water curves according to soil type, as previously explained in Chapter I, sub-section 1.2.3 (page 5).

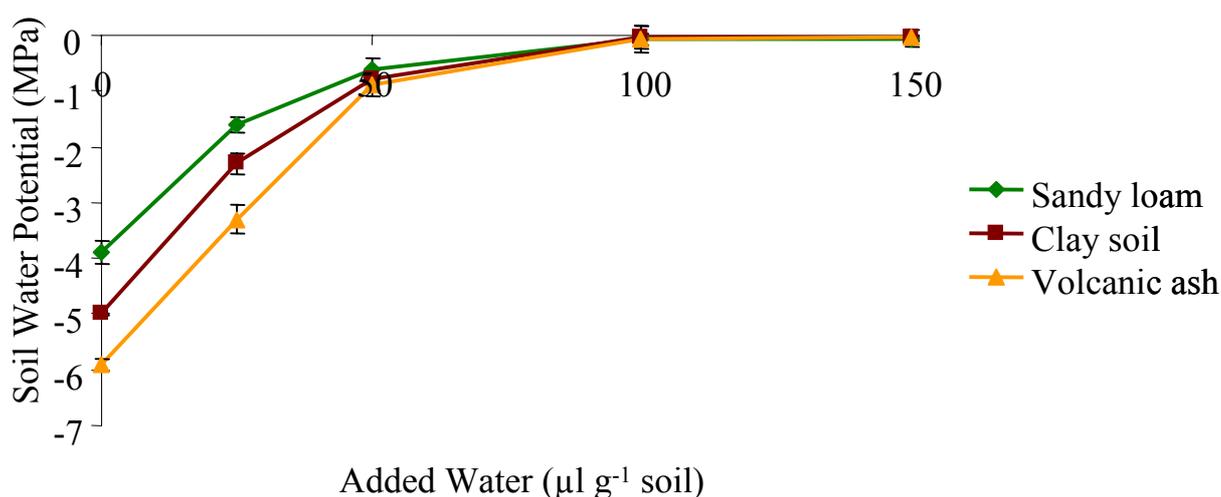


Figure 2.1 Relationship between known volumes of water added to soil (μl) and its water potential (MPa), at 20°C. Vertical bars represent standard errors of means of three replicates.

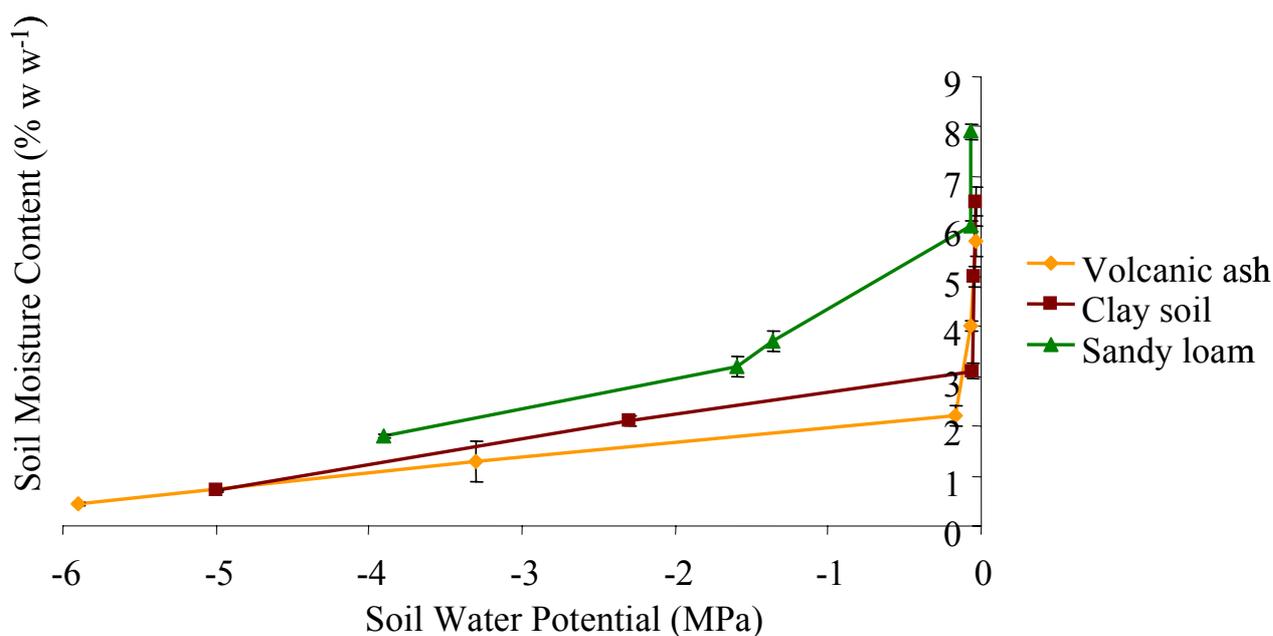


Figure 2.2 Soil–water characteristic curves of soils under study, at 20°C. Soil structure explains differences between soil-water curves according to soil type. Vertical bars represent standard errors of means of three replicates.

2.2.3 Soil microcosms for studying the effects of water potential and temperature

Preparation of treatments

Air-dried soil samples (5 g) were conditioned to the two water potential treatments (-0.7 and -2.8 MPa) by reference to its correspondent soil water potential calibration curve, and the addition of sterile reverse osmosis (RO) water. Samples were then kept for 24 h at 4°C allowing microbial activity to stabilise at the required water potential levels, before returning to ambient conditions for experimental set up. All treatments were applied to the three soil types, in order to obtain five replicates *per* treatment at each sampling time.

Incubation of soil microcosms

The effect of environmental factors and nutrient inputs on soil microbial communities was performed using soil microcosms, as represented in Figure 2.3. Soil samples under the different treatments were contained into sterile Universal Bottles, which were then

placed inside polyethylene boxes previously thoroughly cleaned. A glycerol/water solution (400 ml) was also placed inside each polyethylene box, in order to maintain the equilibrium relative humidity within each microcosms the same as that of the soil treatments.

Samples were incubated at 16, 25 or 37°C in temperature-controlled incubators for 3 hrs, 1, 2, 7 and 10 days, according to the objective of the experiment. During incubation, Universal bottles were left open inside polyethylene boxes, ensuring adequate soil aeration. All treatments involved in this Sub-section are listed in Table 2.2.

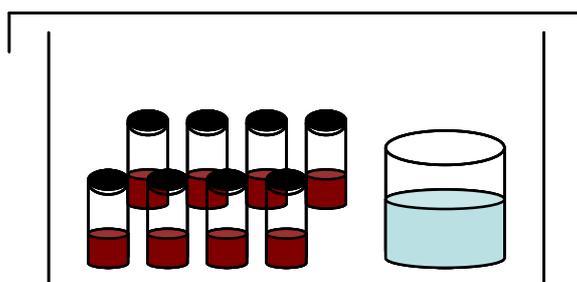


Figure 2.3 Representation of the concept of soil microcosms used throughout this study.

Table 2.2 Summary of the soil environmental conditions involved in Sub-section 2.2.3, for each soil type.

	Ψ (MPa) [*]	Temp (°C)
Soil	-0.7	16; 25; 37
	-2.8	16; 25; 37

^{*} 1 MPa equals 10 bars or 9.87 atm.

2.2.4 Soil microcosms for studying the effects of nutrient addition, type and concentration

Nutrient preparation and soil amendment

Glucose and wheat straw were chosen as nutritional substrates, due to differences between their degradation rates in soil. For selecting the appropriate concentration of glucose and wheat straw to be added to soil, a range of concentrations between 0 and 5 mg nutrient g⁻¹ soil was initially tested based on soil respiration measurements. The mid-point and highest concentrations within that range were selected in both cases.

Glucose was weighed and dissolved in RO water before being added to soil microcosms (West and Sparling, 1986; Lin and Brookes, 1999) at a rate of 2.5 or 5.0 mg g⁻¹ dry soil. The amount of water used for dissolving was the same as that required for the target water potential treatment. The wheat straw was frozen for two weeks prior to use, in order to inactivate mites. It was then milled and mixed thoroughly with air-dried soil, at a concentration of 2.5 or 5.0 mg g⁻¹ dry soil. Due to the fact that straw addition can alter soil water potential depending on its own moisture content, moisture adsorption curves were also produced for each soil type amended with straw at the treatment concentration, based on the procedure described above for un-amended soil. Conditioning of the straw-treated soil to the required water potentials was then done by reference to its corresponded moisture calibration curve. Nutritionally modified soil samples were kept for 24 h at 4°C allowing microbial activity to stabilise at the required water potential levels, before returning to ambient conditions for experimental set up. All treatments were applied to the three soil types, in order to obtain five replicates *per* treatment at each sampling time.

Incubation of nutritionally modified soil microcosms

Incubation of amended soil samples was performed under the same environmental conditions described in Sub-section 2.2.4. All treatments involved in this Sub-section are listed in Table 2.3.

Table 2.3 Summary of the soil treatments involved in Sub-section 2.2.4, for each soil.

	Ref.	Ψ (MPa)	Temp (°C)	Glucose (mg)	Straw (mg)
Soil	S	-0.7	16; 25; 37	-	-
		-2.8	16; 25; 37	-	-
Soil + Glucose	S + G	-0.7	16; 25; 37	2.5; 5.0	-
		-2.8	16; 25; 37	2.5; 5.0	-
Soil + Straw	S + S	-0.7	16; 25; 37	-	2.5; 5.0
		-2.8	16; 25; 37	-	2.5; 5.0

2.2.5 Characterisation of soil volatile fingerprints using a conducting-polymer sensor array

Soil microbial volatile patterns were qualitatively analysed through static sampling using the commercial E-nose system Bloodhound BH-114 (Scensive Technologies Ltd., Leeds, UK) (Plate 2.1). Sample preparation and incubation procedures have already been described in Sub-sections 2.2.3 and 2.2.4.

**Plate 2.1** E-nose Bloodhound BH-114 sampling the headspace of a Universal bottle containing soil.

Sensor technology

The E-nose employs 14 conducting-polymer sensors, comprised of 16 different materials chosen from doped polyaniline, polypyrrole and polythiophene with a variety of substitute groups bound to the polymeric matrix. Polymers were produced by electropolymerisation (cycling the potential between -0.1 and +0.8 V, except for thiophenes for which +1.7 V was used) of the corresponding monomers directly onto clean gold interdigitated transducers (Gibson *et al.*, 1997). The sensor materials themselves, as well as their exact production method and the dopants used remain the subject of confidentiality and copyright of the company. A general description of the production of conducting-polymer sensors is given by Nagle *et al.* (1998) and examples of the typical procedure for developing non-patented polyaniline-, polypyrrole- and polythiophene-based sensors have been described elsewhere (Guadarrama *et al.*, 2000, 2001; Barisci *et al.*, 2002; Hamilton *et al.*, 2005). Nonetheless, what is most important is that sensors obtained using this approach are non-specific. Also that sensors based on different polymer characteristics vary in terms of sensitivity once in contact with the same volatile mixture. Regarding sensor reproducibility, this is assured during manufacture by an accurate control of the production conditions and passing them through a series of quality control assays (Gibson *et al.*, 1997).

E-nose sensing procedures

Soil headspace (2.5 ml) was sampled through a needle into a 6 cm long Teflon tubing segment (Tygon) equipped with a bio-filter (0.45 μm , PTFE Whatman, Hepa Vent). The use of a bio-filter prevented microbial spores reaching and damaging the sensors.

Five replicates of each treatment were analysed in a random pattern. The E-nose sampling profile settings were the following: baseline 5 seconds; absorption 5 seconds; desorption 10 seconds; and flush 5 seconds. The overall sampling time for each replicate was approximately 1 min. The headspace of an empty Universal bottle was also sampled three times at the beginning and at the end of every sampling occasion and after each set of 15 samples, aiming to reduce carry over effects from one sample to the next. It also helped defining a standard reference point and monitoring sensor drift.

This sensory unit uses two calibration points, a baseline and the headspace of a RO water sample. Activated carbon filtered air was set to pass over the sensor surface at a flow rate of 80-160 ml min⁻¹ (Scensive Technologies, pers. communication) generating the baseline and preparing the array for the next sampling cycle (flushing). One minute was allowed between samples (delay) in order to ensure sensors had returned to baseline values, thus reducing carry over effects from one sample to the next. The E-nose was left switched on for over 1 h before sampling in order to allow the sensor array to stabilise. The sensor response corresponds to the change of resistance of the active material ($\Delta R/R$) over time, where R is the baseline resistance value.

Optimisation of E-nose output

Since no standard method has been developed so far for sensing soil headspace using E-nose technology, several different sampling approaches were tested to find the optimum sensor output. This was particularly important in this study, since soil samples were kept under low water potential conditions, thus limiting microbial activity and volatile production.

The approach selected consisted of sampling the headspace directly from the Universal bottle, which contained the soil during the incubation period. Following incubation, bottles were covered with parafilm and left for 3 h at 20°C prior to analysis, in order to ensure an adequate concentration and homogenisation of the volatiles in the headspace above the soil sample.

Studies on reproducibility of sensor response

The variability between sensor responses to 5 replicates of the same treatment was studied for each sampling occasion, following the same procedure and profile settings described for the treatments. Reproducibility tests were performed for every soil temperature and water potential involved in this work.

Other sampling approaches

Other approaches were tested in order to optimise the sensor array response to soil treatments, consisting mainly in using sampling vials and soil amounts of different sizes, as well as different volatile concentration periods before analysis. Among the sampling

vials that were tested were the 50 ml bubble-generating bottles and 50 ml sampling bags (BDH, UK). In both cases, soil treatments were incubated within the sampling vial leaving up to 40 and 25 ml (respectively) of headspace above the soil sample. Also in both cases, soil headspace was sampled through a needle into a 6 cm long Teflon tubing segment (Tygon) equipped with a bio-filter (0.45 μm , PTFE Whatman, Hepa Vent).

Bubble-generating bottles are commonly employed for liquid samples. These are 50 ml centrifuge tubes with modified lids, equipped with two tubing segments: one through which clean air flows above the sample (inlet); and the other, through which the headspace is pulled out by the E-nose vacuum pump (outlet). A 3 h volatile concentration period was also allowed following soil incubation, during which the upper end of both tubing segments were sealed with parafilm. The use of sampling bags involved inflating them with a fixed volume of clean filtered air before incubation and the same 3 h concentration period prior to analysis.

2.2.6 Evaluation of soil microbial activity based on respiration and dehydrogenase activity

Soil basal respiration

Basal respiration in this study refers to total CO₂ evolved from un-amended soils, based on the concept by Schinner *et al.* (1996) and Bloem *et al.* (2006). CO₂ efflux was determined by gas chromatography through static sampling. Following incubation, Universal bottles containing soil samples were sealed and left for 3 h at 20°C prior to analysis, thus ensuring detectable volumes of CO₂ in the soil headspace. The headspace concentration period prior to analysis was tested and optimised in preliminary experiments and was shown to be particularly important in this study where soils were under sub-optimal moisture conditions for microbial activity.

Soil headspace (5 ml) was then injected into a gas chromatographer (GC) equipped with a packed column (Porapak Q packed glass column) and a thermal conductivity detector (Carlo Erba Instruments, GC 8000 Series MFC 800). Five replicates of each treatment were sampled. The experimental details were the following: column and injector

temperatures, 100°C; detector temperature, 180°C; carrier gas (Helium) at a flow rate of 36 ml min⁻¹; calibration gas consisted of a standard mixture (10.01% v v⁻¹ CO₂ in N₂), which was injected three times at the beginning and at the end of every sampling occasion, as well as after each set of 15 samples. The retention time (RT) for CO₂ under these sampling conditions was 0.62 ± 0.01 minutes.

Five replicates of empty Universal bottles (blanks) were also sampled at the beginning of the experiment for determining background CO₂. Soil respiration was assumed to equal the change in concentration over the incubation period minus the background concentration. Soil basal respiration rate was expressed as μg CO₂ g⁻¹ soil h⁻¹.

SIR response to nutrient addition

In this study, SIR was employed as a general term, referring to the potential respiration from soils supplemented with nutritional substrates. This SIR concept was based on Schinner *et al.* (1996) and Bloem *et al.* (2006). It is therefore, important to underline that this is a different approach to the original concept of (short-term) SIR, based on the Anderson and Domsh (1978) approach for estimating biomass.

Following nutrient addition to soil (Section 2.2.4), microbial SIR response to glucose and wheat straw was determined after 3 h, 1, 2, 7 and 10 days of incubation, following the procedure described for basal respiration. SIR was assumed to equal the change in concentration over the incubation period minus the background concentration. SIR rate was expressed as μg CO₂ g⁻¹ soil h⁻¹. Respiration of un-amended soils at the treatment water potentials and incubation temperatures was used as control samples.

Bacterial and fungal relative contributions to respiration

Selective inhibition was used in combination with basal and SIR measurements to determine the relative contributions of bacteria and fungi to total soil respiration for each soil type. Cycloheximide and chloramphenicol were the antibiotic and fungicide used.

Five grams of air-dried soil were weighed into sterile Universal bottles. Soil water potentials were adjusted to -0.7 or -2.8 MPa using sterile RO water with dissolved cycloheximide or chloramphenicol, in order to achieve concentrations of both inhibitors

of 5.0 mg g⁻¹ soil, based on that suggested by Lin and Brookes (1999). Sonication (1 min) of the antibiotic solutions enhanced homogenisation. Soil was then thoroughly mixed for full homogenisation. Both incubation and respiration analyses followed the procedure described in Sub-sections 2.2.3 and 2.2.6 respectively.

Dehydrogenase assay based on INT reduction

The INT method adapted from von Mersi and Schinner (1991) was applied for estimating soil DHA as a response to treatments. The method is based on the incubation of soil with the substrate INT (2 (p-iodophenyl)-3-(p nitrophenyl)-5-phenyl tetrazolium chloride), followed by the extraction and colorimetric estimation of the reduction product INF (iodonitrotetrazolium formazan). Because INT is photosensitive, most of the procedure, in particular the incubation, had to be carried out under low light conditions.

The INT solution was prepared by dissolving 500 mg of INT (Acros) into 2 ml of N,N-dimethylformamide (Sigma), followed by the addition of 50 ml of RO water. The solution was sonicated (2 min) and RO water was again used to bring the volume up to 100 ml. The final concentration of the substrate solution was 0.5% (w v⁻¹). For every sampling occasion, fresh INT solution was prepared and stored in the dark until use.

Soil (0.5 g) at the treatment water potentials was weighed into sterile test tubes and mixed with 740 µl of Tris-HCl buffer (1 M, pH 7.0) and 1 ml of the substrate solution. Test tubes were sealed with sterile sponge stoppers and incubated in the dark at 40°C for 2 h. Following incubation, 5 ml of extraction solution (N,N-dimethylformamide: ethanol in a 1:1 ratio) were added to the mixture and samples were kept in the dark for 1 h. During this time, every sample was vigorously shaken (using the vortex) at 20 min intervals, ensuring an efficient extraction of the product INF. Aliquots of 2 ml were then transferred to Eppendorf tubes and centrifuged for 2 min. Supernatant (200 µl) was introduced into microplate wells and the developed INF (red colour) was determined spectrophotometrically at 450 nm using a Microplate reader (Dynex Technologies Ltd., UK).

Controls were also prepared for estimating the abiotic (chemical) reduction of INT under the same conditions, since INT can also be reduced to INF by ions such as Fe^{2+} present in the soil. For each treatment, controls were prepared using autoclaved soil (121°C, 20 minutes) and were treated like samples. Five replicates of each treatment (including respective controls) were sampled. The INT reduction of the control was then subtracted to that of the samples and dehydrogenase activity was expressed as $\text{ng INF g}^{-1} \text{ soil } 2 \text{ h}^{-1}$.

For developing the INF calibration curve, a standard INF solution ($100 \mu\text{g INF ml}^{-1}$) was used. A range of volumes of standard solution were pipetted into test tubes in triplicate. Tris-HCl buffer (370 μl), RO water and extractant solution (2,5 ml) were added to each tube and mixed thoroughly, in order to obtain a concentration of INF ranging between 0.04 and $0.75 \mu\text{g ml}^{-1}$. Aliquots (200 μl) of this mixture were introduced into microplate wells and the absorbance was read at 450 nm. The calibration curve obtained ($Y = 1.0657x + 0.0061$) showed a good correlation ($r^2 = 0.998$) between the concentration of INF and the optical density of the INF solution.

2.2.7 Assessment of soil microbial population size and fungal community structure

Selective inhibition was used in combination with culture-dependent methods for quantifying viable soil heterotrophic bacteria and fungi under sterile working conditions. This technique also allowed fungal biodiversity studies to be performed, providing a good approach for identifying the most dominant fungal genera in these soils, according to the type of treatment.

Preparation of nutritional media

Nutrient Agar (NA) (Difco Laboratories) and Malt Extract Agar (MEA) (Difco Laboratories) were used for isolation of bacteria and fungi respectively at -0.7 and -2.8 MPa. Water potentials were set prior to sterilisation by adding a known amount of glycerol (Fisher Scientific).

Cycloheximide (Sigma Chemicals) and chloramphenicol (Sigma Chemicals), both at 225 mg L⁻¹ media, were added through sterile 0.2 µm filters to the autoclaved NA and MEA respectively, while still hot. Treated media were poured onto Petri dishes, which were stored in the dark at 4°C while not in use.

Enumeration of culturable bacteria and fungi

A 10⁻¹ dilution was prepared by transferring 1g of soil into a Universal bottle containing 9 ml of sterile RO water. This suspension was shaken for 2 min at 150 rpm using a shaker (Ika Labortechnik, GmgH & Co.) in order to detach microbial cells from the surface of soil particles and allowing better cell distribution. Ten fold dilutions of this soil suspension were prepared in the range 10⁻² and 10⁻⁶ for fungal and bacterial enumeration. In both cases, aliquots of 200 µl were spread onto Petri plates containing the selective media. The diluted soil suspension was dispersed on the Petri dish using a sterile spreader, followed by incubation at 16°C, 25°C and 37°C. Incubation periods of 4 and 7 days were used for bacteria and fungi respectively.

The number of colonies multiplied by the dilution factor provides an estimate of the number of viable microorganisms, and hence population density in the original soil sample. Plates with largely spread bacterial colonies were excluded from counting. Results were expressed in a logarithmic scale as Log₁₀ (CFUs) g⁻¹ soil.

Soil fungal community structure

In this study, fungal community structure refers to the dominant genera and the number of different species growing in MEA. For the identification of the different fungal species, MEA plates were incubated under the treatment environmental conditions for 10 days. During incubation, plates were observed every other day, for identification of the dominant genera and a count of the number of different species found in each soil treatment. Fungal diversity was expressed as the number of fungal species g⁻¹ soil.

2.2.8 Correlations between microbial parameters

Correlations between the different microbial parameters under the treatment environmental conditions were also investigated for un-amended and glucose-amended soil. For correlation purposes, five replicates of each treatment were sampled for volatile fingerprint, CO₂, DHA, microbial population size and fungal community structure analyses every three hours following incubation of 1 and 10 days at the treatment environmental conditions. Regarding the E-nose data, PC1 and PC2 were chosen as representative of the overall data set for correlation analyses with the remaining parameters.

2.2.9 Data handling and statistical treatment

All results presented in this chapter are means of five replicates. Data input and handling, as well as graph plotting (including linear regression analyses) were generally performed using Microsoft Excel 2003 (Microsoft Co.). For comparison between means of treatments in respect to respiration, enzyme activity, population numbers and fungal biodiversity data, analysis of variance (ANOVA) was performed using STATISTICA (Version 7) at a significance level $p = 0.05$. Standard error of means are shown as vertical bars in figures and indicated in tables as \pm SE. For studies on the correlation between different microbial parameters, general regression models for independent variables were applied (STATISTICA). Regarding the Bloodhound BH-114 data, multivariate analysis using a built-in statistical software package (xlStat, Microsoft Excel add-in, version 3.4) was employed for interpreting sensor response.

Among the sensor parameters which comprise a typical sensor response, data for divergence was used, taking into account that the reproducibility of the system was the highest for this parameter. Divergence data were normalised, so that only qualitative information was taken into account (Craven *et al.*, 1996). Then PCA was employed for data reduction and outlier detection, as well as for characterising samples/ treatments, based on their similarities and differences. The first two PCs (PC1 and PC2) were used

throughout this study, as together they explained over 80% of the variance within the data sets. PCs 3 and 4 did not add any additional relevant information.

At the end of each experiment, the loading plots were examined and those sensors contributing most to the overall variability within the data set were selected (never less than 10). Those sensors contributing the least to the information explained by either PC1 or PC2, had their output removed from the data set in order to reduce noise. PCA was performed based on the responses of the selected subset of sensors. In cases where PCA revealed clusters separated along PC2 with this factor contributing with up to 5% for the overall variance, ANOVA was performed on divergence data for evaluating the significance of the cluster separation (Bellamy, pers. communication).

Sensor 13 was found to be a considerable source of noise, presenting the most variable responses to replicates of the same treatment both in preliminary reproducibility tests and throughout the study. This probably reflects damage or a defect in this sensor and therefore its response was not taken into consideration for the results presented. The selection of relevant sensors which can better explain the relationship between data is common practice, particularly when multi-dimensional data are concerned (Jurs *et al.*, 2000).

2.3 RESULTS

2.3.1 Impact of treatments on soil volatile fingerprints

Studies on reproducibility of sensor responses

Replication of the sensor array was firstly examined within the range of temperature and water potentials used. Figure 2.4 shows an example of the low variability obtained between sensor responses to five replicates of the same treatment, in this case for sandy loam incubated at 25°C and -0.7 MPa for 1 day. Similar results were found for all soil types (see Appendix I), demonstrating that the reproducibility of sensor responses was irrespective of soil characteristics, which is an important result when considering the potential applications of this technology to the soil environment. Sensor response was expressed as the change of resistance ($\Delta R/R$) of the sensor material due to binding to the volatiles..

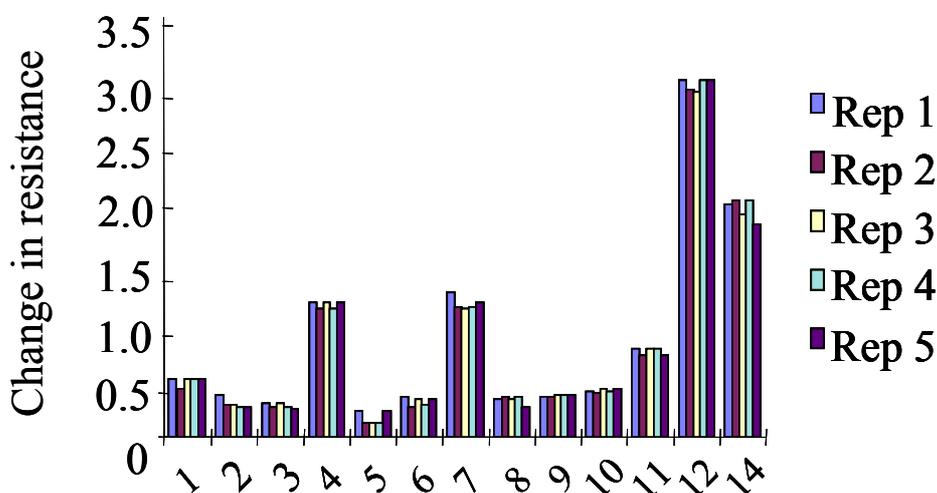


Figure 2.4 Sensor relative responses expressed as changes in resistance showing low variability between five replicates (1-5) of the sandy soil, incubated at 25°C and -0.7 MPa for 1 day.

Influence of soil type

Figure 2.5 shows the PCA plot for the three soils incubated at 25°C and -0.7 MPa for 1 day. It shows that differences in volatile production patterns clearly discriminated between the different soil types, where PCs 1 and 2 accounted for 93% of the variance within the data set. Interestingly, PC2 explained almost on its own the discrimination between the clay and sandy loam soils, whereas these both were separated from the volcanic ash mainly by PC1. Similar results were obtained independently of soil water potential and temperature (data not presented), although the discrimination level was generally higher under wetter and cooler soil conditions.

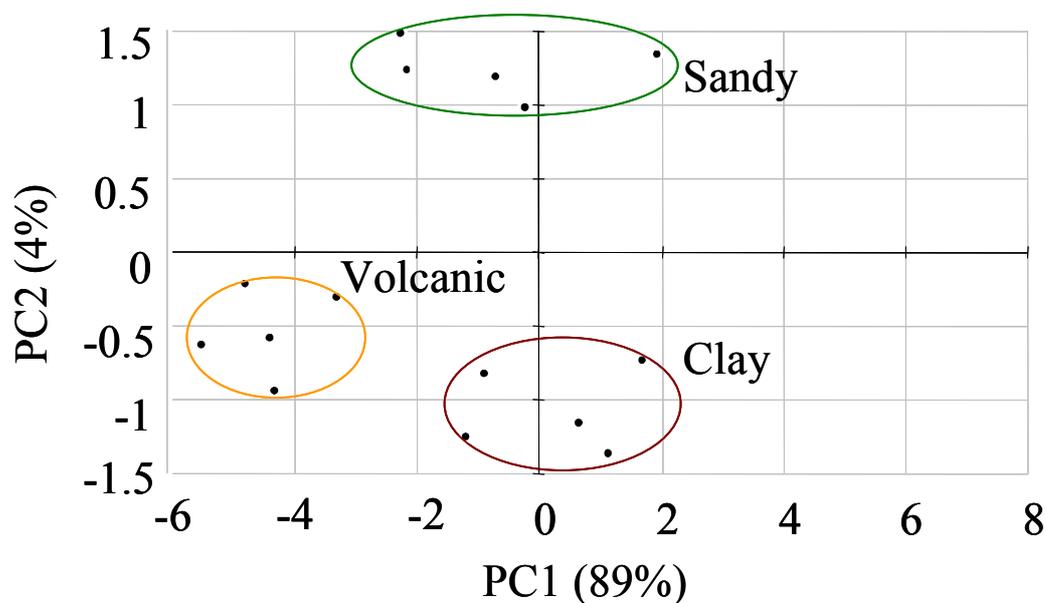


Figure 2.5 PCA plot showing discrimination between samples according to soil type, following 1 day incubation at 25°C, -0.7 MPa. Five replicates of each treatment were used.

Influence of soil water potential and temperature

Soil water potential and temperature had a combined impact on microbial volatile production patterns. For better interpretation of the results and to underline the combined impact of the two factors, the PCA was performed using all the treatments involved in this particular experiment. As an example, Figure 2.6 shows the PCA plot of data for replicates of the clay soil incubated for 7 days at three temperatures (16, 25 and 37°C) and both water potentials (-0.7 and -2.8 MPa). The colour of the line around the clusters represents soil water potential, orange corresponding to the wettest and green to the driest soil treatment, respectively. A clear differentiation between samples at different temperatures as well as at both water potential treatments was obtained, accounting for 87% of the variance within the data. Interestingly, the separation between samples according to temperature was more pronounced under the wettest soil conditions. Similar results were obtained for the sandy loam and volcanic ash soils (see Appendix II for the PCA map of these soils).

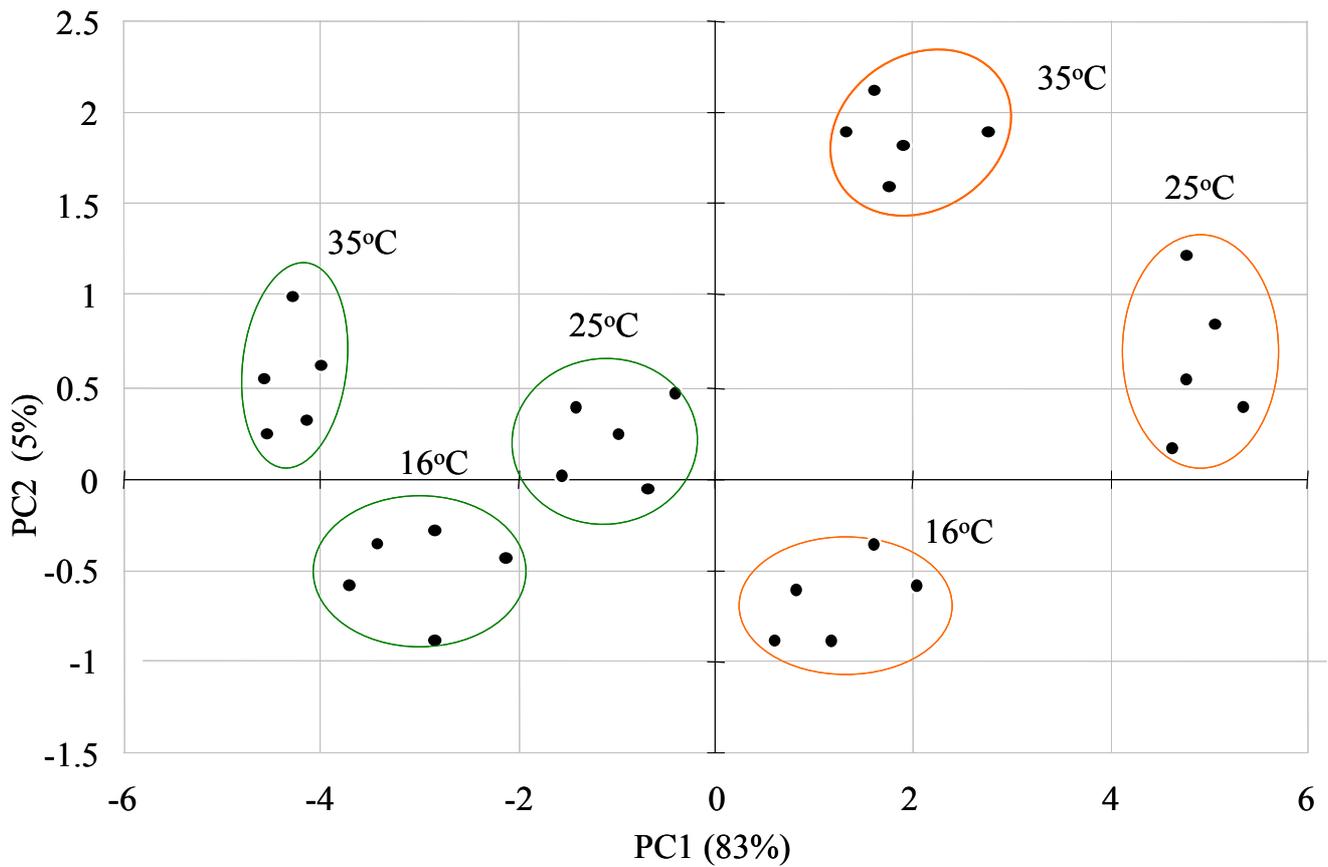


Figure 2.6 PCA map for replicates of the clay soil incubated for 1 day at three temperatures (16, 25 and 37°C) under two moisture regimes, differentiated in the plot by the colour of the line (orange and green for -0.7 and -2.8 MPa respectively). Five replicates of each treatment were used.

Temporal effects of nutrient addition, type and concentration

Figure 2.7 shows the PCA plot of data for samples of calcareous clay soil amended with glucose and wheat straw at two different concentrations and incubated at 25°C for (A) 1 and (B) 10 days. After 1 day of incubation the PCA plot shows two main clusters, corresponding to the glucose-amended samples and the remaining treatments, discriminated along PC1 (Fig 2.7A). On this plot, straw treatments and the untreated controls were grouped together within the same cluster, suggesting similarities between their volatile patterns. However, following a 10 day incubation period, three clusters were attained, indicating that volatile fingerprints of straw-treated soil were now distinctively different from that of the controls (Fig 2.7B). Similar results were obtained for the sandy loam, whereas the volcanic ash produced a higher degree of overlapping, even after 10 day incubation. The temporal separation between clusters of treated soil was the most evident for soil temperatures of 16 and 25°C (data not presented). In terms of the impact of nutrient concentration on soil volatile fingerprints, it was interesting to notice that there was little discrimination between concentrations of the same amendment type independently of soil type, even after a 10 day incubation period.

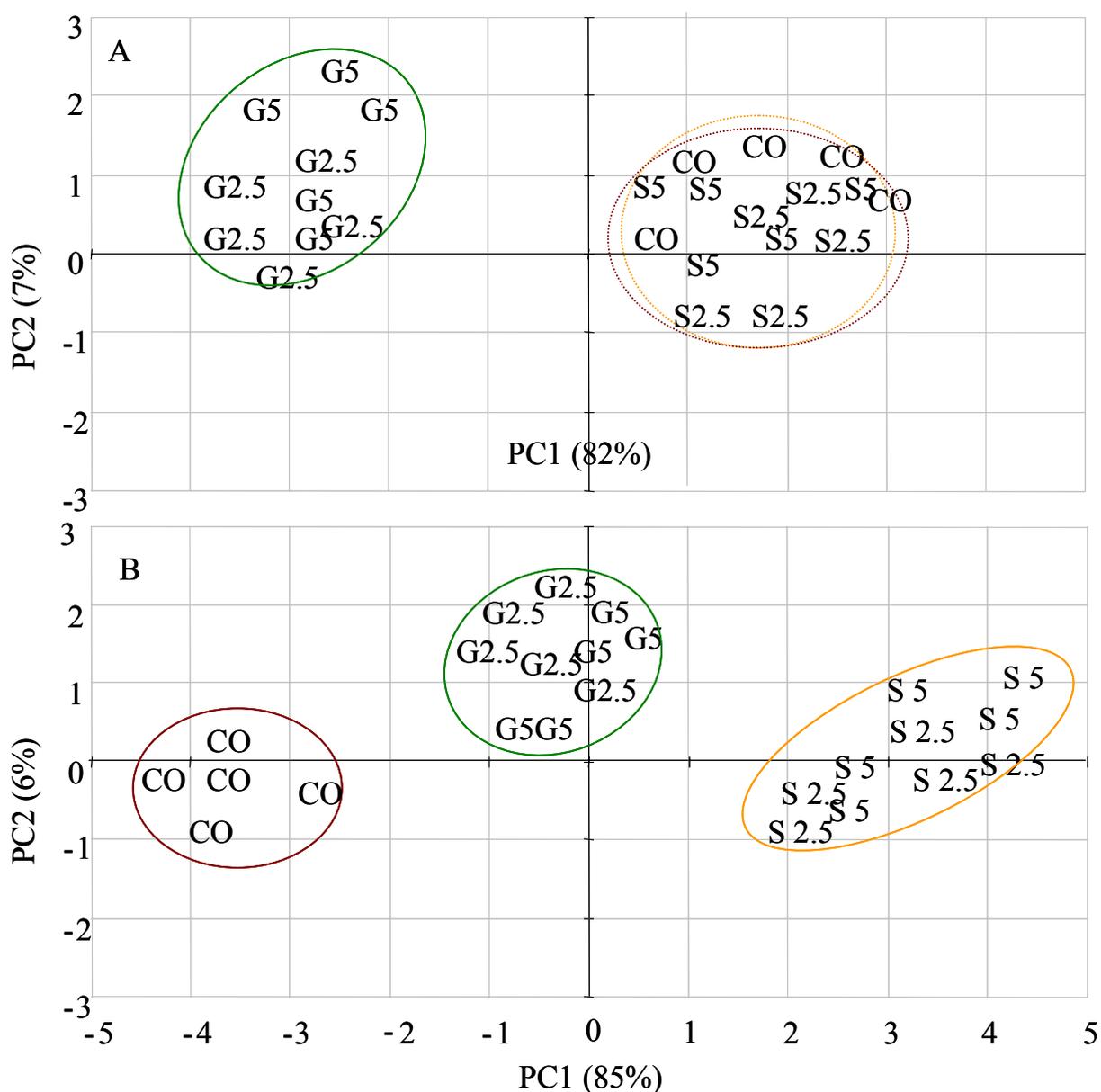


Figure 2.7 PCA map for replicates of clay soil amended with glucose (G) and wheat straw (S) at two concentrations incubated for (A) 1 day and (B) 10 days at 25°C. *Key to treatments:* CO, untreated soil; G 2.5 and G 5, soil treated with glucose at 2.5 and 5 mg g⁻¹ soil; S 2.5 and S 5, soil treated with straw at 2.5 and 5 mg g⁻¹ soil. Five replicates of each treatment were used.

2.3.2 Impact of treatments on soil respiration

Effect of soil water potential and temperature

There was a combined impact of temperature and water potential on soil basal respiratory activity rates, which is shown in Table 2.4. The effect of soil temperature and water potential on respiration was generally significant ($p < 0.01$) for all soil types, but mostly for the sandy loam ($p < 0.001$). However, soil type was the most important ($p < 0.001$) parameter influencing soil basal respiration rate.

Increasing temperatures generally resulted in a significant increase ($p < 0.01$) in soil respiratory activity under the wettest treatment conditions and a decline under the driest treatment. The highest rates were obtained for the sandy loam ($p < 0.001$), particularly under wetter and cooler conditions, reaching a peak ($8.24 \mu\text{g CO}_2 \text{ g}^{-1} \text{ soil h}^{-1}$) at 25°C and -0.7 MPa . In contrast, the clay and volcanic ash soils achieved their highest ($p < 0.01$) respiratory rates under the driest environmental conditions.

Figure 2.8 compares the relative fungal respiration component with that of total soil respiration at both water potential treatments. Under the wettest treatment, 40-50% of total soil respiratory activity derived generally from fungal communities. Under the driest treatment, however, fungal relative activity was found to be the main contribution (over 87%) to soil respiration in the clay and volcanic ash soils. In contrast, total respiration in the sandy loam was predominantly from bacterial relative activity under both water potential treatments, even under the driest soil conditions.

Table 2.4 Summary of the impact of temperature and water potential on soil basal respiration rates, following 1 day incubation. Differences between respiration rates according to temperature and water potential were always statistically significant at $p < 0.05$, $n = 5$. Basal respiration rate was given by the average of five replicates (\pm standard error) and was expressed as $\mu\text{g CO}_2 \text{ g}^{-1} \text{ soil h}^{-1}$.

	-0.7 MPa			-2.8 MPa		
	16°C	25°C	37°C	16°C	25°C	37°C
Sandy loam	6.87	8.24	5.95	6.28	5.26	4.35
	± 0.10	± 0.20	± 0.11	± 0.10	± 0.13	± 0.01
Clay soil	2.98	3.01	3.20	4.58	4.35	4.12
	± 0.09	± 0.02	± 0.08	± 0.02	± 0.06	± 0.10
Volcanic ash	2.52	2.75	3.43	3.66	3.42	4.35
	± 0.07	± 0.09	± 0.13	± 0.09	± 0.08	± 0.09

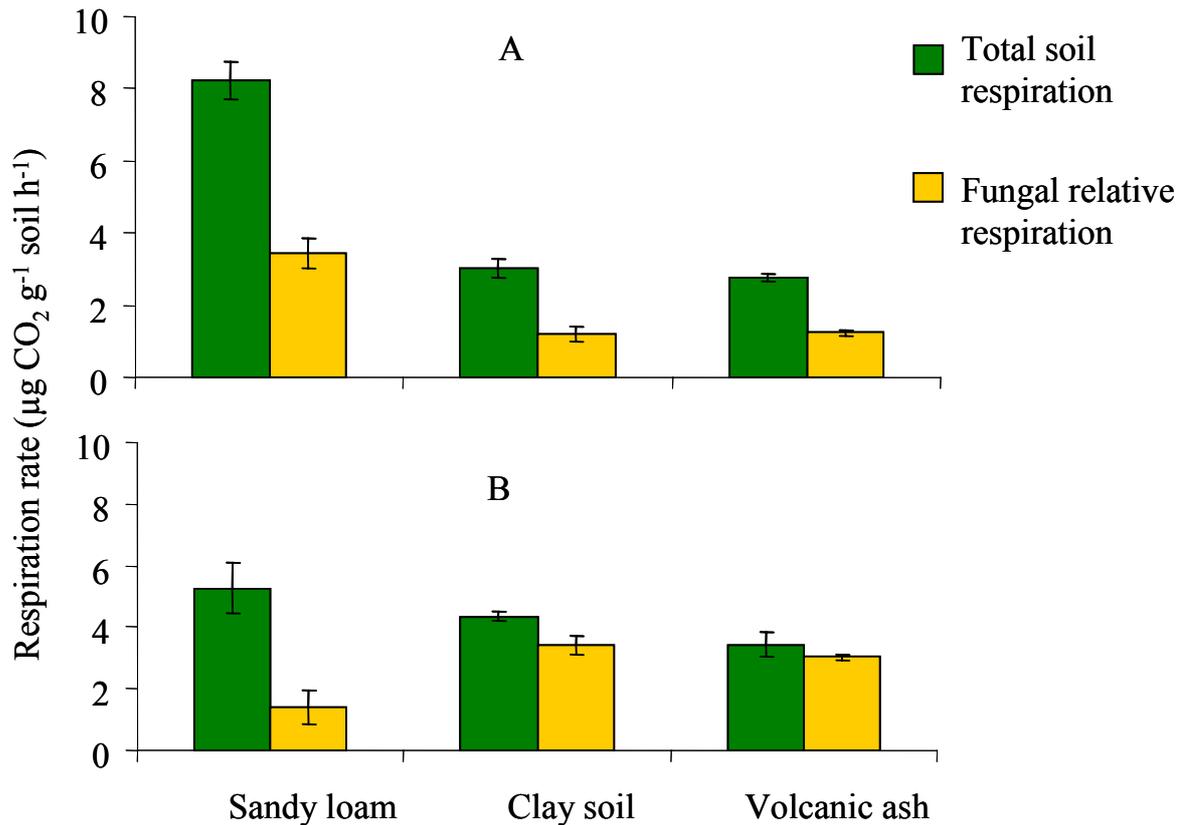


Figure 2.8 Comparison between soil total respiration and that of the fungal relative component at (A) -0.7 MPa and (B) -2.8 MPa following 24 h incubation at 25°C. Vertical bars indicate standard errors of means of five replicates.

Temporal effects of nutrient addition

SIR response of soils to nutrient inputs was evaluated at both soil water potentials, in order to study the status of soil microbial activity in each soil. Two nutrient-related parameters were investigated in terms of potential impact on soil respiration: nutrient type and concentration.

Figure 2.9 compares the cumulative CO₂ production curve between (A) un-amended soil (control), (B) soil amended with glucose (2.5 mg g⁻¹) and (C) soil amended with wheat straw (2.5 mg g⁻¹), following incubation at 25°C and -0.7 MPa over a 10 day period. Figure 2.9A shows the respiration curve for the control treatment, where the highest respiratory activity is achieved by the sandy loam, followed by the other two soils.

Within the first 24h of incubation, the clay and volcanic ash soils do not present significant differences between their respiratory activity rates.

Following glucose addition (Figure 2.9B), it is evident an immediate increase in respiration (of over 20%) for the three soils within the first 3 hours, in particular for the sandy loam presenting a peak ($41 \mu\text{g CO}_2 \text{ g}^{-1} \text{ soil h}^{-1}$) around the first day of incubation. In contrast, CO_2 production by the clay and volcanic ash soils continued rising for up to 10 days, at which time both their respiratory rates had achieved an overall increase of over 75%, when compared to that of the un-amended soil. Interestingly, there was no significant ($p = 0.61$) difference between the respiration rates of these latter two soils within a 10 day incubation period following glucose addition. Unlike glucose, wheat straw addition (Figure 2.9C) did not have a significant effect on the respiration of the three soils within the first day of incubation. By the end of the study period, the overall increase in CO_2 production induced by straw when comparing to un-amended soil was the lowest for the sandy loam (25%) and the highest (81%) for the volcanic ash followed by the clay soil (65%).

At -2.8 MPa, the pattern of response to nutrients was generally similar to that presented, although of a lower magnitude. In terms of nutrient concentration, the impact on soil respiration was generally not significant at the rates studied, regardless of soil water potential.

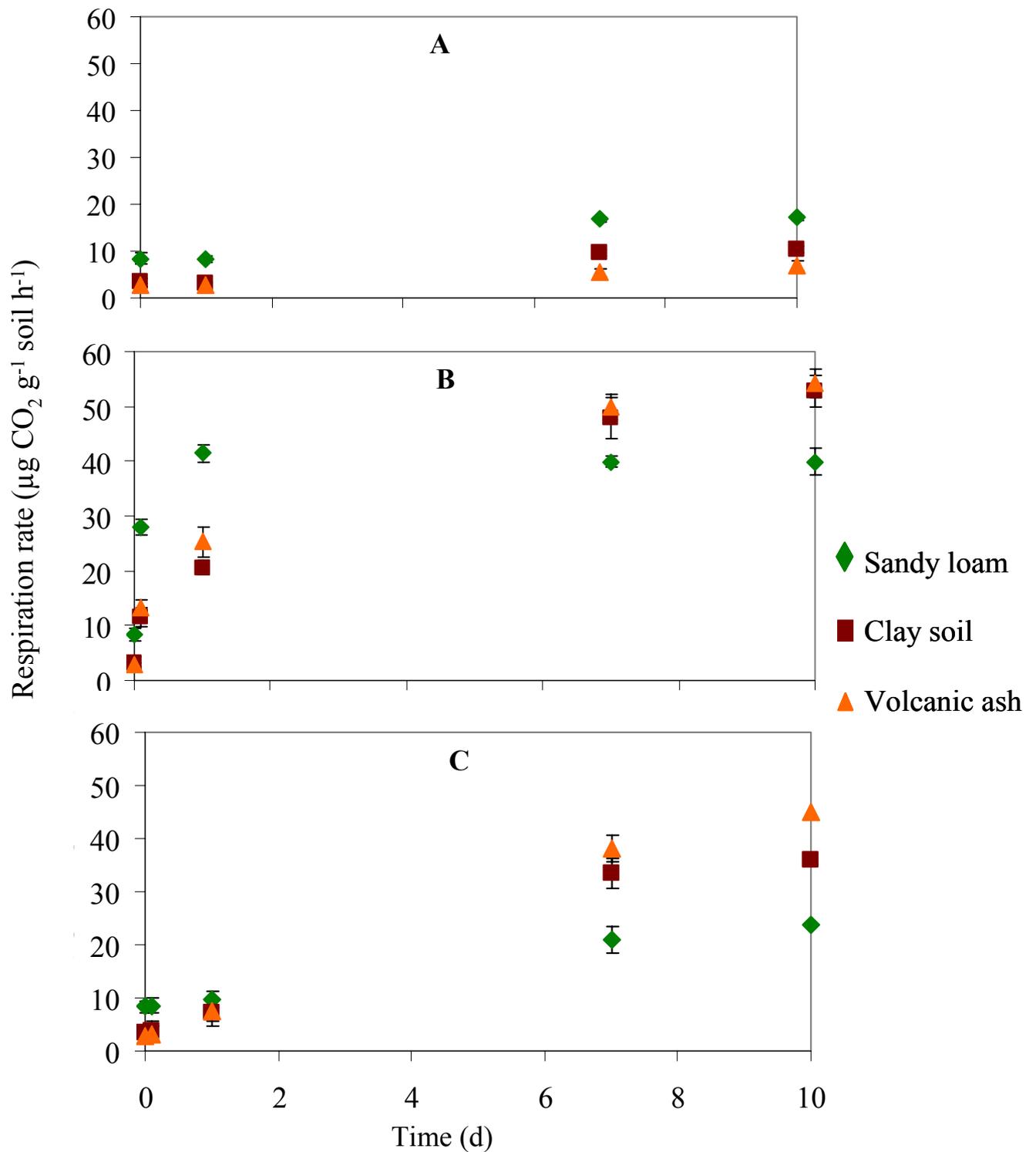


Figure 2.9 Cumulative CO_2 production curve for (A) un-amended soil (control), (B) glucose-amended soil and (C) wheat straw-amended soil, following incubation at 25°C and -0.7 MPa over a 10 day period. Vertical bars represent standard errors of means of five replicates.

2.3.3 Impact of treatments on dehydrogenase activity

Influence of soil water potential and temperature

Table 2.5A summarises the impact of soil temperature and water potential on soil DHA over a 24 h incubation period. The DHA values for the same treatments in the autoclaved control soil are given in Table 2.5B. Overall, water potential had a significant ($p < 0.01$) impact on the activity of this enzyme at all temperatures tested, even after short incubation periods. DHA was generally nearly 30% higher ($p < 0.001$) under the wetter than under the drier soil conditions, regardless of soil type. In contrast, the effect of temperature was often not statistically significant ($0.05 < p < 0.10$), especially for the clay and volcanic ash soils under 16 and 25°C, regardless of soil water potential. The reduction (in over 43%) of activity in the sandy loam when temperatures increased from 25 to 37°C at both water regimes was however significant ($p = 0.02$). Surprisingly, the clay soil presented the highest enzymatic activity irrespective of its temperature and water potential, at least 50% higher than that of the sandy loam.

Table 2.5 Summary of the impact of temperature and water potential on soil DHA following 1 day incubation (Table 2.5A). Differences between DHA as influenced by water potential were always significant at $p < 0.05$, $n = 5$. DHA was obtained after subtraction to that in autoclaved control soil, given as reference in Table 2.5B. DHA was given by the average of five replicates (\pm standard error) and was expressed as $\mu\text{g INF g}^{-1}$ soil 2h^{-1} .

A)

	-0.7 MPa			-2.8 MPa		
	16 °C	25 °C	37 °C	16 °C	25 °C	37 °C
Sandy loam	95 ± 2.86	102 ± 4.88	44 ± 1.07	60 ± 2.54	56 ± 1.22	32 ± 1.28
Clay soil	185* ± 10.9	215* ± 12.2	200 ± 4.75	160* ± 4.74	151* ± 8.03	139 ± 5.90
Volcanic ash	53* ± 1.87	58* ± 2.52	51 ± 1.65	38* ± 2.06	34* ± 2.96	29 ± 1.36

B)

	-0.7 MPa			-2.8 MPa		
	16 °C	25 °C	37 °C	16 °C	25 °C	37 °C
Sandy loam	0.08 ± 0.01	0.08 ± 0.00	0.07 ± 0.02	0.09 ± 0.01	0.08 ± 0.02	0.08 ± 0.02
Clay soil	0.08 ± 0.02	0.07 ± 0.01	0.08 ± 0.03	0.09 ± 0.02	0.08 ± 0.01	0.08 ± 0.00
Volcanic ash	0.06 ± 0.00	0.06 ± 0.01	0.06 ± 0.02	0.06 ± 0.00	0.06 ± 0.02	0.06 ± 0.00

* For each soil water potential value, differences in DHA as influenced by temperature were not statistically significant at $p < 0.05$, $n = 5$.

Temporal effects of nutrient addition

Two nutrient-related parameters were investigated in terms of potential impact on soil DHA: nutrient type and concentration. Nutrient addition induced significant responses in soil DHA within the study period under both water regimes, although no significant differences were found between treatment concentrations at the rates tested (data not presented). As an example, Figure 2.10 compares the cumulative DHA in (A) un-amended soil (control) to that of soil amended with (B) glucose and (C) wheat straw, both at 2.5 mg g^{-1} soil, under 25°C and -0.7 MPa .

Independently of the added nutrient, the clay soil generally had the highest DHA over the 10 day period. In contrast, the volcanic ash had the lowest DHA before nutrient addition, although it was also the treatment showing the highest responses to nutritional amendment over the study period when compared against the control treatments. By the end of the study, this soil had significantly increased its DHA by 71% and 75% as a response to glucose and straw respectively. Over the same time period, straw was the nutrient responsible for the highest activity increases in the clay and volcanic ash soils (34 and 75% respectively). Glucose was on the other hand, particularly favourable for the sandy loam (36%). However, within short periods of time such as 1 day, only the effect of glucose was significant ($p=0.0004$) comparing with that of straw ($p=0.08$).

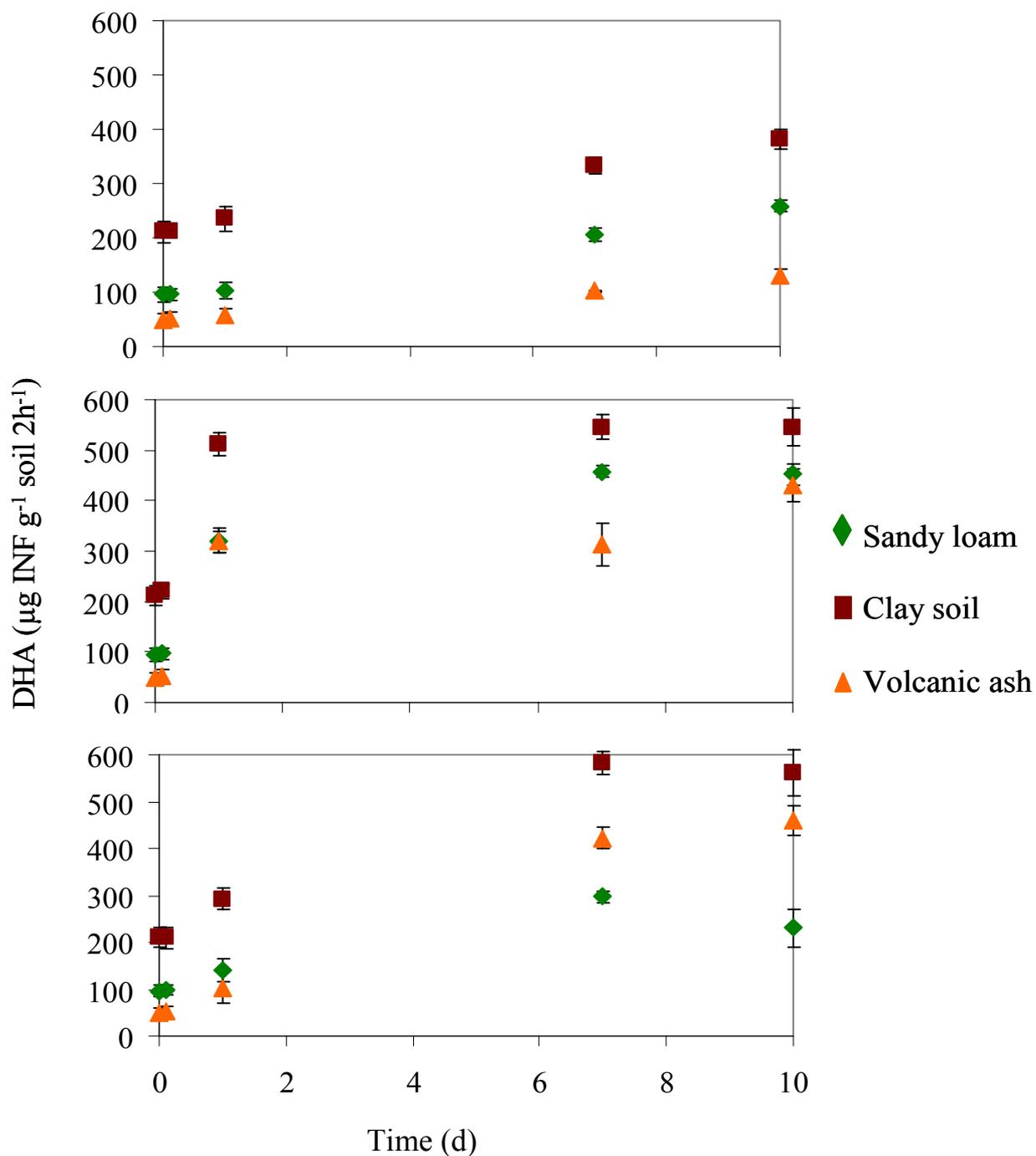


Figure 2.10 Cumulative DHA for (A) un-amended soil (control), (B) glucose-amended soil and (C) wheat straw-amended soil, following incubation at 25°C and -0.7 MPa.

Vertical bars represent standard errors of means of five replicates.

2.3.4 Impact of treatments on soil microbial population size

Influence of soil water potential and temperature

Table 2.6 summarises the combined impact of temperature and water potential on soil total as well as bacterial and fungal relative population sizes, following 7 day incubation. Total microbial population size was assumed to be the sum of the bacterial and fungal relative contributions.

In general, the effect of temperature on total CFUs was not significant ($P > 0.08$), whereas that of water potential was significant ($p < 0.03$) only for the clay and volcanic ash soils. Also, total CFUs in the sandy loam were statistically different ($0.01 < p < 0.04$) from that in the other two soils but only under the wettest treatment. The influence of soil type on this microbial parameter was not significant ($0.05 < p < 0.10$) under the driest soil conditions.

In terms of relative population sizes, bacterial CFUs were generally favoured by cooler and wetter soil conditions, whereas fungal communities thrive best under drier and hotter treatments. This was particularly evident for the clay and volcanic ash soils where surprisingly, differences between bacterial and fungal population sizes were only significant at -0.7 MPa. Interestingly, at -2.8 MPa fungal populations in these later soils were shown to be at least twice as abundant as were at -0.7 MPa.

Table 2.6 Summary of the impact of temperature and water potential on soil microbial CFU (Log_{10} CFUs g^{-1} soil) after a 7 day incubation period. The impact of temperature was generally not statistically significant at $p < 0.05$, $n = 5$. Five replicates (\pm standard error).

		- 0.7 MPa			- 2.8 MPa		
		16°C	25°C	37°C	16°C	25°C	37°C
Sandy loam	Bacteria	7.60 ± 1.41	7.69 ± 1.23	7.58 ± 1.99	6.70 ± 0.16	6.37 ± 1.10	6.22 ± 0.50
	Fungi	6.28 ± 0.98	6.52 ± 0.12	6.00 ± 0.90	6.52 ± 0.80	6.31 ± 0.90	6.31 ± 1.30
	Total	13.9 ± 1.43	14.2 ± 1.21	13.6 ± 1.23	13.2 ± 1.97	12.7 ± 1.83	12.5 ± 2.50
Clay soil	Bacteria	7.30 ± 1.19	7.45 ± 0.36	7.48 ± 0.45	6.70 ± 0.91	6.64 ± 0.90	6.32 ± 1.00
	Fungi	2.64 ± 0.11	3.58 ± 0.13	3.81 ± 0.12	7.82* ± 0.70	7.65* ± 1.14	7.56* ± 0.99
	Total	9.94 ± 1.01	11.0 ± 0.99	11.3 ± 0.79	14.5* ± 1.12	14.3* ± 1.31	13.9* ± 1.04
Volcanic ash	Bacteria	7.00 ± 0.10	7.30 ± 1.24	6.47 ± 0.24	6.22 ± 0.50	6.52 ± 0.60	6.70 ± 0.50
	Fungi	3.43 ± 0.16	3.57 ± 0.14	2.61 ± 0.10	7.62* ± 0.55	7.42* ± 0.55	7.58* ± 0.45
	Total	10.4 ± 1.88	10.9 ± 1.14	9.08 ± 0.87	13.8* ± 1.32	14.0* ± 1.25	14.3* ± 1.08

* Statistically significant at $p < 0.05$, $n = 5$ when comparing to that at -0.7 MPa.

Temporal effects of nutrient addition

The temporal impact of adding 2.5 mg g⁻¹ soil of (A) glucose and (B) wheat straw on soil total microbial population size at 25°C and -0.7 MPa is presented as an example in Figure 2.11. Over the shortest incubation period, nutrient addition generally did not have a significant ($0.12 < p < 0.45$) impact on total microbial CFUs. In the longer-term however, glucose-amended soil showed a generalised increase of over 50% irrespective of soil type when comparing against the control. For the same time period, this nutrient favoured microbial community development in the sandy loam. Similarly, the impact of straw over 10 day incubation was responsible for a considerable increase in soil total CFUs, particularly in the clay and volcanic ash soils where community size was found to have doubled. In contrast, straw addition only caused an approximate 27% increase in CFUs in the sandy loam.

A similar pattern of results was achieved under -2.8 MPa (data not shown). The highest treatment concentration (5 mg g⁻¹ soil) generally did not produce significant differences in terms of total CFUs, when compared with the treatment presented (data not shown).

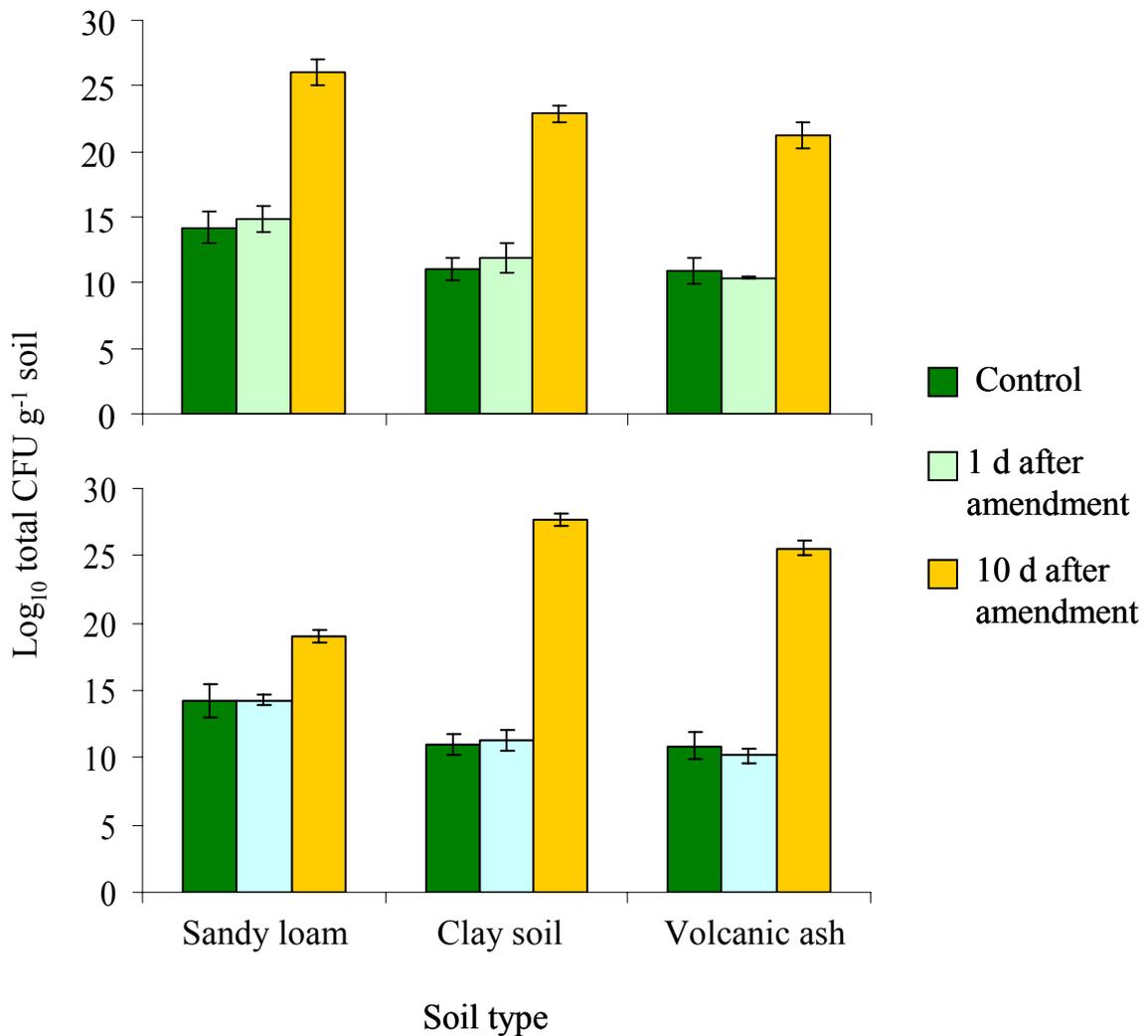


Figure 2.11 Temporal changes in soil total CFUs following nutritional treatment with (A) glucose and (B) wheat straw, both at 2.5 mg g^{-1} soil, after 1 and 10 days of incubation at 25°C and -0.7 MPa . Vertical bars indicate standard errors of means of five replicates.

2.3.5 Impact of treatments on community structure

Impact of temperature and water potential

Table 2.7 and Figure 2.12 show the impact of temperature and water potential on the number of different fungal species growing on MEA and community structure, respectively, in clay soil (given as example), following 10 day incubation. In general, there was a significantly ($p < 0.02$) higher abundance of species under the wettest soil treatments when compared to the drier conditions, independent of soil type. However, irrespective of soil water regime, temperature did not have a significant ($0.05 < p < 0.08$) effect on fungal diversity but rather on the relative abundances of the genera they belonged to. Differences between the number of species found according to soil type, were in the overall significant ($p < 0.03$) for all treatment environmental conditions. The most common genera isolated from the three soils were *Aspergillus* (20-50%), *Penicillium* (20-50%), *Fusarium* (5-10%) and *Rhizopus* (5-10%).

In un-amended soil, those four genera were abundant in the three soils mainly under wetter conditions, although *Fusarium* was not shown to grow under drier and hotter soil conditions. Their relative abundances however were found to vary according to soil type, temperature and water potential. In drier soil conditions, *Penicillium* and *Aspergillus* were abundant and often the only genera found, particularly in volcanic ash soil. Other genera were found exclusively in the sandy loam, including *Alternaria* and *Doratomyces*, with relative abundances ranging between 0-20% according to temperature.

Table 2.7 Impact of temperature and water potential on fungal diversity. Contrary to water potential, temperature did not produce statistically significant differences on fungal diversity at $p < 0.05$, $n = 5$. Diversity was given by the average of five replicates (\pm standard error) and was expressed as the number of fungal species g^{-1} soil growing in MEA for up to 10 days.

	- 0.7 MPa			-2.8 MPa		
	16 °C	25 °C	37 °C	16 °C	25 °C	37 °C
Sandy	7 \pm 0.26	7 \pm 0.33	6 \pm 0.27	6 \pm 0.25	4 \pm 0.22	3 \pm 0.19
Clay	6 \pm 0.17	6 \pm 0.14	5 \pm 0.18	5 \pm 0.11	5 \pm 0.10	5 \pm 0.17
Volcanic	4 \pm 0.19	4 \pm 0.13	4 \pm 0.15	3 \pm 0.12	3 \pm 0.11	2 \pm 0.14

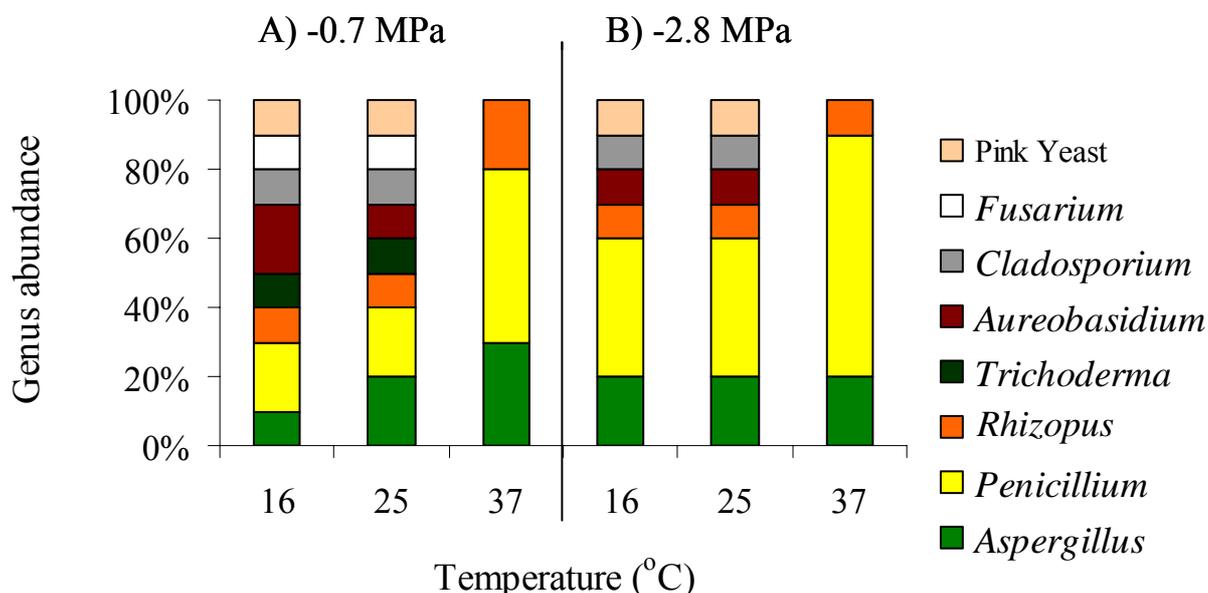


Figure 2.12 Impact of soil temperature and water potential on fungal community structure in un-amended clay soil, following 10 days incubation at 25°C and (A) -0.7 and (B) -2.8 MPa.

Temporal effects of nutrient addition

Table 2.8 and Figure 2.13 show the influence of soil amendment and nutrient type on the number of fungal species isolated on MEA and fungal community structure, respectively, in clay soil (given as example), following 10 days incubation at 25°C and -0.7 MPa. Generally, there was a significant ($p < 0.001$) impact of nutrient addition on the abundance of species when comparing to the control. Between the two, glucose was the nutrient producing the highest increase (over 30%) in diversity on the longer-term, particularly evident for the clay soil. However, when added in excess (5.0 mg g⁻¹ soil) glucose was only favourable for the volcanic ash. Similarly to glucose, fungal diversity also responded positively to the addition of wheat straw at the lowest concentration, compared against the control. Yet, unlike glucose, the impact of excess wheat straw (5.0 mg g⁻¹ soil), was rather small.

Penicillium, *Aspergillus*, *Fusarium* and *Rhizopus* were also identified in amended soil and were still common to the three soil types under the temperatures and nutrient contents examined. *Acremonium* and *Epicoccum* were also found in both clay and sandy loam, predominantly in straw-amended soil. While *Acremonium* was found under both water potentials, *Epicoccum* was common under the hottest treatments. In the sandy loam, *Alternaria* and White yeasts also showed to be present but only at 16 and 25°C in wetter soil.

Table 2.8 Impact of adding glucose (+G) and straw (+S) at two concentrations (2.5 and 5.0 mg g⁻¹ soil) on fungal diversity, compared to untreated control soil (S) for the three soil types. Diversity was given by the average of five replicates (\pm standard error) and was expressed as the number of fungal species g⁻¹ soil growing in MEA for up to 10 days.

	Sandy loam	Clay soil	Volcanic ash
S	7 \pm 0.23	6 \pm 0.09	4 \pm 0.40
S+G (2.5 mg g ⁻¹)	13* \pm 0.11	14* \pm 0.30	6* \pm 0.15
S+G (5.0 mg g ⁻¹)	9* \pm 0.08	14* \pm 0.57	11* \pm 0.22
S+S (2.5 mg g ⁻¹)	10* \pm 1.08	11* \pm 0.81	10* \pm 0.09
S+S (5.0 mg g ⁻¹)	10* \pm 0.46	10* \pm 0.62	10* \pm 0.43

* Statistically significant at $p < 0.05$, $n = 5$.

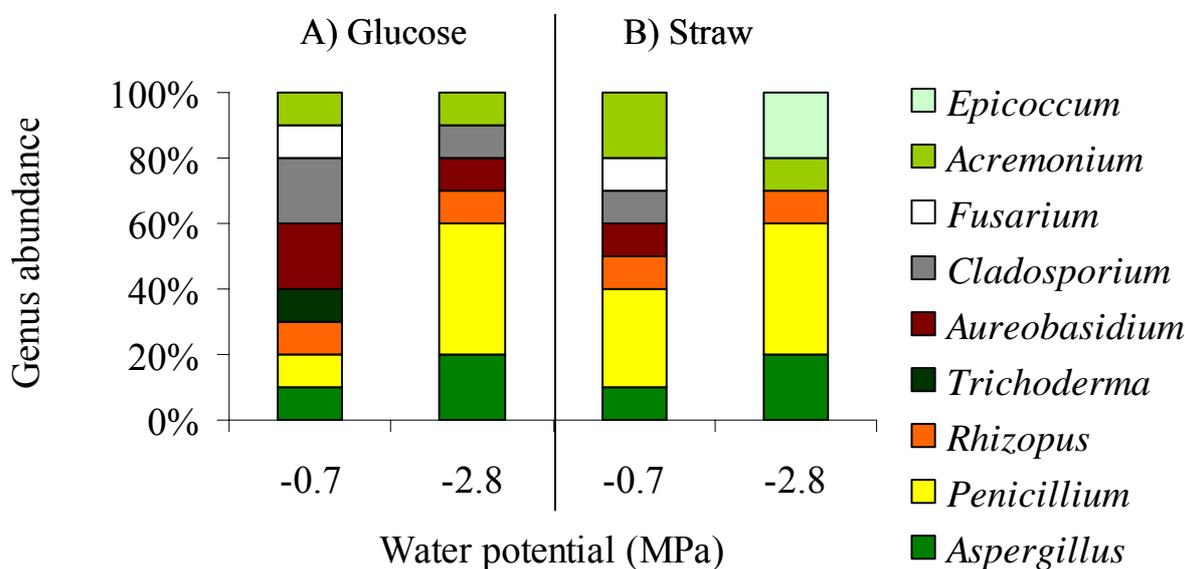


Figure 2.13 Impact of nutrient addition (A) glucose and (B) straw on fungal community structure in clay soil, following 10 days incubation at 25°C under -0.7 or -2.8 MPa.

2.3.6 Correlations between microbial parameters

Statistical correlations between the different microbial parameters were explored at the different treatments using general regression models. Table 2.9 (A;B) show the correlation coefficients between the different microbial parameters tested in un-amended and glucose-amended soil (respectively) at 25°C and -0.7 MPa over the study period. In the results presented, PC1 was used as representative of the overall information leading to discrimination between treatments.

In untreated soil, all traditional parameters (respiration, DHA and total CFUs) were found to be significantly correlated. PC1 was also found to be strongly correlated to those three parameters in the longer-term, but not over the shortest incubation time. Yet, a strong link between PC1 and respiration was found independent of the time period.

In glucose-amended soil incubated for 1 day, SIR was shown to be correlated to DHA as well as to PC1 but no other significant relationships were found under such soil conditions. In contrast to that in untreated soil, CFUs were not related to the remaining parameters over the short-term, although over the study period this situation changed. Also unlike in untreated soil, PC1 was poorly correlated with both CFUs and DHA, independent of the incubation period.

Another significant ($p < 0.05$) result was obtained by looking at the relationship between PC1 and the number of fungal species present in both untreated and glucose-treated soil (Table 2.10). Generally, these parameters were negatively correlated independently of the time period, particularly for the sandy loam and clay soils.

Table 2.9 Correlations at $p < 0.05$ between different microbial parameters in (A) unamended and (B) glucose-amended soil under 25°C and -0.7 MPa over the study period. Black and green values correspond to 1 and 10 days incubation periods. *S*, *C* and *V* refer to sandy loam, clay soil and volcanic ash, respectively.

A)

Correlation coefficient (r)				
	Respiration	DHA	Total CFUs	PC1
Respiration	1	<i>S</i> 0.854* 0.881*	<i>S</i> 0.547* 0.507*	<i>S</i> 0.823* 0.853*
		<i>C</i> 0.536* 0.640*	<i>C</i> 0.522* 0.539*	<i>C</i> 0.809* 0.811*
		<i>V</i> 0.793* 0.862*	<i>V</i> 0.585* 0.441	<i>V</i> 0.871* 0.825*
DHA	-	1	<i>S</i> 0.653* 0.520*	<i>S</i> 0.430 0.604*
			<i>C</i> 0.612* 0.579*	<i>C</i> 0.317 0.521*
			<i>V</i> 0.598* 0.511*	<i>V</i> 0.285 0.577*
Total CFUs	-	-	1	<i>S</i> 0.287 0.583*
				<i>C</i> 0.317 0.604*
				<i>V</i> 0.285 0.533*
PC1	-	-	-	1

B)

Correlation coefficient (r)				
	SIR	DHA	Total CFUs	PC1
SIR	1	<i>S</i> 0.781* -0.854*	<i>S</i> 0.331 -0.586*	<i>S</i> 0.837* 0.225
		<i>C</i> 0.750* 0.799*	<i>C</i> 0.201 0.552*	<i>C</i> 0.894* 0.283
		<i>V</i> 0.673* 0.608*	<i>V</i> 0.262 0.559*	<i>V</i> 0.666* 0.309
DHA	-	1	<i>S</i> 0.424 0.499*	<i>S</i> 0.490 0.402
			<i>C</i> 0.402 0.576*	<i>C</i> 0.385 0.428
			<i>V</i> 0.371 0.492*	<i>V</i> 0.312 0.433
Total CFUs	-	-	1	<i>S</i> 0.230 0.319
				<i>C</i> 0.216 0.367
				<i>V</i> 0.207 0.398
PC1	-	-	-	1

* Statistically significant at $p < 0.05$, $n = 5$.

Table 2.10 Correlation between PC1 and the number of different fungal species in un-amended and glucose-amended soil under 25°C and -0.7 MPa over the study period. Black and green values correspond to 1 and 10 days incubation periods.

Correlation coefficient (r)				
PC1 vs. number of fungal species				
	Un-amended soil		Glucose-amended soil	
Sandy loam	-0.833*	-0.851*	-0.854*	-0.551*
Clay soil	-0.719*	-0.798*	-0.792*	-0.698*
Volcanic ash	0.705*	-0.734*	0.680*	-0.622*

* Statistically significant at $p < 0.05$, $n = 5$.

2.4 DISCUSSION

Because soil is such a complex environment, numerous factors may influence microbial growth and activity. The fact that soil microorganisms respond much quicker to changes in soil conditions than any other soil component, makes them an early and reliable indicator of such changes. It is thought that the quick response to altering soil conditions consist of adjusting population size, activity rate, community structure and diversity (Bloem *et al.*, 2006).

Temperature, water potential and nutrient inputs were the three main factors tested in this research in terms of their short term (considered to be up to 10 days) impact on soil basal and potential metabolic activity rate, volatile production patterns, microbial population size and fungal diversity. The potential use of qualitative soil volatile fingerprints for characterising soil microbial activity was explored in detail, as was the correlation between this and traditional microbial parameters. Also, the comparison between all these parameters in different soils, allowed gathering information on the way soil type determines the characteristics of its native microbial communities and the way they respond to such key environmental factors. Finally, data obtained for the volcanic ash soil throughout this study was highly relevant, since very little research has been done using this soil at the microbial level.

2.4.1 Soil volatile fingerprints

This study examined the potential of using an array of non-specific conducting polymer sensors for discriminating between soil types and different soil environmental conditions and treatments, based on qualitative volatile fingerprint analysis. Its feasibility to be employed for detecting changes in soil microbial activity as influenced by environmental factors was explored.

Differences in volatile production patterns clearly discriminated between the different soil types by PCA, even over very short incubation periods such as 1 day. Such

discrimination is likely to reflect differences between their physical and chemical properties. As previously mentioned, soil characteristics are known for determining not only the characteristics of native microbial communities (in terms of community size, diversity and metabolic activity) but also that of native soil organic matter (van Gestel *et al.*, 1991; Amato and Ladd, 1992; Jarvis *et al.*, 1996; Saggiar *et al.*, 1996; Brady and Weil, 2002; Verheijen *et al.*, 2005). Therefore, it may consequently influence the type and amount of volatiles produced by microbial activity. This hypothesis is also supported by Stotzki and Schenck (1976), who have suggested that the type of volatiles produced by soils with differing characteristics may differ markedly even when subjected to the same soil conditions.

Generally, PC1 accounted for most of the variance within the data, suggesting that for the soil treatments involved in this study, headspace volatiles can be characterised by PC1 alone, without much loss of variance (Abbey *et al.*, 2001). As an exception, PC2 explained almost on its own the discrimination between the clay and sandy loam soils, following 1 day incubation at 25°C, whereas both were separated from the volcanic ash mainly by PC1. This may indicate that differences between headspace volatiles in the volcanic ash and in the other two soils are probably related to volatile intensity, perhaps reflecting its poor content of readily-available organic matter when compared to that in the other two soils. The relationship between PC1 and volatile intensity is discussed in further detail in the Discussion section in Chapter IV.

Soil temperature and water potential were shown to have a considerable impact on soil volatile patterns, irrespective of the soil matrix. This was clear by the differentiation obtained between samples at different temperatures as well as at both water potential treatments for all soil types. Interestingly, the impact of both factors on soil volatile fingerprints was clearly correlated. This was obvious given the separation between clusters according to temperature being more pronounced under the wettest rather than the driest soil conditions.

The differential temporal response of soil volatile patterns to the addition of a labile and a recalcitrant nutrient was equally interesting, and is likely to arise from differences between their nutritional composition and subsequent assimilation rates by soil microbial

communities. After very short incubation periods (1 day) the PCA plot showed only two main clusters, corresponding to the glucose-amended samples and the remaining treatments respectively. On this plot, straw-amended soil and untreated controls were clearly overlapping within the same cluster, suggesting similarities between their volatile patterns. Over an extended incubation period (10 days) however, three clusters could be seen on the plot, indicating that volatile fingerprints of straw-treated soil were now distinctively different from that of the controls. The fact that straw degradation is a considerably slower process when compared to that of glucose, explain the lag phase in microbial activity observed immediately after its addition to soil. In terms of soil volatile fingerprints, this lag is represented by the overlapping between straw-treated soil and the controls within short incubation periods.

Interestingly, although the differentiation between soil types by PCA was possible under all soil temperatures and water potentials studied, the discrimination level was generally higher for the wetter and cooler soil conditions. Similarly, the temporal discrimination between soil volatile patterns as a response to nutrient addition was dependent on soil type and the treatment conditions, being the most evident for the sandy and clay soils under -0.7 MPa at 16 and 25°C. This is probably due to the enhanced microbial activity resulting in a wider range of volatiles being produced under higher water availability and lower soil temperatures, as supported by data on respiration and DHA. It may also be a reflection of the higher microbial density and fungal community structure under such conditions, corroborated by microbial counts and fungal diversity analysis. In the same way, the differential assimilation of nutrients according to soil treatment is not surprising. Sommers (1981) and Magan (1988) have both demonstrated that nutrient decomposition and utilisation can vary markedly according to soil type and its environmental conditions.

Changes in soil water and temperature regimes as well as nutrient addition and consequent utilisation are likely to have induced changes in the structure of soil microbial community by determining which microorganisms are metabolically active (as well as their relative abundance) under such conditions (Wardle and Parkinson, 1990; Mackie and Wheatley, 1999; Williams and Rice, 2007). Thereby, a change in microbial community structure is probably a key factor for explaining dissimilarities between soil

volatile production patterns as response to treatments. For example, the immediate effect of glucose was probably due to enhanced activity of glucose-responsive microorganisms such as bacteria, whereas fungi are known to be the first colonisers of straw (Potthoff *et al.*, 2001). Alternatively, changes in microbial activity may also be responsible for variations in the volatile pattern as a response to treatments. For example, it was clear in this study that changes in microbial metabolic activity may also occur rapidly as a response to a variety of factors, without that resulting in growth or a change in community structure.

Interestingly, there was very little discrimination between concentrations of the same amendment (at the rates studied) independently of soil type, even after a 10 day incubation period. It is likely that nutrient type rather than concentration influences soil volatile patterns the most, at least under low water potential conditions. One reason for this may be related to the fact that higher nutrient concentration probably results in a higher amount of volatiles being produced rather than in an alteration of the volatile fingerprint.

Very limited number of studies has focused on the study of how soil environmental factors influence soil microbial volatile production patterns and it is therefore difficult to compare data obtained in this study with that available in the literature. Yet, beside Stotzki and Schenck (1976), two other studies seem to corroborate these findings, since they also suggest that soil volatile patterns are likely to be influenced by the same key environmental factors influencing soil microbial activity. For example, Wheatley and co-workers (1996) identified a total of 35 VOCs in the headspace of a silt/clay loam soil using GC-MS. The predominant groups of volatiles found as well as their relative concentrations were different according to soil aeration status during incubation. More recently, Sheppard and Lloyd (2002, 2004) used membrane-inlet-mass spectrometry (MIMS) for analysing soil depth profiles of gases such as O₂, CO₂, Ar, CH₄, N₂ and NO_xs. They found that gas profiles were not only associated with soil depth but also changed following nutrient addition (sewage sludge or lime) into soil. Similar results have also been reported by Stotzki and Schenck (1976), who found that the addition of substrates into soil may give rise to a variety of volatiles, depending on the nutrient

itself, on the microbial species using it and on whether that is performed aerobically or anaerobically.

Variations in the patterns of VOC production may thus function as early indicators of changes in soil conditions. Corroborated by those authors, it is thereby likely that changes in volatile production patterns are closely related to changes in microbial activity and community structure as a response to soil environmental factors, suggesting a relevant correlation between these different aspects of soil microbial community. Even more relevant is the indication that soil volatile pattern analysis can be employed as a complementary tool for assessing and characterising microbial community response to changes in soil conditions. Moreover, while a direct identification of soil microorganisms as well as of specific metabolites is difficult, volatile fingerprints can easily and rapidly be assessed, and are sensitive enough to detect variations within very short incubation periods. This may be particularly useful in situations where other microbial parameters do not respond fast enough, or for monitoring specific microbial activities.

2.4.2 Soil basal respiration

Basal respiration from soil under standardised conditions was used as an indicator of the whole microbial heterotrophic activity, thus providing a measure of soil metabolic status. It has been traditionally employed for purposes such as determining the ability of microbial populations for degrading native organic matter (Schinner *et al.*, 1996; Bloem *et al.*, 2006) and for comparing between microbial metabolic activity in soils of different regions (Grisi *et al.*, 1998).

The effect of both soil temperature and water potential on basal respiration was generally significant ($p < 0.01$) for all soil types. In fact, both factors resulted in a combined effect, supporting data obtained by Conant *et al.* (2004). Increasing temperatures generally resulted in a significant increase ($p < 0.01$) in soil respiratory activity under the wettest treatment conditions and a decline under the driest treatments. Conant *et al.* (2004) reported similar observations and suggested that temperature

increase may cause a further reduction in soil water availability. In contrast, Brady and Weil (2002) refer that respiration rates typically more than double for every 10°C rise in temperature up to an optimum of about 35 to 40°C. Such substantial rise was not observed in this study, probably because it only applies to soils under no water availability limitations. In fact, those authors did not discuss respiration rates under drier soil conditions. A large number of studies have been developed in the last decades, focusing on the impact of temperature and soil moisture on microbial activity. Very few however, have looked at soil water availability and even fewer have studied the impact of those factors combined.

Soil water availability is critical for microbial life in soil, due to its impact on cell osmotic potential and metabolism, nutrient diffusion, spore germination and bacterial motility (Sommers *et al.*, 1980; Magan *et al.*, 1988; Williams and Rice, 2007). But on the other hand, high soil water potentials corresponding to near soil saturation (100% water filled porosity) generally have detrimental effects on microbial growth and activity by limiting gas exchange and O₂ supply (Wilson and Griffin, 1975; Skopp *et al.*, 1990). In the same way, temperature can also have a marked impact on soil microbial communities. According to Orchard and Cook (1983) and Paszczynski and Crawford (2000), most soil microorganisms, due to the fact of being mesophilic in their temperature requirements, have optimum growth and activity between 25°C and 30°C. However, this is a generalisation and may not always be the case. This research has shown that the optimum temperature required for optimum microbial activity rates is very dependent on both soil water potential and on soil characteristics combined.

In fact, when comparing to the significance of the impact of those two environmental factors, 'soil type' was found to be the most relevant ($p < 0.001$) parameter influencing soil basal respiration rate. This was evident due to the differences observed between respiratory rate responses of the three soils to temperature and water regime simultaneously. The sandy loam was the soil presenting the highest respiratory activity under the wettest conditions, but suffered a marked decrease in respiration under the lowest water potential treatment as a response to increasing temperatures. In contrast, the clay and volcanic ash soils achieved their highest ($p < 0.01$) respiratory rates under the driest environmental conditions, with respiration peaks at 16°C and 37°C respectively.

The importance of soil type in determining basal respiration rates has already been reported by Rusted *et al.* (2001).

The fact that the sandy loam exhibited significantly higher respiration rates under the wettest conditions, when compared to that of the other two soils, probably reflects its high organic matter content, since basal respiration in moist soil has been found to be tightly correlated to the amount of available carbon (Shen-Min *et al.*, 1987). It thus strongly suggests that water potential was the limiting factor influencing sandy loam's basal respiration under lower temperatures. On the other hand, its respiratory decrease as a result of increasing temperatures under low water availability may be easily explained, as native microbial populations are well adapted to moister and cooler soil conditions. Conant *et al.* (2004) supports this hypothesis by referring that soils from cooler climates are more sensitive to increases in soil temperature than those from warmer climates. Similarly, microbial populations in the clay and volcanic ash soils are probably better adapted to drier and hotter soil conditions. One mechanism that many microorganisms (both bacteria and fungi) have developed for resisting osmotic stress in such environments is by accumulating intracellular organic and inorganic solutes such as carbohydrates and polyols, which are compatible with cellular metabolic processes (Brown, 1979; Halverston *et al.*, 2000).

In order to further explore the reasons behind the different responses of soils to temperature and water potential, the relative fungal component was assessed for all soil types at both water potential treatments. Relative bacterial activity was considered to equal the difference between total respiration and that of the fungal component. Under wetter conditions, bacterial and fungal relative contributions to total respiration were generally equivalent. Under the driest treatment, however, fungal relative activity was found to be the main significant contribution to soil respiration (over 87%) in the clay and volcanic ash soils, agreeing with previous studies (e.g. Wilson and Griffin, 1975). On the contrary, under the same conditions, total respiration in the sandy loam still evolved mainly from bacterial relative activity. This is an anomalous result since bacterial activity is known to be particularly vulnerable to low water regimes (Wilson and Griffin, 1975; Harris, 1981). Most bacteria are thought to become inactive when soil water potential drops below -0.5 to -0.7 MPa (Magan *et al.*, 1988). Perhaps most of this

CO₂ arose from relative activity of actinomycetes populations, which have found to be relatively more resistant to osmotic stress (e.g. Halverson *et al.*, 2000).

Changes in the relative contributions of bacteria and fungi to soil total respiration as influenced by soil water potential has previously been described by Wilson and Griffin (1975) and Orchard and Cook (1983). One reason for these changes may have to do with limitation of movement and consequently reduced activity due to nutrient exhaustion. This would affect bacteria more strongly, since bacterial movement in soil is water-dependent and they can only remain active while nutrients are able to diffuse through the soil matrix. In contrast, fungal activity is not restricted in this way, since hyphae extensions allow fungi to bridge water-filled pores and actively find better local conditions (Magan, 1997).

pH is another key feature of soil, which may generally affect microbial activity and heavily contribute in determining soil native microbial community structure. For example, it is well known that fungi are better adapted to low soil pH than are bacteria (Kearney and Roberts, 1998; Gadd, 2001). However, in this study pH may have not been a significant parameter in explaining such dissimilarities, since all soils used had a pH value within the range of that for which microbial activity is optimal (± 0.3), according to Gravilescu (2005).

2.4.3 Substrate-induced respiration

Although useful for studying soil response to environmental factors and for comparing microbial activity in different soil types, traditional basal respiration measurements may not always provide enough information on the characteristics of soil microbial communities. An additional and widely used approach for assessing microbial activity consists of measuring its response to nutrient addition (Meli *et al.*, 2003). Anderson and Domsch (1973) have earlier suggested that microbial communities respire more when fresh nutrients (which are limiting) are added to soil.

The cumulative respiratory response curves developed in this study allowed comparing between the responses of each soil to nutrient addition. It provided clear indications of the different response times and response magnitudes of microbial activity for the three soils following the addition of a soluble and a recalcitrant nutrient under the treatment environmental conditions. Although a vast body of research has studied the impact of nutrients such as glucose (e.g. Nordgren, 1988; Nannipieri *et al.*, 2003) and straw (Brookes *et al.*, 1991; Thiet *et al.*, 2006) on soil microbial respiration in moist soils, very limited data are available for drier soil conditions.

Heterotrophic soil microorganisms acquire C and N for maintenance and growth by decomposing plant residues and other organic materials added to soil (Haney *et al.*, 2002). Soluble nutrients, in particular sugars and amino-acids, are more easily taken up by the microbial community than the more recalcitrant ones (Meli *et al.*, 2003). On the contrary, wheat straw consists of crop remains after that has been harvested (Magan, 1988), with cellulose and hemicellulose being its major components (75-80%), as well as lignin (10-14%) (Harper and Lynch, 1985). This is likely to explain the immediate response of respiration within the first 3 hours following the addition of glucose, comparing against the lag phase observed within the first day after that of straw. An immediate increase in respiration after 2-6 hours following the addition of glucose has also been reported by others (e.g. Anderson and Domsh, 1973; Wardle and Parkinson, 1990).

The pattern of response to glucose and straw was generally similar at both water potential treatments, although at much lower magnitude at -2.8 MPa. Corroborated by findings of Wilson and Griffin (1975), this may indicate that under such low water availability, nutrients may have been poorly distributed throughout the soil and also perhaps most microorganisms have not been able to utilise the fresh nutrients. This also probably explains why nutrient concentration had generally no significant impact on respiration at the rates studied, regardless of soil water potential.

Glucose addition was generally the most favourable for the sandy loam, which achieved a respiration peak around day 1, followed by a steady phase. This is in agreement with Wilson and Griffin (1974), who demonstrated that the maximum respiration rate in a dry

(-0.6 and -2.0 MPa) sandy loam soil was achieved within 20-40 h following glucose addition. According to Gray and Williams (1971), in field moist soil (near 0 MPa), glucose added at small amounts have been utilised to 100% by microbial populations following approximately 100 h. It can thus be hypothesised that at the peak rate obtained in this study, the microbial populations in the sandy loam may have only used around 20-25% of the glucose added. In contrast, CO₂ production by the clay and volcanic ash soils increased steadily for up to 10 days, at which both their respiratory rates had achieved an overall increase of over 75%, when compared to that of un-amended soil. It thereby suggests that under the treatment environmental conditions, microbial populations in these latest soils might have used more glucose than those in the sandy loam. This might be a reflection of the naturally lower contents of readily-available organic matter in these two soils, when comparing against that in the sandy loam.

Contrary to glucose, the addition of wheat straw at a similar rate did not have a significant effect on the respiration of the three soils in the very short-term. Also, by the end of the study period, the overall increase induced by straw addition when comparing against that in un-amended soil was the lowest for the sandy loam (25%) and the highest (81%) for the volcanic ash followed by the clay soil (65%). This may indicate that the sandy loam consists of microbial communities which are more responsive to readily available nutrients than to those more slowly degradable. In turn, although there seems to be a large amount of glucose (fast)-responsive microorganisms in the clay and volcanic ash soils, the contribution of slow-responsive microorganisms is also high, reflecting their relevant role in the overall metabolic activity in such environments.

So far, it is unclear from the literature which proportion of the microbial community responds immediately to glucose, although there are suggestions that these are either the bacterial populations or rather those microorganisms (from both bacterial and fungal groups) which are generally more active (Wardle and Parkinson, 1990). According to this hypothesis, data in this study suggests that microbial populations may be naturally more active in the sandy loam, whereas the clay and volcanic ash soils may naturally contain a higher proportion of inactive (or dormant) microorganisms. It also supports the results regarding the dominance of bacterial activity over that of fungi in the sandy loam

under both water potential conditions, whereas fungal activity clearly dominated the clay and volcanic ash soils under the driest soil conditions.

The steady state reached by the sandy loam following the respiratory peak may be a result of other essential nutrients becoming limiting after such enhanced period of activity. In contrast, respiration in the clay and volcanic ash soils was maintained high, which might suggest that the addition of a readily available substrate may have triggered microorganisms to start using the more recalcitrant organic matter dominant in those soils (the so called “priming effect”). This effect has also been reported by Brookes *et al.* (1991) and Thiet *et al.* (2006) in a range of different soil types as response to glucose and to straw addition respectively. However, the mechanisms triggering this effect are still not clearly understood (Brookes *et al.*, 1991).

2.4.4 Dehydrogenase activity

Dehydrogenase activity is considered to be one of the most reliable and generally used microbial parameter, not only as an indicator of the overall status of microbial activity but also for determining the impact of soil pollutants as well as the degree of recovering of degraded soils (Gil-Sotres *et al.*, 2005). It is considered to be a sensitive indicator of microbial activity (Garcia *et al.*, 1994) and yet, very little information exists regarding the way it responds to key environmental factors and nutrient inputs under dry soil conditions.

Overall, water potential had a significant ($p < 0.01$) impact on the activity of this enzyme at all temperatures tested, over short incubation periods. Dehydrogenase activity was generally nearly 30% higher ($p < 0.001$) under the wetter than under the drier soil conditions, regardless of soil type. Besides its direct influence on microbial cells, soil water potential is also thought to indirectly influence metabolic activity, slowing down enzyme reaction rates, by limiting nutrient diffusion (Adebayo *et al.*, 1971 in Wilson and Griffin, 1975). In this sense, these data corroborate that obtained for respiration measurements.

Unlike water potential, the effect of soil temperature in determining the level of dehydrogenase activity in soil was only significant for the sandy loam when temperatures increased from 25 to 37°C at both water regimes. Recently, Quilchano and Marañón (2002) looked at the impact of two contrasting seasons (dry and hot summer *vs.* wet and cool autumn) on DHA in Mediterranean soils. Corroborated by Garcia *et al.* (1994), those authors have registered nearly double as much DHA under cooler and wetter soil conditions as that under drier and hotter conditions.

Once again, soil type was the most responsible factor explaining the differences observed in dehydrogenase activity for the studied temperature and water potential values, agreeing with that discussed by Nannipieri *et al.* (1990). Surprisingly, the clay soil consistently presented the highest enzymatic activity, nearly 50% higher than that in the sandy loam, irrespective of its temperature and water potential. This is an unexpected result, since several authors, including Beyer (1992), have found a good correlation between nutrient content and the activity of this enzyme. Alternatively, the explanation might be related to other soil characteristics rather than to its organic matter content. Yet, the estimation of the abiotic INT reduction (page 59) ensured that the high enzymatic activity in this soil cannot be explained by the ability of common clay minerals such as Cu^{2+} , $\text{Fe}^{2+, 3+}$ and $\text{Mn}^{3+, 4+}$ to oxidise organic matter and catalyze electron transfer reactions (Beyer *et al.*, 1992, 1993; Nannipieri *et al.*, 2002). One can thereby hypothesise that microbial communities in this soil are able to keep their organic matter oxidation machinery high under low water availability conditions, without that being related to CO_2 production. The fact that several soil physical and chemical features can influence DHA, is the reason why Nannipieri *et al.* (1990) recommend DHA to be used for comparing only between microbial activities in soils of similar characteristics.

Nutrient addition induced significant responses in soil dehydrogenase activity over 10 day incubation under both water regimes, although no significant differences were found between treatment concentrations. The volcanic ash was the soil with the lowest DHA before nutrient addition but was the one showing the highest increase in activity (by 71% and 75%) as a response to glucose and straw respectively. Several other reports exist on the response of DHA to nutrient addition. For example, Sparling *et al.* (1981) have also found an increase in DHA in different soil types following glucose addition at a range of

concentrations, including that used in this study. But contrary to what has been found in this study, those authors reported that the magnitude of response was correlated to the amount of glucose added. The reason why there was no significant difference in responses between nutrient concentrations in the present study was probably related to the low soil water potential, this being the limiting factor to nutrient assimilation by microbial populations, rather than the amount of nutrient itself.

In the longer-term, straw was the nutrient responsible for the highest activity increases in the clay and volcanic ash soils (34 and 75% respectively) when compared against the control. Glucose was on the other hand, particularly favourable for the sandy loam over the same time period. Differential responses of activity according to the nature of the nutrient added are probably related to different rates of nutrient assimilation and has been registered by others, among who Sparling *et al.* (1981) and Perucci (1992). Sparling and co-workers (1981) have found an increase in DHA in different soil types within three days following glucose addition, whereas Perucci (1992) reported that the effect of compost addition was only significant a month after the nutrient was added to soil. The fact that glucose and straw induced different responses in DHA according to soil type may be best explained by dissimilarities between the characteristics of their microbial populations.

2.4.5 Microbial community size

When analysed individually, the effect of temperature on total CFUs was generally not significant ($P>0.08$), contrary to that of water potential, which was significant ($p<0.03$) for the clay and volcanic ash soils. Total CFUs were consistently higher for the sandy loam and the lowest for the volcanic ash at all temperatures examined, which might be explained by its high organic matter content. But interestingly, differences between soil types in terms of total microbial counts were only significant under the wettest conditions, suggesting that water potential was the main limiting factor determining total microbial population size in the three soils.

In terms of relative population sizes, bacterial CFUs were generally favoured by cooler and wetter soil conditions, whereas fungal communities thrive best under drier and hotter conditions, as discussed previously. On the other hand, soil type was also found to be an important factor determining the bacterial and fungal relative population sizes as well as their responses to soil temperature and water potential. Bacteria were generally more abundant at -0.7 MPa and although this was observed in the three soils, was markedly so for the sandy loam. In contrast, in the clay and volcanic ash soils, fungal populations were at least twice as abundant at -2.8 MPa, as they were under the wettest conditions. This data is therefore in agreement with results obtained for bacterial and fungal relative respiratory activities as influenced by the combined effects of soil type, temperature and water potential.

Several soil features have been considered responsible for the differential bacterial and fungal relative population sizes in different soil types (e.g. Miller and Donahue, 1995; Schinner *et al.*, 1996; Gadd, 2001). For example, bacteria have been described to thrive in nutrient-rich soils dominated by labile organic matter with high N concentration (Schinner *et al.*, 1996). On the contrary, fungi have shown to grow best in soils rich in recalcitrant and humified nutritional compounds and tolerating a much wider range of environmental conditions, in terms of oxygen levels, soil temperature and water potential (e.g. Miller and Donahue, 1995; Schinner *et al.*, 1996; Gadd, 2001). It supports the hypothesis that fungal biomass dominates the metabolism in soils naturally low in nutrient and water availability such as those in Mediterranean-like regions, playing a relevant role on soil functioning in these ecosystems (Orchard and Cook, 1973; Anderson and Domsh, 1975; Mulder *et al.*, 2003). Mulder *et al.* (2003) go a step further, suggesting that the bacteria/fungi population density ratio in any given soil deserves more research as it may be a potential indicator of soil nutrient status.

Similar pattern of response to nutrient addition was obtained irrespective of soil water potential in all soil types. Following very short incubation periods (1 day), nutrient addition generally did not have a significant ($0.10 < P < 0.40$) impact on total microbial CFUs. On the longer term however, a generalised increase in total microbial numbers was obtained as response to both nutrients irrespective of soil type. Sparling *et al.* (1981) and Potthoff *et al.* (2001) are among the authors who have also registered an increase in

microbial biomass due to glucose and straw addition, although that increase was faster compared to that in this study due to no water limitation conditions. Another interesting result was the fact that glucose favoured the most microbial communities in the sandy loam, whereas the impact of straw was the strongest for the clay and volcanic ash soils. This may again have to do with differential microbial community structure, therefore strongly agreeing with SIR data. In fact, a review study by Bloem *et al.* (2006) refers to the addition of glucose into soil as causing an increase in bacterial numbers, unlike straw which favours fungal communities the most.

2.4.6 Fungal community structure

Little is still known about fungal population structure in agricultural soils in general (Hagn *et al.*, 2003) and even less under dry soil conditions. There was significantly ($p < 0.02$) higher abundance of species under the wettest and cooler soil conditions, as well as following the addition of nutritional substrates for all soils. Also, soil type has shown to have a significant ($p < 0.03$) impact on the number of genera found at all temperature and water potential treatments. These data agree with other already published. For example, a relevant study by Hagn *et al.* (2003) used molecular-based techniques to find that fungal diversity in an agricultural soil was markedly influenced by seasonal changes, as well as by soil characteristics and management practices.

The most common genera isolated from these soil types were *Aspergillus*, *Penicillium*, *Fusarium*, *Cladosporium* and *Acremonium*, which according to Paszczyński and Crawford (2000) and Hagn *et al.* (2003) is a typical result for arable soils. In un-amended soil, *Penicillium* (20-50%), *Aspergillus* (20-50%), *Fusarium* (5-10%) and *Rhizopus* (5-10%) were common to the three soil types. Other genera were found exclusively in the sandy loam under the studied water potential conditions, including *Alternaria* and White Yeast, but generally, there was very little difference between fungal community structure in the sandy loam and clay soil using the Plate Count method. This is partially unexpected, since the sandy loam contains higher nutritional content and diversity. Therefore, the reason behind this observation may be related to the low soil water potential, which might be limiting the growth and activity of organisms of

other genera. In drier soil, *Penicillium* and *Aspergillus* were abundant and often the only genera found, particularly in the clay and volcanic ash soils, which is an expected result according to Griffin (1963). *Penicillium* was also the most abundant genera isolated in the sandy loam by Fragoeiro (2004) under -2.8 MPa.

The addition of nutrients resulted in a significant ($p < 0.0001$) increase in the abundance of species when comparing against the control, and the number of species isolated from amended soil varied mainly according to nutrient and soil types but also to soil environmental conditions. This again reflects a shift in microbial community structure as response to nutrient addition. Comparing between responses to glucose and wheat straw over the study, the first resulted in a higher abundance of species over this time period, probably due to its readily-available nature. However, when added in excess (5.0 mg g^{-1} soil) both nutrients had either an insignificant or a detrimental effect on fungal diversity, particularly in the sandy loam. This might be related to increased competition interactions between microorganisms rather than to toxicity. Recently, Willcock and Magan (2001) have suggested that the colonisation and utilisation patterns of straw by mixed soil populations may vary as result of antagonistic interactions between species.

2.4.7 Correlations between microbial parameters

Volatile patterns, respiration, DHA and total CFU have in common the feature of allowing assessing active microorganisms, and therefore together providing a global picture of the status of microbial communities in soil. Statistical correlations between the different parameters were explored using general regression models. Finding hidden relationships between microbial volatile patterns (through PC1) and some of the most relevant traditional methods for characterising soil microbial activity allowed for further investigating the potential use of this technique for soil applications. In contrast to PC1, PC2 was found not to be correlated to any of the remaining parameters. This is probably a reflection of PC2 generally explaining only up to 7% of the overall variance within the data sets.

Interestingly, PC1 was found to be strongly correlated to respiration, DHA and total CFUs under the study soil environmental conditions over a 10 day incubation period. While in the case of both DHA and CFUs that relationship was time-dependent, in the case of basal respiration, its correlation with PC1 was independent of the incubation period. Those correlations suggest that under the treatment environmental conditions, the type and amount of volatiles produced as result of microbial activity are related to respiration, oxidative metabolism of native organic matter and generation of biomass, although being time-dependent. It also suggests that, like respiration and unlike CFUs, volatile patterns respond quickly to changes in soil conditions. The choice of using PC1 as representative of the overall variance within the data set was therefore probably correct under the treatment conditions. It may also support the suggestion that PC1 may be related to volatile intensity.

In glucose-amended soil over 1 day incubation, SIR was shown to be correlated to potential DHA and to PC1 but none of the latest parameters were significantly correlated to total CFUs under those specific soil conditions. Over 10 days however, total CFUs were already significantly correlated with SIR and DHA. Nannipieri *et al.* (2002) agree with these findings. The authors reported that the response of microbial activity to glucose addition may not be related to microbial growth, particularly in the short-term basis. However, they are usually correlated in the longer-term, since then the biomass would have had time to adjust to the new conditions (Nannipieri *et al.*, 2002). Unlike respiration and DHA, biomass produces a much slower response to changes in soil conditions. It probably indicates that in the short-term, the added substrate was not used for biomass generation, although over longer periods the general increase in total CFUs might have already been a result of nutrient use for cell generation. Similar conclusions were produced by Tsai *et al.* (1997) regarding the use of microbial biomass measurements for detecting the response of microbial communities to glucose within 9 day study periods. Hence, it supports the hypothesis that microbial biomass is probably a more useful indicator of long-term changes in microbial populations, rather than that of short-term as employed in this study. Nevertheless, this parameter has shown to be suitable for assessing microbial population size under low water availability conditions.

Another interesting result was the fact of the correlation described above between PC1 and the remaining parameters not having been valid for glucose-amended soil, over the study period. The most likely explanation for this might be that the amount and type of volatiles produced in the presence of added substrates are independent of CO₂ evolution, oxidative metabolism and growing biomass. It can be hypothesised that this is another consequence of a shift in microbial community structure due to nutrient addition or even a result of specific interactions between microorganisms, rather than having to do with metabolism of the added substrate itself. In fact, PC1 was shown to be tightly correlated to the number of fungal species present in both un-amended and glucose-treated soil, irrespective of soil type. The fact that this was a negative correlation further suggests that an increase in fungal species at any given moment may reduce volatile emission, probably due to an increase in competitive interactions.

However, it is worth underlining that the fact that PC1 was correlated at some extent to respiration, DHA, total CFUs and fungal diversity in un-amended soil under 25°C and -0.7 MPa, does not necessarily mean that that relationship is valid for every soil condition. In the same way, the lack of a significant correlation between PC1 and the traditional microbial parameters in glucose-amended soil does not mean that volatile patterns should not be used for characterising potential microbial activity, but rather demonstrates that further research is needed for evaluating its feasibility for such an application.

In addition, the correlations found between the traditional microbial parameters at low soil water potentials were also interesting. In untreated soil, respiration, DHA and total CFUs were found to be significantly correlated under the treatment environmental conditions, irrespective of soil type, which is not an entirely surprising result. Although few other studies have reached similar conclusions using moist soils, in general, the results are often contradictory, which is probably due to the variety of soil types and soil treatments employed in the literature, making it difficult to establish comparisons.

For example, Nannipieri *et al.* (1990) discuss a few studies where correlations between DHA and both microbial biomass and respiration have not been found using both INT and TTC as artificial electron acceptors and a variety of different methodologies. Yet,

strong correlations were found in this study between basal respiration and DHA in the sandy ($r = 0.881$) and volcanic ash ($r = 0.862$) soils, supporting the hypothesis that intracellular enzymatic activity can be used as indicators of microbial activity under dry soil conditions. It thus agrees with data obtained in other studies using forest and agricultural moist soils as well as soils of semi-arid climates (e.g. Garcia *et al.*, 1994; Jimenez *et al.*, 2002). Similarly, von Mersi and Schinner (1991) have been able to find positive correlations between INT reduction and respiration and between that and microbial biomass, using the same protocol followed in this study.

However, DHA and basal respiration were not always in agreement in the clay soil reflected by a much lower correlation coefficient found between both parameters in this soil ($r = 0.540$ and $r = 0.638$ over 1 and 10 days respectively), when comparing against that in the other two. These contradictory results may indicate that the correlation between DHA and basal respiration may not be valid for all soil types under all environmental conditions. In addition, it also suggests that dehydrogenase should not be applied alone, but rather in combination with other methods for a more adequate characterisation of soil microbial activity, as suggested by Nannipieri *et al.* (1990).

CHAPTER III

Using Volatile Fingerprints as a Monitoring Tool for Atrazine Bioremediation in Soil by a White- rot Fungus

CHAPTER III

USING VOLATILE FINGERPRINTS AS A MONITORING TOOL FOR ATRAZINE BIOREMEDIATION IN SOIL BY A WHITE-ROT FUNGUS

3.1 INTRODUCTION AND OBJECTIVES

Health losses in agricultural soils are very often related to the difficulty of native soil microbial populations to degrade chemical contaminants, particularly pesticides and herbicides, therefore increasing their persistency in soil and the potential for reaching water and groundwater resources (Gil-Sotres, 2005). As referred to before, one of the reasons behind this recalcitrance may be related to environmental conditions, which do not promote biodegradation.

Insufficient attention has been paid to bioremediation processes of low concentrations of pesticides. Low concentrations of pesticides, likewise most soil pollutants, are usually the norm in soil, with high concentrations generally being very localised and occurring only for a limited period of time (Nannipieri *et al.*, 1990). Atrazine is mainly subject to microbial degradation with reported half-life concentrations in soil ranging from several days to months depending on soil conditions and history of previous atrazine exposure (Rhine *et al.*, 2003).

Microbial populations in a wide range of agricultural soils worldwide inhabit soils under low water potential conditions and which also contain low amounts of readily available nutrients; such is the case of soils in Mediterranean-like regions. Yet, very little quantitative and qualitative data are available regarding the effect of soil water potential on the rate of pesticide degradation under such conditions (Morreno *et al.*, 2007) either by native or introduced microorganisms (e.g. white-rot fungi).

Most of the studies done so far involving the application of white-rot fungi (in particular *T. versicolor*) for bioremediation of environmental contaminants were performed using liquid culture media, very often in bioreactors. In soil, where other factors influence pesticide degradation apart from the fungus degrading capabilities, our knowledge is more limited (Šašek *et al.*, 2003). Previous studies have shown that *T. versicolor* is able to grow in non-sterile soil under low water availability conditions (Fragoero, 2004; Fragoero and Magan, 2005). However, in order for that to happen, *T. versicolor* as any other white-rot fungus species needs to be pre-grown on a ligninolytic substrate for 5 to 10 days in order to achieve adequate biomass and enzyme production (Meysami and Baheri, 2001). Commonly used woody materials include straw, sawdust and woodchips (Harvey and Thurston, 2001).

In this research, *T. versicolor* was inoculated into an atrazine-containing soil, which was then incubated for up to 24 weeks. Periodic HPLC analysis and the assessment of soil microbial activity, population size and fungal community structure were used for monitoring the extent of atrazine biodegradation under the study conditions. Laccase activity was also determined as an indicator of relative *T. versicolor* activity.

The main objectives of the work described in this chapter include:

- (1) Exploring the potential of qualitative soil volatile fingerprints for detecting atrazine in soil at usual field application rates and as a monitoring tool for bioremediation of atrazine in this environment;
- (2) Investigating possible correlations between volatile fingerprints and traditional microbial parameters throughout biodegradation under the treatment environmental conditions;
- (3) Investigating the potential of *T. versicolor* for enhancing bioremediation of atrazine under such conditions, based on laccase production;
- (4) Studying the impact of adding low concentrations of atrazine into soil, (corresponding to usual field rates) on microbial populations, under low water potential conditions and as influenced by soil type.

3.2 MATERIALS AND METHODS

3.2.1 Fungal inoculant

Trametes versicolor (R26) was the species of white-rot fungi selected for the bioremediation study (Plate 3.1). In previous studies this species exhibited good tolerance to water stress conditions as well as to triazine herbicides. The isolate was supplied by Dr Mike Challen (HRI-Warwick, Wellesbourne, Warwick, UK). The pure culture was kept as plates on MEA at 4°C for up to 3 months before use. Streak plating was performed before use to ensure that there was no contamination during storage.

Preparation of the inoculum involved growing the isolate within a sterile jar on wet sterile sawdust (50% water content) (used as carrier) at 25 °C for up to 3 weeks, until the sawdust was completely colonised by mycelium. The jar had a ventilated cap to allow an adequate aeration. In order to avoid the isolate drying out during growth, the jar was then placed inside a polyethylene box, where the equilibrium relative humidity was maintained by a glycerol/water solution (400 ml).

This procedure, in which the isolate is allowed to grow under optimal conditions prior to use was shown to be critical in bioremediation processes (Bumpus, 1993; Levanon, 1993). It aims to improve fungal growth in contaminated soil, as well as the production of extracellular enzymes, therefore, enhancing the pesticide breakdown process.



Plate 3.1: Pure culture of *T. versicolor* (R26) on MEA kept at 4°C.

3.2.2 Soil microcosms for the soil treatments

The concept of soil microcosms described in Chapter II was applied for the bioremediation studies. All treatments described below were applied to 5 g of each soil type, in order to obtain five replicates per treatment at each sampling time. In the first three treatments described below, the various components of the soil microcosms system (atrazine, sawdust and *T. versicolor*) were added to soil individually, in order to assess their impact on soil microbial communities.

Atrazine addition to soil

Atrazine (Sigma Aldrich) was dissolved in RO water and the solution was sonicated for 1 min until complete dissolution of the herbicide. The amount of water used for dissolving was the same as that required for setting the target soil water potential treatment. The solution was then added to soil microcosms in order to obtain a concentration of atrazine in soil of $0.5 \mu\text{g g}^{-1}$. This concentration corresponds to the usual field application rate of this herbicide, according to Ghani *et al.* (1996) and Abdelhafid (2000). The fortified soils were thoroughly homogenised by grinding with a mortar and pestle and were kept for 1 day at 4°C allowing microbial activity to stabilise at the required water potential levels, before incubation and analysis.

Soil amendment with sterile sawdust

Wet (50% w w⁻¹) sterile sawdust was left equilibrating overnight at 4°C, prior to its addition to soil. The moist sawdust was then added to air-dried soil in order to obtain a concentration of 5% (w w⁻¹) and samples were left equilibrating overnight at 4°C. Soil-sawdust moisture calibration curves were developed for each soil type at the treatment concentration similarly to what has been done before for the wheat straw-amended soil. Conditioning of the treated soil to the required water potentials was then done by reference to its correspondent calibration curve based on the procedure described in Sub-section 2.2.2 (Chapter II). Soil samples were kept for 1 day at 4°C allowing microbial activity to stabilise at the required water potential levels before incubation and analysis.

Inoculation of *T. versicolor* into soil

Sawdust colonised by the test isolate (0.5 g) was added to air-dried soil under sterile conditions in order to obtain a concentration of 5% (w w⁻¹). Care was taken in order to ensure that each replicate received equivalent amounts of fungal mycelium. It was then mixed thoroughly with the soil until a homogeneous mixture was obtained. The procedure followed that described for the soil-sawdust treatment.

Incorporation of *T. versicolor* into atrazine-containing soil

The preparation of the homogeneous mixture of *T. versicolor* with sawdust and its incorporation into air-dried soil (5% w w⁻¹) was already described. Conditioning of the soil to the treatment water potentials was done by reference to a soil-sawdust water potential calibration curve and the addition of sterile RO water supplemented with atrazine. The procedure followed that of the soil-atrazine treatment.

Incubation of soil microcosms

Treated soil samples and untreated controls were incubated at 20°C in a temperature controlled chamber for 0, 6, 12 and 24 weeks. Incubation conditions were as described in Sub-section 2.2.3 (Chapter II). All treatments involved in this chapter are summarised in Table 3.1.

Table 3.1 Summary of the soil treatments involved in this Chapter, for each soil type. Concentrations of the amendments are expressed *per gram* of soil.

	Ref.	Ψ (MPa)	Atrazine (μg)	Sawdust (%)	Sawdust + <i>T. versicolor</i> (%)
Soil	S	-0.7; -2.8	-	-	-
Soil + Atrazine	S+A	-0.7; -2.8	0.5	-	-
Soil + Sawdust	S+S	-0.7; -2.8	-	0.5	-
Soil + <i>T. versicolor</i>	S+T	-0.7; -2.8	-	-	0.5
Soil + Atrazine + <i>T. versicolor</i>	S+A+T	-0.7; -2.8	0.5	0.5	0.5

3.2.3 Temporal characterisation of soil volatile fingerprints

The Bloodhound BH-114 was the E-nose employed for assessing soil volatile fingerprints throughout bioremediation. It was done following the optimised procedure and using the same sampling profile settings described in Sub-section 2.2.5 (Chapter II).

3.2.4 Temporal evaluation of metabolic activity in the soil microcosms based on respiration, dehydrogenase and laccase activities

Soil respiration

CO_2 evolved from total soil microbial respiration was determined by GC according to the optimised procedure described in Sub-section 2.2.6 (Chapter II). *T. versicolor*

relative respiration was assumed to equal the difference in CO₂ concentration between fungi-inoculated soil and that of sawdust-amended soil.

Dehydrogenase activity based on INT reduction

The INT method was again applied for estimating soil potential dehydrogenase activity. The experiment followed as described in Sub-section 2.2.6 (Chapter II). *T. versicolor* relative enzymatic activity was assumed to equal the difference in the concentration of the product INF between fungi-inoculated soil and that of sawdust-amended soil.

Laccase activity

Estimating soil laccase potential activity involved 2 steps: 1) laccase extraction from soil; 2) quantification of enzyme activity.

1) Laccase extraction from soil

For a more sensitive and accurate measurement of laccase activity in soil, it was chosen to extract the enzyme from soil before quantification. Working with an enzyme extract, not only allows overcoming problems related to undetectable concentrations of the enzyme in soil, but is also more convenient to handle, since small sample volumes can be used. The extraction method employed was based on the protocol described by Criquet *et al.* (1999) with adaptations by Fragoeiro (2004).

Sub-samples (2 g) of treated soil and untreated controls were weighed into sterile test tubes and 8 ml of phosphate buffer in water (10 mM, pH 6.0) were added. The suspension was kept under agitation (incubator shaker, 250 rpm) at 4°C for approximately 1 h. Aliquots of 1 ml were then passed into 1.5 ml Eppendorfs and centrifuged (3800 rpm) for 6 min at room temperature. The supernatant containing the enzyme was stored at -18°C while not in use.

2) Quantification of laccase activity

The method for determining laccase activity using an enzyme extract was based on the protocol described by Buswell *et al.* (1995) with adaptations by Fragoeiro (2004).

The reaction mixture representing a total of 300 μl was contained into a 96 well microtitre plate. It was prepared with 150 μl sodium acetate buffer (0.1 M, pH 5), 50 μl of the redox substrate ABTS (2,2-azino-bis-ethylbenthiazoline-6-sulphonic acid) (0.55 mM) and 100 μl of enzyme extract. The acetate buffer is mostly effective in the pH range of 4-5.5, at which degradation is generally promoted (Gadd, 2001). ABTS is a low-molecular weight compound and has been widely recognised as an effective laccase artificial mediator (Pointing, 2001).

The procedure was carried out at ambient temperature, although the substrate ABTS and the buffer were at 40°C when added to the reaction mixture. The incubation was performed at 40°C for 1 h. A positive laccase activity was indicated by a green colourisation of the reaction mixture, characteristic of the ABTS oxidised form. Product was determined spectrophotometrically at 405 nm using a Microplate reader set in the Endpoint reading mode, with 5 seconds of agitation at the beginning (optimised procedure based on Fragoeiro, 2004).

Control samples were prepared using boiled enzyme (15 min) and were treated like samples. Five replicates of each treatment including the respective controls were used. One enzyme activity unit (U) was defined as the amount of enzyme required for producing a 0.001 increase in the optical density of the reaction mixture per minute, under the conditions of the assay. Results were expressed as U g^{-1} soil.

The procedure described was firstly calibrated and optimised using purified commercial laccase from *Rhus vernificera* in crude acetone powder (50 Units mg^{-1} solid, minimum) as standard. Triplicate volumes ranging from 0 to 100 μl of enzyme standard were introduced into microplate wells. Sodium acetate buffer (150 μl) and RO water were added in order to obtain enzyme concentrations ranging between 0 and 1 mg ml^{-1} . The calibration curve obtained ($Y = 26.33x + 1.643$) showed a good correlation ($r^2 = 0.971$) between the concentration of commercial purified laccase (mg ml^{-1}) and laccase activity (U).

3.2.5 Temporal assessment of soil microbial population size and fungal community structure

Preparation of nutritional media, as well as enumeration of culturable bacteria and fungi and characterisation of soil fungal community structure as influenced by treatments was performed as described in Sub-section 2.2.7 (Chapter II).

3.2.6 Monitoring atrazine concentration in soil microcosms

A two-step procedure was involved in assessing the rate of atrazine degradation in soil microcosms: 1) pesticide was extracted from soil at the end of 0, 6, 12 and 24 weeks of incubation at 20°C; 2) extracts were analysed by HPLC for atrazine detection and quantification. For each soil type, fungal-inoculated soil as well as two sets of non-inoculated controls (SA and SS), both at -0.7 and -2.8 MPa water potentials, were tested. The use of both sets of controls allowed measuring the rate of atrazine degradation by native microbial populations in the absence (SA) and presence of added sawdust (SS).

Atrazine extraction from soil

The atrazine extraction method employed was adapted from that of Elyassi (1997) and Fragoeiro (2004) for an optimised simazine extraction, being based on the similarity between the properties and behaviour of both pesticides in soil. Triplicate soil samples corresponding to the SA, SS and SAT treatments were weighed (2 g) into test tubes. Aliquots (3 ml) of methanol (100%) were added to soil, after which tubes were sealed and shaken at 300 rpm in a circular motion shaker (IKA-LABORTECHNIK, KS 250 basic) for 24 hours in the dark and at room temperature. Following agitation and still under the same ambient conditions, soil was allowed to settle down until a clear supernatant was obtained (30 min approx.). Aliquots of supernatant (extract) was then withdrawn with a syringe and filtered using a nylon 0.2 µm syringe filter.

HPLC analysis of soil extracts

Pesticide analysis method and HPLC settings used were based on the procedure by Elyassi (1997) and Fragoeiro (2004). Extracts were diluted with acetonitrile (75% sample: 25% acetonitrile) prior to injection into the HPLC system (Gilson HPLC, UK). A volume of 50 μl was injected into the system, which was equipped with a UV detector operating at 215 nm (117 UV Detector, Gilson), sampling injector (model 231XL, Gilson), pump (model 306, Gilson), dynamic mixer (model 811C, Gilson) and a 5 μm wide column (model C18, Altima). The column operated at ambient temperature with a flow rate of 1.5 ml min^{-1} .

An isocratic mobile phase system was established using acetonitrile:water at a ratio of 70:30. Each HPLC run was up to 20 min, with atrazine eluting after approximately 9.75 min.

3.2.7 Data handling and statistical treatment

All results presented in this chapter were means of five replicates unless referred otherwise. Statistical treatment of data obtained from microbial volatile fingerprint analyses, soil respiration dehydrogenase and laccase activities, and microbial CFUs were as described in Sub-section 2.2.8 (Chapter II). Similarly, HPLC data regarding atrazine quantification was also analysed for comparison between means of treatments, using three-way ANOVA at a significance level $p = 0.05$.

3.3 RESULTS

3.3.1 Characterisation of soil volatile fingerprints

1) Time-independent response of volatile profiles to treatments:

Discrimination between untreated and atrazine-treated soil

Figure 3.1 is the PCA map for untreated and atrazine-treated (2.5 ppm) clay soil after 1 day incubation (week 0) at 20°C, -0.7 MPa. A solution of atrazine in RO water at the treatment concentration was used as a control only. A clear discrimination between the atrazine solution control and the remaining treatments, as well as between untreated and atrazine-contaminated soil was obtained under the study environmental conditions, accounting for 84% of the overall information. PC1 explained 71% of the variance within the data set. Similar results were obtained for the sandy loam and volcanic ash soils at -0.7 MPa (see Appendix III).

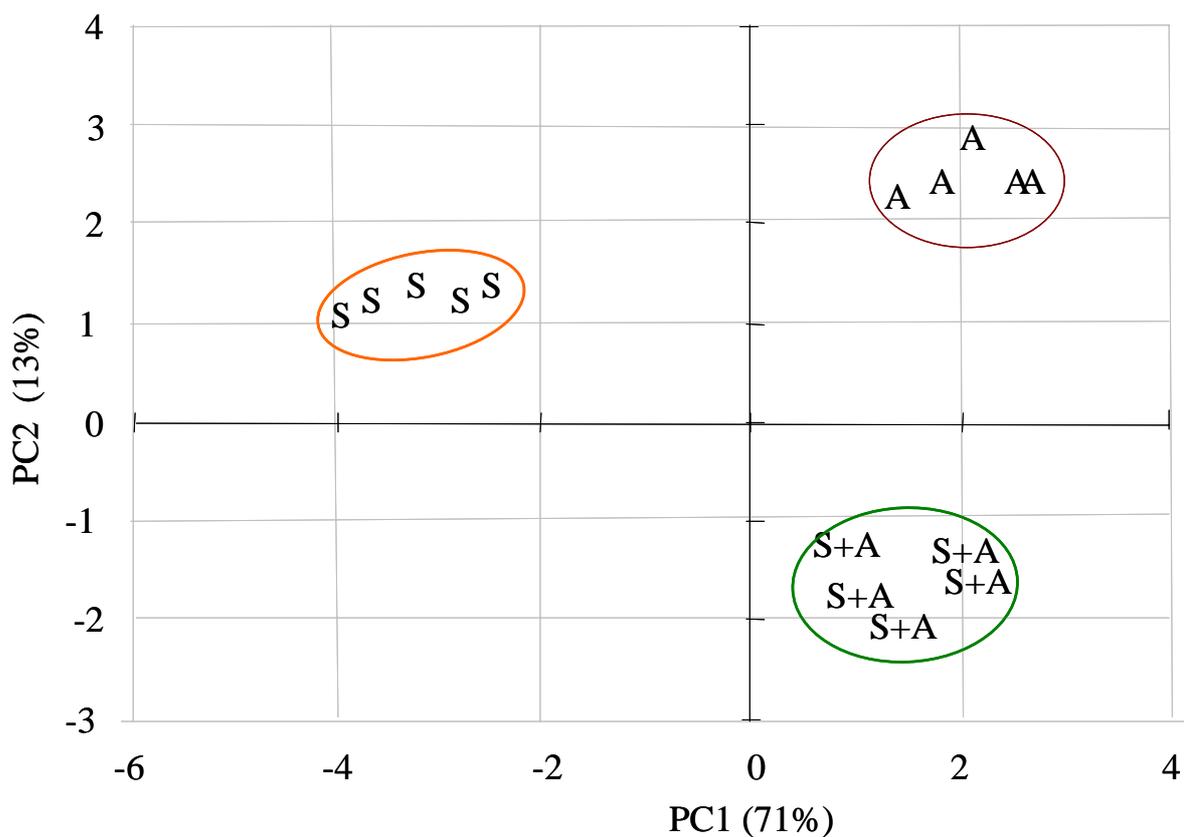


Figure 3.1 PCA map showing clear discrimination between untreated (S) and atrazine-treated (S+A) (2.5 ppm) clay soil after 1 day incubation at 20°C, -0.7 MPa. An atrazine solution in RO water (A) was used as a control only.

Discrimination between soil treated with atrazine and sawdust

Figure 3.2 is the PCA map for both treatments in sandy loam after 5 days incubation at 20°C, -0.7 MPa. Soil containing atrazine and that amended with sawdust were successfully discriminated based on volatile patterns, accounting for 79% of the variance. Although PC1 was the main contribution to the result presented, PC2 explained approximately 1/3 of the overall information. Likewise, a clear discrimination between treatments was found for the clay and volcanic ash soils under both water potential treatments.

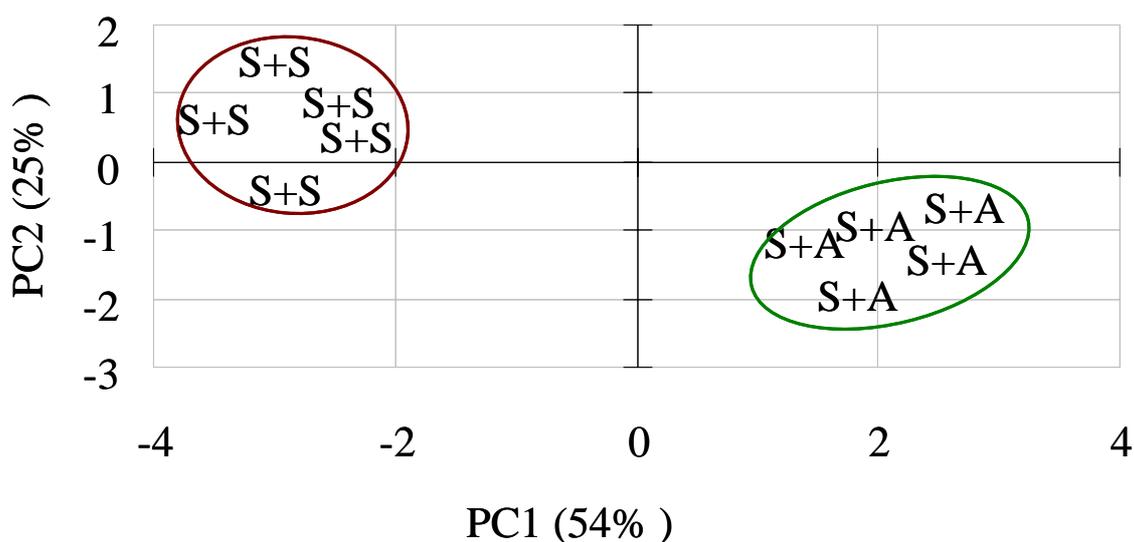


Figure 3.2. PCA map for the sandy soil treated with atrazine (S+A) (2.5 ppm) and with sawdust (S+S) (5%) after 5 days incubation at 20°C, -0.7 MPa.

Discrimination between un-inoculated soil and that inoculated with *T. versicolor*

It was possible to discriminate between un-inoculated soil and that carrying the test isolate, using 82% of the information contained within the data. The PCA map (Figure 3.3) shows the differentiation obtained between the headspace volatiles of volcanic ash soil in the absence and in the presence of *T. versicolor*, following 5 days incubation at 20°C, -0.7 MPa. Similar discrimination was obtained using the sandy loam and clay soils at both -0.7 and -2.8 MPa, where PC1 was also the main contribution for the end result.

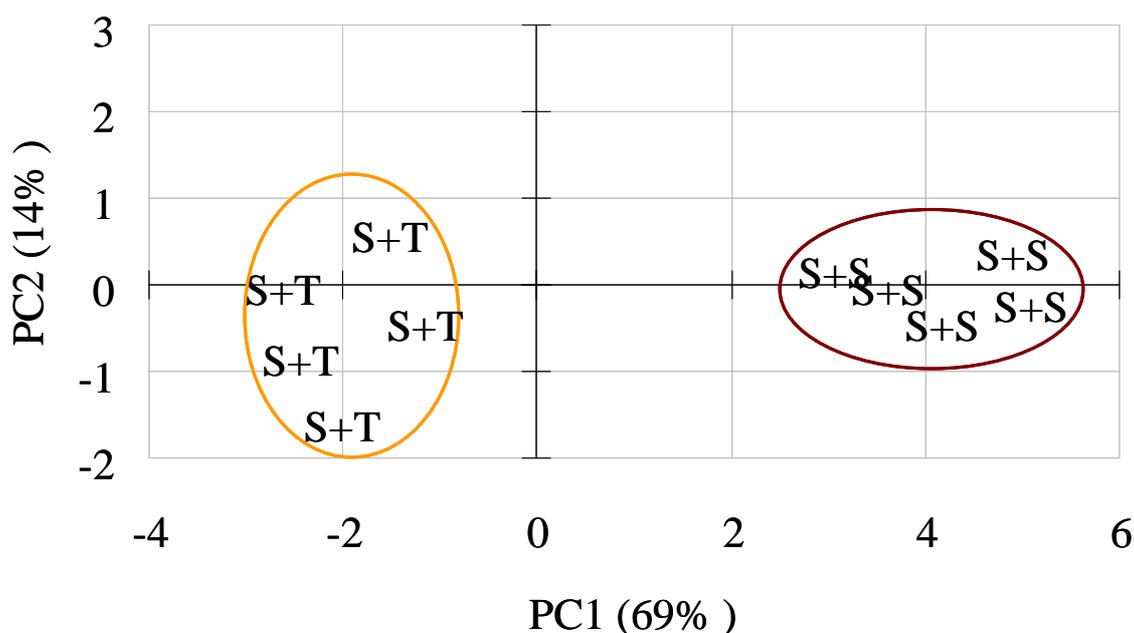


Figure 3.3. PCA map showing differentiation between clusters of volcanic ash soil in the absence (S+S) and in the presence of *T. versicolor* (S+T), following 5 days incubation at 20°C, -0.7 MPa.

Soil inoculated with *T. versicolor* as influenced by atrazine

There was a clear discrimination between volatile fingerprints in soil inoculated with *T. versicolor* as influenced by atrazine. Figure 3.4 is the PCA map showing the differentiation obtained between the headspace volatiles of sandy loam inoculated with *T. versicolor* in the absence and in the presence of atrazine (2.5 ppm), following 5 days incubation at 20°C, -0.7 MPa. The contribution of PC1 for the discrimination obtained was nearly three times more than that of PC2. Similar results were found using clay soil and volcanic ash, independently of soil water potential.

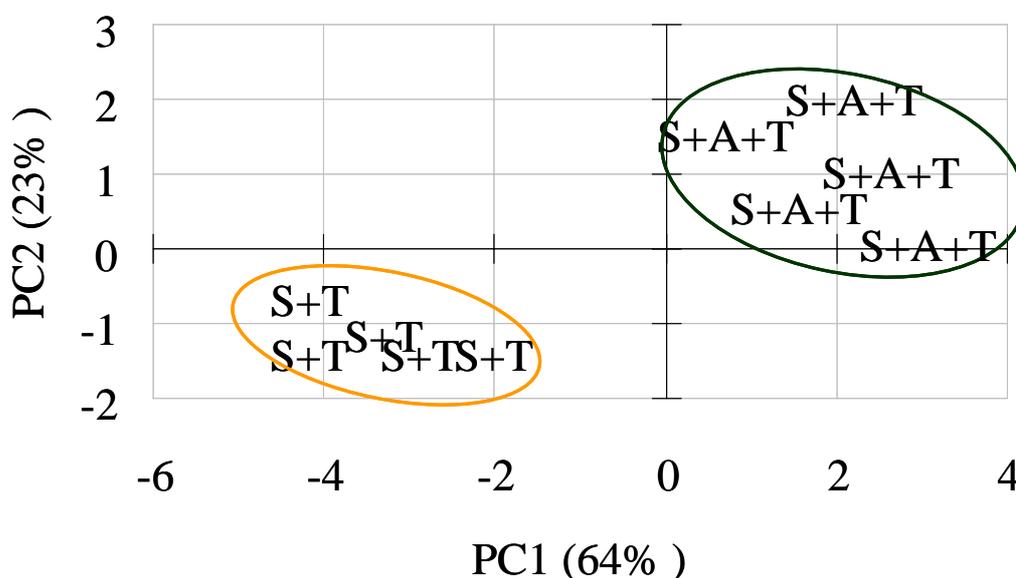


Figure 3.4. PCA map showing differentiation between clusters of sandy loam inoculated with *T. versicolor* in the absence (S+T) and in the presence (S+A+T) of atrazine, following 5 days incubation at 20°C, -0.7 MPa.

2) Temporal effects on the response of volatile profiles to treatments

Untreated soil over time

Figure 3.5 is the PCA map for samples of untreated clay soil incubated for 0, 6 and 24 weeks at 20°C, -0.7 MPa. The cluster corresponding to 0 weeks incubation was clearly separated from the remaining two. In contrast, there was an overlapping between treatments in clusters corresponding to 6 and 24 weeks incubation. PC1 alone explained 75% of the information used for the result obtained. Similar results were achieved using the sandy and volcanic ash soils.

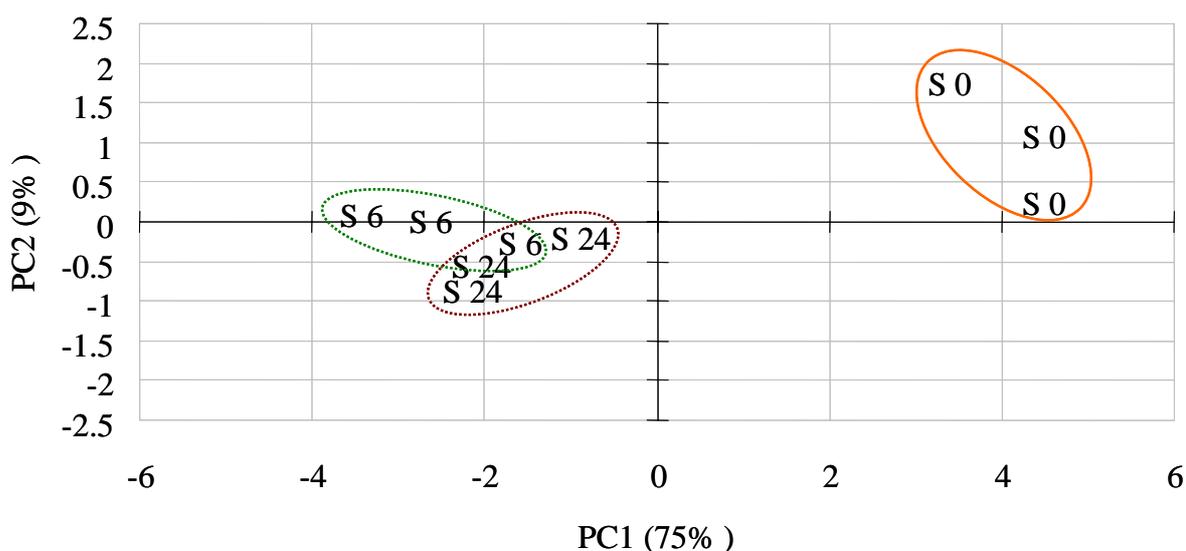


Figure 3.5 PCA map for samples of untreated clay soil incubated for 0 (S 0), 6 (S 6) and 24 (S 24) weeks, respectively, at 20°C, -0.7 MPa.

Atrazine-treated soil over time

Figure 3.6 is the PCA map for samples of atrazine-treated (2.5 ppm) volcanic ash soil incubated for up to 24 weeks at 20°C, -0.7 MPa. Differentiation between clusters according to incubation period was possible using 88% of the variance within data, with PC1 contributing with the majority (77%) of the information. Although the treatments corresponding to 0 weeks incubation were clearly separated from the remaining treatments, clusters relating to 6 and 24 incubation weeks were found to be closer together. Similar results were achieved using the clay and the sandy loam with more or less discrimination according to soil type.

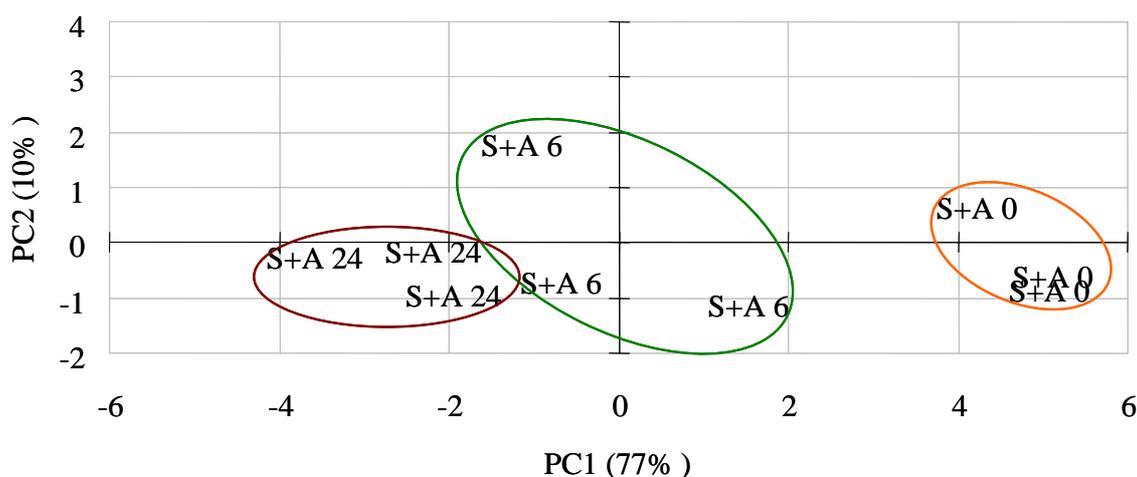


Figure 3.6 PCA map for samples of atrazine-treated (2.5 ppm) volcanic ash soil incubated for 0 (S+A 0), 6 (S+A 6) and 24 (S+A 24) weeks, respectively, at 20°C, -0.7 MPa.

Sawdust-amended soil over time

It was possible to clearly discriminate between treatments of soil containing sawdust according to the incubation period. Figure 3.7 is the PCA map for samples of sawdust-treated clay soil incubated for up to 24 weeks at 20°C, -0.7 MPa. Using 82% of the variance, the separation between the three clusters show dissimilarities between volatile patterns of sawdust-amended soil incubated for 0, 6 and 24 weeks, with PC1 contributing the most (69%) for the discrimination. Equally clear separation between clusters was obtained using the sandy and the volcanic ash soils.

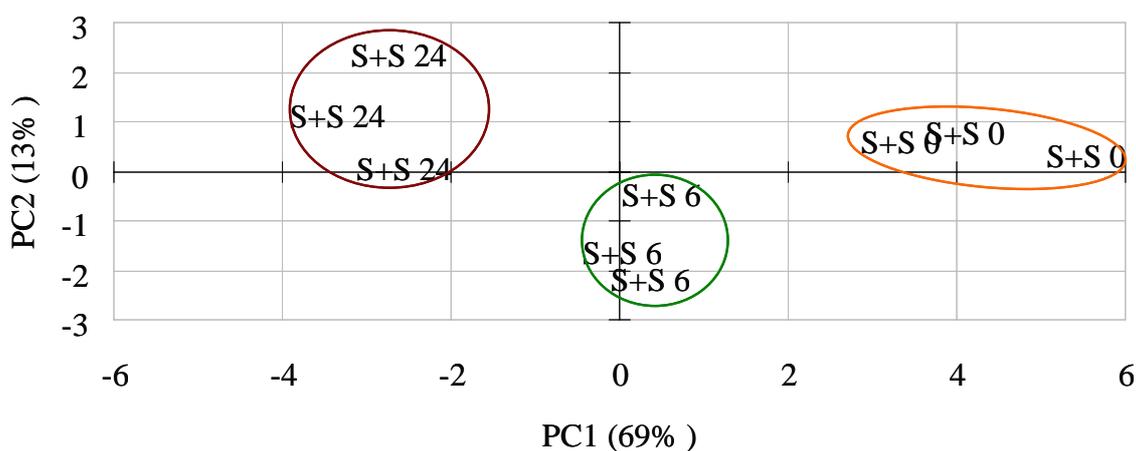


Figure 3.7 PCA map for samples of sawdust-treated clay soil incubated for 0 (S+S 0), 6 (S+S 6) and 24 (S+S 24) weeks, respectively, at 20°C, -0.7 MPa.

Soil inoculated with *T. versicolor* over time

The PCA map of sandy soil samples inoculated with the test isolate and incubated for up to 24 weeks at 20°C, -0.7 MPa is shown in Figure 3.8. The three clusters were separated on the plot based on their volatile fingerprints, with clear differentiation between the cluster corresponding to 0 weeks and the remaining two. In contrast, treatments incubated for 6 and 24 weeks were grouped into clusters closer together. The greater part of the information (74%) was explained by PC1. Similar results were obtained with the clay and volcanic ash soils.

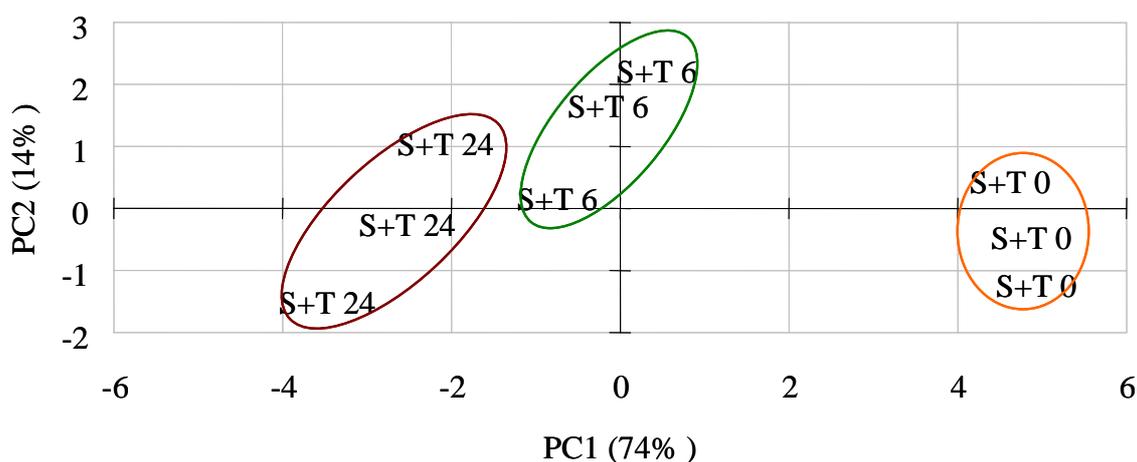


Figure 3.8 PCA map for sandy soil inoculated with *T. versicolor* and incubated for 0 (S +T 0), 6 (S+T 6) and 24 (S+T 24) weeks, respectively, at 20°C, -0.7 MPa.

Atrazine-treated soil inoculated with *T. versicolor* over time

Figure 3.9 shows the PCA map for clay soil inoculated with *T. versicolor* in the presence of atrazine (2.5 ppm) and incubated for up to 24 weeks at 20°C, -0.7 MPa. There was a clear differentiation between treatments according to the incubation period. Map shows that volatile fingerprints of soil containing atrazine and carrying the inoculum are distinctively different at 0, 6 and 24 weeks incubation, using 87% of the overall information. PC1 was the factor holding the majority of the variance (77%) within the data set. Likewise, similar differentiation between clusters was obtained using the sandy loam and volcanic ash soils.

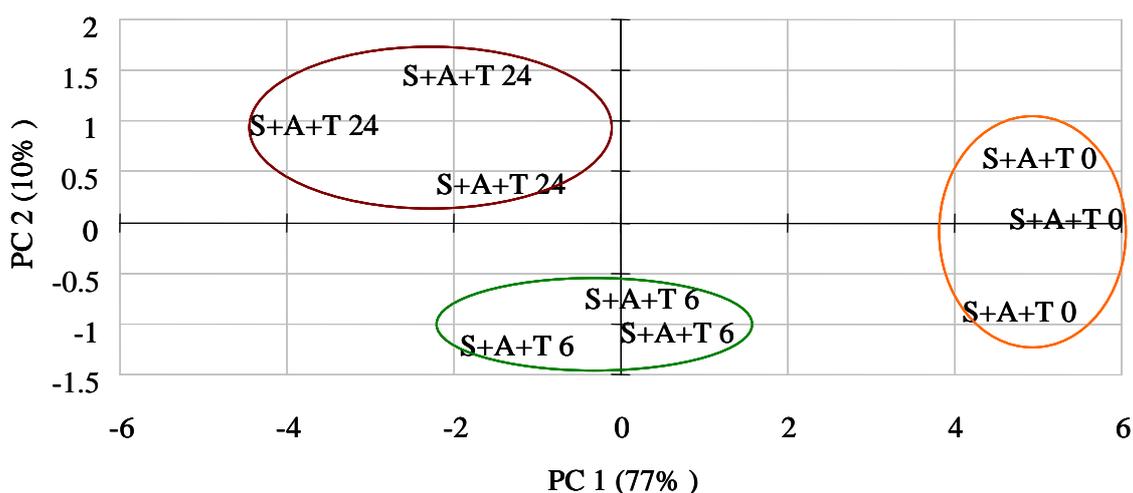


Figure 3.9 PCA map for clay soil inoculated with *T. versicolor* in the presence of atrazine (2.5 ppm) and incubated for 0 (S+A+T 0), 6 (S+A+T 6) and 24 (S+A+T 24) weeks, respectively, at 20°C, -0.7 MPa.

3.3.2 Temporal evaluation of metabolic activity in the soil microcosms based on respiration, dehydrogenase and laccase activities

Soil respiration

Cumulative respiration curves allowed studying and comparing between the rates of CO₂ evolution as response to soil treatments, for up to 24 weeks. Figure 3.10 shows the cumulative respiratory curve for the sandy loam incubated under (A) -0.7 MPa and (B) -2.8 MPa at 20°C, as an example, although similar behaviour was obtained for the three soil types.

Under the wettest soil conditions, untreated control soil (S) presented the lowest respiratory activity rates ($p < 0.003$) within the time study, when comparing against that of the other treatments. For example, soil treated with atrazine has shown to have produced 41.6% more CO₂ than pristine soil, following 6 weeks of incubation. Further, atrazine contaminated soil carrying the inoculum was the treatment generally presenting the highest CO₂ evolution rates ($0.01 < p < 0.04$), at least 20% higher than in the absence of the fungi, suggesting enhanced microbial activity by the fungus. Surprisingly, differences between respiration rates of soil treated with atrazine, sawdust and *T. versicolor* individually, were often not significant ($0.05 < p < 0.16$) under the treatment soil conditions, regardless of soil type. The maximum respiration achieved at -0.7 MPa occurred around week 6 for all soil treatments, after which it started decreasing at a slow but consistent rate (see Appendix IV for more information on the respiration rate of treatments as measured at weeks 0, 6, 12 and 24). Under drier conditions, respiration rates as response to treatments were generally not statistically different ($0.05 < p < 0.27$) throughout the study.

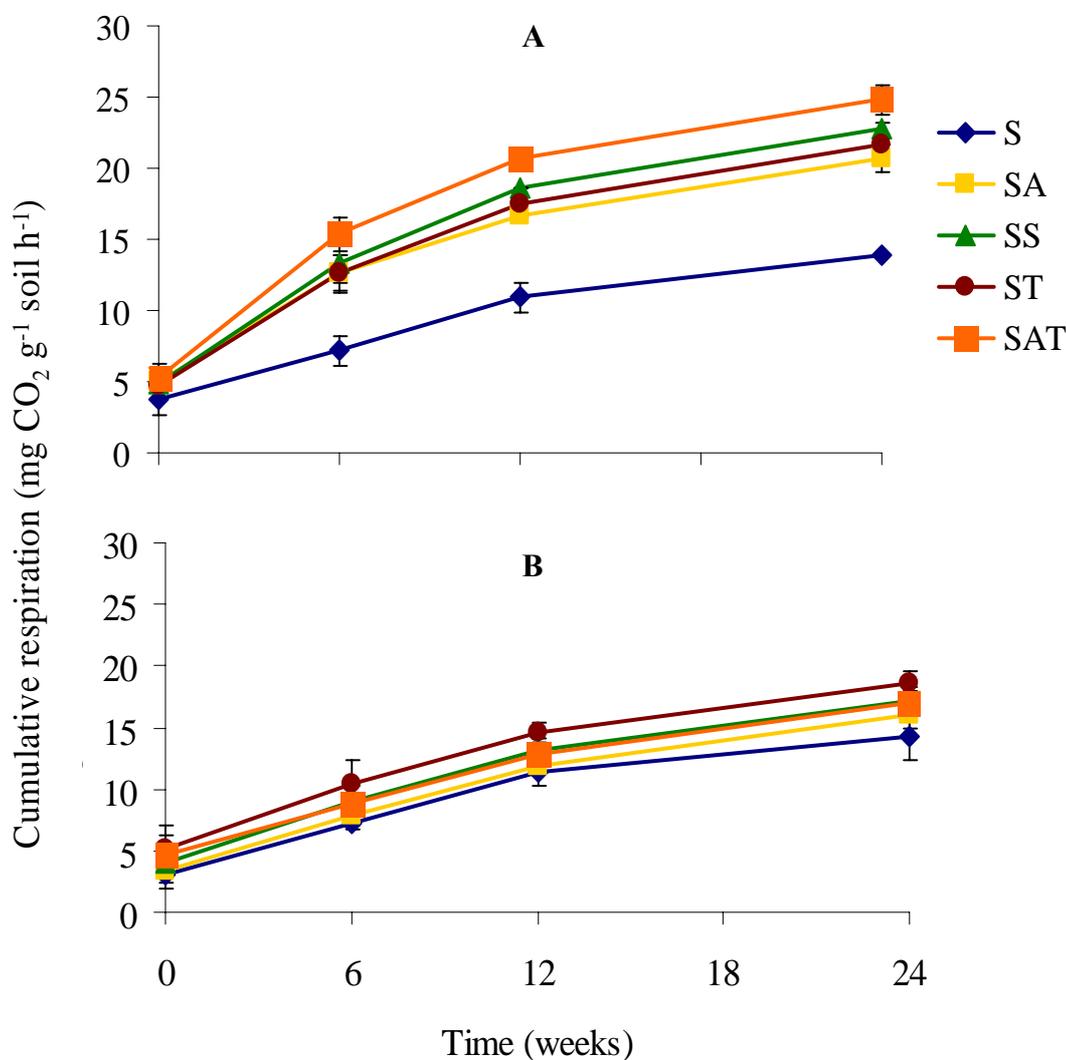


Figure 3.10 Cumulative respiration curve for the sandy loam under different soil treatments incubated for up to 24 weeks at 20°C under (A) -0.7 and (B) -2.8 MPa. Key to treatments: S, untreated control soil; SA, soil contaminated with atrazine (2.5 ppm); SS, soil with sawdust (0.5%); ST, soil inoculated with *T. versicolor* (0.5%); SAT, soil contaminated with atrazine (2.5 ppm) and inoculated with *T. versicolor* (0.5%).

Dehydrogenase activity

Cumulative DHA curves were produced for each soil type in order to study the way this enzymatic activity varied throughout the study, according to the treatment given to soil. As an example, DHA is shown in Figure 3.11 for the clay soil, which presented the highest activity rate of this enzyme within 24 weeks compared against that of the other two soils (data not shown).

Regardless of soil treatment, the highest DHA levels were achieved under the wettest conditions ($p < 0.001$). Untreated soil had the lowest DHA, but the addition of atrazine as well as the inoculant (individually or combined) enhanced activity in over 40% under both water potentials ($p < 0.001$). Soil amended with sawdust produced the highest DHA ($p < 0.003$), at least 53% higher than untreated soil, independently of soil water regime. Surprisingly, and also irrespective of water potential, there was no significant difference between soil inoculated with *T. versicolor* in the presence and absence of atrazine.

However, it was interesting to notice that unlike that observed in the absence of *T. versicolor*, inoculated soil showed the highest DHA at the end of the study (following 24 weeks of incubation). For the remaining treatments, the highest activity levels of this enzyme were obtained after 6 weeks of incubation, although differences between that and those obtained after 12 weeks were not always significant ($0.002 < p < 0.009$) under the conditions studied (see Appendix V for more information on DHA of treatments as measured at weeks 0, 6, 12 and 24).

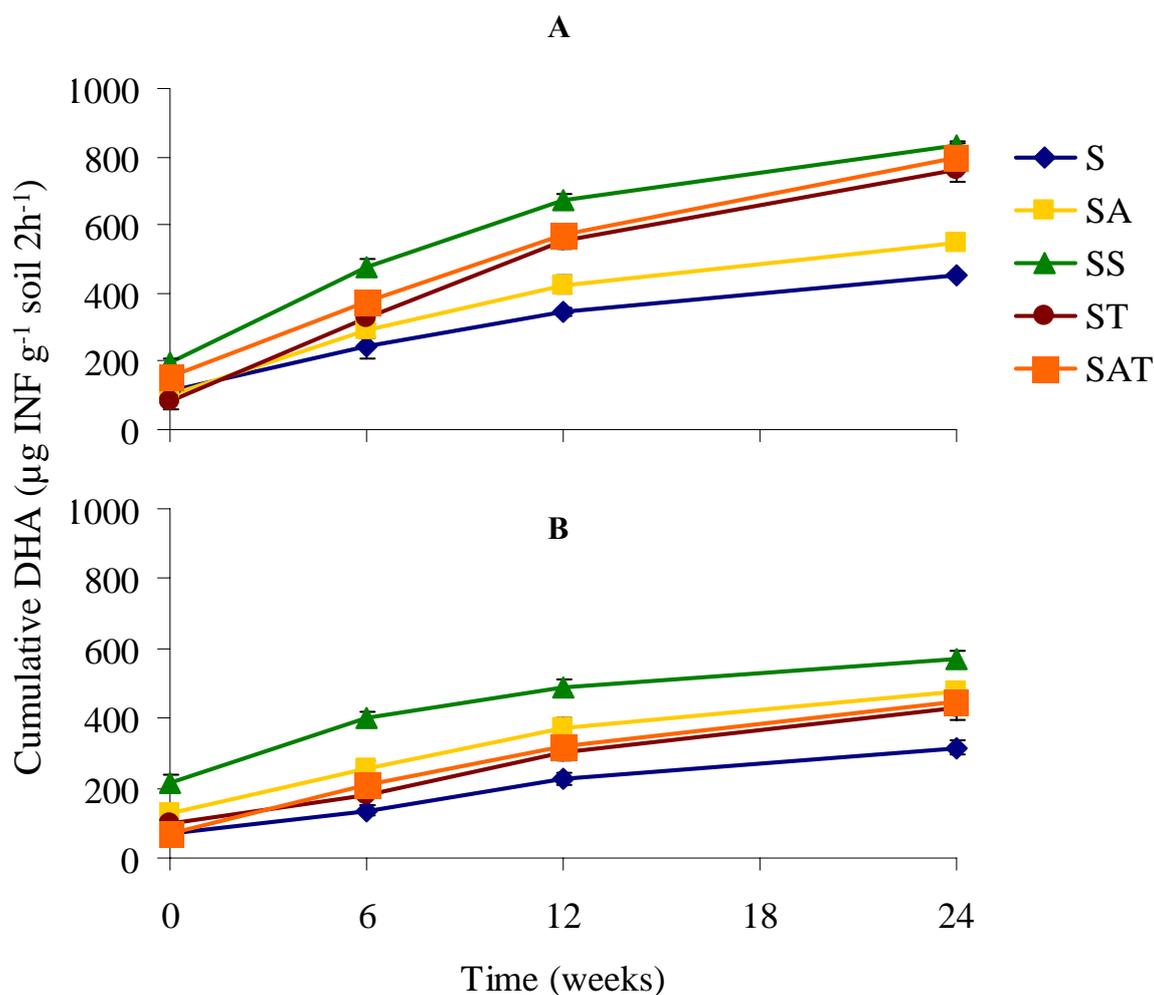


Figure 3.11 Cumulative DHA curve for the sandy loam under different soil treatments incubated for up to 24 weeks at 20°C under (A) -0.7 and (B) -2.8 MPa. *Key to treatments:* S, untreated control soil; SA, soil contaminated with atrazine (2.5 ppm); SS, soil with sawdust (0.5%); ST, soil inoculated with *T. versicolor* (0.5%); SAT, soil contaminated with atrazine (2.5 ppm) and inoculated with *T. versicolor* (0.5%).

Laccase activity

Laccase activity was the parameter showing the highest variability according to soil type. For that reason, temporal laccase activity under -0.7 and -2.8 MPa for both sandy loam and clay soil are presented in Tables 3.2 and 3.3 respectively.

In the sandy loam, laccase activity was only registered in soil without amendment or inoculant under the driest soil conditions. The presence of pesticide, sawdust and fungal inoculant significantly ($0.001 < p < 0.04$) enhanced the activity of this enzyme, particularly at -0.7 MPa. Soil inoculated with the test isolate in the absence of atrazine produced the highest ($p < 0.01$) laccase activity levels under the wettest conditions, although significantly less ($p < 0.001$) activity was found in the presence of atrazine. Surprisingly, sawdust-containing soil generally had the lowest ($p < 0.04$) LAC activity, independently of water potential.

In contrast to the sandy loam, clay soil without amendment or inoculant was shown to have some LAC activity, especially under the wettest conditions. Nevertheless, similarly to the previous treatment, enhanced LAC production occurred ($p < 0.001$) as a response to amendments under both water regimes. In this case, soil containing sawdust was the treatment presenting the highest LAC activity under the wettest conditions, contrary to the S+A+T treatment which had the lowest. Surprisingly, the opposite behaviour was obtained under the driest soil conditions.

The S+A+T treatment gave the most inconsistent results as influenced by soil type and water potential. Likewise, peaks in activity over time were highly dependent on soil treatment, especially marked in the sandy loam. In the clay soil, however, the highest LAC production was generally obtained after 6 weeks incubation. Very little activity was often registered over the study period (24 weeks), independently of the treatment conditions.

Table 3.2 Temporal laccase activity (U g^{-1} soil) in sandy loam incubated for up to 24 weeks under 20°C at (A) -0.7 and (B) -2.8 MPa, as response to different soil amendments. *Key to treatments:* S, untreated control soil; S+A, soil contaminated with atrazine (2.5 ppm); S+S, soil with sawdust (0.5%); S+T, soil inoculated with *T. versicolor* (0.5%); S+A+T, soil contaminated with atrazine (2.5 ppm) and inoculated with *T. versicolor* (0.5%).

A)

	Incubation time (weeks)			
	0	6	12	24
S	0	0	0	0
S+A	19.0 ± 4.92	14.1 ± 1.76	6.63 ± 2.47	0
S+S	1.50 ± 0.43	8.50 ± 0.71	3.92 ± 1.41	0
S+T	36.5 ± 9.19	50.0 ± 17.7	18.0 ± 4.24	0
S+A+T	4.50 ± 1.12	8.00 ± 0.35	10.9 ± 0.84	11.2 ± 1.06

B)

	Incubation time (weeks)			
	0	6	12	24
S	5.50 ± 0.71	16.6 ± 1.06	7.76 ± 2.12	0
S+A	0	0	0	0
S+S	2.50 ± 1.41	0	0	0
S+T	22.4 ± 7.71	4.50 ± 0.35	3.43 ± 1.77	0
S+A+T	7.08 ± 1.98	34.0 ± 10.8	29.8 ± 3.54	0

Table 3.3 Temporal laccase activity (U g^{-1} soil) in clay soil incubated for up to 24 weeks under 20°C at (A) -0.7 and (B) -2.8 MPa, as response to different soil amendments. *Key to treatments*: S, untreated control soil; S+A, soil contaminated with atrazine (2.5 ppm); S+S, soil with sawdust (0.5%); S+T, soil inoculated with *T. versicolor* (0.5%); S+A+T, soil contaminated with atrazine (2.5 ppm) and inoculated with *T. versicolor* (0.5%).

A)

	Incubation time (weeks)			
	0	6	12	24
S	0.30 ± 0.29	2.55 ± 1.06	1.88 ± 0.88	0.67 ± 0.41
S+A	9.04 ± 1.41	12.0 ± 3.53	7.92 ± 1.57	0
S+S	9.50 ± 0.77	24.5 ± 9.60	16.4 ± 3.04	0
S+T	8.49 ± 3.53	21.2 ± 9.11	13.3 ± 2.89	0
S+A+T	18.5 ± 7.66	0.51 ± 0.22	0.50 ± 0.51	0

B)

	Incubation time (weeks)			
	0	6	12	24
S	0.72 ± 0.11	1.97 ± 0.80	0.86 ± 0.23	0
S+A	0	21.5 ± 4.32	15.7 ± 2.03	0
S+S	6.50 ± 0.51	11.1 ± 3.88	0	0
S+T	10.0 ± 1.06	14.6 ± 3.63	9.88 ± 1.54	0
S+A+T	14.2 ± 1.90	23.1 ± 5.69	13.4 ± 2.01	0

3.3.3 Temporal assessment of soil microbial population size and fungal community structure

Microbial population numbers

Overall, there was a tendency for microbial numbers to decrease over time, although this was not always a significant result. Surprisingly, there were no significant ($p < 0.05$) differences in terms of total population size as response to treatments and incubation period. There were, however, few but significant differences in terms of the relative responses of bacterial and fungal relative contributions to total population numbers influenced by these two factors. This observation was common to the three soil types. Data presented here as an example corresponds to that obtained for the sandy loam.

When comparing against the control, bacterial numbers were the most abundant ($p < 0.001$) in atrazine-amended soil, although this result was only significant under -0.7 MPa. This was the only treatment for which bacterial population size achieved a peak (8.85 and 6.81) after 6 weeks incubation before starting to decrease up to the end of the study. Interestingly, this behaviour was observed under both soil water potentials. In contrast, sawdust-amended soil has shown the lowest number of bacterial CFUs both in the presence and absence of the fungal inoculum and at both water regimes. Atrazine-amended soil containing the inoculum was generally favourable to bacterial numbers when comparing to that in soil containing the inoculum alone (e.g. 7.06 vs. 6.44). This was particularly evident under the wettest soil conditions.

Similarly to bacteria, fungal numbers were also higher in atrazine-containing soil than in any other soil treatment, for which a peak (8.46 and 6.77) was achieved around the 6th week of incubation, irrespective of water potential. Soil amendment with sawdust alone was also favourable to fungal numbers. However, numbers decreased significantly when soil was inoculated with the test isolate, whether atrazine was present or not. Nevertheless, and like that observed for bacteria, the presence of atrazine in soil containing the inoculum was generally favourable to fungal numbers compared to that

in soil containing the inoculum alone (e.g. 6.89 vs. 6.07) (although this result was not statistically significant at $p < 0.05$).

Table 3.4 Total population size and relative bacterial and fungal contributions as response to different soil treatments of sandy loam incubated for up to 24 weeks under 20°C at (A) -0.7 and (B) -2.8 MPa. Population size was given by the average of five replicates (\pm standard error) and was expressed as Log CFUs g^{-1} soil h^{-1} . *Key to treatments*: S, untreated control soil; S+A, soil contaminated with atrazine (2.5 ppm); S+S, soil with sawdust (0.5%); S+T, soil inoculated with *T. versicolor* (0.5%); S+A+T, soil contaminated with atrazine (2.5 ppm) and inoculated with *T. versicolor* (0.5%).

A)

		Incubation time (weeks)			
		0	6	12	24
S	Bacteria	7.58 \pm 1.14	7.22 \pm 0.33	7.39 \pm 0.89	6.31 \pm 0.22
	Fungi	6.50 \pm 0.98	6.37 \pm 0.16	6.49 \pm 1.05	6.60 \pm 0.58
	Total	14.1 \pm 1.07	13.6 \pm 1.11	13.8 \pm 1.56	12.9 \pm 1.16
S+A	Bacteria	7.63 \pm 1.13	8.85 \pm 0.09	8.54 \pm 0.36	7.71 \pm 1.22
	Fungi	7.08 \pm 1.01	8.46 \pm 1.20	8.12 \pm 0.45	7.93 \pm 0.96
	Total	14.7 \pm 1.10	17.3 \pm 1.13	16.7 \pm 1.47	15.6 \pm 1.18
S+S	Bacteria	7.39 \pm 1.07	6.41 \pm 0.88	6.37 \pm 1.01	6.19 \pm 1.05
	Fungi	7.75 \pm 0.57	7.71 \pm 0.07	7.59 \pm 0.66	7.68 \pm 0.38
	Total	15.1 \pm 1.02	14.1 \pm 1.14	14.0 \pm 1.08	13.9 \pm 1.14
S+T	Bacteria	7.56 \pm 0.28	6.44 \pm 0.22	6.48 \pm 1.08	6.49 \pm 0.71
	Fungi	6.82 \pm 0.13	6.07 \pm 0.83	6.31 \pm 0.96	6.40 \pm 0.02
	Total	14.4 \pm 1.67	12.5 \pm 1.48	12.8 \pm 1.03	12.9 \pm 1.63
S+A+T	Bacteria	7.50 \pm 1.19	7.06 \pm 0.09	6.52 \pm 0.96	6.66 \pm 1.30
	Fungi	7.31 \pm 1.07	6.89 \pm 0.54	7.40 \pm 0.81	7.54 \pm 0.86
	Total	14.8 \pm 1.12	13.9 \pm 1.01	13.9 \pm 1.17	14.2 \pm 0.98

B)

		Incubation time (weeks)			
		0	6	12	24
S	Bacteria	6.25 ± 0.99	6.13 ± 0.79	6.15 ± 0.84	5.98 ± 0.78
	Fungi	6.18 ± 0.98	5.90 ± 0.56	5.87 ± 0.96	5.80 ± 0.69
	Total	12.4 ± 1.03	12.0 ± 1.63	12.0 ± 1.01	11.8 ± 1.40
S+A	Bacteria	6.43 ± 1.13	6.81 ± 0.19	6.65 ± 0.43	6.61 ± 0.55
	Fungi	6.29 ± 1.01	6.77 ± 1.20	6.40 ± 0.62	6.32 ± 0.67
	Total	12.8 ± 1.10	13.6 ± 1.13	13.5 ± 1.22	12.9 ± 1.59
S+S	Bacteria	6.76 ± 1.07	6.19 ± 0.88	6.15 ± 0.63	5.88 ± 0.90
	Fungi	7.43 ± 0.57	7.38 ± 1.07	7.07 ± 0.48	6.96 ± 0.39
	Total	14.2 ± 1.02	13.9 ± 1.94	13.2 ± 1.05	12.8 ± 1.11
S+T	Bacteria	7.01 ± 1.28	5.27 ± 0.10	5.83 ± 0.94	5.74 ± 0.91
	Fungi	6.22 ± 1.13	5.49 ± 0.83	6.10 ± 0.80	6.23 ± 0.52
	Total	13.2 ± 1.37	10.8 ± 1.48	11.9 ± 1.61	12.0 ± 1.63
S+A+T	Bacteria	7.44 ± 1.19	5.82 ± 1.00	5.43 ± 0.42	5.31 ± 0.83
	Fungi	7.38 ± 1.07	5.99 ± 1.14	6.26 ± 0.66	6.24 ± 0.77
	Total	14.9 ± 1.12	11.8 ± 2.01	11.7 ± 1.58	14.6 ± 1.23

Fungal community structure

Fungal community structure has shown to be markedly influenced by soil treatments in the three soil types. As an example, data for the clay soil is presented in Table 3.5 and Figure 3.12, since amendment induced in this soil the highest diversity increase, when comparing against that in the other two. Table 3.5 shows the number of species isolated in clay soil as a response to soil treatment. Figure 3.12 shows the changes in fungal community structure as influenced by amendment at (A) -0.7 and (B) -2.8 MPa. Data shown was obtained over 6 week incubation, when most studied parameters suggested higher microbial activity.

In un-amended soil, there was a reduction by one species ($p < 0.03$) as a response to drier soil conditions. *Aspergillus*, *Penicillium* and *Rhizopus* were the only genera found in this soil under the study conditions, irrespective of soil water potential. In atrazine-amended soil there was an increase of at least 50% compared to un-amended soil and was responsible for the highest number of species and different genera isolated in this soil over the study period. Sawdust amendment has also shown to be favourable to fungal diversity within this time period, causing (at least) a 35% increase in the number of species isolated under both water regimes. The number of genera found as result of sawdust addition was at least 50% higher than in untreated soil, although 30% smaller than that resulted by the addition of atrazine under the wettest conditions. However, when comparing to the latest, the presence of *T. versicolor* significantly decreased both the number of species ($p < 0.001$) and that of genera ($p < 0.05$) found, particularly under the driest conditions. In soil treated with both atrazine and the test isolate, a small but significant improvement was found in relation to the previous. In this case, it was evident an increase in diversity in terms of genera ($p < 0.001$) comparing against soil containing the inoculum alone, specially under low water potentials.

Table 3.5 Influence of soil treatment on the number of species growing onto MEA isolated from clay soil incubated for 6 weeks at 20°C. It was given by the average of five replicates (\pm standard error). *Key to treatments*: S, untreated control soil; S+A, soil contaminated with atrazine (2.5 ppm); S+S, soil with sawdust (0.5%); S+T, soil inoculated with *T. versicolor* (0.5%); S+A+T, soil contaminated with atrazine (2.5 ppm) and inoculated with *T. versicolor* (0.5%).

	- 0.7 MPa	-2.8 MPa
S	6 \pm 0.17	5 \pm 0.43
S+A	13 \pm 0.12	9 \pm 0.11
S+S	10 \pm 1.09	8 \pm 0.22
S+T	6 \pm 0.08	6 \pm 0.41
S+A+T	7 \pm 0.64	6 \pm 0.09

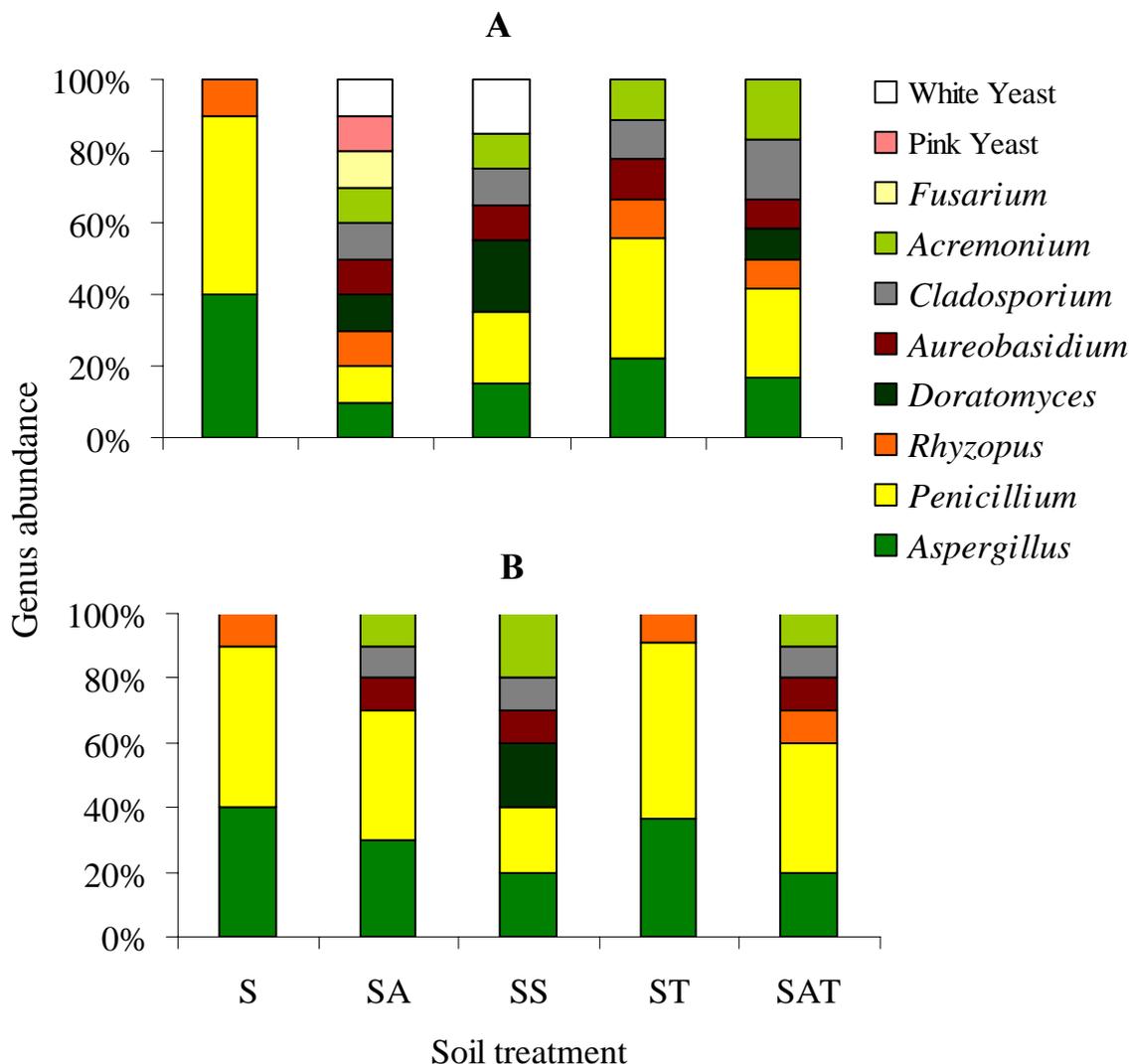


Figure 3.12 Influence of soil treatment on fungal community structure in clay soil incubated for 6 weeks at (A) -0.7 and (B) -2.8 MPa at 20°C, given by the average of 5 replicates. *Key to treatments:* S, untreated control soil; SA, soil contaminated with atrazine (2.5 ppm); SS, soil with sawdust (0.5%); ST, soil inoculated with *T. versicolor* (0.5%); SAT, soil contaminated with atrazine (2.5 ppm) and inoculated with *T. versicolor* (0.5%).

3.3.4 Monitoring atrazine concentration in soil microcosms

Table 3.6 shows the remaining atrazine, added at an initial concentration of 2.5 ppm, in sandy loam soil incubated for up to 24 weeks at 20°C, -0.7 MPa, in the absence (S+A) and in the presence (S+A+T) of *T. versicolor*. The concentration of atrazine extracted from soil decreased with the incubation period in both treatments, achieving its fastest rate within the first 6 weeks.

In the absence of the fungus, 0.403 ppm of atrazine was recovered from the treated soil incubated for 6 weeks, corresponding to 16.1% of the initial concentration of the herbicide. For the same time period, only 0.104 ppm of atrazine (i.e. 4.2% of the initial concentration) was extracted from soil containing the inoculum. By the end of the study, residues of the herbicide in soil were down to 0.128 and 0.056 ppm in the absence and in the presence of the inoculum, respectively.

However, the impact of *T. versicolor* on the rate of atrazine disappearance from soil, was only significant ($p=0.00039$) within the first 6 weeks incubation. On the contrary, there was no significant ($p=0.28$) difference between both treatments after 24 incubation weeks under the study conditions.

Table 3.6 Remaining atrazine, added at an initial concentration of 2.5 ppm, in sandy loam soil incubated for up to 24 weeks at 20°C and -0.7 MPa in the absence (S+A) and in the presence (S+A+T) of *T. versicolor*.

Incubation (weeks)	Treatment	Remaining atrazine (ppm)
6	S+A	0.403* \pm 0.001
	S+A+T	0.104* \pm 0.002
24	S+A	0.128 \pm 0.041
	S+A+T	0.056 \pm 0.019

* Statistically different at $p < 0.05$

3.4 DISCUSSION

It has been recognised the role of soil and the influence of its properties on the recycling of a wide range of potential environmental contaminants such as pesticides through the activity of microbial populations (Bridges, 1998; Doran, 2002; Stengel and Gerin, 2003; Crawford *et al.*, 2005). There is enough evidence in the literature that key characteristics of soil microbial populations, such as activity and size, can be useful indicators of changes in soil conditions (Bloem *et al.*, 2006). In this chapter, they proved to be useful for evaluating and monitoring the status of atrazine biodegradation in soil by *T. versicolor* under low water potentials. They also allowed assessing the impact of the presence of atrazine at field application rate on soil microbial populations under such low water regimes.

Most studies done so far involving the application of white-rot fungi for bioremediation of environmental contaminants were performed using liquid culture media (Šašek *et al.*, 2003). In soil, where other factors influence pesticide degradation apart from the fungus degrading capabilities, knowledge is more limited (Ghanni *et al.*, 1996).

The development of the appropriate methodology for the bioremediation experiments required a detailed previous knowledge on the capabilities of native soil microbial communities (i.e. microbial status, in terms of numbers and activity). A key aspect that was taken into consideration while designing the experiment, was to ensure an optimal performance of *T. versicolor* in terms of growth and activity, which is dependent on its capability to compete with native microflora in contaminated soil. According to Šašek *et al.* (2003), this is an important aspect since the interaction between both parts can result in either inhibition or cooperation in the degradation process. This study employed two strategies in order to enhance *T. versicolor* colonisation and activity in non-sterile soil: growth on sawdust prior to inoculation into soil in order to maximise growth and enzyme production; use of sawdust as carrier but also as nutrient source selective for the fungus.

In previous studies (Fragoieiro, 2004) *T. versicolor* exhibited good tolerance to water stress conditions as well as to triazine pesticides (simazine, in particular). *T. versicolor* has been used widely in liquid culture for bioremediation of a wide range of compounds, among which chlorophenols, synthetic dyes and pesticides (Šašek *et al.*, 2003). There are also suggestions that contrary to the widely used *P. chrysosporium*, *T. versicolor* produces insignificant amounts of toxic metabolites (Lamar *et al.*, 1993). However, up to now, this species has been studied scarcely in the soil environment (Gadd, 2001; Šašek *et al.*, 2003). In this study, the test isolate was grown on moist sawdust for up to three weeks at 25°C prior to inoculation into soil. Extensive colonisation of the sawdust was observed by the end of the cultivation period. However, according to Boyle (1995), biomass growth is not necessarily correlated to a biodegradation-promoting physiological state.

3.4.1 Temporal soil volatile fingerprints

1) Time-independent response of volatile profiles to treatments:

A clear discrimination between untreated and atrazine-treated soil was obtained after 1 day incubation under the treatment environmental conditions, using 84% of the overall information. The addition of pesticides into soil, such as atrazine, is likely to have had an impact on microbial community structure, determining which microorganisms were metabolically more active or perhaps by changing the metabolic pathway of suitable microorganisms. In order to establish whether the differentiation between untreated and treated soil was caused by the impact of atrazine on microbial activity rather than on the atrazine odour itself, a control solution of atrazine in RO water was used as reference.

The discrimination was obtained regardless of soil type and water potential, although higher discrimination levels were always achieved at -0.7 MPa. This may indicate that atrazine can be detected in soil using this approach, independently of soil characteristics (e.g. organic matter content) and environmental conditions. It has been thus

demonstrated that the E-nose threshold detection level for atrazine in these three soil types at 20°C and -0.7 MPa is below 2.5 ppm. This is a relevant result from an environmental point of view, particularly when considering that the amount of atrazine used in this study was equivalent to field application rates.

From a health point of view, this is also important. The widespread use of atrazine, its moderate persistence and mobility in soil has led to its frequent detection in soil and water resources (Gil-Sotres, 2005; Rhine *et al.*, 2003). In potable water, the presence of atrazine can be detected by the human nose at concentrations above 9200 ppm, with its odour being described as “plastic-” and “weak bleach-like” (Young *et al.*, 1996). It is likely that the human threshold level for atrazine in soil is even higher. However, at these concentrations atrazine is already considered a serious hazard when ingested (Young *et al.*, 1996). The Environment Protection Agency (EPA) and the World Health Organisation (WHO) have determined maximum contaminant levels of 3 and 2 ppm (respectively) for atrazine in surface and groundwater (Young *et al.*, 1996; Rhine *et al.*, 2003), many times inferior than its human OTC in water. This suggests that the use of a trained E-nose for the detection of atrazine and perhaps other herbicides in both water and soil could be a valuable tool for Environmental and Health screening purposes.

Up to now, very few studies have employed sensor arrays for the detection of low levels of pesticides in water, and even fewer in soil. Commonly applied insecticides (such as lindane and nitrobenzene) have been recently detected in water at 1 and 500 ppm using a sensor array (Baby *et al.*, 2000).

It has been shown previously (Chapter II) that commonly applied soil amendments such as cereal straw markedly influence soil volatile fingerprints, as they are used by microbial populations as carbon and energy sources. In this Chapter, there was also discrimination between un-amended and sawdust-amended soil (data not shown). But in this case, the relevant result is the differentiation between volatile patterns of soil treated with atrazine and that with sawdust, independently of soil type, using at least 75% of the variance. Soils were incubated at 20°C, -0.7 MPa, for 5 days considering that sawdust is a slowly-degradable nutrient. This result suggests that this approach is sensitive enough

to discriminate between soil containing herbicides at concentrations similar to field rates and soil amended with carbon-rich supplements. It is known that generally, herbicides are added to soil in combination with other organic supplements. In this sense, it is important that atrazine detection in soil is possible in the presence of other organic substrates.

It was possible to discriminate between un-inoculated soil and that carrying the test isolate following 5 days incubation at 20°C, -0.7 MPa. This shows that the headspace volatiles of soil in the absence and in the presence of *T. versicolor* are distinctively different (using 82% of the information contained within the data), independently of soil type. The genera *Trametes* are known to produce a wide range of halogenated organic compounds, most of which are chlorinated products (Paszczynski and Crawford, 2000). The release of these compounds into the headspace probably explains the discrimination obtained. This is an interesting result, since it indicates that *T. versicolor* was metabolically active at the time of analysis, even when inoculated into non-sterile soil. However, care should be taken when using this approach on its own for determining whether the inoculant is metabolically active or not. For example, it is also likely that the presence of the test isolate may influence the metabolism of native microbial populations due to interaction competitions. In this case, a change would also be expected a change in soil volatile fingerprints, without that being directly related to the metabolism of the inoculant. For this reason, volatile fingerprint analysis on its own may not be a sufficient tool for assessing the status of the fungal inoculant in non-sterile soil, but as a complementary approach to other microbial methods such as LAC production.

The presence of atrazine clearly influenced volatile fingerprints of soil inoculated with *T. versicolor*. The PCA map showed a marked differentiation between the headspace volatiles of sandy loam inoculated with *T. versicolor* in the absence and in the presence of atrazine (2.5 ppm), following 5 days incubation at 20°C, -0.7 MPa. This is highly relevant for the objectives of this chapter, suggesting that the presence of atrazine changes the metabolism rate and pathway of the inoculated fungus when a ligninolytic substrate (sawdust) is provided.

2) Temporal effects on the response of volatile profiles to treatments

Headspace volatile patterns of all treatments were shown to change over time. Even the volatile profiles of un-amended soil were different throughout the study period, demonstrated by the PCA map of untreated clay soil incubated for 0, 6 and 24 weeks at 20°C, -0.7 MPa. In fact, while the cluster corresponding to 0 weeks incubation was markedly separated from the remaining clusters, the latest two were found to be overlapping. That overlap was observed using the three soils, although its extent varied slightly with soil type. The highest degree of overlapping was shown by the volcanic ash. It may be a reflection of the selective effects of nutrient exhaustion, accompanied by a decrease in nutritional diversity within the first 6 weeks of incubation. It is likely that nutrient exhaustion within the first 6 weeks would be responsible for a shift in microbial community structure, favouring slow-growing microorganisms and those capable of degrading the more recalcitrant fraction of native organic matter. This hypothesis may explain the similarity observed between the volatile fingerprints of soil incubated for 6 and 24 weeks.

Volatile fingerprints in atrazine-treated soil were also shown to vary throughout the experiment, shown by the PCA map for samples of volcanic ash soil incubated for up to 24 weeks at 20°C, -0.7 MPa. Differentiation between clusters according to incubation period was possible using 88% of the variance within data. The map shows that treatments corresponding to 0 weeks incubation were clearly separated from the remaining treatments, whereas clusters relating to 6 and 24 incubation weeks were found to be closer together. There was however, no overlapping between these latest two clusters, which was also true when using the sandy and clay soils. It is likely that dissimilarities between treatments over time indicate atrazine metabolism by native microbial populations. A study by Wünsche *et al.* (1995) corroborates this hypothesis. Authors used the Biolog microplates for studying the substrate utilisation patterns of microbial communities in a wide range of soil types artificially contaminated with hydrocarbons. They have reported a temporal shift within the substrate utilisation patterns of the overall community as response to oil metabolism, suggesting an alteration in total metabolic activity and changes in community structure.

Another interesting result in the current study was that soil volatile fingerprints after 24 weeks were still distinctively different from that in the beginning of the experiment in all soil types, suggesting that atrazine has not been completely mineralised by the end of the study. Even though some atrazine was probably converted to CO₂ throughout the incubation period, some of it may have just been transformed. In fact, pesticide metabolism and mineralization are thought not to be linked. For example, Boyle *et al.* (1997) has found that while mineralization of some organopollutants in soil by white-rot fungi had not occurred, their metabolism was clearly observed by the formation (and detection by GC/MS) of a wide range of polar metabolites.

A temporal discrimination between treatments of soil containing sawdust was also possible, using 82% of the variance. The separation between the three clusters was clear, indicating great dissimilarities between volatile patterns of sawdust-amended soil incubated for 0, 6 and 24 weeks. The fact that clusters corresponding to 0 and 24 weeks were so markedly separated on the plot suggested that sawdust was being metabolised by the native populations, although at a slow rate. In Chapter II, results concerning the temporal discrimination between clusters of glucose or wheat straw-amended soil and the control suggested that volatile fingerprint analysis could be used for monitoring organic matter degradation in soil for a period up to 10 days. In this Chapter, the results obtained for the sawdust treatment confirm that hypothesis. Moreover, it shows that long-term monitoring can also be performed using this approach, independently of the nature of the organic amendment added to soil. The fact that similar results were obtained for the other two soil types further indicate that soil monitoring with this purpose can be performed irrespective of soil type. One useful application for this result would perhaps be for studying the impact of substrate addition on microbial community structure, in particular when combined with methods for taxonomic identification.

Dissimilarities between volatile profiles at the beginning and end of the study period were also found for soil inoculated with the test isolate, independently of soil type. The PCA map of sandy soil samples inoculated with *T. versicolor* and incubated for up to 24 weeks at 20°C, -0.7 MPa has shown three distinct clusters. There was a clear

differentiation between the cluster corresponding to 0 weeks and the remaining two, whereas in contrast, treatments incubated for 6 and 24 weeks were grouped into clusters closer together with no overlapping. As previously suggested, this may reflect metabolism of the test isolate due to colonisation of the sawdust and use of the polysaccharides (mainly cellulose and hemi-cellulose) embedded in the lignin matrix. The presence of sawdust in the medium aimed to enhance the fungus activity, offering it advantage over the indigenous populations. Therefore, the separation between clusters may also be due to interactions between the inoculant and the native microbial population.

A clear differentiation between soil treatments containing both the inoculum and atrazine, according to the incubation period, was also achieved for all soil types using at least 85% of the overall information. The PCA map for clay soil inoculated with *T. versicolor* incubated for up to 24 weeks in the presence of atrazine, showed that the treatment volatile fingerprints are distinctively different at 0, 6 and 24 weeks incubation. This may indicate that the inoculum (probably in combination with native microorganisms) was metabolising atrazine. The fact of volatile profiles being clearly different in the beginning and at the end of the time study suggests that a great part of the atrazine present was mineralised, or at least transformed. For example, Boyle *et al.* (1997) has found that metabolism of some organopollutants in soil by white-rot fungi may occur, even though no mineralization is registered. Their metabolism was identified by GC/MS through the detection of a wide range of polar metabolites. In addition, they realised that these polar metabolites were more rapidly degraded by soil microflora than the original contaminant. This supports the idea that although white-rot fungi may be key for starting the degradation process of the contaminant, it is likely that metabolism (and perhaps mineralization) occur from the activity of the whole microbial community. However, this technique by its own should be used carefully when predicting whether the bioremediation process has occurred or not.

Although the discrimination between treatments was observed for the three soil types under both water potentials, small variations in the discrimination level were obtained as influenced by soil type and water potential. For example, the level of discrimination

obtained between untreated and atrazine- and sawdust-treated soil was generally lower for the -2.8 MPa treatment. Likely, this reflects a poorer distribution of the amendment in soil under such low water availability.

PC1 was generally the factor explaining the majority of the variance within the data set. By looking specifically at the discrimination of treatments as influenced by incubation period, that suggests that PC1 may probably be linked to volatile intensity.

3.4.2 Temporal soil respiratory activity

Temporal soil respiration was used as an indicator of microbial activity and pesticide mineralization throughout the study. Cumulative respiration curves allowed studying and comparing between the rates of CO₂ evolution as response to soil treatments, for up to 24 weeks. High CO₂ production levels in atrazine-treated soil as result of enhanced microbial activity, suggested atrazine mineralization. In this study, maximal CO₂ evolution was generally attained after 6 weeks incubation for the sandy loam and clay soils, independently of soil water potential. For this time period, atrazine contaminated soil carrying the inoculum was the treatment generally presenting the highest respiratory activity, at least 20% higher than in the absence of the fungi. Similar results were obtained by Fragoeiro (2004), who employed three different white rot fungi, among which *T. versicolor* (R26), for bioremediation of a pesticide mixture in soil. Using a sandy loam under the same water potential conditions, Fragoeiro (2004) has also reported a peak in respiration around week 6 of incubation and maximal CO₂ evolution from soil treated with a mixture (5 ppm) of simazine, dieldrin and trifluraline, as well as *T. versicolor* (R26). In contrast, the respiratory peak found by Ghani (1996) and Houot *et al.* (1998) following atrazine addition into moist soil was obtained after 15 and 14 days respectively. Both authors also explained the presence of the peak as indicating atrazine mineralization.

When compared to un-amended soil, all treatments have shown an increase in soil respiration as a result of the amendment, although surprisingly, differences between

respiratory activity in atrazine-, sawdust- and *T. versicolor*- amended soil (alone) were generally not significant. This may indicate that the activity of *T. versicolor* in soil microcosms was lower than expected, probably due to interactions with native microbial populations. In this case, results do not agree with those obtained by Fragoeiro (2004), where soil inoculated with *T. versicolor* alone has shown improved respiration when compared to pesticide contaminated soil or that amended with wood chips.

The higher respiratory activity in soil treated with sawdust or with atrazine (under the treatment concentrations) compared to un-amended soil is not surprising, as both may be used as nutrient sources by native microbial communities. The impact of adding C-rich nutrients on soil microbial respiration has been widely discussed in Chapter II. Another possible benefit of the addition of sawdust, which could also result in enhanced microbial activity, may be an improvement in soil aeration. This effect has been suggested by Boyle (1995) using wood chips.

According to Ghani *et al.* (1996), the application of herbicides to soils can induce two types of response from microbial communities: inhibitory through toxicity or stimulatory when used as nutrient resources by suitable microorganisms. Nitrogen most often controls the rate of many soil processes such as organic matter degradation, since it is the limiting nutrient (Miller and Donahue, 1995). Atrazine is a poor source of C and energy but is a valuable source of N for microorganisms able to utilise the side chain and ring-N atoms (Haney *et al.*, 2002). According to Rhine *et al.* (2003), the amount of available N provided by the addition of 2.5 ppm of atrazine is approximately 0.8 ppm. Other studies have also reported an enhancement in microbial respiration (e.g. Haney *et al.*, 2002) or rather no effect at all (e.g. Moreno *et al.*, 2007) as a result of atrazine addition, comparing to untreated soil. For example, Moreno *et al.* (2007) reported that respiration was more or less constant throughout the study in soil containing 1 ppm of atrazine, incubated at 28°C, although the increase in respiration induced by atrazine addition has shown to be dependent on the concentration of the herbicide (Moreno *et al.*, 2007).

Alexander (1984) goes further suggesting that the increase in microbial activity following atrazine addition may be even more accentuated when soil organic matter is scarce. However, this has not been confirmed by this study, since no significant ($P>0.06$) difference was found between the increase in microbial activity after atrazine addition in the volcanic ash (40.8%) and that in the sandy loam (39.8%). Similarly to the effect of atrazine, other potential soil contaminants have also stimulated soil microbial activity. For example, Bundy *et al.* (2002) reported an immediate increase in respiration of two sandy and a silt loam soils following the addition of diesel.

Following the peak around week 6, soil respiration generally started decreasing at a slow but consistent rate up to the end of the study, probably due to nutrient exhaustion. This was obtained for all treatments, even for those containing atrazine or sawdust, but the decrease was faster for untreated soil. For the atrazine treatment in particular, this is a normal trend for respiratory activity when comparing to other studies where the herbicide (Haney *et al.*, 2002) or other potential soil contaminants such as diesel (Bundy *et al.*, 2002) were added to soil. In both studies, respiration had returned to near-control values by the end of the study at day 56 and 62 respectively.

Unlike that obtained under the wettest treatment, respiration rates between treatments were generally not statistically different ($0.05 < p < 0.27$) under the driest conditions, throughout the study. This is probably because water potential rather than nutrient content was the limiting factor to soil respiration, as previously discussed in further detail in Chapter II. The reason may be directly linked to the inhibitory effects of desiccation on microbial cells but also (indirectly) to limited diffusion of nutrients and pesticide molecules throughout the soil (Kearney and Roberts, 1998). A similar influence of drier soil conditions on soil respiration throughout a bioremediation program of a mixture of pesticides has also been recently reported by Fragoeiro (2004).

The use of respiration analysis for monitoring pesticide biodegradation in soil may therefore provide relevant information regarding the status of total soil microbial activity, as well as an insight to that of the relative contribution of the inoculated fungus. However, respiration alone does not provide the overall picture. For example,

respiration data suggests that *T. versicolor* relative activity was lower than that expected, although it does not explain why that may be.

Respiration has been employed by several authors for studying the impact of herbicides on soil microflora (e.g. Wainwright, 1978; Schinner *et al.*, 1996; Margesin *et al.*, 2000). However, although in some studies on respiration have shown that it can be employed as an early indicator of stress (Bloem *et al.*, 2006), there is evidence that it can be insensitive for monitoring the impact of certain soil contaminants on microbial communities, unless they are present at very high concentrations (Brookes, 1984; Landi *et al.*, 2000). For this reason, some authors prefer using the “lag time” approach to respiration analyses, by measuring its response to nutrient addition.

The change in soil respiration over time (sigmoid curve) can provide important information when studying the early impact of pollutants on soil microbial activity by comparing the “lag time” following the addition of nutrients between contaminated and un-contaminated (control) soil (Nordgren, 1988; Shen and Bartha, 1997). The lag time, rather than the amount of CO₂ respired, has been used in these cases as a more sensitive indicator of stress. For example, Nordgren *et al.* (1988) and Shen and Bartha (1997) reported an increase in the lag time prior to glucose, cellulose and amino acid degradation following the addition of heavy metals or biodegradable plastics into soil. They interpreted changes in the lag time as being a reflection of changes in the physiological status of the microbial population, perhaps due to toxicity, and consequently, a delay in organic matter degradation. Brookes (1995) supports this suggestion. Another interpretation of the lag time may be that added soil pollutants, such as heavy metals, may cause a shift in microbial community structure (from a prokaryote- towards an eukaryote-dominant microbial population), as suggested by Landi *et al.* (2000).

Basal respiration has also been used in combination with substrate-induced respiration in a diverse range of studies for evaluating the effects of contaminants and stress factors on soil microbial populations. For example, Perucci *et al.* (2000) employed respiration in order to monitor over 30 days, the impact of an herbicide on microbial activity in

untreated and compost-treated soil. Using this approach authors concluded that the addition of compost into soil could overcome the detrimental effect of the herbicide on soil microbial activity.

3.4.3 Temporal dehydrogenase activity

Intracellular enzymatic activity has been associated to key soil functions such as organic matter oxidation and xenobiotic degradation (Min *et al.*, 2001; Acosta-Martinez *et al.*, 2003). Cumulative DHA curves were produced for each soil type in order to study the way the activity of this enzyme varied throughout the study, according to the treatment given to soil. In this way, it was possible to investigate the response of the native microbial populations, as well as the introduced *T. versicolor* to atrazine addition into soil. The clay soil presented the highest activity rate of this enzyme within the time study when compared against that of the other two soils (data not shown).

Regardless of soil treatment, the highest DHA levels were achieved under the wettest conditions. The impact of water potential on soil oxidative metabolism has already been discussed in Chapter II. Overall, soil amendment enhanced DHA in all soil types, regardless of soil water potential. For example, soil amended with sawdust produced the highest DHA, which was at least 53% higher than in untreated soil, corroborating results obtained Moorman *et al.* (2001). Similarly, in soil containing atrazine the DHA levels obtained were higher compared to un-contaminated soil. Previous studies reviewed by Kearney and Roberts (1998) have also achieved similar results using a range of atrazine concentrations and soil types, when compared to other pesticides which decreased microbial activity. For example, Moreno *et al.* (2007) registered an increase in DHA with the incubation period for atrazine-treated soil in the range of 0.2 to 1000 mg kg⁻¹ soil incubated at 28°C.

The inoculation of *T. versicolor* into soil (alone and in the presence of atrazine) enhanced DHA levels by over 40% when compared to untreated soil, under both water

potentials. Surprisingly, there was no significant difference between DHA in soil inoculated with *T. versicolor* and atrazine and that of soil carrying the inoculant alone.

In most treatments, the highest activity levels of this enzyme were obtained after 6 weeks of incubation, although differences between that and those obtained after 12 weeks were not always significant ($0.002 < p < 0.009$) under the conditions studied. Fragoeiro (2004) has also found maximal DHA levels in sandy loam after 6 weeks of incubation independent of the treatment applied to soil, although the levels decreased rapidly between 6 and 12 weeks. The fact that high levels of this enzyme were still recorded in this study following 12 weeks of incubation may have to do with differences in soil characteristics and those of native microbial communities between both studies.

Surprisingly, unlike that observed in the remaining treatments, soil containing atrazine as well as *T. versicolor* has generally showed the highest ($p < 0.05$) DHA at the end of the study (following 24 weeks of incubation), suggesting enhanced oxidative metabolism for this time period. This result is not in agreement with respiration data for the same treatments, where CO₂ rates started decreasing after 12 weeks of incubation. The discrepancy found between respiration and DHS activity data in this specific treatment suggests that atrazine degradation by white-rot fungi may not be coupled to oxidative metabolism of organic matter.

Other studies have also support this suggestion, that there may not be a link between pesticide mineralization and DHA. For example, McGrath and Singleton (2000) monitored PCP biodegradation in a clay loam. After 6 weeks incubation at 25°C, they found that PCP concentration had decreased from 250 to 2 mg kg⁻¹, whereas DHA had dropped following the addition of PCP into soil and remained low throughout the study. Interestingly, they have also found that inoculating the soil with *P. chrysosporium* did not enhance PCP degradation rates at the concentration studied. It supports the idea that certain soil contaminants (perhaps concentration-dependent) or, alternatively, the generation of toxic biodegradation products of the original contaminant may be inhibitory for DHA and under those circumstances, it may be difficult to establish a link

between DHA and pesticide breakdown. According to Somerville and Greaves (1987), DHA probably depends upon the nature and concentration of available substrates in soil and their potential and strength as alternative electron acceptors.

Several authors have employed dehydrogenase assays for evaluating the impact of soil pollutants on soil health. Corroborated by this study, others have also demonstrated that pesticide addition into soil may enhance oxidative metabolism of soil organic matter. Fragoeiro (2004) has recently reported similar results in a sandy loam containing a mixture of simazine, dieldrin and trifluraline (5 and 10 ppm). Likewise, Min *et al.* (2001) have previously registered an increase in DHA activity as response to increasing concentrations (up to 22 ppm) of butachlor being added into soil. Nevertheless, other soil contaminants have shown to have the opposite effect. For example, Brookes (1984) reported a significant decrease in DHA in metal-contaminated soil, when compared to pristine soils. Contradictory results on the effects of the soil contaminant on microbial oxidative metabolism may be explained by the type and concentration of contaminant (Brookes, 1984; Min *et al.*, 2001).

3.4.4 Temporal laccase activity

The direct measurement of fungal growth in soil is difficult, since it is not possible to clearly differentiate the hyphae from the carrier and organic matter (Novotny, 1999), therefore, fungal colonisation in soil is usually measured indirectly through enzyme activity. The ability of white-rot fungi to degrade pesticides has been associated with the production of extracellular ligninolytic enzymes such as LAC in the presence of ligninolytic substrates (Novotný *et al.*, 2004; Fragoeiro and Magan, 2005). LAC is non-specific with regard to its substrate (Pointing, 2001) and it has been shown that the enzyme is able to co-metabolise lignin-like compounds (e.g. herbicides, among many common soil contaminants) with lignin through similar oxidative mechanisms (Hägglom, 1992; Paszczyński and Crawford, 2000; Gadd, 2001). Lignin-extracellular metabolism therefore explains the ability of white-rot fungi to tolerate such a wide range of environmental organic pollutants, even when present at high concentrations

(Kearney and Roberts, 1998; Gadd, 2001; Baldrian and Šnadjr, 2006). On the other hand, since the production of LAC is only dependent on the presence of ligninolytic substrates (and not of the contaminant), there is no need for pre-conditioning the fungi to it. And for the same reason, white-rot fungi have the ability to degrade the contaminant even when present at very low concentrations (Canet *et al.*, 2001).

Amongst the parameters used in this study, LAC activity showed the highest variability and inconsistency as influenced by the soil treatment. For example, in untreated sandy loam soil, LAC was not detected under the wettest treatment, although surprisingly, some activity was registered under the driest conditions. In contrast, untreated clay soil presented LAC activity, independently of soil water potential. Extracellular LAC activity is expressed in various genera of fungi (e.g. *Aspergillus*, *Rhizopus*), some actinomycetes (e.g. *Streptomyces*) and bacteria (e.g. *Pseudomonas*, *Bacillus*), but is mostly found in white-rot fungi (Kearney and Roberts, 1998). Those results, therefore, suggest that native wood-degrading fungi may be a key contribution to soil overall metabolic activity in soils dominated by recalcitrant organic matter and those under osmotic stress conditions.

The presence of atrazine, sawdust and *T. versicolor* individually enhanced significantly ($0.001 < p < 0.04$) the activity of this enzyme in both soil types. The addition of atrazine did not impair LAC production in either soil, but rather showed to have stimulated it, particularly at -0.7 MPa. In contrast, the effect of sawdust addition was less clear and appeared to be related to soil type. The sawdust treatment generally presented the lowest LAC activity in the sandy loam and the highest in the clay soil. It again might be a reflection of a more active wood-degrading fungi community in the clay soil, which meanwhile would have colonised the sawdust.

Surprisingly, both soils generally produced higher LAC when inoculated with the test isolate alone, than that in the presence of atrazine. It seems to indicate that atrazine impaired LAC production in this treatment, which is an unexpected result. Given the low concentration of herbicide applied in this study and comparing to data from other studies in the literature, it is unlikely to be a result of toxicity. Considering the effect of

atrazine and the test isolate individually, one explanation for this could be competition interactions between *T. versicolor* and native microbial populations in the presence of atrazine. Previously, other studies have also reported LAC inhibition in the presence of some pesticides using different species of white-rot fungi. For example, Sannino *et al.* (1999) found that *Cerrena unicolor* did not produce LAC in the presence of simazine (0.5-7 ppm concentration range). Among others, Boyle *et al.* (1992, 1997) have suggested that the presence of additional N-sources (in this case the pesticide) may in some cases inhibit the lignin-degrading system of white-rot fungi, preventing wood degradation although probably enhancing its growth. In contrast, Fragoeiro (2004) reported the highest LAC activity in a sandy loam containing *T. versicolor* and a pesticide mixture (5 and 10 ppm), when compared to soil containing the inoculum alone.

The treatment containing both atrazine and *T. versicolor*, has also presented the most inconsistent results over time. For example, in the sandy loam, this treatment presented a peak in laccase activity after 24 weeks incubation at -0.7 MPa, whereas in clay soil the same treatment has shown the maximal LAC levels at the beginning of the experiment (0 weeks). In contrast, the remaining treatments generally presented the highest enzymatic activity after 6 weeks, followed by a consistent decrease up to the end of the study.

Water potentials of -0.7 and -2.8 MPa have shown to have some influence on the activity of LAC, although the effects have little consistency and appeared to be dependent on soil type and treatment. Similar observations have been made by Fragoeiro (2004) and Schmidt *et al.* (2005), in respect to the effects of water potential. Other authors have also studied the influence of soil water potential on LAC activity, including Boyle (1995). This author has found that *T. versicolor* produced maximal LAC levels in soil around -0.4 and -0.9 MPa, with no activity having been registered at and below -3.4 MPa.

However, the inconstancy in results continues in the literature. According to Novotný (1999), *T. versicolor* generally produces relatively low levels of LAC in non-sterile soil.

In contrast, Fragoeiro and Magan (2005) reported extremely high LAC activity (797.8 units ml⁻¹) by *T. versicolor* growing in a sandy loam soil at 25°C, -0.7 MPa, in the presence of pesticide mixture (simazine, dieldrin and trifluralin) in the range of 5-30 ppm. On the other hand, Mougín *et al.* (1996) employed *P. chrysosporium* for the degradation of the insecticide lindane in soil and found it to be independent on LAC production by the fungus.

Contradictory results have led to the role of LAC production in the co-metabolism of pesticides with lignin by white-rot fungi not yet to be well understood. Novotný (1999) defended that LAC production and activity may be dependent on the nature and concentration of the potential contaminant. Also, there is evidence that these may be dependent on the white-rot strain and even perhaps on the carrier used (Mougín *et al.*, 1996; Boyle, 1997).

Contrary to the other microbial parameters used in this study, LAC activity was the only one for specifically monitoring white-rot fungi colonisation and activity in non-sterile soil. However, LAC activity was also shown to be present in uninoculated clay soil. This contradictory evidence indicates that the use of this parameter as a measure of white-rot activity in non-sterile soil should be used carefully as proposed by Boyle (1995), since it may be dependent on the combined effect of a wide range of factors.

3.4.5 Temporal microbial population size

Surprisingly, there were no significant ($p < 0.05$) differences in terms of total population size as response to treatments and incubation period. This may be due to the low water potential levels used in this study limiting the extent of the response to those factors. Alternatively, this may be related to the difficulty in isolating some representative microbial groups through the plate count method. There were, however, few but often significant ($p < 0.05$) differences in terms of the relative responses of bacterial and fungal relative contributions to total population size.

When comparing with the control, both bacterial and fungal numbers were the most abundant in atrazine-amended soil, comparing with the remaining treatments (although the differences were not always significant). This result (8.85 and 8.46 Log CFUs g⁻¹ soil respectively) was the most significant ($p < 0.001$) after 6 weeks of incubation, when bacterial and fungal numbers were near 25 and 19% (respectively) higher than in untreated soil. It is likely that these microbial groups are those able to use the atrazine-N. However, the increase in the overall microbial population size in response to atrazine exposure was not always significant ($P > 0.05$). The explanation for this may be related to the concentration of the herbicide applied. This study used atrazine concentrations in the range of that applied in the field. At this rate, it is likely that atrazine does not influence microbial population size, especially under low water potential conditions.

Similar results were achieved by Ghani *et al.* (1996), who assessed the impact of atrazine application (5 or 10 µg g⁻¹ soil) on microbial biomass of soils of different types over 81 days using the SIR Method (based on the approach by Anderson and Domsch, 1978). They found no changes in soil microbial biomass content, contrary to what previous studies have shown. As a possible explanation, they suggested that the low water solubility of atrazine (33 µg ml⁻¹), which was added to soil as a powder, resulted in low bioavailability of the compound in soil. In this study atrazine was added as a solution, similarly to what Haney *et al.* (2002) have done, but the low availability of the compound under low soil water potential is still a valid explanation. Contrasting these results, other studies have found a significant increase in population size in the presence of potential soil contaminants. For example, Wünsche *et al.* (1995) demonstrated that total microbial population size in oil-contaminated soil increased over time, suggesting substrate utilisation for biomass generation.

Sawdust-amended soil has shown the lowest number of bacterial CFUs both in the presence (6.44 Log₁₀ CFUs g⁻¹ soil) and absence (6.41 Log₁₀ CFUs g⁻¹ soil) of the fungal inoculum. In contrast, fungal numbers were generally enhanced when soil was amended with sawdust alone, but decreased significantly when soil was inoculated with the test isolate. In the sawdust treatment, soil generally contained up to nearly 20% more fungi than bacteria. This is in agreement with previous data relating to fungi

thriving in soils with recalcitrant nutrients, whereas bacteria are more abundant in nutrient-rich soils dominated by labile organic matter (Orchard and Cook, 1973; Anderson and Domsh, 1975; Mulder *et al.*, 2003). The fact that both microbial groups had their population size reduced in the presence of *T. versicolor* clearly indicates competition interactions between the native populations and the introduced fungus. For example, it is known that white-rot fungi can affect bacteria in many different ways, either through the production of lignolytic enzymes which may directly attack the bacterial membranes, through the production of toxic metabolites or by reducing soil pH during metabolism due to production of organic acids (Gadd, 2001; White and Boddy, 2001; Tornberg *et al.*, 2003). A recent study by Tornberg *et al.* (2003) has also revealed that the inoculation of *P. cryosporium* into a PAH-contaminated sandy loam induced a significant decrease in the number of bacterial CFUs.

Soil carrying both atrazine and the inoculum generally contained more bacteria and fungi than soil containing the inoculum alone (e.g. 7.06 and 6.89 vs. 6.44 and 6.07 respectively). Although this result was usually not statistically significant at $p < 0.05$, it is not unexpected, since the addition of atrazine constitutes a relevant source of N for suitable microorganisms.

Surprisingly, there was an overall tendency for both bacterial and fungal numbers in the three soil types to decrease over time, although this result was often not significant. Soil treated with atrazine was the only treatment for which bacterial and fungal population sizes achieved a peak after 6 weeks incubation before starting to decrease up to the end of the study. Perhaps during the first 6 weeks, the partial degradation of atrazine produced an increase in organic substrates which could be used by the wider fraction of the population for biomass generation. Similar pattern of response to atrazine addition (1 ppm) was reported by Moreno *et al.* (2007), who registered a peak in microbial biomass after an incubation of 16 days at 28°C, before a decrease by over 20% after 45 days.

3.4.6 Temporal fungal community structure

Fungal community structure has shown to be markedly influenced by soil treatments in the three soil types, although the highest diversity increase due to amendment was found in clay soil, comparing with the other two. This corroborates data obtained in Chapter II, on the impact of glucose and wheat straw amendment on fungal community structure in the three soils. The greater changes in community structure comparing to that at the beginning of the experiment were found after 6 weeks incubation, when data from the remaining parameters suggested the highest microbial activity.

Aspergillus, *Penicillium* and *Rhizopus* were the only genera found in untreated clay soil under the study conditions, irrespective of soil water potential. Atrazine amendment induced an increase in over 50% compared to un-amended soil and was responsible for the highest number of species and different genera isolated in this soil over the study period. The addition of the herbicide into soil at low concentrations is likely to have caused a shift in microbial community structure towards the increase in the amount of microorganisms able to utilise atrazine partially or in full, such as White and Pink Yeast, *Fusarium*, *Acremonium*, *Cladosporium*, *Aureobasidium* and *Doratomyces*. Results by Rhine *et al.* (2003) support this hypothesis. These authors compared the rate of atrazine mineralization in soil with and without previous exposure to the herbicide. They have reported an increase in atrazine mineralization rate in soil with previous exposure to the herbicide shortly after the incubation has started, suggesting an atrazine-adapted microbial community. Following 12 weeks incubation at 25°C, the rate of atrazine degradation in both soils were now similar, suggesting that the microbial community structure in pristine soil had shifted towards an increase in the number of atrazine-degrading microorganisms.

Similarly to atrazine, sawdust amendment has also shown to be favourable to fungal diversity within this time period, causing (at least) a 35% increase in the number of species isolated under both water regimes. The number of genera found as result of sawdust addition was at least 50% higher than in untreated soil, but 30% lower than that resulted by the addition of atrazine. Surprisingly, White Yeast, *Acremonium*,

Cladosporium, *Aureobasidium* and *Doratomyces* have also grown in the sawdust treatment, suggesting that are also able to utilise some of the most recalcitrant fraction of organic matter.

The presence of *T. versicolor* had a marked detrimental effect on both the number of species ($p < 0.001$) and that of genera ($p < 0.05$) found. Interestingly, the genera found in this treatment were similar to that found in untreated soil. It is likely that in the presence of the exogenous fungus, only the highly competitive microorganisms are able to thrive, and those which are not affected strongly by the metabolism of the fungus. For example, microorganisms which have low nutritional requirements were probably able to grow and remain metabolically active, as well as those less susceptible to toxic metabolites/volatiles emanating from white-rot's metabolic activity. Among these are *Penicillium*, *Aspergillus*, *Acremonium*, *Cladosporium*, *Aureobasidium* and *Rhizopus*. Similarly, a study by Tornberg *et al.* (2003) revealed that the inoculation of *P. cryosporium* into PAH-contaminated sandy loam induced significant changes in both the number of bacterial CFUs but also in the number and frequency of species found. In soil treated with atrazine and the test isolate, a small but significant improvement was found in the number of species and genera present in relation to the treatment containing the inoculant alone. It is likely that the presence of atrazine provided some nutritional advantage to those microorganisms capable of using the triazine-N.

Surprisingly, the selective effects of low water potential conditions had the highest impact on the number of fungal species in the atrazine and sawdust-treated soil. It was probably due to the fact that drier soil conditions restricted the bioavailability of the added nutrients, giving advantage to those microorganisms capable of fast use of the nutrients (e.g. fast-growing).

3.4.7 Monitoring atrazine concentration in soil microcosms

Pesticide degradation in soil was estimated by determining the amount of herbicide extracted from treated soil after 6 and 24 weeks incubation at 20°C, -0.7 MPa, compared to its initial concentration. There was a decrease in the atrazine extracted from soil over time, in both the control soil (atrazine-treated soil) and in soil containing both atrazine and *T. versicolor*.

The decrease in recovered atrazine in the control soil throughout the study can be explained by the presence of an active native microbial population, capable of degrading the herbicide under the treatment conditions. This was corroborated by data obtained from qualitative soil volatile fingerprint analysis as well as by the traditional microbial parameters. For example, 83.9% of atrazine was degraded in this treatment within 6 weeks incubation. This was consistent with the enhanced microbial metabolic activity, increase in bacterial and fungal CFUs and the shifts in fungal community structure found for the same time period. Furthermore, the CO₂ production rates from this soil, obtained for the same time period, suggest that most of the atrazine that was metabolised was also mineralised. Yet, it is likely that some of the atrazine degraded in this treatment was only transformed. Volatile fingerprint analysis appear to confirm this hypothesis, by showing that after 6 weeks, volatile patterns of atrazine-treated soil were still distinctively different from that in the beginning of the experiment.

Other studies have reported similar potential of soil native populations to biodegrade atrazine added at low concentrations. For example, Moreno *et al.* (2007) have recently demonstrated that 50% of the atrazine added (5 ppm) to a moist sandy loam (c.a. 0 MPa) had been degraded by day 16 of incubation (28°C), and that after 45 days, no herbicide had been recovered. In the same way, Haney *et al.* (2002) had previously not been able to detect any atrazine in a silt loam soil, added at similar rates, following 56 days incubation at 25°C with no moisture availability restrictions. The faster rates of atrazine degradation in these earlier studies, when compared to the present, can probably be explained by the optimum incubation conditions that were used in both cases, in respect to soil temperature and water potential.

Nevertheless, when compared to soil treated with atrazine only, it was clear that the rate of herbicide disappearance was significantly ($p = 0.00039$) enhanced in soil containing the inoculum, corresponding to 95.8% of the initial concentration, within the first 6 weeks of incubation. It suggests that *T. versicolor* was able to grow and remain metabolically active in the non-sterile soil, and also that sawdust was an adequate carrier, which is comparable to what Boyle (1995) found. Similarly, it suggests that the fungus was able to degrade atrazine in this environment, under the study conditions. This is supported by volatile fingerprint analysis. There was a shift in volatile patterns from soil containing both the inoculum and atrazine within 6 incubation weeks, suggesting that the fungus (perhaps in combination with native populations) was metabolising atrazine. This is also in agreement with respiration data, where this treatment presented the highest respiratory activity after 6 incubation weeks.

Furthermore, considering the population size and community structure data, one could even suggest that the relationship established between the inoculum and native degraders was, in this case, mainly cooperative. The fact that its action is mostly extracellular suggests that the fungus probably played an important role in the first attack on the herbicide, then followed by the rest of the microbial community which action is essentially intracellular. These results contrast others in the literature (e.g. Tornberg *et al.*, 2003), in which *T. versicolor* has failed to grow or remain metabolically active once inoculated in non-sterile soil. High rates of triazine degradation by *T. versicolor* were also found in other studies, both in liquid culture and in soil. For example, Bending *et al.* (2002) reported that 86.2% of atrazine was degraded by this species in liquid culture, after 42 days of incubation. Recently, Fragoeiro and Magan (2005) reported that *T. versicolor* was responsible for > 48% more degradation of simazine (5 and 10 ppm) than in the control soil, using a sandy loam under low soil water potential conditions.

However, in the present study atrazine degradation by the fungus did not appear to be linked to the fungus oxidative metabolism. Also, it did not appear to involve contribution of LAC activity. Bending *et al.* (2002) reached similar conclusions using *T.*

versicolor for biodegradation for atrazine in liquid culture, for up to 42 days. This may suggest that other enzymes may be involved in atrazine degradation by this species under the treatment conditions.

The rate of atrazine degradation slowed down up to 24 weeks, whether *T. versicolor* was present or not. Although the overall rate of atrazine degradation was possible in up to 94.8% (in soil containing atrazine only) and 97.8% (in soil containing both the herbicide and the inoculum), the contribution of *T. versicolor* by the end of the study was found to be no longer significant ($p=0.36$). The slowing down of the degradation rates was also consistent with the respiration data for both treatments. This may be related to nutrient exhaustion, and/or to the fact that as the concentration decreases the herbicide becomes less bioavailable (Gavrilescu, 2005).

The fact that data from volatile fingerprint analysis and the traditional microbial parameters were often consistent with HPLC results, confirms their potential for monitoring pesticide biodegradation in soil. High variability was found between atrazine degradation rates in replicates of the same treatment. The same has also been reported by Fragoeiro (2004) under similar soil environmental conditions. The reason may be related to the spatial heterogeneity of soil and to the poor distribution of atrazine, given the low soil water potential.

CHAPTER IV

Volatile Fingerprints: Potential for Early Detection of *Streptomyces* Activity and Geosmin Production in Water and Soil

CHAPTER IV

VOLATILE FINGERPRINTS: POTENTIAL FOR EARLY DETECTION OF STREPTOMYCES ACTIVITY AND GEOSMIN PRODUCTION IN WATER AND SOIL

4.1 INTRODUCTION AND OBJECTIVES

Geosmin is a VOC of critical importance since it can have detrimental effects on the organoleptic quality of our water supply and aqua-cultured food products as well as crops and vegetables, and yet it has not been well documented. According to Lloyd (1998) and Watson *et al.* (2000), geosmin can be detected in the air by the human nose at concentrations ranging between 4 and 100 ppb. Whereas in surface water it is commonly found at 50-200 ppb levels (Lin *et al.*, 2002), in soil, geosmin may be present at even higher concentrations although very little research has focused on this point.

Actinomycetes, in particular streptomycetes, have long been recognised as responsible for the production of off-odours and flavours in rivers, lakes and reservoirs, as they are washed in from the surrounding soil and decaying marginal vegetation (Wood, 1982; Wood *et al.*, 2001). A number of attempts have been previously made for correlating *Streptomyces* activity and geosmin production. For example, Stahl and Parkin (1994) extracted geosmin from two different soils using purge-and-trap extraction and found that the one with the highest geosmin extraction levels also had the highest number of actinomycete filaments. Later, Schöller *et al.* (2002) corroborated by other authors found a relationship between streptomycetes differentiation, more precisely sporulation, and geosmin production. Both these studies therefore suggest that geosmin may be used as an indicator of metabolic activity of these microorganisms.

Unfortunately, there are no efficient means of removing such off-flavours and odours from water (Wnorowski, 1992). Contrary to most water contaminants, geosmin is

resistant to chlorination even when applied at high concentrations, although activated carbon is generally considered the best approach for its attenuation (Wnorowski, 1992; Whitmore and Denny, 1998). Therefore, a quick detection of geosmin at an early stage of production in both soil and water, based on identifying and monitoring *Streptomyces* differentiation and activity, may be essential in preventing off-odour occurrences. Two water types (RO and tap water) were used in this study for evaluating whether the background water matrix was significant in defining the sensor response pattern.

The work described in this chapter aimed to test the E-nose potential for:

- (1) Early detecting and monitoring *Streptomyces* differentiation and activity in water and soil;
- (2) Detecting and discriminating between different geosmin concentrations in water and soil within the range of its OTD level;
- (3) Examining the potential correlation between sensor response and the concentration of *Streptomyces* spores and geosmin in water and soil;
- (4) Investigating the potential of volatile fingerprint analyses for identifying geosmin production at an early stage in both water and soil.

In order to achieve these objectives two sets of experiments were developed using spores and cells respectively. The first used different concentrations of spores from *Streptomyces aureofaciens* and *Streptomyces griseus*, as well as geosmin solutions at different concentrations for inoculating water and soil samples. The objective was to test the E-nose capability for discriminating between the different spore and geosmin concentrations in both sets of samples and for investigating a possible correlation between the response of sensor array and the inoculant concentration. The second set of experiments aimed to use viable cells taken from growing mycelia from both species (rather than spores) for inoculating the water and soil samples. This was to investigate the potential of using the E-nose to detect geosmin production by *Streptomyces* activity, by examining the relationship between volatile fingerprints of samples inoculated with cells and those spiked with geosmin solutions. However, it is likely that both viable cells and spores were harvested in the second set of experiments due to the inherent difficulty in isolating viable *Streptomyces* cells only.

4.2 MATERIALS AND METHODS

Both water and soil treatments used in this Chapter are listed in Table 4.1 (A and B). The soil was the sandy loam used previously at -0.1 MPa.

Table 4.1. List of the (A) water and (B) soil treatments involved in this Chapter. *Key to treatments: RO and Tap, reversed osmosis and tap water; G, geosmin; S, soil; Spore & cell, Streptomyces biological material harvested for use in the second set of experiments.*

(A)

Water treatment	Treatment reference	Spore & cell conc. (units)	Geosmin (ppb)
RO	RO	-	-
Tap	Tap	-	-
RO + Spore & Cell	10 ² , 10 ⁴ , 10 ⁵	10 ² ; 10 ⁴ ; 10 ⁵	-
Tap + Spore & Cell	10 ² , 10 ⁴ , 10 ⁵	10 ² ; 10 ⁴ ; 10 ⁵	-
RO + Geosmin	G 0.5, 10, 100	-	0.5; 10; 100
Tap + Geosmin	G 0.5, 10, 100	-	0.5; 10; 100

(B)

Soil treatment	Treatment reference	Spore & cell conc. (units)	Geosmin (ppb)
Soil	S	-	-
Soil + Spore & Cell	10 ² , 10 ⁴ , 10 ⁵	10 ² ; 10 ⁴ ; 10 ⁵	-
Soil + Geosmin	G 0.5, 10, 100	-	0.5; 10; 100

4.2.1 Spore and cell collection from pure cultures of *Streptomyces*

Spores and cells of both (A253) *S. aureofaciens* and (A26) *S. griseus* were obtained from each pure culture and suspended in 9 ml of sterile water with Technical Agar and Tween 80, contained in Universal bottles. Spore and cell concentrations were calculated using a haematocytometer and a microscope.

4.2.2 Preparation and incubation of samples inoculated with *Streptomyces*

Spore and cell inoculation into RO and tap water

Spores and cells of both species were used to spike RO and tap water in order to prepare 10^2 , 10^4 , 10^5 spores or cells ml^{-1} in each water type. The 10 ml liquid samples were transferred to 50 ml polypropylene bubble-generating bottles, leaving 40 ml of headspace above the water in each bottle. These were then tightly closed *in-situ* and both upper ends of the tubing segments sealed with parafilm, in order to prevent any loss of volatile compounds. Samples were then incubated at 25°C for a period of 24 or 48 h prior to E-nose analysis, after which were left at room temperature for 3 h for headspace saturation with volatiles.

Spore and cell inoculation into soil

Before inoculation into soil, spores and cells of both species were suspended in RO water. The volume corresponded to that necessary for setting a soil water potential of -0.1 MPa, by reference to the soil moisture calibration curves produced in Sub-section 2.2.3 (Chapter II).

Ten grams of soil were weighed into 50 ml polypropylene bubble-generating bottles, leaving 40 ml of headspace above the soil in each bottle. Spore or cell- inoculated water was added to soil and mixed thoroughly, in order to obtain a final spore and cell concentration of 10^2 , 10^4 and 10^5 spore or cell g^{-1} . Bottles were then tightly closed with both upper ends of the tubing segments sealed with parafilm and incubated at 25°C for a

period of 2 or 7 days. A 3 h volatile accumulation period was allowed prior to E-nose analysis.

4.2.3 Preparation and incubation of geosmin-spiked samples

Geosmin incorporation into RO and tap water

Geosmin up to a minimum of 98% purity (Sigma) was incorporated into 10 ml RO and tap water samples in order to obtain geosmin solutions at 0.5, 10 and 100 ppb concentrations.

Samples and respective controls were transferred to propylene bubble-generating bottles and kept incubated at 25°C for 48 h before headspace analysis. A 3 h volatile accumulation period (at 20°C) was allowed for following incubation, during which the upper end of both tubing segments were kept sealed with parafilm.

Geosmin incorporation into soil

An aliquot of pure solution of geosmin was diluted in sterile RO water and added to 10 g soil. The volume used for dilution was that necessary to set the target soil water potential. The final concentration of geosmin in soil was 0.5, 10 or 100 ppb. Samples and respective controls were incubated as described for the water samples.

4.2.4 Water and soil volatile fingerprints

All experiments involved in this Chapter employed the Bloodhound E-nose system, using the same sampling procedure and profile settings described in Sub-section 2.2.5 (Chapter II). Reproducibility of sensor array response to treatments was also tested for both sample types as described previously.

4.2.5 Data handling and statistical analyses

For both sample types, five replicates of each treatment were prepared and sampled, including respective controls. Apart from PCA, the DFA technique was also employed in this Chapter for interpreting sensor response using normalised divergence data. PCA and DFA were performed based on the responses of a subset of sensors, corresponding to those contributing the most to the overall variance within the data set. The response of sensor 13 was excluded from analysis. Any correlations found within data were examined using one-way analysis of variance (ANOVA), STATISTICA (Version 7) at a significance level $p = 0.05$.

4.3 RESULTS

4.3.1 Reproducibility of sensor responses

Firstly, the reproducibility of the sensor array response to treatments of both water and soil was studied. Figure 4.1 shows low variability (< 5%) between sensor responses to five replicates of 10^2 spore ml^{-1} treatments of *S. griseus* in (A) RO water and (B) soil, based on divergence data. Similar variability was obtained between RO and tap water. The reproducibility found for both water and soil treatments and the fact of being independently of the contaminant species, enhances the confidence level in subsequent results.

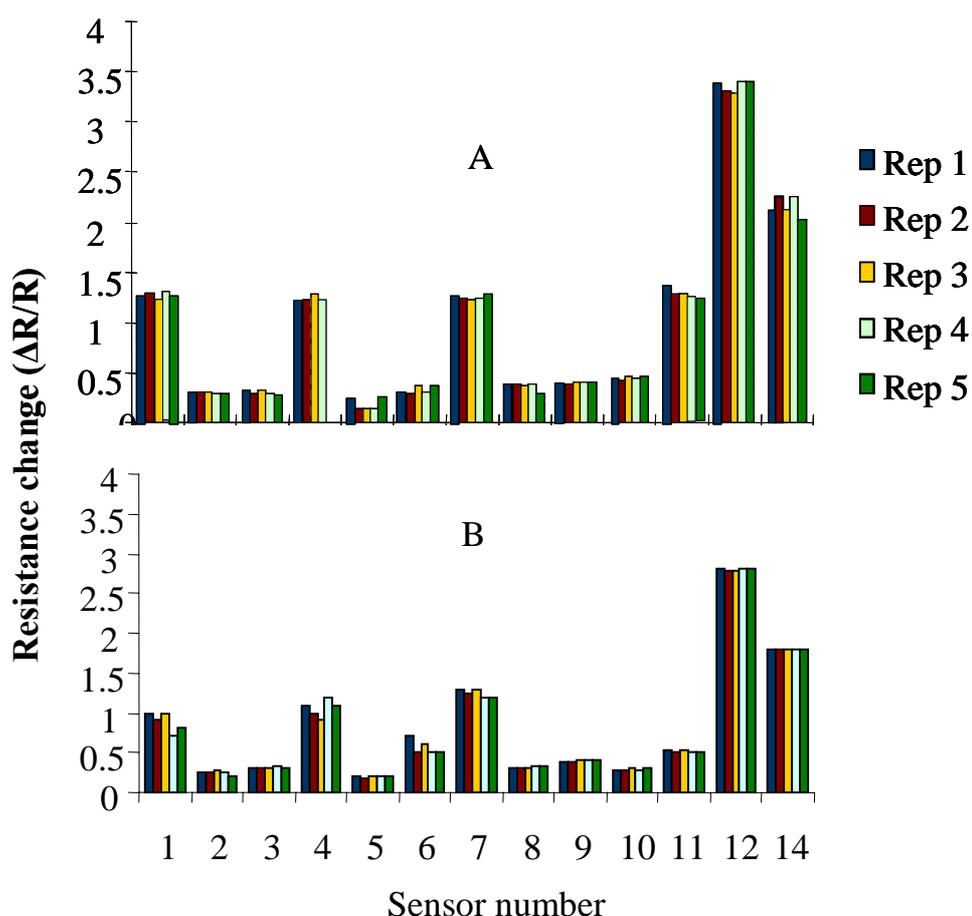


Figure 4.1 Sensor array response based on divergence data showing low variability between 5 replicates of *S. griseus* 10^2 spore ml^{-1} treatment in (A) RO water and (B) soil.

4.3.2 Discrimination between treatment concentrations in water and soil

In water

Figure 4.2 shows a PCA map of different spore treatments of *S. aureofaciens* in tap water after 24h incubation at 25°C. Data shows a good discrimination between different spore treatments and between tainted and untainted water samples (control), where most of the variance is explained by both PC 1 (95%) and PC 2 (2%). Similar results were obtained using spores of *S. griseus* and for both water types.

Likewise, the potential of the E-nose for differentiating between different geosmin concentrations in both RO and tap water was tested. The PCA map for RO water is given as example of the results obtained (Figure 4.3), where 92% of the overall information was used for discrimination. Different geosmin treatments could be clearly differentiated using this approach, even for concentrations below its OTD level.

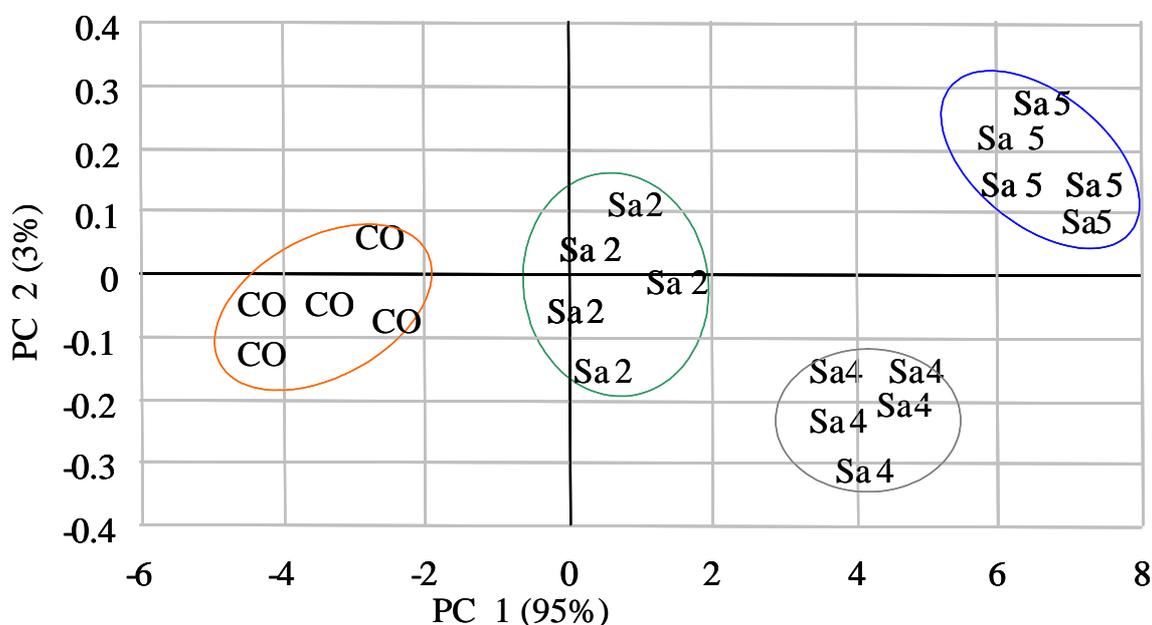


Figure 4.2 PCA map of different *S. aureofaciens* spore treatments in tap water after 24h incubation at 25°C. Key to treatments: CO, tap water control; Sa 2, Sa 4, Sa 5, *S. aureofaciens* at 10^2 , 10^4 , 10^5 spores ml^{-1} respectively.

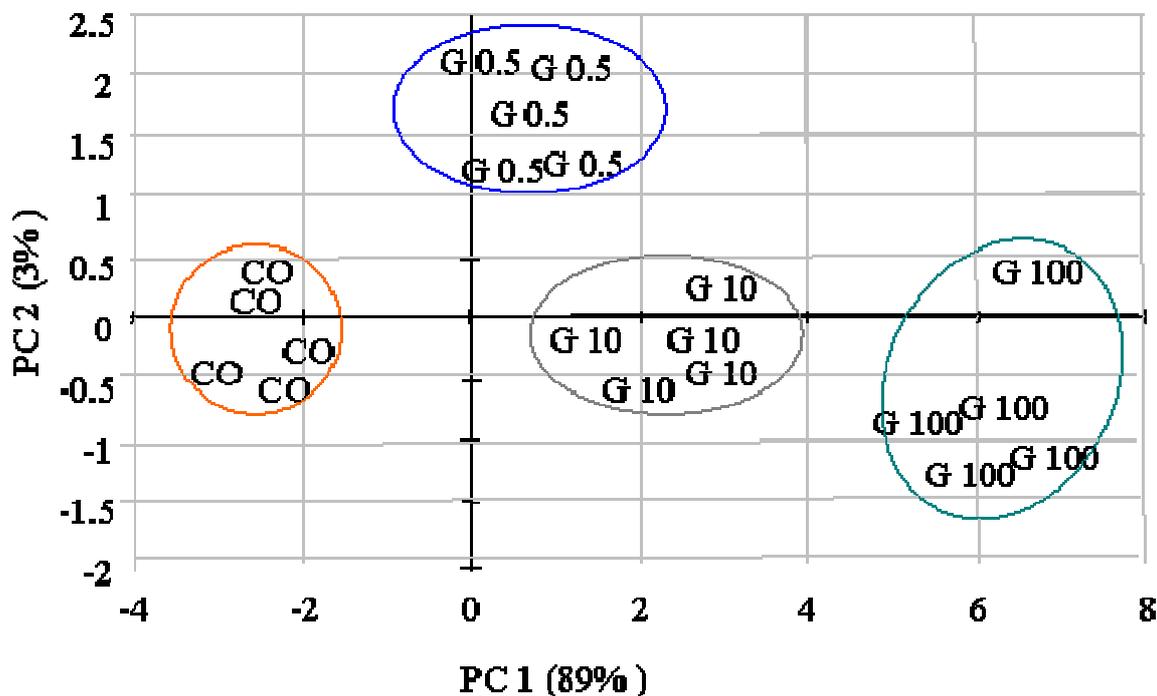


Figure 4.3 PCA map showing discrimination between different geosmin concentrations in RO water. *Key to treatment:* CO, RO water control; G 0.5, G 10 and G 100, geosmin at 0.5, 10 and 100 ppb respectively. Geosmin tainted water was stored at 4°C for 24h prior to analysis.

In soil

Similarly studies aimed to evaluate the E-nose ability for discriminating between different spore, as well as geosmin concentrations in soil using PCA. Figure 4.4 shows the discrimination between different spore treatments of *S. aureofaciens* following 48h incubation at 25°C. PC1 and PC2 explained 91% of the information within the data set. In this case, it is evident the overlapping between the control samples and those inoculated with the lowest *S. aureofaciens* spore concentration, even after 48h incubation. In contrast, it shows a good differentiation between this later cluster and those corresponding to 10^4 and 10^5 spore ml^{-1} . Headspace analysis of soil inoculation with *S. griseus* produced similar results.

Regarding the soil samples inoculated with geosmin, it was not possible to discriminate between the lowest treatment concentrations and the controls (Figure 4.5), when using 95% of the variance within the data. In contrast, the highest geosmin concentration (100 ppb) was effectively discriminated from the remaining treatments using this approach. It therefore shows that the E-nose was not able to detect geosmin in soil within the lower range of its ODT level.

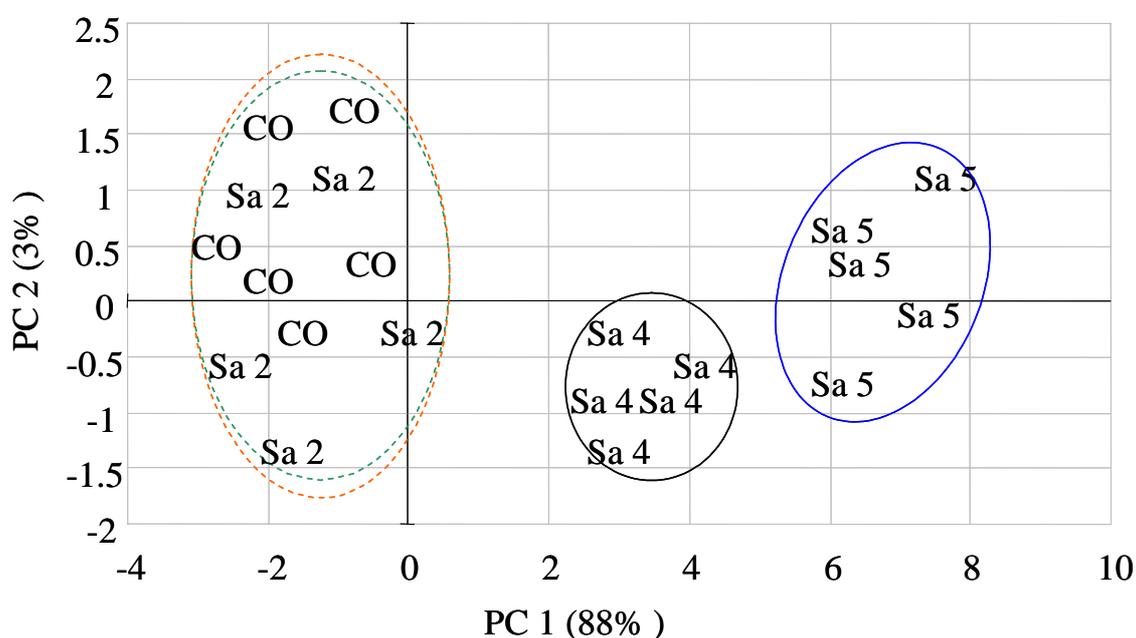


Figure 4.4 PCA map of different spore treatments of *S. aureofaciens* in soil after 48h incubation at 25°C. Key to treatments: CO, control soil; Sa 2, Sa 4, Sa 5, *S. aureofaciens* at 10^2 , 10^4 , 10^5 spores g^{-1} respectively.

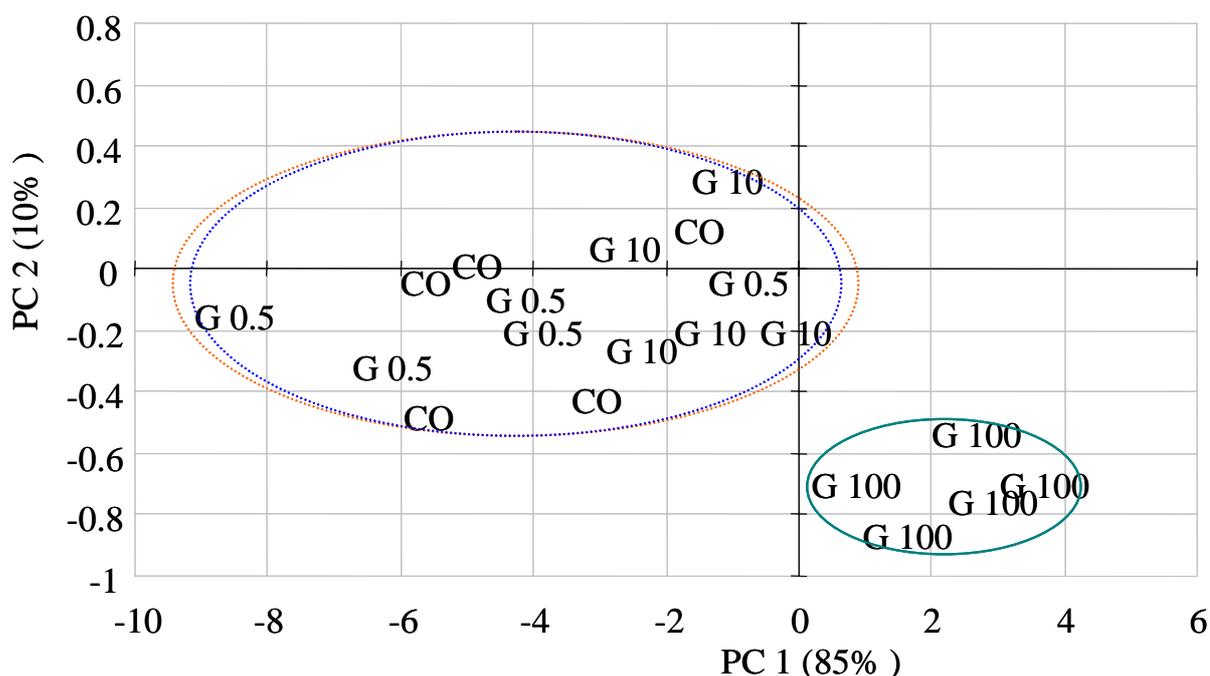


Figure 4.5 PCA map of the discrimination between different geosmin concentrations in soil. Key to treatments: CO, control soil; G 0.5, G 10 and G 100, geosmin at 0.5, 10 and 100 ppb respectively. Geosmin tainted soil was stored at 4°C for 24h prior to analysis

4.3.3 Temporal effects on detection and differentiation between *S. aureofaciens* treatments in water and soil

In water

DFA was employed on divergence data for studying the impact of incubation period on the discrimination between two tap water treatments of *S. aureofaciens* spores incubated for 24 and 48h at 25°C. Figure 4.6 shows that the level of discrimination between 10^2 and 10^4 spore ml^{-1} treatments varies over time, being greater after longer incubation periods. DF 1 explains the majority of the information (67%), although the contribution of DF 2 (30%) for the result obtained was also significant. Similar results were achieved for *S. griseus* in both water types.

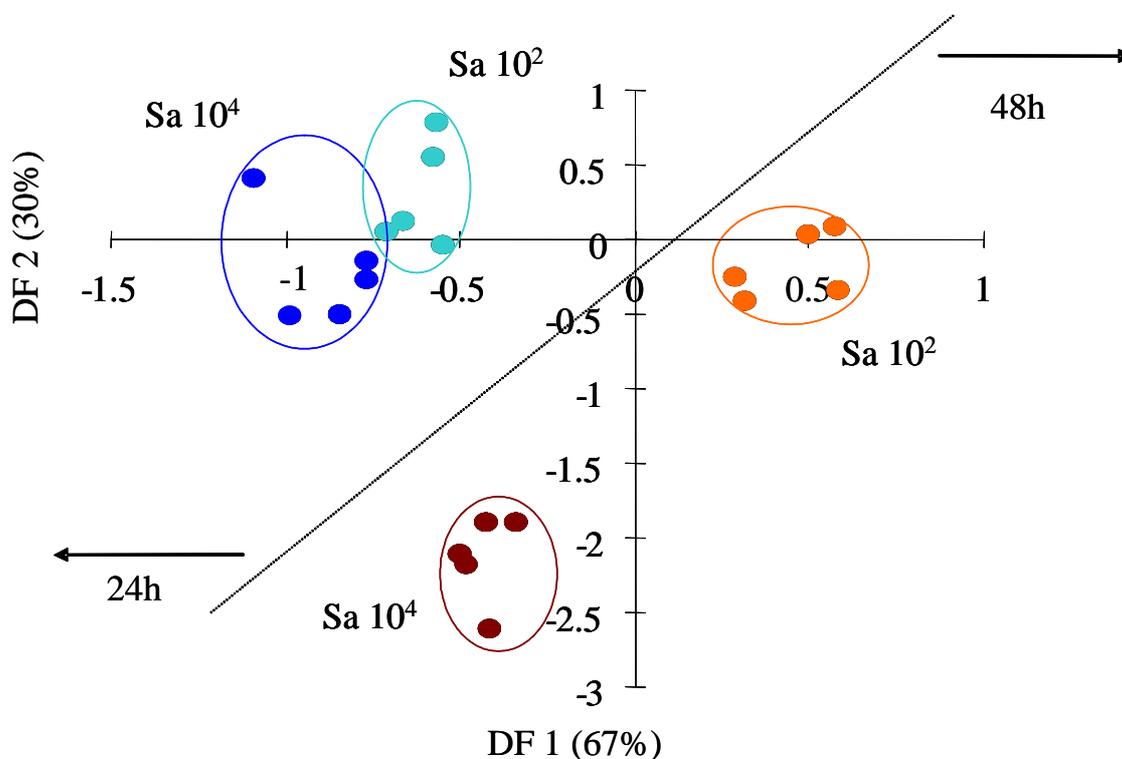


Figure 4.6 DFA plot of tap water samples inoculated with *S. aureofaciens* spores at two concentrations and incubated for 24 and 48h at 25°C. Key to treatments: Sa 10² and Sa 10⁴, *S. aureofaciens* 10² and 10⁴ spores ml⁻¹ respectively.

In soil

Figure 4.7 shows the DFA map for the differentiation between 10² and 10⁴ streptomycetes spore ml⁻¹ treatments following 24 and 48h incubation at 25°C. Similarly, the level of discrimination between both spore concentrations increased with the incubation period, regardless of the inoculated streptomycetes species. Interestingly, the discrimination level between these treatments in soil was higher than that obtained in water, which was particularly evident following the 24h incubation period.

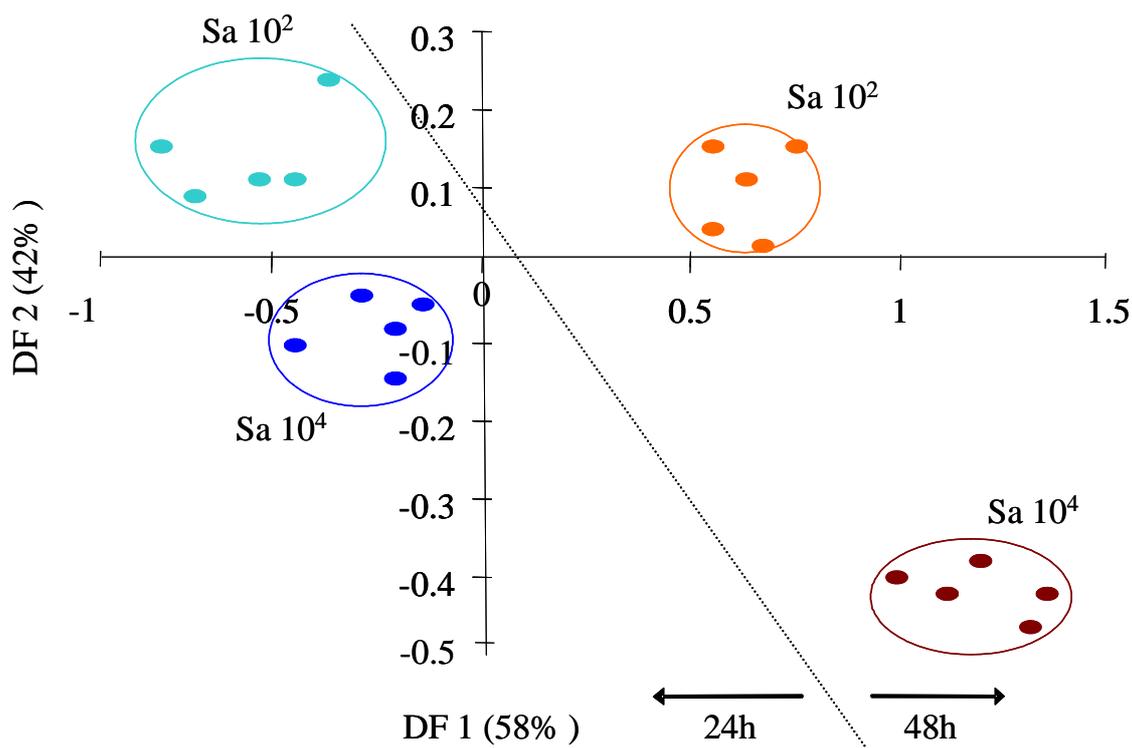


Figure 4.7 DFA plot of soil samples inoculated with *S. aureofaciens* spores at two concentrations and incubated for 24 and 48h at 25°C. Key to treatments: *Sa 10²* and *Sa 10⁴*, *S. aureofaciens* 10² and 10⁴ spores ml⁻¹ respectively.

4.3.4 Differentiation between microbial contaminant species in water and soil

In water

An attempt at early discrimination between *S. aureofaciens* and *S. griseus* spores in both types of water was also performed using DFA. Figure 4.8 shows that both species can be clearly differentiated on tap water based on their volatile production patterns, using this approach. Similar results were obtained for RO water. Although DF 1 is the factor contributing the most (85%) for the results obtained, it can not explain alone the differentiation between clusters, due to the significant (13%) contribution of DF 2.

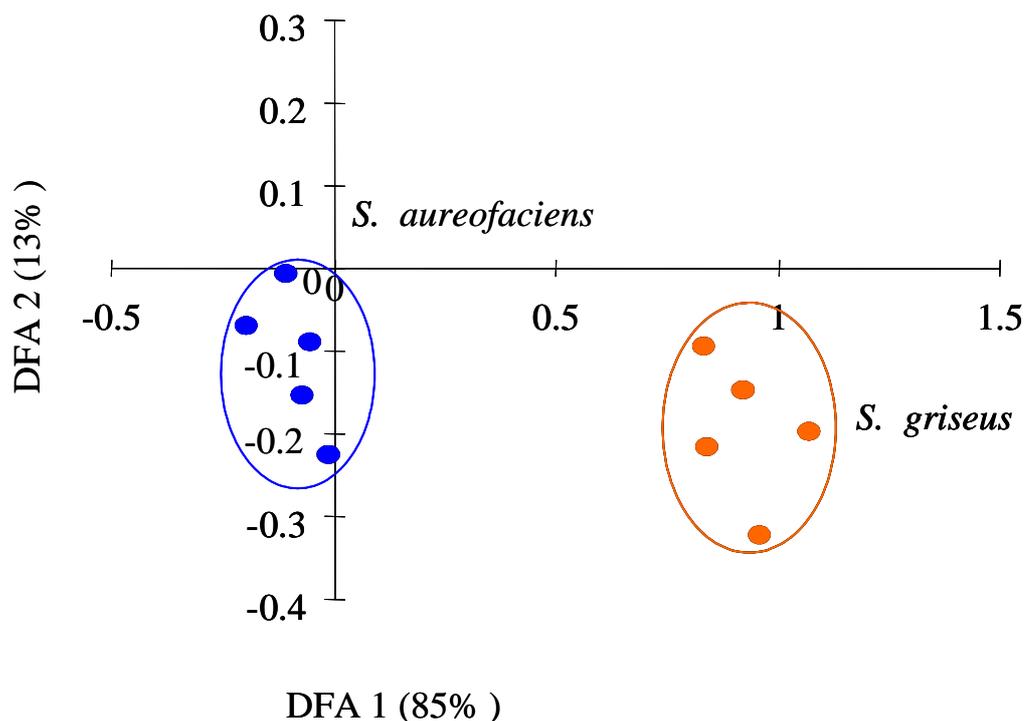


Figure 4.8 DFA map differentiating between tap water samples inoculated with *S. aureofaciens* and *S. griseus* at 10^2 spores ml^{-1} incubated for 24h at 25°C .

In soil

Figure 4.9 indicated clear dissimilarities between volatile fingerprints of *S. aureofaciens*- and *S. griseus*- inoculated soil. Together, DF 1 and DF 2 represented 87% of the overall information within the data set, with both factors contributing significantly for the discrimination.

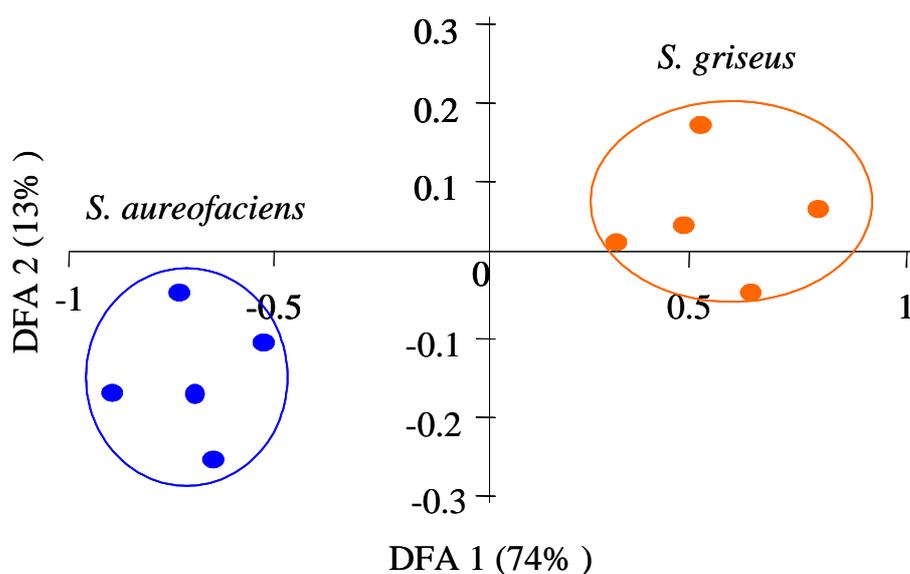


Figure 4.9 DFA map differentiating between soil samples inoculated with *S. aureofaciens* and *S. griseus* at 10^4 spores ml^{-1} incubated for 48h at 25°C .

4.3.5 Potential for early detection of geosmin production in water and soil

In water

PCA was employed in order to investigate a possible correlation between geosmin solutions in RO water and samples consisting of RO water inoculated with *Streptomyces* cells. Fig. 4.10 indicates that there is no clear discrimination between geosmin and *S. aureofaciens* tainted samples incubated for 48 h at 25°C . The control samples were obviously separated from the remaining treatments. In contrast, the lack of discrimination between microbial and geosmin tainted water treatments seems interesting, since it can indicate that this species was producing geosmin at the time of analysis. PC1 accounts for the majority of the variance (79%). However, there was very little differentiation between the different geosmin concentrations or the different streptomycetes spore concentrations.

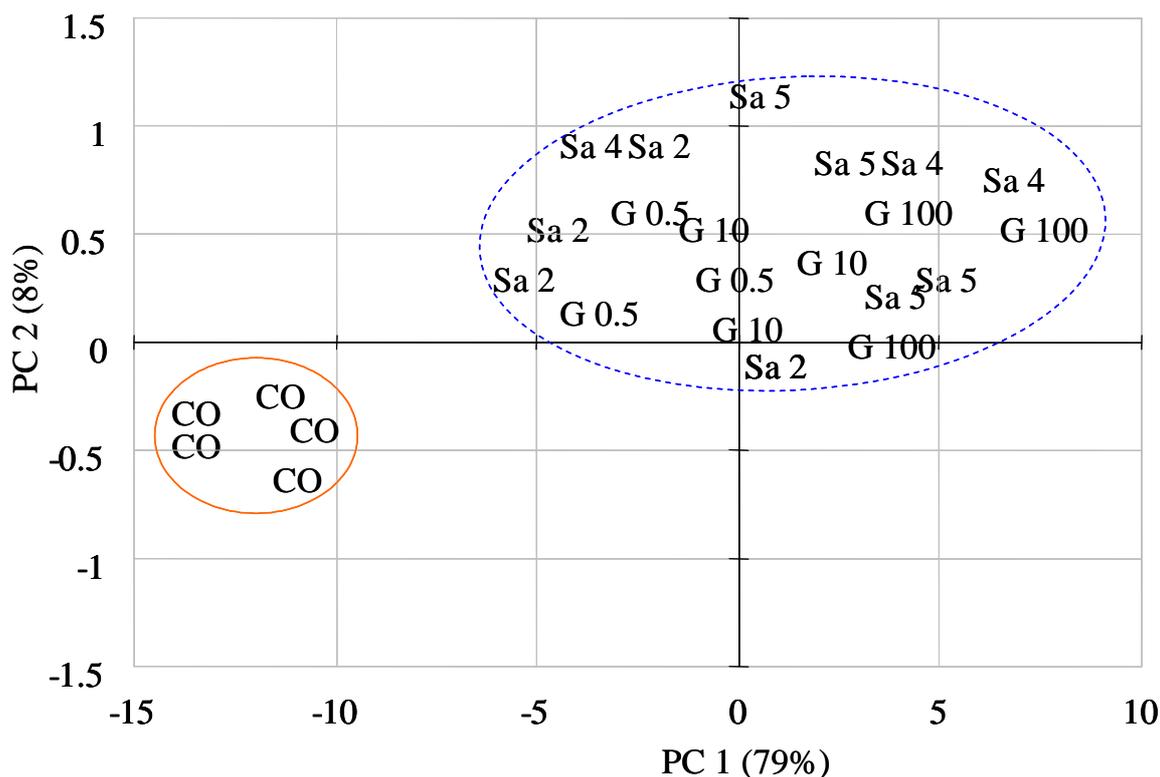


Figure 4.10 PCA map for geosmin and *S. aureofaciens* tainted RO water samples incubated for 48 h at 25°C. Key to treatments: CO, RO water control; G 0.1, 0.5 and 10, geosmin solutions at 0.5, 10 and 100 ppb respectively; Sa 2, Sa 4 and Sa 5, *S. aureofaciens* at 10^2 , 10^4 and 10^5 cells ml^{-1} respectively.

In soil

Similarly, Figure 4.11 is the PCA plot for the possible correlation between geosmin-tainted soil samples and those inoculated with *Streptomyces* cells at different concentrations. It is clear that there is a high degree of overlapping between the different soil treatments, where PC 1 and PC 2 together explain 73% of the overall variance. There is little discrimination between the lower geosmin and streptomycetes tainted soil and the highest treatment concentrations.

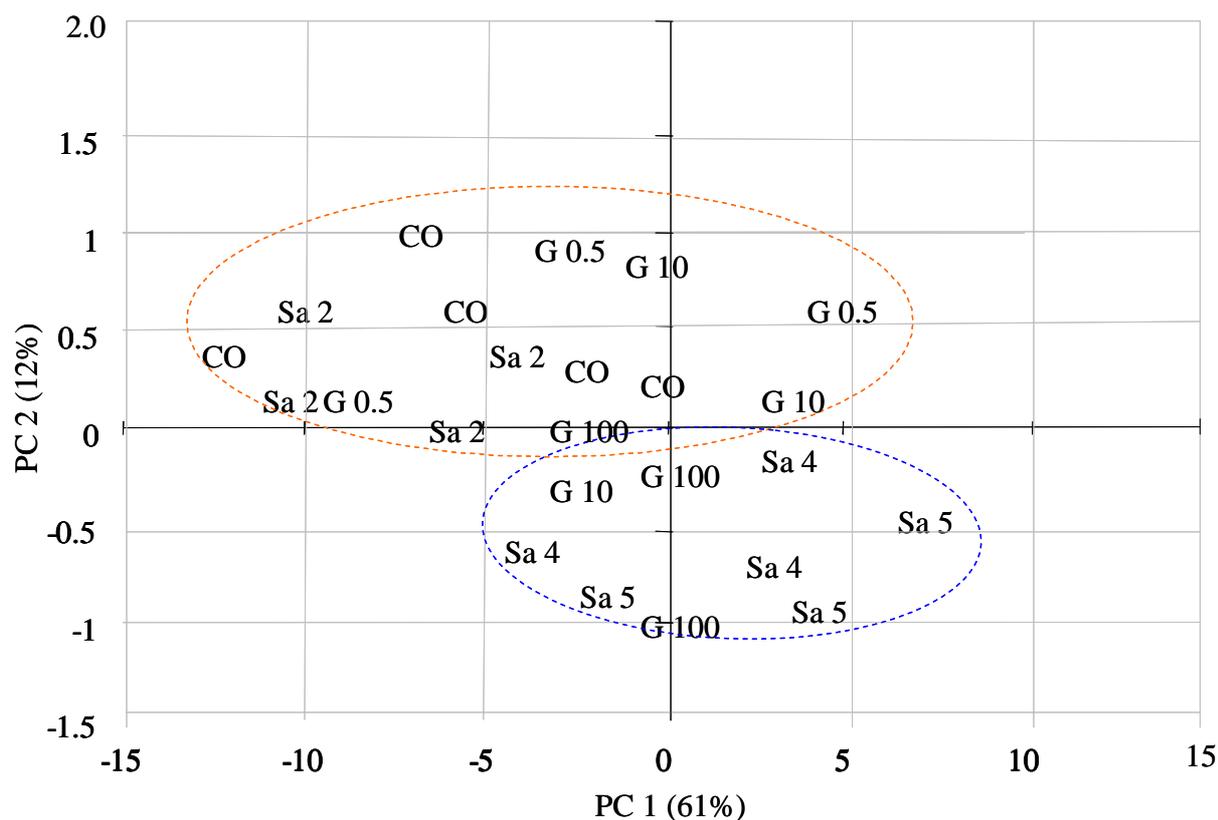


Figure 4.11 PCA map for geosmin and *S. aureofaciens* inoculated soil samples. *Key to treatments:* CO, soil control samples; G 0.1, 0.5 and 10, geosmin solutions at 0.5, 10 and 100 ppb respectively; Sa 2, Sa 4 and Sa 5, *S. aureofaciens* at 10^2 , 10^4 and 10^5 cells g^{-1} respectively.

4.3.6 Correlation between sensor array response and volatile concentration

Separation of clusters corresponding to different spore or geosmin treatments both in water and soil, suggested a possible correlation between sensor response and volatile intensity, which is in turn closely related to the concentration of that compound as well as that of the bacterial contaminant. PC1 was generally found to explain on its own at least 85% of the overall variance for this discrimination, with small contribution of PC2, and therefore was chosen as representative of the sensor array response.

Table 4.2 shows the correlation at $p < 0.05$ between PC1 and \log_{10} concentration of *S. aureofaciens* spores (\log_{10} spore ml^{-1}) and that of geosmin (ppb) in (a) tap water and (b) soil, after 24h and 48h incubation (respectively) at 25°C . Clearly, there is a significant correlation between PC1 and the concentration of the bacterial contaminant in both water and soil. Similarly, PC1 was also found to be correlated to geosmin concentration.

Table 4.2 Correlation at $p < 0.05$ between PC1 and concentration of *S. aureofaciens* spores (\log_{10} ml^{-1}) and that of geosmin (ppb) in (A) tap water after 24h incubation, and (B) soil after 48h incubation, at 25°C .

A)

Correlation coefficient (r)		
	Spore concentration (\log_{10} spore ml^{-1})	Geosmin concentration (ppb)
PC1	0.9997*	0.9823*

B)

Correlation coefficient (r)		
	Spore concentration (\log_{10} spore ml^{-1})	Geosmin concentration (ppb)
PC1	0.6914*	0.7972*

* Statistically significant at $p < 0.05$

4.4 DISCUSSION

4.4.1 Discrimination between treatment concentrations

Besides geosmin, a wide range of VOCs are commonly produced by streptomycetes isolated from water/soil (Schöller *et al.*, 2002). Based on VOC fingerprints, a clear discrimination was obtained by PCA between untainted water samples (control) and those inoculated with *Streptomyces* spores after only 24h of growth. The discrimination was possible for both water types, regardless of the inoculated bacterial species. This indicates that early microbial activity prior to visible growth could be detected in water using this approach.

Nevertheless, it was interesting to realise that for most of the tested samples the level of discrimination between different microbial concentrations was different, when using RO or tap water. This observation is corroborated by Stuetz *et al.* (1998), who also found that for some contaminants such as alcohols, the level of discrimination between volatile patterns was different when using raw or treated water samples. This may be explained by the fact that tap water contains dissolved solutes, among which is residual chlorine, which could interfere with the readings. It indicates that for all or perhaps only some taints, the background water matrix may be significant in defining the sensor response pattern. Similarly, the level of discrimination was different when taints were due to the presence of *S. aureofaciens* or *S. griseus*, demonstrating that the pattern of sensor response was also taint-specific. The detection of this bacterium in tap water at an early stage of differentiation seems particularly important, since it could help to prevent the production of off-flavours and tastes in the water supply system.

Moreover, the separation into distinct clusters according to spore concentration was possible even at 10^2 spore ml^{-1} . Considering that such a clear discrimination was obtained between the control treatments and 10^2 spore ml^{-1} , suggests that the detection of *Streptomyces* spores in both tap and RO water, under the treatment environmental conditions would have been possible at even lower concentrations, although this was not tested in this research. It therefore supports previous studies by Canhoto and Magan

(2003) which showed that 10^2 CFUs ml⁻¹ was the lowest level at which microbial contaminants were detected in water using volatile production patterns.

PCA was equally applied to soil for studying the influence of inoculating different spore concentrations on soil volatile fingerprints. Following 24h incubation, and contrary to what was observed in water for the same incubation period, there was no differentiation whatsoever between the different bacterial concentrations or between these and the untaunted controls (data not shown). Following 48h incubation, however, PCA revealed the presence of three clusters: one comprising both control samples and those inoculated with the lowest spore treatment, and two others corresponding to 10^4 and 10^5 spore ml⁻¹ concentrations respectively. This indicates that the limit of detection of the E-nose for *Streptomyces* spores in soil is probably between 10^3 and 10^4 spore ml⁻¹, under the treatment environmental conditions. Data also suggests that longer incubation periods may be required for any differentiation between treatments to be achieved in this environment, when compared to that in water. However, further studies are necessary for evaluating whether the incubation period is significant for decreasing the threshold detection level of these microorganisms in soil using this technology.

It was also possible to detect and discriminate between different geosmin concentrations in the range of its OTD in both water types. In water, the sensor array was able to detect geosmin at 0.5 ppb, which is a lower concentration than that detected by the human nose. This technique is therefore feasible to be used for both early detection of off-odorous agents in water and for setting threshold odour levels for specific water screening purposes. In contrast, data in this study seem to indicate that 100 ppb is the lowest geosmin concentration that can be detected in soil using this technique. So far, the lowest detectable concentration of this compound (ca. 0.2 ppb) in the soil environment was reported by Stahl and Parkin (1994) using a purge-and-trap method. However, there are suggestions that the concentration at which it can be detected in soil may be dependent on its initial concentration (i.e. that produced by native actinomycete populations). In turn, the amount of naturally produced geosmin by soil microorganisms is probably dependent on other soil factors which influence both microbial activity and volatile production. In this study, the soil was at 25°C and had a water potential of -0.1 MPa, conditions which are favourable for enhanced microbial activity. On the other

hand, the fact that geosmin is present in a given agricultural soil does not necessarily mean off-odorous vegetables and other soil-related food products, since there is probably a threshold concentration for the contamination of these products. For this reason, more research involving geosmin detection in soil is necessary, using different soil types under a wider range of environmental conditions, in order to explore E-nose applications for such soil screening purpose.

4.4.2 Correlation between sensor response and volatile concentration

The separation between clusters corresponding to different streptomycetes and geosmin treatments by PCA and DFA suggested that the individual odour profiles measured by the E-nose can be correlated with the contaminant compound concentration over short time sample incubation periods. In order to verify this observation, the correlation between PC1 and contaminant concentration was investigated in both environments. The fact that PC1 explained almost on its own the separation between clusters based on taint concentration meant that this component could be considered representative of the overall variance within the sensor array response. In this way, there was no significant loss of information for not considering the contribution of PC2 for finding that relationship. The significant correlation found was a relevant result, indicating the feasibility for setting odour detection limits and perhaps recommending ranges for specific water and soil screening purposes. A similar relationship has been previously found by Fenner and Stuetz (1999) when plotting DF 1 against odour intensity from the headspace of wastewater.

Finding a significant correlation between the sensor array response and the concentration of the volatile responsible for the off-odour is particularly interesting, since it suggests that a non-specific sensor array can potentially provide an objective quantitative measure of odours as perceived by humans. This represents an enormous advantage of E-nose systems over conventional analytical techniques for odour evaluation (Gostelow and Stuetz, 2001). In the case of geosmin, the E-nose would need to distinguish between acceptable and un-acceptable levels of this compound, if to be employed for off-odour monitoring. This probably means that the device would need to

be calibrated against traditional human sensory panels (Fenner and Stuetz, 1999). But once calibrated, the E-nose offers the potential for complementing or even replacing such conventional methods for preventing, screening and early control of geosmin derived off-odours and flavours in potable water and also perhaps in crops and vegetables.

4.4.3 Temporal effects on detection and differentiation between *S. aureofaciens* treatments

The sample incubation period was another factor markedly influencing microbial volatile fingerprints in water and soil. In both environments, it was clear that the discrimination between different *Streptomyces* treatments into distinct clusters by DFA was greater after 48h than after 24h of growth. For both types of treatments, DFA plots suggest that DF 1 discriminates mostly according to odour intensity. However, apart from volatile concentration, the discrimination obtained is also likely to be explained by the stage of microbial differentiation, agreeing with the fact that DF 2 had a considerable contribution (30 and 52%) for the four-group separation. This observation therefore suggests that off-flavour producing agents can be detected at any stage of differentiation, and that volatile fingerprint analysis can be a useful tool for monitoring *Streptomyces* differentiation (and perhaps that of other microbial species) in both environments. Nevertheless, the usefulness of the E-nose for such purpose needs to be further evaluated, in particular for soil applications.

4.4.4 Differentiation between microbial contaminant species

The differentiation between *S. aureofaciens* and *S. griseus* into distinct clusters by DFA, both in water and soil was another relevant result. The successful application of conducting-polymer sensor arrays for differentiation between different microbial species (or strains) has been previously reported (Canhoto *et al.*, 2004). Similarly, Schöller *et al.* (2002) has recently demonstrated that VOC profiles can vary considerably among species and even strains of streptomycetes, particularly between *S.*

aureofaciens and *S. griseus*. When analysing two different strains of each of these species for VOC composition they also realised that while both strains of *S. aureofaciens* produced geosmin, only one of the strains of *S. griseus* produced this compound. Corroborated by such studies, data in this research suggest that VOC profiling can be a useful tool for specifically detecting and monitoring geosmin-producing microorganisms, and perhaps other off-flavour and off-odour causing agents, in water and soil.

4.4.5 Potential for early detection of geosmin production

Interestingly, there was evidence of the potential for detecting the production of geosmin (and therefore perhaps other volatile compounds) in both soil and water using volatile fingerprint analyses. This was clearly observed in water, where PCA indicated a close relationship between geosmin and *S. aureofaciens* inoculated samples, while clearly separating these from the corresponding controls. This may indicate that this species was producing geosmin at the time of analysis, suggesting that this compound may be a useful indicator for detecting streptomycetes activity in this environment.

In soil, although there was also generally little discrimination between geosmin and streptomycetes tainted soil, there was no differentiation whatsoever between tainted and untainted controls. This is probably a reflection of the background geosmin in soil, naturally produced by soil actinomycetes, but also by other bacterial as well as fungal species (reviewed by Wood *et al.*, 2001). Going a bit further, one can even suggest that this lack of discrimination can also mean that geosmin was already present in soil at similar concentrations to the lower treatments used in this study (0.5 and 10 ppb). In contrast, there was some discrimination between the later cluster (corresponding to the lower geosmin and streptomycetes tainted soil treatments) and that corresponding to the highest of these treatment concentrations. It seems to indicate that the production of geosmin in soil may only be possible to detect if high concentrations of the compound are being produced (at least 100 ppb) at the time of analysis.

With the intention of confirming such results, an attempt for detecting geosmin in the headspace of *S. aureofaciens* tainted water samples was performed using gas chromatography. Geosmin and *S. aureofaciens* tainted water treatments were analysed using a GC (GC 8000 series, Carlo Erba Instruments Ltd, Paradise, Hemel Hempstead, Herts, HP2 4TG, UK) linked to a DP Integrator. Unfortunately, the columns available were not the most appropriate for geosmin detection, and time constraints did not allow for a more adequate column to be obtained. In water, the peak obtained which most likely corresponded to geosmin was detected after approximately 24.6 min. In soil, GC results were inconclusive in this aspect, probably due to the higher volatile background.

However, the probability that these microorganisms produced geosmin at the time of analysis was further investigated, at 25°C. In the literature, there is evidence that temperature is one of the most relevant factors influencing geosmin production (Wood *et al.*, 2001). In an earlier study, Wood (1982) noted that the characteristic earthy odour of geosmin developed in reservoir and river waters when temperatures exceeded 15°C. On the other hand, streptomycetes are known for their ability to grow and differentiate on both eutrophic and oligotrophic substrates (Wood *et al.*, 2001), which probably indicates that they are also able to produce geosmin under both nutrient availability conditions. For example, Wood (1982) reported that the highest concentration of available carbon found to support geosmin production by streptomycetes in water was as low as 80 µg L⁻¹. These studies suggest that there is a possibility of both species producing geosmin at the time of analyses, even in the water treatment given its low nutrient availability.

4.4.6 Treatments in water vs. in soil

Throughout this research, data suggested that although VOC fingerprinting can be used for detecting and monitoring streptomycetes differentiation, as well as geosmin production in water, they may be ineffective in the presence of mixed microbial populations and of a much higher volatile background concentration such as those in the soil environment. Here, other factors may influence streptomycete activity and geosmin production (Wood, 1982; Wood *et al.*, 2001), this approach may not be sufficient on its

own but rather as a complementary tool with other methods for detecting and monitoring off-flavour- producing compounds.

Generally, there were differences between the level of discrimination of streptomycetes inoculated-water and-soil (for the same treatment). This probably reflects the impact of other factors such as interactions between the inoculated streptomycetes and native soil populations, whereas in water such interactions probably happen on a much smaller scale. Microbial interactions may be the reason why following 24h incubation, the clusters corresponding to 10^2 and 10^4 streptomycetes spore treatments were further apart in soil than in water (pages 167 and 168), when the opposite should be expected. Despite this observation, the level of differentiation between all treatments was generally higher in water than in soil.

CHAPTER V

Summary of Results and Integrative Discussion

CHAPTER V

SUMMARY OF RESULTS AND INTEGRATIVE DISCUSSION

5.1 SUMMARY OF RESULTS

This study has explored the potential application of E-nose systems in soil science and agriculture as well as for off-odour monitoring in potable water, based on microbial volatile fingerprint analysis. In combining a set of sensitive and compatible parameters, it has also gathered relevant knowledge on the characteristics of microbial communities in a sandy loam (from a temperate region), a clay soil (from a Mediterranean-like region) and a volcanic ash (Martian simulant soil), as influenced by low water potentials and other key environmental parameters. Similarly, this study has offered a sound basis for the further exploitation of white-rot fungi for bioremediation of atrazine herbicides in non-sterile soil of different types under low water regimes.

5.1.1 Main findings of Chapter II - Evaluating the E-nose potential for differentiating between soil types and conditions

- 1) Dissimilarities between volatile production patterns, clearly discriminated between the three soils, even after short incubation periods such as 1 day, independently of soil water potential and temperature;
- 2) Temperature and water potential (at the values studied) showed a combined impact on soil volatile fingerprints independently of soil type;
- 3) The patterns of soil volatile production responded to nutrient addition and showed a temporal differentiation according to whether it was a labile (glucose) or a more recalcitrant substrate (straw). Volatile patterns were, however, weakly influenced by nutrient concentration at 2.5 and 5.0 mg g⁻¹ soil within 10 incubation days at 25°C at the treatment water potentials;

- 4) Soil basal respiration was found to be significantly influenced by soil type, temperature and water potential combined. Under wetter and cooler conditions, the sandy loam presented the highest respiratory activity, arising mainly from bacterial respiration; under drier and hotter soil conditions, the clay and volcanic ash soils showed the highest respiration rate, mainly from fungal contribution;
- 5) The patterns of SIR as a response to glucose and wheat straw was generally similar under both water potentials for all soil types, although at a lower magnitude under -2.8 MPa. Glucose induced the highest respiration increase (25%) in the sandy loam within 1 day of incubation, whereas wheat straw induced the highest respiratory increase in the clay (65%) and volcanic ash (81%) soils after 10 incubation days. Overall, following nutrient addition, respiration was kept at a high rate for longer in the latter two soils, independent of nutrient type;
- 6) DHA was highly affected by soil water potential (30% higher under the wetter than under the drier conditions) and soil type (clay soil presenting at least 50% higher activity than the other two soils), although the effect of temperature on the enzymatic activity was generally not significant. The addition of both glucose and straw induced the highest activity increases (at least in 34%) in the clay and volcanic ash soils, although no significant differences were found between nutrient concentrations;
- 7) Total microbial population size was in general significantly influenced by water potential ($p < 0.03$) but not by temperature ($p > 0.08$), whereas soil type had a significant impact only under wetter conditions. Under such conditions, bacterial CFUs were the highest contribution to total population size in the sandy loam, whereas fungal communities were dominant under the drier soil conditions in the clay volcanic ash soils. Nutrient addition induced an increase in total population size in the longer term (10 days) with bacterial and fungal communities responding the most to glucose and straw respectively;
- 8) The most common genera isolated in the three un-amended soils were *Aspergillus*, *Penicillium*, *Fusarium* and *Rhizopus* with relative abundances depending on soil type temperature and water potential. Generally, there was little difference between fungal community structure in the sandy and clay soils using the plate count method. Nutrient addition at 2.5 mg g^{-1} of soil resulted in

an increase of at least 30% in the number of fungal species when compared to un-amended soil, with glucose inducing the highest diversity after 10 incubation days;

- 9) PC1 was found to be significantly correlated ($p < 0.05$) to traditional microbial parameters in un-amended soil; it was strongly correlated ($r > 0.81$) to basal respiration independently of the soil treatment and even after 24h incubation; it was also highly correlated ($r > 0.70$) to the number of fungal species in all soil types; it was moderately correlated to DHA and total CFUs ($r > 0.52$ and $r > 0.53$, respectively), although over longer incubation periods (10 days). This suggests that similar to respiration, and unlike CFUs, shifts in soil volatile patterns change quickly to changes in soil conditions. In glucose amended soil, PC1 was only correlated to the number of fungal species ($r > 0.79$);

5.1.2 Main findings of Chapter III - Using volatile fingerprints as a monitoring tool for atrazine bioremediation in soil by a white-rot fungus

- 10) The E-nose was able to discriminate between untreated and atrazine-treated soil at usual field application rates, even after 1 day of incubation. This means that the E-nose threshold detection for this herbicide in soil is < 2.5 ppm at 20°C and at -0.7 MPa (the human threshold detection level of this herbicide in soil has not been established, although in water the herbicide can be detected by the human nose at approximately 9.2 ppm). Volatile fingerprints of atrazine-containing soil changed throughout the study in all soil types, suggesting atrazine metabolism by suitable native microorganisms;
- 11) It was possible to discriminate between un-inoculated soil and that carrying *T. versicolor* after 5 incubation days. Volatile patterns in soil containing the inoculant alone were also distinctively different from that containing both the inoculum and the herbicide;
- 12) A temporal discrimination based on volatile fingerprints was obtained for the treatment containing both *T. versicolor* and the herbicide, although over 24

- weeks, volatile patterns were still distinctively different from that in the beginning of the study;
- 13) Soil respiration was also deeply influenced by the presence of atrazine and *T. versicolor* (alone and combined) during the bioremediation programme. The highest respiratory rates were generally achieved over a 6 week incubation period under the wettest conditions. The presence of atrazine at usual field application rates has shown to generally increase respiratory activity in all soil types by at least 41%, compared to that in untreated soil. However, the highest respiration rates were achieved by soil containing both atrazine and the inoculant, being at least 20% higher than in the absence of the fungus;
 - 14) The highest DHA was generally achieved between 6 and 12 weeks of incubation under the wetter conditions. For this time period, the presence of atrazine increased enzyme activity in over 40% irrespective of water potential and soil type, although there was no significant ($p>0.06$) difference between DHA in the presence and in the absence of *T. versicolor*;
 - 15) LAC activity presented the most inconsistent results from all the traditional microbial parameters used. LAC was expressed in soil whether *T. versicolor* was present or not. Instead, its activity appeared to be dependent on soil type, water potential and soil amendment, strongly suggesting that it may not be a good indicator of *T. versicolor* relative activity in non-sterile soil;
 - 16) Generally, there were no significant differences between total microbial population size as a response to atrazine (both in the presence and absence of *T. versicolor*) throughout the study, although significant responses were often registered in terms of its bacterial and fungal relative components. Both populations were the most abundant ($p<0.05$) in atrazine-amended soil; even in the presence of *T. versicolor*, atrazine was responsible for an increase in population size of both groups when compared to that of soil carrying the inoculum alone;
 - 17) The number of different fungal genera found was the highest in atrazine-amended soil, independently of water potential (e.g. 10 compared to 3 in un-amended clay soil at -0.7 MPa). The inoculation of *T. versicolor* into soil only had a detrimental effect on species diversity of native fungi under the drier

conditions, although generally soil containing both atrazine and the inoculum showed at least 14% higher diversity than that containing the inoculum alone;

- 18) The role of atrazine disappearance was significantly ($p=0.00039$) enhanced in soil containing *T. versicolor*, compared to the control soil. By the end of the study, up to 97.8% and 94.8% of the herbicide was degraded in soil containing both the atrazine and the inoculum and in the control soil, respectively. However, the contribution of *T. versicolor* was only significant within the first 6 weeks of incubation.

5.1.3 Main findings of Chapter IV - Volatile fingerprints: potential for early detection of *Streptomyces* activity and geosmin production in water and soil

- 19) The E-nose was able to discriminate between *Streptomyces* spore treatments and different geosmin concentrations (in the range of its odour threshold detection level) in both water types and in soil, as well as between tainted and untainted controls. However, whereas in water that discrimination was possible even for the lowest treatment concentrations (10^2 spore ml^{-1} and 0.5 ppb of geosmin), in soil that discrimination was only possible for greater treatment concentrations (10^4 spore g^{-1} and 100 ppb of geosmin);
- 20) The detection of geosmin in water at 0.5 ppb mean that the E-nose threshold detection for this compound is many times lower than it's human threshold detection in air (4-100 ppb);
- 21) The differentiation between *S. aureofaciens* and *S. griseus* based on their volatile production patterns was possible in both water types and in soil over a 24h and 48h incubation, respectively;
- 22) The level of the discrimination between 10^2 and 10^4 spore ml^{-1} treatments varied over time independently of the contaminant species, being greater after longer incubation periods for both water types and soil treatments.
- 23) There is potential for the E-nose to be used for detecting geosmin production by *Streptomyces* activity in water and soil, given the overlapping obtained between

samples tainted with geosmin and those with *Streptomyces* cells. However, the production of gesomin in soil may only be possible to detect using this approach if high concentrations of the compounds (> 100 ppb) are being produced at the time of analysis and it may be dependent on other soil environmental conditions.

5.2 INNOVATIVE ASPECTS AND LIMITATIONS

The innovative aspects of this study, which have been extensively discussed throughout this thesis, are summarised in Sub-section 5.2.1. Similarly, the main limitations encountered during this research are listed and briefly discussed in Sub-section 5.2.2.

5.2.1 Innovative aspects

- Applying the E-nose based on volatile fingerprint analysis for detecting changes in soil microbial activity, as influenced by key environmental factors;
- Investigating relationships between soil volatile fingerprint and traditional microbial parameters for characterising soil microbial populations;
- Detecting and monitoring *Streptomyces* activity and differentiation, as well as geosmin production in potable water and in soil based on volatile production patterns;
- Using the concept of soil water potential as a more accurate measure of soil water availability than gravimetric/volumetric soil moisture content or percentage of saturation;
- Providing a deep and valuable insight into the characteristics of microbial populations native to the Martian simulant soil JSC-MARS 1 under different soil conditions (see Appendix VI for a brief integrative discussion on the importance of the microbiological data obtained for the martian simulant soil).

5.2.2 Limitations

- Comparing between results in this study and others in the literature was often difficult in respect to soil microbial activity and population size, for two main reasons. Firstly, in most studies, soil water content is often expressed as percentage of saturation or of maximum field capacity (*i.e.* percentage of pores filled with water). Secondly, a very limited number of studies have used soils with comparable properties to those in this study and under similar treatments and environmental conditions.
- There is an inherent difficulty in assessing individually the impact of factors such as temperature, water potential, nutrient and pesticide inputs on soil microbial populations. This is mainly due to heterogeneous nature of soil, where physical, chemical, and biological factors all act combined on soil microbial communities.
- The need for air-drying the soil samples followed by rewetting in order to set low water potentials, identical for all soils, may influence soil microbial activity rates to some extent. However, it is likely that this effect is more pronounced in the very short term.

5.3 SOIL MICROCOSMS AS AN APPROACH TO SOIL STUDIES

As previously mentioned, soil physical, chemical, and biological characteristics are highly variable in time and space (Nannipieri *et al.*, 1990). *In situ*, this combination of factors may be problematic in studies where the main focus is on the comparison between microbial characteristics in different soil treatments (Bloem *et al.*, 2006). Furthermore, measurements of soil respiration and dehydrogenase activity as influenced by environmental factors, when conducted *in situ*, allow determining the overall soil

biological activity rather than microbial activity due to the contributions of mesofauna and plants (Harris and Steer, 2003).

The microcosms approach to soil studies in laboratory-based analyses offers a way of standardising environmental conditions, thus allowing for more reliable comparisons to be made between samples and different soil treatments (Nannipieri *et al.*, 1990, 2002). This means that they allow reducing the number of different variables that have to be considered when characterising soil microbial populations. Also, the use of consistent methodology for sample preparation and analysis throughout the research, as well as carefully assessing data reproducibility for each set of soil conditions and after each set of experiments, contributes to reduce noise in data.

The non-specific sensor array was found to work well for soil volatile pattern characterisation under controlled environmental condition in soil microcosms. The use of microcosms in this case provided the background studies necessary if this technology is later to be used in the field for *in situ*, real-time characterisation of soil volatile fingerprints (*e.g.* aiming for rapid detection of herbicide-contaminated sites). The potential application of this technology in the field is likely to encounter difficulties related to background noise (Kurup *et al.*, 2006), which may not only arise from microbial activity but also from the presence of other soil contaminants, as well as from the influence of the soil matrix. They also allow for the necessary training of the sensor array, odour recognition and calibration to be performed previous to field analysis.

On the other hand, the use of soil microcosms in bioremediation research is also advantageous in view of *in situ* processes (Mollea *et al.*, 2005). According to Šašek *et al.* (2003), most studies done so far, involving the use of white-rot fungi for bioremediation of environmental contaminants, have been performed in liquid culture, where conditions differ greatly from those in the multi-phase soil environment (Boopathy, 2000). Additionally, studies in liquid culture do not consider factors such as soil properties, water potential, competition with indigenous microorganisms, and colonisation and survival of the introduced fungi (Šašek *et al.*, 2003). In this sense, the use of microcosms is a useful tool for understanding the factors influencing the biodegradation of the contaminant, the interactions between the introduced fungi and

the surrounding environment and the impact of the contaminant itself on the native microflora (Nerud *et al.* 2003).

Although microcosm-based research using homogenised soil samples under controlled environmental conditions can provide very useful and reproducible information such as that provided by this study, extrapolating results to the *in situ* conditions in undisturbed-soil may be difficult, and in some cases misleading. The main differences between undisturbed and disturbed soil samples are related to loss of heterogeneity and soil structure, resulting in changes in the patterns of gas exchange, nutrient availability and microbial interactions (Nannipieri *et al.*, 1990), *e.g.* promoting niche overlapping.

5.4 E-NOSE TECHNOLOGY: PROSPECTS FOR FUTURE APPLICATIONS IN SOIL SCIENCE AND AGRICULTURE

The potential of the E-nose to be employed as a tool for detecting changes in microbial activity and community structure in response to changing soil conditions has been studied in detail in the present study for the first time. Key factors such as environmental conditions, soil type, nutrient addition, the presence and metabolism of herbicides (such as atrazine), have all been shown to markedly influence soil microbial volatile fingerprints. These data therefore suggest that soil volatile patterns are likely to be influenced by the same parameters influencing soil microbial activity. Moreover, the correlation found between PC1 and traditional microbial methods indicates that volatile fingerprinting can complement and perhaps replace some of the currently employed methods for assessing microbial metabolic activity and community structural changes. Based on rapid and non-invasive soil volatile fingerprint analysis, the application of this technology in soil science and agriculture looks promising.

The temporal impact of added nutrients into soil microbial volatile production patterns was a relevant result. It indicates that volatile fingerprints, evolved from microbial

activity, can be used for monitoring the impact of organic matter inputs, but also for studying microbial substrate utilisation patterns and perhaps even replacing existing techniques for their assessment. Substrate utilisation patterns of the whole microbial community can be considered as the sum of patterns of the individual microbial groups. Thus, it is highly influenced by the structure of the microbial community, as well as by its metabolic potential (utilisation of the available substrates). Substrate utilisation patterns as used by the methods currently available (*e.g.* Biolog microplates) reflect the metabolic activities of only a small proportion of the overall population (Wünsche *et al.*, 1995). For example, it excludes most fungi, anaerobic microorganisms and all of those that are not able to grow in culture due to more specific nutritional and environmental requirements. An interesting study by Bundy *et al.* (2002) used the Biolog approach followed by PCA analysis of data for looking at the substrate utilisation patterns of three different soil types after 1 day incubation at 25°C. They found no discrimination between clusters according to soil type, in contrast to that found in the present study using volatile fingerprint analysis. They suggested that the lack of discrimination was probably due to disturbance of the soils caused by homogenisation and microcosms preparation, in this way attenuating differences between them. It is likely therefore, that the use of volatile fingerprints analysis using sensor arrays provides a more sensitive and reproducible way of looking at substrate utilisation patterns of the whole microbial community, independently of the nature of the substrates.

The array of non-specific polymer sensors was also able to detect atrazine in soil at usual field application rates. The discrimination between atrazine-containing soil and treatments free of the herbicide was clear, through a fast, simple and reproducible analysis of its odour. In this study, atrazine was dissolved in water prior to its addition to soil. Usually, pesticide sprays are obtained by dissolving the pesticide powder in an organic solvent (such as methanol or acetone), which are easily evaporated, leaving behind the solid residue (Baby *et al.*, 2000). It is likely that the E-nose would still have been able to detect the herbicide if it had been applied to soil microcosms in this way.

Besides herbicides there is a growing need for rapid and cost-effective lab-based and on-site methods for detecting and monitoring a wide range of other soil contaminants. Data in this study suggest that the E-nose is a suitable instrument to serve this need due

to its sensitivity, reproducibility, speed and potential for portability (Roberts and Kearney, 1995). But, besides being mainly qualitative, it can also provide reliable quantitative results in terms of determining the concentration of a specific contaminant. This can be done based on the construction of calibration curves where the change of sensor resistance due to interaction with the volatile is plotted against the contaminant concentration. Barisci *et al.* (2002) developed an array of 8 conducting polymer sensors for identifying and differentiating between benzene, toluene, ethyl benzene and xylene. Authors were able to quantify these compounds based on such calibration curves, although this may provide only an approximate value.

Alternatively, the change of resistance can be plotted against the sensor number producing a fingerprint in the form of a histogram for that volatile at a certain concentration, which is then used for building up a fingerprint database. This “library” can then be employed for class prediction of unknown samples using supervised techniques such as DFA or ANN. This has been done before for identification of common environmental contaminants (Kurup *et al.*, 2006), although not many studies have applied it to soil and so far, none have applied it for monitoring bioremediation of contaminants. ANNs consist of a series of pattern recognition algorithms and are an alternative way of data for processing and interpreting sensor response. Similarly to DFA (although more complex), this is a supervised (or “trained”) technique, meaning that once enough background data has been collected, this method can be used to effectively and accurately predict the group to which an “unknown” sample belongs to. Among others, Craven *et al.* (1996) and Turner and Magan (2004) supply further information on ANNs and suggest that they may offer advantages over multivariate techniques. Craven *et al.* (1996) go further suggesting that this technique should perhaps be used in combination with multivariate methods.

However, the detection of herbicides/pesticides in soil would need to involve extensive preliminary laboratory-based training of the system, involving a wide range of different soil conditions and matrices. Also, very often, herbicides and pesticides are applied to soil as mixtures rather than as single compounds at different concentrations (Schoen and Winterling, 1987). The E-nose would need to be able to detect the compounds and even possibly discriminate between them. The training of the system would probably involve

the use of reference standards of the pure compounds and as part of a mixture at different concentrations and wide range of different soil characteristics, in order to obtain an odour library. Clearly, in some occasions the use of sensor arrays developed to respond to specific volatiles is useful. However, the use of non-specific sensor arrays based on pattern analyses has the advantage of allowing much more general gas sensor applications for characterising, detecting and identifying a much broader range of compounds.

Another potential application of E-nose technology is in the field of the so-called 'precision agriculture'. The basic objectives of site specific management are to increase crop yields, improve product quality and at the same time protect the environment (soil and water resources specifically) (Adamchuk *et al.*, 2004). According to these authors, the lack of information about the overall soil attributes at a specific site is the major limitation of precision agriculture, along with the inability for rapidly assessing soil characteristics, which are essential for adequate soil management. Combining data relating to the different key soil parameters in a field allow dividing it into smaller areas (management zones), which are relatively homogeneous in terms of soil features. Standard soil parameters that need to be assessed by exhaustive sampling include nutrients such as phosphorus (P), potassium (K), calcium (Ca), magnesium (Mg), soil texture, water potential, and perhaps salinity and heavy metals (Foth and Ellis, 1988; Adamchuk *et al.*, 2004). Traditionally, these data come from a variety of sources using conductivity, electromagnetic and optical sensors (Adamchuk *et al.*, 2004). This is where a non-specific E-nose system would provide a valuable alternative. Through a rapid and non-invasive assessment of soil volatile fingerprints throughout a field, an equally valuable mapping of the soil characteristics could be obtained. This would provide essential information on soil differences and similarities within the field, this way aiding prediction of selected soil attributes and support site specific management. However, further investigations need to be performed on the use of this approach for optimising precision soil and crop management.

Meanwhile, it can be expected that soil mapping using volatile pattern analysis can also be of importance at a broader scale. Data from this research suggests that the E-nose as a tool can offer a way of characterising and comparing between different soil systems,

sites, or agricultural areas, and perhaps even their responses to changes in climate, crop management (*e.g.* pesticide/herbicide application), soil management (*e.g.* tillage operations, lime applications), restoration of degraded environments (*e.g.* afforestation, reforestation, presence of contaminants). Versatility, simplicity of use, and the potential for automation and portability for on-site applications are additional qualities that make this technology even more appealing, especially when taking into consideration the associated low sampling costs (Bougeois *et al.*, 2003; Kurup *et al.*, 2006).

At a purely academic level, the contribution of the E-nose in soil research may also be substantial. The possible link between soil volatile profiles and the characteristics of the underlying microbial populations suggest that this tool can be relevant in complementing studies on soil microbial community structure and functional diversity, as well as for examining their role in the overall health of agricultural soil. Additionally, it may offer a way of further investigating the hypothesis of Wheatley *et al.* (1996) and other, who defend that VOCs can play a key role in microbial communication during soil processes. This study has also shown that volatile production patterns shift as a response to the addition of non-indigenous microorganisms into soil and consequent interactions with native microbial populations, in the absence as well as in the presence of atrazine. This specific result indicates an ability of the E-nose as a relevant tool for monitoring processes such as bioremediation of soils containing pesticides, either involving the inoculation of appropriate microorganisms or through the stimulation of native populations. There is even potential for the E-nose to participate in climate change research. For example, current concerns regarding the 'greenhouse effect' have increased the interest in evaluating the production of N₂O and CH₄ from soil. For instance, earlier studies have shown that an estimated 50% of production and consumption of these gases, which together explain approximately 25% of global warming increase, occur in soil. It is, therefore, essential to obtain a further understanding of the factors that influence gases and volatile fluxes in, to and from soils (Sheppard and Lloyd, 2002), which could be done quickly and non-invasively using this approach, particularly when real-time monitoring is required.

Taking into consideration what has been done so far in terms of technological development, as well as the changes still under progress, it is fair to say that the future

looks promising for the E-nose systems to be used for environmental purposes. E-noses are developing at a rapid pace regarding both hardware and software; longer life sensors are being developed and sensor drift related problems are being solved; calibration techniques are also being optimised (Harper *et al.*, 2001). Nevertheless, widening the application of such sensor arrays for environmental monitoring perhaps relies on the development of systems evidencing a stronger relationship between sensor output and volatile intensity, rather than volatile type (Gostelow *et al.*, 2000).

5.5 TRADITIONAL PARAMETERS FOR CHARACTERISING SOIL MICROBIAL COMMUNITIES

No single microbiological criterion is ideal to be used individually for characterising soil microbial communities (Brookes, 1995; Gil Sotres, 2005). Instead, the consensus in the literature is to use a set of parameters focusing on different general as well as more specific features of the community. For example, Margesin *et al.* (2000) found that the number of microbial counts increased over time in soil contaminated with oil derivatives, both in the absence and in the presence of nutritional amendments. Those authors concluded that although this increase suggests that oil biodegradation is occurring, used alone it does not provide any information on the biodegradation ability of those microorganisms to degrade hydrocarbons, the process rate, or which microbial groups are more likely to be involved.

In this study, the combined use of parameters associated with volatile production patterns (page 55), respiratory (page 59) and enzymatic activities (pages 60 for dehydrogenase and 109 for laccase), population size (page 62) and fungal community structure (page 63), provided a reliable insight into the status of microbial communities native to these soils, as well as the way they respond to the different soil treatments.

Generally, parameters associated with microbial activity (soil volatile fingerprints, respiration and dehydrogenase activity) were found to be the most reproducible and responsive for assessing changes in microbial characteristics as influenced by the different treatments, opposed to microbial population size and fungal community structure. For example, the three microbial activity parameters were significantly ($p < 0.05$) different between soil types after only 24 h of incubation in un-amended atrazine-free soil, independently of temperature and water potential. In contrast, microbial counts and fungal community structure did not appear to be useful for discriminating between the three soils under the study conditions. Other authors, including Tsai *et al.*, (1997) reached similar conclusions after using a set of different microbial parameters, among which respiration and biomass, for determining microbial response to glucose addition in moist soil. There are also suggestions that soil microbial activity, rather than biomass, offers the potential to be used as an early and sensitive indicator of stress factors imposed on soil (*e.g.* low water availability and the presence of contaminants). Brookes (1995) explained that the presence of stress factors may decrease or inhibit some microbial processes without necessarily causing the death of microbial biomass.

Generally, the static sampling method used for CO₂ measurements (page 59) of basal and substrate-induced respiration was adequate for the three soil types under the treatment environmental conditions. It was highly sensitive allowing very small concentrations of this gas to be detected and quantified even under soil conditions which limited substantially microbial respiration rates. It was also highly reproducible between replicates of the same treatment, with standard errors of the mean below 1%. In addition, it was fast, reliable and showed to be suitable for routine analyses purposes.

Up to now, studies involving the measurement of soil microbial activity response to nutrient addition have been carried out usually using moist soils, mainly for ensuring that all added substrates can be efficiently distributed throughout the soil (West and Sparling, 1986). This study demonstrates that respiration response to substrates can also be assessed under low water potentials for these soil types, considering that glucose is added as an aqueous solution rather than as a powder. However, there are suggestions that the extent to which it relates to actual processes in soil is very limited since

microbial populations are rarely confronted with such an availability of single substrate additions (Meli *et al.*, 2003). For the purpose of this study, respiration response to nutrient inputs provided important complementary information regarding the status of microbial activity as well as an insight to the microbial community structure (*e.g.* proportion of fungal and bacterial relative components) in each soil under drier soil conditions. Since the relevant information obtained by SIR was based on very short-term periods (within 1 day of incubation), it ensured that CO₂ measured arose from native soil microbial communities rather than by contribution from those carried in by non-sterile substrates. Nevertheless, according to Degens and Harris (1997) comparing between SIR response patterns in different soil types should be done cautiously.

The Selective Inhibition method (pages 58 and 60) was employed for assessing bacterial and fungal relative components, in respect to activity and population size. Although Selective Inhibition is a well established and widely employed technique, Anderson and Domsh (1975) and Lin and Brookes (1999) provided a good discussion of its disadvantages when applied to moist soil. These authors suggested that firstly, it is difficult to measure the extent to which the antibiotics used do not interfere with the activity of the group of interest. For this reason, a lower dosage may be advantageous, however, it may then act as nutritional source for the non-suppressed microorganisms, thereby enhancing their activity and growth. Inhibitors are also prone to be inactivated by binding to soil particles and organic matter, which explains why some authors prefer using them at higher dosages, with possible detrimental effects for the group of interest. Lin and Brookes (1999) recommended optimal concentrations of 4-8 and 8-12 mg g⁻¹ moist soil of streptomycin (antibiotic) and cycloheximide (fungicide) respectively, although the concentration of inhibitors used in the literature vary widely. It is also probable that those concentrations vary with soil type and water potential, in the case of the inhibitors being added to soil directly. In order to ensure an adequate distribution of the inhibitor throughout the soil, these were previously dissolved in the water used to set the appropriate soil water potential. Due to the lack of data in the literature regarding the addition of inhibitors into soil at low water potentials, the dosage used for chloramphenicol and cycloheximide was the same as that recommended by Lin and Brookes (1999).

The protocol used for determining dehydrogenase activity (page 60) using INT as artificial substrate was based on the method by von Mersi and Schinner (1991) developed for optimal determination levels of dehydrogenase activity. However, considering that the original method has been standardised for field moist arable soils (von Mersi and Schinner, 1991) a series of preliminary experiments had to be performed in order to test and optimise that procedure for soils under drier conditions and of different characteristics. The assay parameters which received particular attention were the amount of soil used, the concentration of both substrate INT and the extraction solvent, the temperature and period of incubation. The resulting optimised procedure has shown to be adequate for the purpose of this study and for the soil treatments involved. Supported by Fragoeiro (1994) and Garcia *et al.* (1994), it therefore strongly disagrees with authors such as Nannipieri *et al.* (1990) who suggested that this method can not be effectively employed for determining the activity of this enzyme under dry soil conditions. On the contrary, it has shown to be a sensitive, reliable and reproducible indicator of microbial activity even under such conditions. Furthermore, it was generally consistent with respiration data obtained. An advantage of using this particular protocol is that, unlike equivalent methods described in the literature, it takes into consideration the abiotic reduction of INT (Von Mersi and Schinner, 1991). Moreover, unlike those based on TTC reduction, INT-based methods ensure that soil microbial metabolism is not suppressed due to toxicity effects (Trevors, 1982).

Bacterial and fungal colony counts, as well as fungal diversity studies, traditionally have been determined using culture-dependent techniques (page 62 and 63), although they are known for carrying disadvantages (Nannipieri *et al.*, 1990; Schinner *et al.*, 1996; Paszczyński and Crawford, 2000). The major weakness of these methods is that they allow assessing only microorganisms that are able to grow on the nutrients supplied (by the nutritional media) and under the incubation temperatures and period used. Nannipieri *et al.* (2003) argues that culture-dependent techniques allow estimating only between 1-10% of the whole population. They defend that this discrepancy is mainly due to the fact that some species are only cultivable under very specific environmental conditions, and that no media can reproduce the unique environment that is soil. Nevertheless, for the purpose of this study, this method provided relevant

information on the microbial population size as well as its bacterial and fungal relative components and the structure of fungal community in these soil types. Additionally, these results are a valuable contribution for the understanding of the way these parameters shift as a response to low water potentials, nutrients and atrazine inputs and throughout a bioremediation programme using an introduced white-rot fungus. Currently, the structure of microbial populations is usually determined with molecular techniques such as fatty acid profiles and DNA characterisation, instead of classical enumeration (Nannipieri *et al.*, 2002), but these techniques are costly and require specialised staff, therefore, they have little use for routine diagnostics (Gil-Sotres *et al.*, 2005).

5.6 AN ENVIRONMENTAL PERSPECTIVE ON THE USE OF FUNGI IN BIOREMEDIATION

Traditional methods for soil remediation use physical (e.g. containment, vapour extraction, incineration) or chemical (e.g. soil washing, solvent extraction, oxidation-reduction based reactions) processes that attempt to quickly and effectively isolate, reduce or eliminate the contaminant (Ralebitso *et al.*, 2002). However, such traditional chemical/physical soil remediation techniques are being less and less acceptable from a health, environmental and economic perspectives, whereas bioremediation is gaining interest as an alternative approach to soil clean-up (Tornberg *et al.*, 2003; Mollea *et al.*, 2005).

The end goal of bioremediation is the mineralisation of the contaminant to CO₂ and water, or in the case of metals to immobilise them by sorption (Pointing, 2001). Bioremediation aims for the least disturbance of the contaminated site (Gavrilescu, 2005), the preservation of the environment (Margesin *et al.*, 2000) as well as allowing to decrease human exposure to the xenobiotic (Gavrilescu, 2005). Biostimulation approaches are generally performed *in situ*, taking advantage of the degrading capability of indigenous microflora, through the optimisation of parameters such as temperature,

pH, water content, nutrients and oxygen concentrations (e.g. Yare, 1991; Weaver and Rhyllkerd, 1996; Pointing, 2001). Similarly, bioaugmentation using introduced microorganisms also offers the advantage of being an *in situ* technique (Pointing, 2001). These processes are also generally cheaper, requiring low cost technology as well as low maintenance (Kearney and Roberts, 1998). The simplest, and perhaps the most attractive, method for performing it is through biostimulation of indigenous microorganisms (Šašek *et al.*, 2003; Mollea *et al.*, 2005), which has already proved effective for enhancing degradation of some pesticides, oil and derivatives and BTEX mixtures (Yare, 1991; Weaver and Rhyllkerd, 1996).

Most bioremediation approaches are conducted using prokaryotes based on intracellular biodegradation, since bacteria generally utilise the pollutants as a nutritional source (Pointing, 2001). Recently, a great amount of research has shown the advantages of using white rot fungi over bacteria, given the much wider range of compounds that they are able to oxidise, as well as their extracellular lignin-degrading enzymatic complex (Gadd, 2001; Mollea *et al.*, 2005). Also, the fact that they are generally tolerant to low water potential conditions and have the ability to thrive using recalcitrant substrates (Gadd, 2001), makes them attractive as bioremediation agents, particularly in Mediterranean-like and semi-arid ecosystems. However, their application to non-sterile soil may be limited by inadequate environmental conditions for growth and colonisation, as well as by interactions with indigenous microbial populations, as described by Radtke *et al.* (1994).

One way of overcoming this problem could be by adding to soil previously isolated ligninolytic enzymes instead of the fungal inoculant. However, the enzyme extraction and purification processes are costly and extracellular enzyme activity has already shown to decrease rapidly due to sorption by soil colloids (Libra *et al.*, 2003). Alternatively, another group of ligninolytic fungi, the litter-decomposing fungi (LDF), are physiologically related to white-rot fungi and also show good lignin-degrading features. Recently, Baldrian and Šnadr (2006) compared the performance of LDF against that of white-rot fungi for degradation of synthetic dyes. Despite lower production of ligninolytic enzymes, LDF were shown to be better degraders for some of the dyes used, which can be considered a promising result. On the other hand,

Streptomyces are also capable of solubilisation and mineralization of lignin-like compounds. Therefore, they are expected to be powerful competitors with white-rot fungi for bioremediation applications. One reason for that is the fact that they are prokaryotes and thus, they can colonise the soil more quickly without needing the pre-inoculum preparation step. A diverse collection of actinomycetes has already been screened for production of extracellular enzymes, among which LAC, with several strains showing high extra-cellular activity (Paszczynski and Crawford, 2000).

The development of genetic manipulation techniques has given the potential ability to develop strains with the desired characteristics for bioremediation programs of a wide range of environmental contaminants (Paul *et al.*, 2005). However, genetically engineered microorganisms still have very limited use in soil bioremediation. The main interest has been developing superior pesticide-degrading strains with expanded metabolic capabilities, as well as competition and adaptability strategies (Kearney and Roberts, 1998). Yet, substantial barriers posed by gene-technology as well as public un-acceptance are preventing this technology from progressing at this level (Hägglum, 1992). The potential of using these genetically-manipulated microorganisms immobilised in bioreactors could eventually overcome many of the concerns involving the wide spread of these super-organisms in the environment (Kearney and Roberts, 1998; Paul *et al.*, 2005). The use of bioreactors also ensures that certain parameters (*e.g.* aeration, temperature and nutrient levels) can be controlled in order to optimise the bioremediation process, but its this same feature that turns their use into a high cost and maintenance approach (Pointing, 2001; Gavrilescu, 2005).

Nevertheless, in the quest to explore other resources, it is important firstly to assess the unexplored 'unculturable' microbial diversity native to our environment. It has been estimated that 1 g of soil may contain between 1,000 and 10,000 species of unculturable microorganisms (Torsvik and Ovreas, 2002) and there is likely to be further diversity within each species. There is a whole genetic potential which has not been explored at its maximum (Paul *et al.*, 2005), because traditional culture-dependent techniques underestimate the great microbial diversity in environmental samples. Developments and applications of molecular techniques for studies on microbial diversity may be key

to further discovery and exploitation of microorganisms (fungi in particular) for bioremediation purposes of a much wider range of environmental contaminants.

According to Šašek *et al.* (2003) two major limitations still need to be overcome in order to establish bioremediation as an effective and reliable field-remediation technique. Firstly, this is a much slower process of clean up when compared to the traditional remediation techniques (Gavrilescu, 2005). Secondly, there are practical problems concerning the handling and behaviour of the introduced fungus in soil and a lack of knowledge on the influence of soil properties, environmental parameters and microbial interactions on the bioremediation process (Mollea *et al.*, 2005).

With increasing concerns about the impact of climate change and increased temperatures on soil processes, it has become important to investigate the success of bioremediation of contaminated soils under drier and hotter soil conditions. This is not only important in the case of temperate, agricultural soils, such as the sandy loam used in this study, but also and mostly in the case of naturally dry and nutritionally poor soils, such as the clay and volcanic ash soils, where this gap of knowledge is even more evident (Ghanni *et al.*, 1996). This study has provided relevant preliminary information about the effect of low water availability on the biodegradation of a widely used herbicide in soil through a bioaugmentation programme.

CHAPTER VI

Conclusions and Recommendations for Future Work

CHAPTER VI

CONCLUSIONS AND RECOMMENDATIONS FOR FUTURE WORK

6.1 CONCLUSIONS

This study has demonstrated that:

- ✓ A non-specific polymer sensor array was able to detect variations in the patterns of VOC production from soil, functioning as indicators of changes in microbial activity and community structure as a response to key environmental factors (alone and in combination), such as water potential, temperature, nutrient inputs, as well as their differential assimilation according to their type. This was possible independently of soil physical, chemical and biological characteristics with high reproducibility (standard error < 5%);
- ✓ Strong and significant ($p < 0.05$) correlations were found between soil volatile patterns (through PC1) and traditional microbial parameters of respiration, DHA, population size and number of fungal species. This suggests that soil volatile fingerprinting using an E-nose can complement and perhaps replace some of these traditional parameters when a quick, non-invasive and routine approach is required for the qualitative and semi-quantitative evaluation of the impact of any given external factor on microbial activity and community structure in soil. The close relationship ($r > 0.80$) between PC1 and soil respiration is particularly relevant, since it indicates that similarly to this parameter, volatile fingerprints respond quickly to changes in microbial activity in un-amended soil.
- ✓ The array of non-specific sensors was also able to detect atrazine in soil at usual field application rates, and differentiate between the odour of soil containing atrazine and that containing an organic substrate, independently of the soil matrix. Similarly, it showed to be sensitive enough to detect temporal variations in volatile production patterns of atrazine-containing soil, due to herbicide

mineralisation/transformation by either native or introduced microorganisms. It is, therefore, likely that similarly to atrazine, the metabolism of a wide range of soil contaminants can be monitored using this approach.

- ✓ There is also potential for the E-nose to be employed as a monitoring tool for the activity of white-rot fungi (and perhaps other microorganisms) following introduction in non-sterile soil for the purpose of bioremediation of usual soil contaminants. Additionally, it can offer a sound basis as a complementary tool for studies intending to explore microbial interactions, particularly between native and introduced microorganisms, during a bioremediation programme in non-sterile soil;
- ✓ This study has demonstrated that an E-nose consisting of a non-specific conducting polymer sensor array was able to detect and differentiate between potential sources of off-odours, at different concentrations, in both RO and treated water, as well as in soil, based on volatile production patterns. However, the detection of geosmin at concentrations below its OTD was only possible to achieve in water, probably due to lower volatile background when comparing against that in soil. A significant correlation was also found between PC1 and inoculant concentration indicating that quantitative results can be achieved and potentially used in specific water and soil off-odour screening purposes. Besides proven to be fast and highly reproducible, this approach also offers potential for on-site applications in both environments, although additional studies are needed for further evaluating its feasibility for early off-odour detection and monitoring in soil.

6.2 RECOMMENDATIONS AND FUTURE WORK

6.2.1 GC/GC-MS analysis of soil volatiles

It would have been interesting to identify and quantify the main groups of volatiles responsible for the discriminations obtained between headspace volatile fingerprints of the different soil treatments. This could not only provide a highly relevant insight into the microbial response to changes in environmental conditions, but also be used for monitoring atrazine degradation/transformation in soil. Also important is the wide range of applications arising from using it in combination with E-nose based soil qualitative volatile fingerprint analysis. Additionally, it could be used for developing or improving sensor design for detection and monitoring of specific groups of volatiles in soil atmosphere, and perhaps enable establishing correlations between specific volatile groups and soil quality.

6.2.2 Alternative methods for assessing microbial population size and community structure

This study has shown very few significant differences in total microbial population size as influenced by soil treatments, using the plate count method. Similarly, changes in fungal community structure throughout the bioremediation study were generally not significant using this approach. For that reason, it is suggested that alternative methods for assessing population size and community structure be performed in order to confirm those results. Methods based on extracting from soil and determining phospholipid fatty acids (PLFA) are recommended, as they not only serve as a quantitative measure of biomass, but also provide a reliable profile of the structure of the total community. Alternatively, nucleic acid based analysis also offer a way of looking at both parameters simultaneously. Protocols of both methods are widely available in the literature.

6.2.3 Long-term respiration response to nutrient addition

Continuous long-term investigation on the response of the three soils to nutrient addition would have allowed for further understanding of differences in the decomposition rates of both types of substrates under low water potential conditions, and the way they are influenced by soil type. Additionally, one can even investigate the impact of the added nutrient on the degradation of native organic matter. In the latest case, it is common to use ^{14}C -labelled nutrients and the ^{14}C - CO_2 evolved is subsequently quantified, in opposition to measuring that obtained subtracting CO_2 in the control soil from that in the treated soil (Shen and Bartha, 1997; Nannipieri *et al.*, 2003). This happens due to the ‘priming effect’ (or the lag time) in the treated soil and can actually function as an indicator of any acceleration or inhibition of mineralization of the native organic matter due to nutrient addition (Shen and Bartha, 1996). An example of this effect is also very common in contaminated soil (Brookes, 1995).

Extensive and long-term activity monitoring is probably the most realistic approach to effectively and reliably evaluate microbial response to changes in soil conditions and formulate appropriate conclusions (Bloem *et al.*, 2006). Such changes may also be easier to interpret than momentary values, which very often present some subjectivity.

6.2.4 Soil toxicity tests

According to Rhine *et al.* (2003), atrazine transformation is more commonly observed than its mineralization. In this case, any pesticide bioremediation program should not only guarantee the disappearance of the original pesticide in soil, but also make sure that its degradation products, and others resulting from co-metabolic reactions, are not an environmental and health hazard.

In part, this is assured by previous knowledge on the pesticide itself. Nevertheless, toxicity tests are recommended to be performed in the three soil types following bioremediation under the treatment environmental conditions. Among toxicity tests available, microbiotests (small-scale bioassays using microorganisms as test

organisms), are strongly advised for their reproducibility, and time and cost-effectiveness when compared against conventional direct-contact tests such as phytotoxicity tests (Šašek *et al.*, 2003). In particular, Toxalert®10 (Merck), which uses the aquatic bioluminescent bacterium *Vibrio fischeri*, has showed in previous studies to be suitable for soil toxicity analysis (e.g. Kovats *et al.*, 2003; Fragoeiro, 2004). Bioluminescence is directly associated with respiration. Once in contact with potentially contaminated soil, any reduction in light emission is proportional to the strength of the toxic compound, which can then be detected by a luminometer. For more information on the principles and applications of Toxalert®10 (Merck) see Kovats *et al.* (2003).

6.2.5 Measurement of other enzyme activities possibly linked to pesticide degradation by *T. versicolor*

Although it is generally assumed that pesticide degradation is related to the production of LAC, few recent studies have demonstrated the key contribution of other ligninolytic enzymes such as lignin- and Mn-peroxidase for the metabolism of other xenobiotics. Besides the results from the present study, Bending *et al.* (2002) also found no correlation between LAC activity and atrazine degradation in liquid culture using *T. versicolor*. The activity of those enzymes may explain this lack of correlation found by these authors between atrazine degradation and LAC activity. Further studies should be performed using this species, in order to evaluate the contribution of those two extracellular enzymes in atrazine degradation in soil.

6.2.6 Pesticide ‘aging’ in soil

In some studies, the pesticide is allowed to age in soil before the bioremediation takes place. Over a short period of time following pesticide addition, sorption processes at soil surface are reversible and therefore may not strongly influence the bioremediation success. However, with time, more pesticide gets strongly absorbed forming stable complexes with soil colloids, resulting in a decrease in its bioavailability even by extracellular enzyme activity (Kearney and Roberts, 1998; Gadd, 2001; Moorman *et al.*,

2001). It would have been interesting to redesign a bioremediation programme of atrazine in soil under the treatment conditions using aged atrazine-containing soil treatments and compare to the results obtained in the present study.

6.2.7 *In situ* bioremediation

Bundy *et al.* (2002) suggested that bioremediation of soil based on microcosm studies, which by necessity use disturbed soils may not be representative of *in situ* processes. The true test of any bioremediation program is in field application, but because of the high costs and practical experimental aspects involved (e.g. survival of the fungus) there are limited reports on its application. Bioremediation through biostimulation of indigenous populations has seen relatively successful results in the field, but the attempts of using bioaugmentation by fungi are very limited up to now. Nevertheless, it would be interesting to explore this possibility. Walter *et al.* (2005) designed and conducted a successful field scale bioremediation of an aged PCP-contaminated site using *T. versicolor*. The initial concentration of PCP was 800 mg kg⁻¹ soil, and it was reduced to less than 50 mg kg⁻¹ soil within 74 weeks. Another interesting result was that irrigation was not required throughout the 2.5 year study, as metabolic water from decaying woodchips (added as aid to the fungus) was enough to maintain an adequate soil moisture content.

Obviously, the use of introduced fungi would only be justified if they were shown to degrade the pesticide more effectively and rapidly than native microflora. However, in the field, a more reliable method for monitoring the growth and colonisation of the white-rot fungus would have to be developed, instead of using LAC as an indicator of activity. For example, Johnston and Aust (1994) used molecular techniques for successfully detecting the fungus among the mixed microbial population. A complete assessment and monitoring of toxicity by metabolites of the initial compound would also be essential (Pointing, 2001).

One possible way of reducing the expenses related to the production of fungal inocula for addition into soil could be the use of waste fungal mycelium from industry. For

example, spent oyster mushroom substrate and spent sawdust culture of *shiitake* mushroom both have shown to degrade PCP at some extent (Okeke *et al.*, 1993). On the other hand, the inoculation of cheap but effective soil amendments such as straw, sawdust and corn cobs can also be used as aid for the colonisation of the fungus in the soil. A detailed review on the potential application of white-rot fungi for *in situ* soil bioremediation is given by Gadd (2001).

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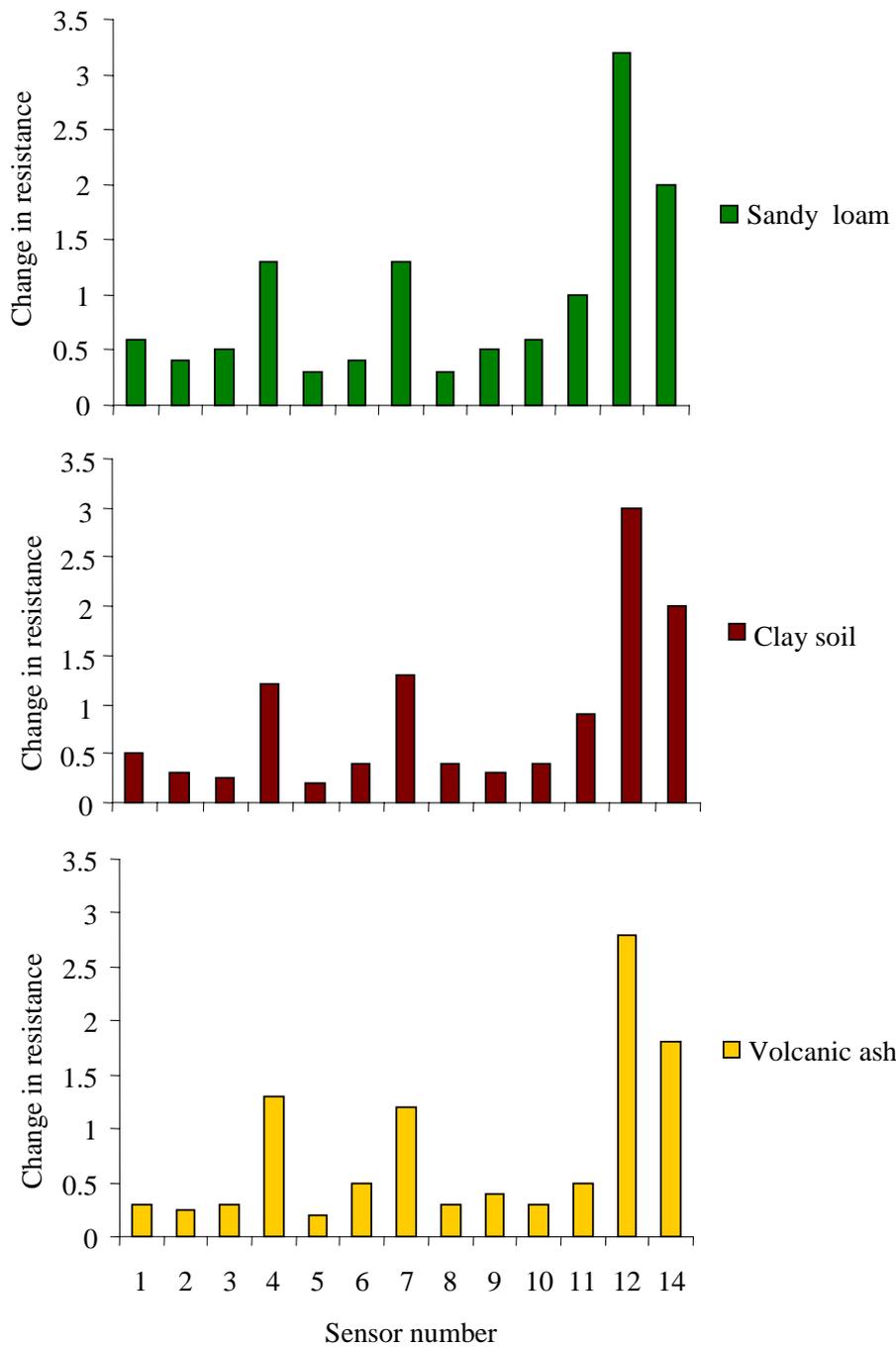
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APPENDIX I

**Reproducibility in sensor relative responses for
all soil types expressed as changes in resistance**

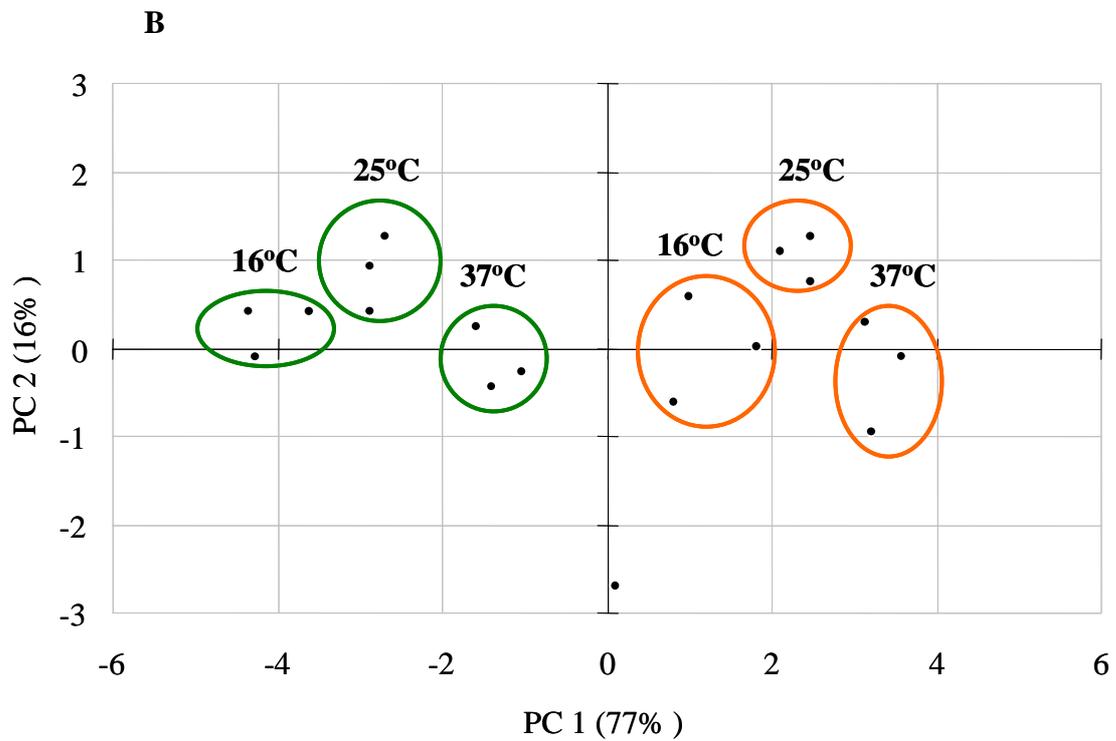
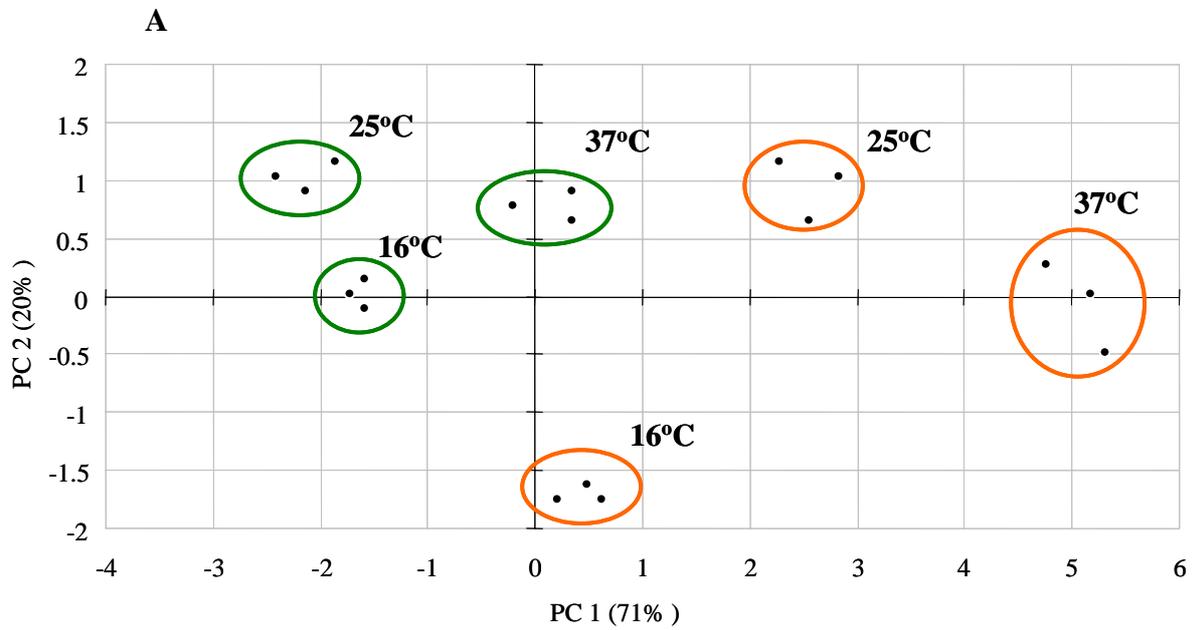
Sensor relative responses for all soil types, expressed as changes in resistance (1 day incubation at 25°C and -0.7 MPa). For each sensor, responses are the average of five replicates.



APPENDIX II

**PCA map for replicates of (A) sandy loam and
(B) volcanic ash at 16, 25 and 37°C under two
moisture regimes, -0.7 and -2.8 MPa**

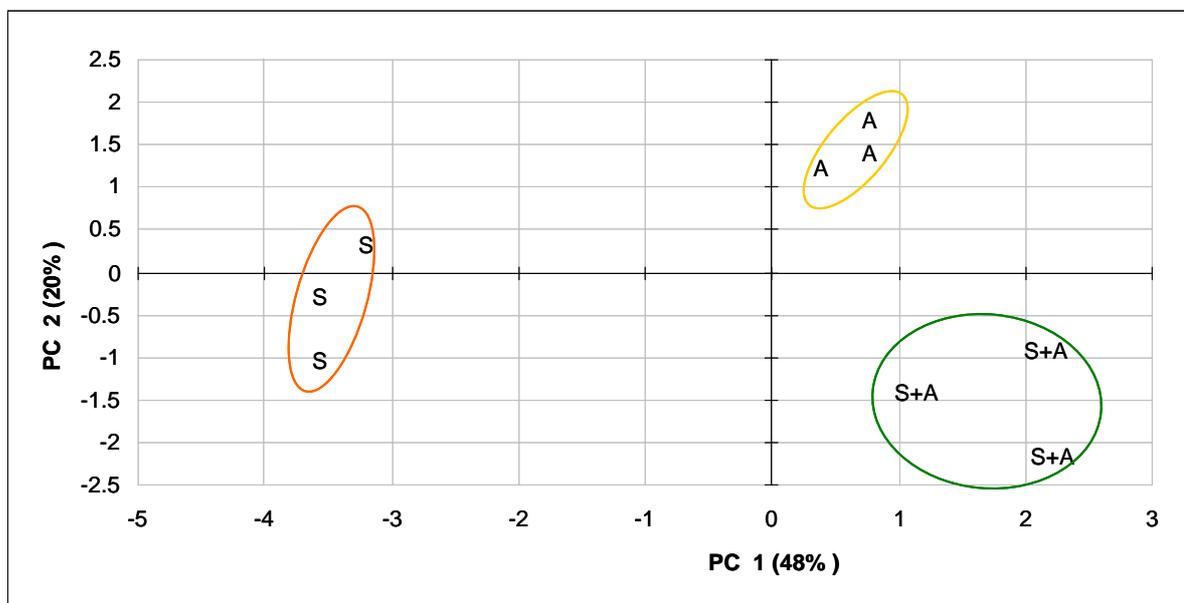
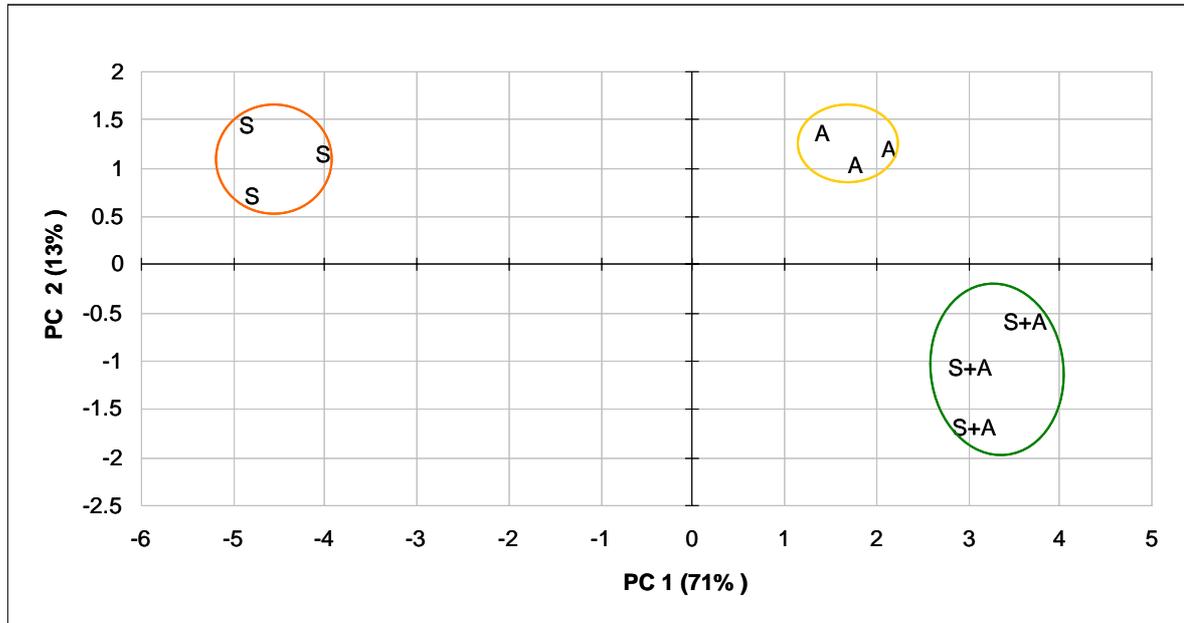
PCA map for replicates of (A) sandy loam and (B) volcanic ash (1 day incubation at 16, 25 and 37°C, under two moisture regimes differentiated in the plot by the colour of the line: orange and green for -0.7 and -2.8 MPa respectively).



APPENDIX III

**PCA maps discriminating between untreated
and atrazine-treated soil for the sandy loam and
volcanic ash**

PCA maps discriminating between untreated (S) and atrazine-treated (S+A) (2.5 ppm) for the sandy loam and volcanic ash soils respectively (1 day incubation at 20°C, -0.7 MPa). In both cases, an atrazine solution in RO water (A) was used as control only.

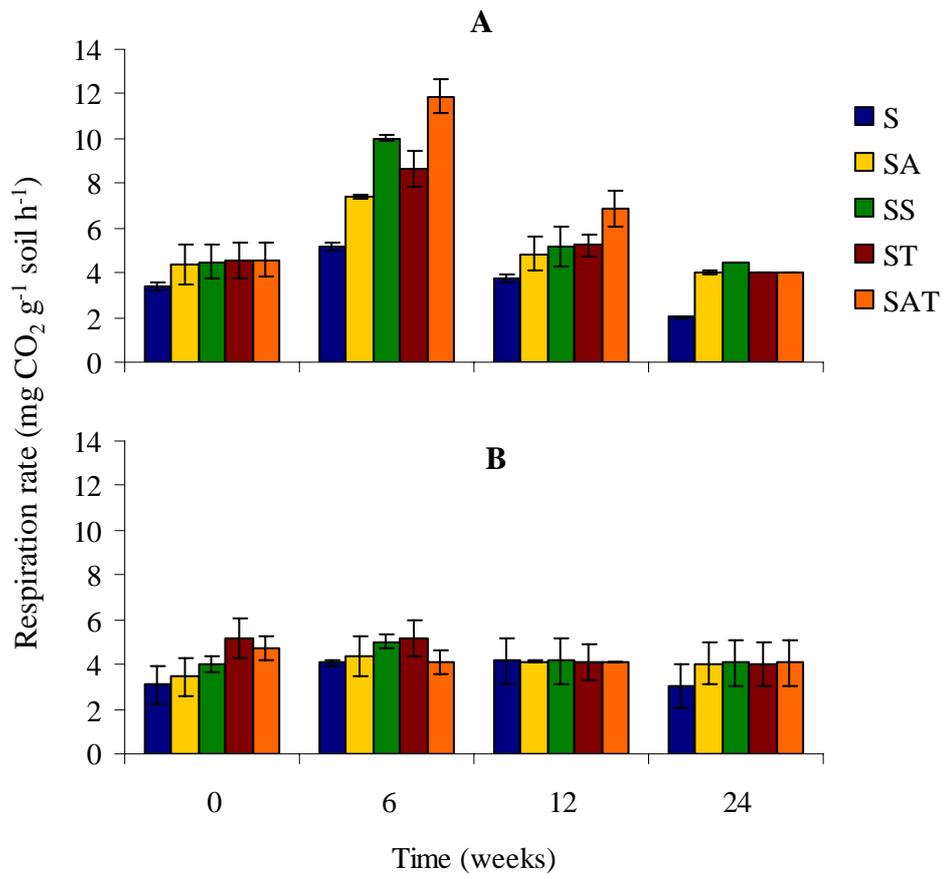


APPENDIX IV

**Respiration rate for the sandy loam under
different treatments as measured at weeks 0, 6,
12 and 24**

Respiration rates for the sandy loam under different treatments at (A) -0.7 and (B) -2.8 MPa as measured at weeks 0, 6, 12 and 24

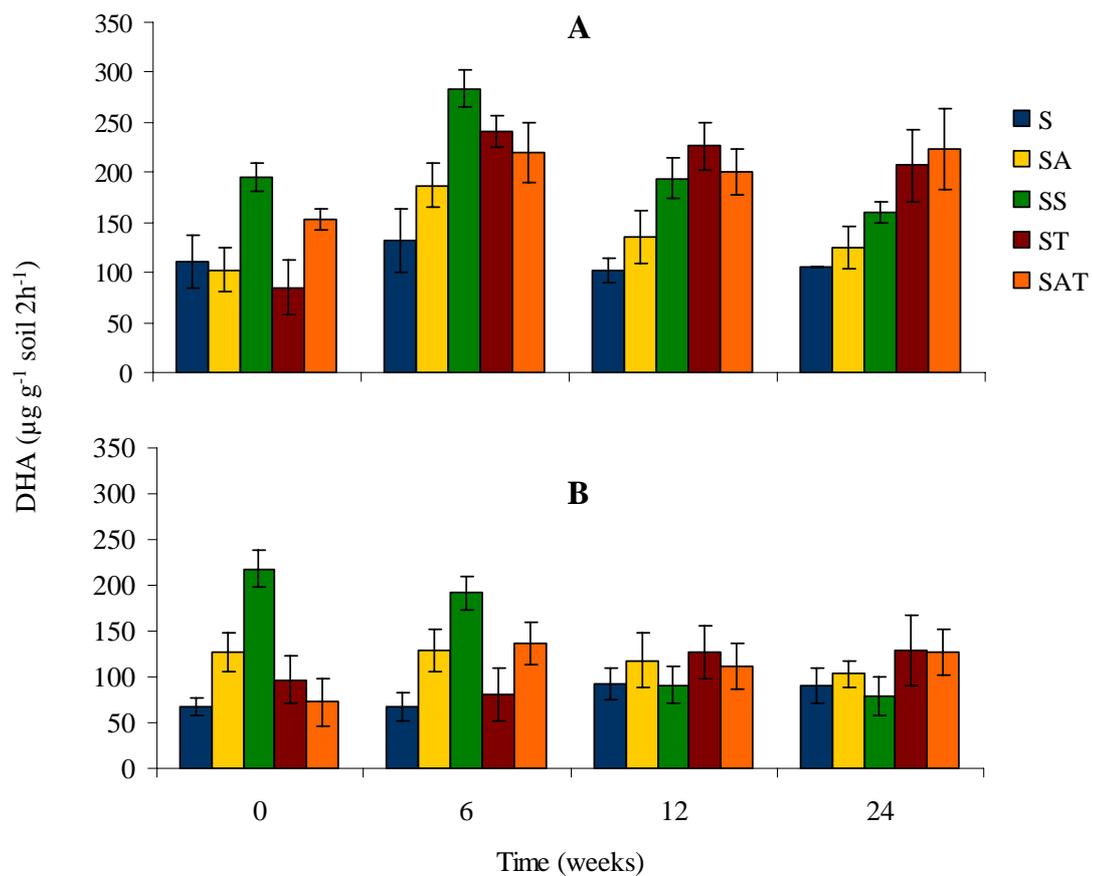
(incubation at 20°C). *Key to treatments:* S, untreated control soil; SA, soil contaminated with atrazine (2.5 ppm); SS, soil with sawdust (0.5%); ST, soil inoculated with *T. versicolor* (0.5%); SAT, soil contaminated with atrazine (2.5 ppm) and inoculated with *T. versicolor* (0.5%).



APPENDIX V

DHA values for the sandy loam under different treatments as measured at weeks 0, 6, 12 and 24

DHA values for the sandy loam under different treatments at (A) -0.7 and (B) -2.8 MPa as measured at weeks 0, 6, 12 and 24 (incubation at 20°C). *Key to treatments:* *S*, untreated control soil; *SA*, soil contaminated with atrazine (2.5 ppm); *SS*, soil with sawdust (0.5%); *ST*, soil inoculated with *T. versicolor* (0.5%); *SAT*, soil contaminated with atrazine (2.5 ppm) and inoculated with *T. versicolor* (0.5%).



APPENDIX VI

**Exploring microbial activity in JSC-Mars 1
simulant soil**

Exploring Microbial activity in JSC-MARS 1 simulant soil

During this research, the volcanic ash was regarded as a regular soil, but it is now time to differentiate it from the sandy loam and clay soils used. Contrary to these latter agricultural soils, the volcanic ash, known as JSC-MARS 1, was developed and distributed by NASA for supporting research and as a teaching/learning resource mainly concerning the planet Mars (Allen *et al.* 1997). This material was chosen because of its similarity to the Martian soil in physical and chemical composition as analysed by the Viking lander 1 (Mendez *et al.*, 2005). Yet, very little microbiological-based research has been done involving this soil.

This study has found that JSC-MARS 1 contains a total microbial population size varying between 2.51×10^{10} and 1.99×10^{14} CFUs / g dry soil according to temperature and water potential, following 7 incubation days. Native microbial communities were clearly active given by the respiration rate, dehydrogenase activity, and shifts in volatile fingerprints in both un-amended and nutrient-treated soil. Interestingly, bacterial and fungal abundances, as well as the way microbial community responded to changes in soil conditions followed similar patterns that in the clay soil, which is considered a typical agricultural soil in dry and hot Mediterranean-like regions (DRAALG, 2000).

Bacterial populations growing on NA were the most abundant and metabolically active under the wetter conditions with colony counts varying between 1.7×10^6 to 2.0×10^7 with temperature. Similarly, fungal populations growing on MEA thrive best under drier and hotter conditions, with community size ranging between 4.07×10^2 and 4.17×10^7 CFUs g^{-1} soil according to treatment. In terms of species diversity, fungal community presented low diversity in un-amended soil, containing only *Aspergillus*, *Penicillium*, and *Rhizopus*, but the number of species (g^{-1} soil) increased from 4 up to 11 following nutrient addition.

Allen and co-workers (2000) used 250 mg of JSC-MARS 1 suspended in 5 ml of sterile water and incubated on different media for 7 days at 27°C, in order to isolate bacteria and fungi. They counted 1.8×10^4 and 3.1×10^3 bacterial (on NA) and fungal

APPENDIX VII

Scientific Publications and Presentations

Scientific Presentations

A. C. BASTOS and N. MAGAN. *Soil volatile fingerprints: rapid discrimination between soil environmental conditions*. British Soil Science Society Conference – Soil, Vegetation and Climate Change, 14-17 September 2006, Leeds University, Leeds, UK.

A. C. CATARINA BASTOS and N. MAGAN. *Electronic nose technology for monitoring soil microbial activity*. British Mycological Society's Annual Meeting – Promoting Fungal Science, 4-7 September 2006, Birmingham, UK.

A. C. BASTOS and N. MAGAN. *Microbial volatile fingerprints: potential for monitoring soil microbial activity*. 7th Postgraduate Research Conference, 16-17 June 2005, Cranfield University, Silsoe, UK.

A. C. BASTOS. *Comparing microbial activity in different soil types based on respiration and volatile fingerprint analyses*. Institute of BioScience and Technology's Seminar, on the 27th September 2005, Cranfield University, Silsoe, UK.

A. C. BASTOS and N. MAGAN. *Monitoring changes in soil microbial status based on volatile fingerprints*. British Mycological Society's Annual Meeting – Exploitation of Fungi, 5-8 September 2005, Manchester, UK.

A. C. BASTOS and N. MAGAN. *Soil microbial activity as a function of soil type and environmental factors*. 6th Postgraduate Research Conference, 16-17 June 2005, Cranfield University, Silsoe, UK.

A. C. BASTOS and N. MAGAN. *Comparisons of fungal biodiversity in different soil types in relation to environmental factors*. British Mycological Society's Annual Scientific Meeting - Fungi in the Environment, 13-15 September 2004, University of Nottingham, UK.

Potential of an electronic nose for the early detection and differentiation between *Streptomyces* in potable water

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Abstract

Much attention has been focused on the production of musty-aroma compounds such as geosmin and their impact on the quality of fresh water and water-cultured raised fish and sea-food. At present, there are no efficient means of removing these off-flavours from water. Therefore, the rapid detection of geosmin-producing microorganisms, in particular the genus *Streptomyces*, at early stages of differentiation, is still the best option in preventing deterioration of water quality. We investigated the potential of an electronic nose consisting of an array of 14 conducting polymer sensors for the rapid and early detection of *Streptomyces* spores in reverse osmosis and tap water. Geosmin solutions in water at different concentrations were also prepared for headspace analysis in order to study the e-nose potential for detecting geosmin production in this environment. Normalised divergence data were analysed using principal component analysis (PCA) and discriminant function analysis (DFA). Data indicates that an e-nose could be employed to detect and monitor early activity of *Streptomyces* in water at different stages of differentiation, as well as to discriminate between different species based on their volatile production patterns. It also suggests that it could be used for monitoring geosmin production in water and possibly set threshold odour levels, as a routine task for specific water-screening purposes.

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Keywords: Off-flavours; Geosmin; Water quality; *Streptomyces*; Polymer sensors; Electronic nose

1. Introduction

Off-flavours and odours in potable water and aquaculture-raised fish and sea-food are a major source of consumer complaints all over the world [1]. Taste and odour occurrences are usually caused by the presence of trace organic compounds such as geosmin [2]. This volatile has been isolated and identified as having a muddy, musty odour discernible by the human nose when present at concentrations above 4–20 mg L⁻¹ [3]. Actinomycetes, particularly from the genus *Streptomyces*, are known to be the mainly responsible organisms for these occurrences in the water supply system. They produce geosmin as a secondary metabolite at a certain stage of differentiation prior to sporulation and therefore, the presence of spores in water can be correlated with geosmin production [4]. Unfortunately, at present, there

are no efficient means of removing such off-flavours and odours from water, although activated carbon is still considered the best solution for their attenuation [1]. Therefore, the rapid detection of geosmin-producing microorganisms at early stages of differentiation is still the best option in preventing deterioration of water quality.

Gas chromatography (GC) and gas chromatography–mass spectrometry (GC/MS) are traditionally used to identify and quantify these compounds but although they are effective, reliable and low cost, they can be time consuming, particularly if many replicates are necessary. Other current methods for detection and quantification at similar threshold levels require large sample volumes and intensive sample concentration procedures [5,6].

Rapid developments in sensor technology allowed the production of devices known as electronic noses, which represent an attempt to mimic the human sense of smell, with the same sensitivity and accuracy [7]. The basic principle of electronic nose technology involves exposing a range of non-specific sensors to volatile compounds, resulting in a change in con-

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ductivity of the sensor due to binding between that and the volatile [8]. Signals are detected and amplified by a software package system and may then be interpreted using a variety of methods such as principal component analysis (PCA) and discriminant function analysis (DFA). The pattern of the overall response generated by the sensor array is then used to characterise the odour. The obtained data are mainly comparative, since different samples or treatments may be characterised and discriminated based on their volatile production patterns [9].

E-nose technology has been widely employed for early detection of microorganisms causing food spoilage [10] and for biomedical purposes [11]. Some reports suggest that the application of e-nose technology for environmental diagnostics has been limited because of the inherent variability of environmental samples. Nevertheless, it has been successfully employed for detecting cyanobacteria in water [12] as well as heavy metals and pesticides [13]. Due to the growing interest in environmental monitoring, the early detection of microbial activity in producing volatile compounds such as geosmin is of increasing economic importance.

We have examined the potential of using an array of 14 conducting polymer sensors to detect and monitor early activity of *Streptomyces* in potable water prior to visible growth. Moreover, we tested the e-nose potential for differentiating between *Streptomyces aureofaciens* and *Streptomyces griseus* based on their volatile production patterns and for detecting and monitoring geosmin production in this environment.

2. Material and methods

2.1. Initial procedures

These included preparing pure solid cultures of two species of *Streptomyces*, *S. aureofaciens* (A253) and *S. griseus* (A26). Solid media was prepared by adding 11 g of Actinomycete Isolation Media (OXOID) to 500 ml of distilled water. Media was autoclaved for 15 min and while still hot, distributed on Petri dishes, which were kept at 4 °C while not in use. After inoculation, plates were incubated at 25 °C for 17 days.

2.2. Spore collection and sample preparation

Spores of both species were collected from each pure culture and suspended in 9 ml of sterile water with Technical Agar and Tween 80, contained in Universal bottles. Spore concentration was calculated using a haematocytometer and a microscope. Spores of both species were used to taint RO and tap water in order to prepare 10^2 , 10^4 , 10^5 spores ml^{-1} in each water type. The 10 ml liquid samples were transferred to 50 ml polypropylene bubble-generating bottles, leaving 40 ml of headspace above the water sample in each bottle. These were then tightly closed in-situ to prevent loss of volatile compounds and then incubated at 25 °C for a period of 24 or 48 h prior to e-nose analysis, according to the objective of the experiment. During incubation, headspace became saturated with the volatiles pro-

duced, which was expected to increase the e-nose output. Five replicates of each treatment and controls were prepared.

2.3. Preparation of geosmin and ethanol solutions

Geosmin up to a minimum of 98% purity (Sigma) was used to prepare geosmin solutions in sterile RO and tap water at different concentrations (5, 10 and 100 ppb). Ethanol (Sigma) solutions at 10 ppb were also prepared in both water types to be used as second controls. Five replicates of each treatment and RO and tap water controls were transferred to propylene bubble-generating bottles for headspace analysis. Aqueous solutions were stored in the fridge for 48 h prior to sampling.

2.4. E-nose analyses of sample's headspace

Headspace analysis of all treatments followed the same procedure. These were analysed randomly using an electronic nose (BH-114 Bloodhound sensors Ltd., Leeds, UK), incorporating a non-specific array of 14 conducting polymer sensors. It had been left switched on for over 1 h before sampling in order to allow the sensor array to stabilise. A needle was connected to a system of 6 cm long Teflon tubing segments (bubble-generator), a bio-filter (0.45 μm , PTFE Whatman, Hepa Vent) preventing sensor contamination by spores, and an activated carbon filter. Sampling was performed at room temperature using the following profile settings: injection 9 s, desorption 5 s, baseline length 5 s and time delay 5 s. Activated carbon filtered air (flow rate 4 ml min^{-1}) was automatically passed over sensors surface after each sampling to set the baseline. Two calibration reference points were used: the baseline and the headspace of a control sterile RO water sample. One minute was allowed between samples to ensure that the sensor array had returned to baseline, preventing carry over effects between one sample and the next.

2.5. Statistical analysis

The program XLSTAT (Microsoft Excel add-in) was used and PCA and DFA were the statistical methods employed for analysing and interpreting sensor response using normalised divergence data.

3. Results

3.1. Replicability of sensor response

The replicability of sensor response was firstly studied. Fig. 1 shows low variability between five replicates of 10^2 spore ml^{-1} treatments of *S. griseus* in RO water. Similar variability was obtained for all treatments in both water types, thus enhancing the confidence level for the results obtained. Sensor 13, which had the most variable responses, was unstable and its response was excluded from analysis.

3.2. Discrimination between different spore treatments

The potential of an e-nose for discriminating between tainted samples and untainted controls as well as for differentiating

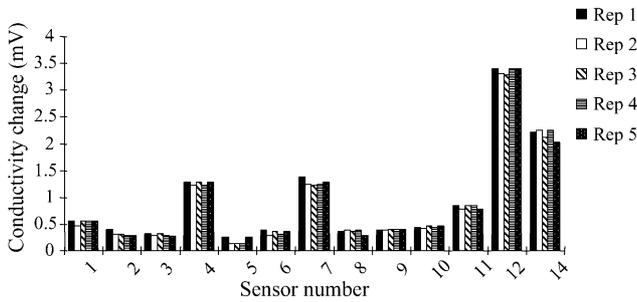


Fig. 1. Sensor array response showing low variability between five replicates of *S. griseus* 10² spore ml⁻¹ treatment in RO water.

between different spore treatments was tested. PCA, as a non-supervised technique, was employed to look at general relationships between samples while retaining most of the variance within data. Fig. 2 shows a PCA map of different spore treatments of *S. aureofaciens* in RO water after an incubation of 48 h at 25 °C. Data shows a good discrimination between different spore treatments, where most of the variance is explained by PC1 (95%) and PC2 (2%). PC1 contains the information on odour intensity, which is closely related to sample concentration. Similar results were obtained using spores of *S. griseus* and for both water types.

3.3. Temporal effects on detection and differentiation between treatments

DFA was employed on the raw data values to study the impact of the incubation period for discriminating between two tap water treatments of *S. aureofaciens* spores. Fig. 3 shows that the level of discrimination between 10² and 10⁴ spore ml⁻¹ treatments varies over time, being greater after longer incubation periods. Function (axis) 1 contributes with the majority of the information (67%) and seems to discriminate mostly according to odour intensity. However, the discrimination obtained is not only due to odour intensity but also by the stage of microbial differentiation, which may explain the fact that function (axis) 2 also contributed considerably (30%) for the four-group separation.

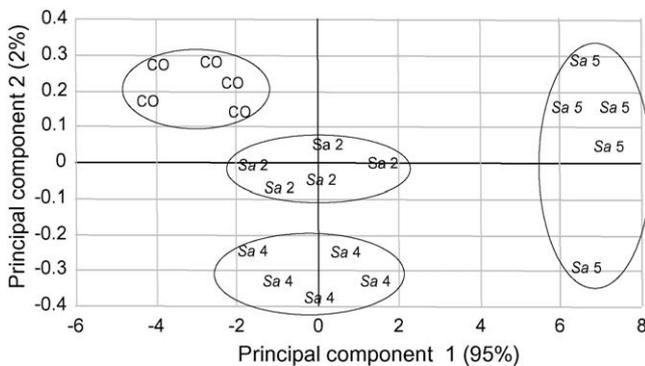


Fig. 2. PCA map of different spore treatments of *S. aureofaciens* in RO water after 48 h incubation at 25 °C. Key to treatments: CO, RO water control; Sa 2, *S. aureofaciens*, 10² spores ml⁻¹; Sa 4, *S. aureofaciens* 10⁴ spores ml⁻¹; Sa 5, *S. aureofaciens* 10⁵ spores ml⁻¹.

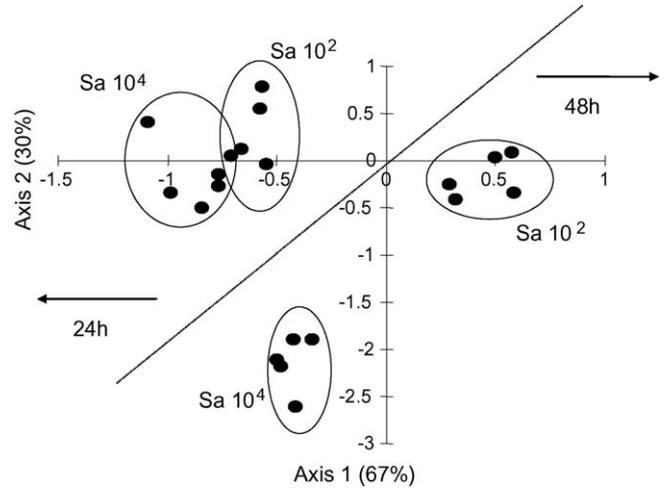


Fig. 3. DFA map for untainted and tainted samples of tap water using different concentrations of *S. aureofaciens* spores incubated for 24 and 48 h at 25 °C. Key to treatments: Sa 10², *S. aureofaciens* 10² spores ml⁻¹, Sa 10⁴, *S. aureofaciens* 10⁴ spores ml⁻¹.

Similar results were achieved for all spore treatments in both water types.

3.4. Discrimination between the two species

An attempt to early discriminate between *S. aureofaciens* and *S. griseus* spores in tap water was also performed using DFA. Fig. 4 shows that both species can be clearly differentiated based on their volatile production patterns using this approach. It is evident that function 1 contributes almost totally (98%) to the discrimination, indicating that it may explain alone differences in volatile intensity and odour type.

3.5. Potential for detecting geosmin production in water

PCA was employed in order to investigate a possible correlation between geosmin solutions in RO water and samples consisting of *Streptomyces* cells, without losing much of the variance. For this particular experiment, *Streptomyces* tainted water samples were prepared using viable cells instead of spores,

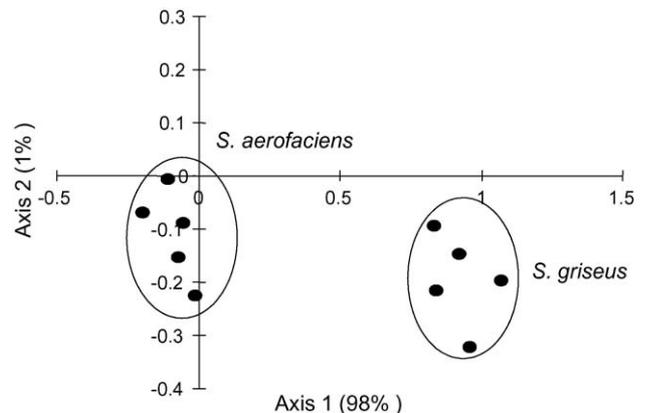


Fig. 4. DFA map for *S. aureofaciens* and *S. griseus* tainted tap water treatments (at 10⁴ spores ml⁻¹), both incubated for 24 h at 25 °C.

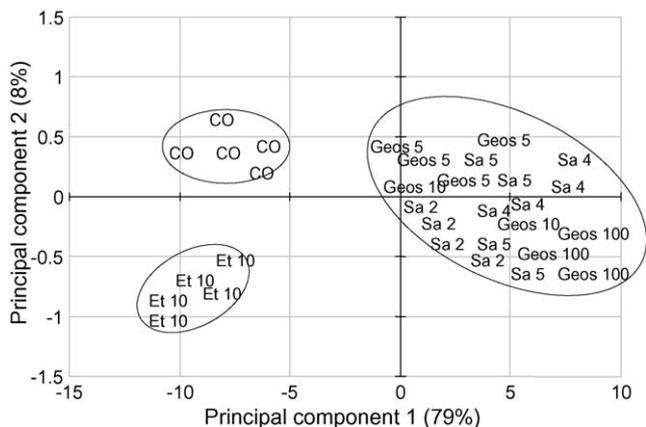


Fig. 5. PCA map for geosmin, ethanol and *S. aureofaciens* tainted RO water samples. Key to treatments: CO, RO water control; Et 10, ethanol solution in RO water at 10 ppb; Geos 5, Geos 10 and Geos 100, geosmin solutions at 5, 10 and 100 ppb, respectively; Sa 2, Sa 4 and Sa 5, *S. aureofaciens* at 10^2 , 10^4 and 10^5 cells ml^{-1} .

since those are the effective producers of geosmin. Fig. 5 indicates that there is no clear discrimination between geosmin and *S. aureofaciens* tainted samples. Note that both control treatments (RO water and ethanol solutions) were obviously separated between each other and from the remaining treatments. The lack of discrimination between microbial and geosmin tainted water treatments seems interesting, since it can indicate that this specie was producing geosmin at the time of analysis. PC1 accounts for the majority of the variance (79%), arising mostly from the discrimination between both control treatments and the group of interest, whereas the remaining 8% variance comes mainly from the separation between both controls.

4. Discussion

Data have demonstrated that an e-nose consisting of a non-specific conducting polymer sensor array was able to detect microbial taints in different water types in a quick and reproducible way, based on volatile production patterns. Therefore, e-nose technology shows potential to be used as a monitoring tool for changes in water quality. In this study, the use of two water types was intended to demonstrate that the detection and discrimination of taints is independent of the contained solutes, being particularly important for monitoring drinking water.

PCA analysis showed clear discrimination between untainted and *Streptomyces* tainted water samples after 24 h of growth, indicating that early microbial activity could be detected in this way. The detection at an early stage seems particularly important, since it could help preventing the production of off-flavours and tastes in the water supply system. Moreover, separation into distinct clusters according to spore concentration was possible even at 10^2 spore ml^{-1} , suggesting that it may also be used to achieve set threshold odour levels for specific water screening purposes. So far, according to Canhoto and Magan [11], 10^2 CFUs ml^{-1} was also the lowest level at which microbial contaminants were detected in water using volatile production patterns.

It was clear that the discrimination between different *Streptomyces* treatments into distinct groups was greater after 48 h than after 24 h of growth. It suggests that off-flavour producing agents can be detected in water at any stage of differentiation. Nevertheless, further studies are needed to evaluate the usefulness of the e-nose in such an application.

The differentiation between *S. aureofaciens* and *S. griseus* into distinct clusters by DFA, could indicate that this technique can be used to specifically detect geosmin-producing species or strains in water. Very recently, Canhoto et al. [1,2] also reported the successful application of a conducting polymer sensor array for discrimination between different strains of bacteria.

Furthermore, it was interesting to note that there is evidence of potential for detecting the production of volatile compounds such as geosmin in the water environment. PCA analysis indicated a close relationship between water samples tainted with geosmin and with *S. aureofaciens* viable cells, while clearly separating them from both controls. The inability to differentiate between those treatments could suggest that this species was producing geosmin at the time of analysis. In fact, the presence of geosmin in the headspace of *S. aureofaciens* tainted water was confirmed by gas chromatography. Samples were analysed using a GC (GC 8000 series, Carlo Erba Instruments Ltd, Paradise, Hemel Hempstead, Herts, HP2 4TG, UK) linked to a DP Integrator and geosmin was detected (~ 1.3 ng ml^{-1}) after approximately 24.6 min. However, further studies should be performed in order to evaluate the potential of using e-nose technology for this specific purpose.

5. Conclusions

Potential exists for an electronic nose to detect early microbial activity in water as well as for monitoring geosmin production in different water types, thus help preventing off-odours and tastes occurrences. E-nose technology also offers potential for replacing existing techniques for environmental applications, in a quick and highly reproducible way.

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Soil volatile fingerprints: Use for discrimination between soil types under different environmental conditions

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Abstract

A conducting-polymer sensor-based electronic nose (E-nose) was employed for sampling the headspace volatiles of three soils (a sandy loam, a calcareous clay soil and a volcanic ash) at two soil water potentials (−0.7 and −2.8 MPa), three temperatures (16, 25 and 37 °C) and following the addition of nutrients such as glucose or wheat straw. Based on qualitative soil volatile fingerprint analysis, the aim was to test the E-nose potential for evaluating the influence of soil type and soil environmental conditions, as well as monitoring the impact of nutrient addition on soil microbial volatile production patterns. Principal component analysis (PCA) on normalised divergence data showed a clear discrimination between soil treatments accounting for 90% of the variance within the data set, irrespective of soil type. The non-specific sensor array was able to differentiate between soil types, and between soil samples under different temperature and water potential conditions. Following the addition of glucose or wheat straw into soil, a temporal discrimination between soil volatile fingerprints was obtained as response to nutrients, as well as between treated and untreated controls. This suggests that such non-specific sensor arrays for headspace monitoring may provide a rapid and non-invasive method for characterising soil microbial activity, as influenced by environmental factors and nutrient inputs.

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1. Introduction

There is strong evidence that most of the gases and volatile organic compounds (VOCs) in the soil atmosphere are probably produced by microbial metabolic activity and have been found to vary widely in type and relative concentrations [1–4]. Soil fungi, bacteria and actinomycetes are known to produce a wide variety of VOCs including alcohols, aliphatic and aromatic aldehydes, amines, esters, methylated halogens, terpenoids and volatile fatty acids [5]. Soil microbial activity is markedly influenced by key factors such as soil temperature, water potential (i.e. water availability), nutrient inputs and soil physical properties (e.g. its capacity to store water and nutrients). The impact of soil environmental factors on soil microbial activity has been widely explored and documented in previous studies [e.g. [41–46]]. Factors which influence soil microbial activity are therefore very likely to influence microbial volatile production patterns.

Very few studies have focussed on the profiles of VOCs and other gases which may occur in the soil atmosphere [3,4,6,7]. For example, Wheatley et al. [3] identified a total of 35 VOCs in the headspace of a silt/clay loam soil using GC–MS. The predominant groups of volatiles found as well as their relative concentrations were different according to soil aeration status during incubation. Sheppard and Loyd [4,7] used membrane-inlet-mass spectrometry (MIMS) for analysing soil depth profiles of gases such as O₂, CO₂, Ar, CH₄, N₂ and NO_xs. They found that gas profiles were not only associated with soil depth but also changed following nutrient addition (sewage sludge or lime) into soil.

Sensor arrays for the detection of volatiles have been commercialised as electronic nose (E-nose) devices. These consist of three main elements: (a) a sampling conditioning unit, which delivers the volatiles from the sample headspace to the sensor array; (b) a reaction chamber containing the sensor array and (c) a processing unit involved in the interpretation of sensor response and pattern recognition. The basic principle of sensing technology is based on the measurement of the variation of mass, optical or electrical properties of the sensor active material due to the interaction between that and volatile compounds

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[8–10]. Conducting-polymer based E-nose systems are among those more widely used [8,9,11,12]. The sensor surface in contact with the volatile mixture experiences a change in the electrical resistance, which can then be amplified, normalised and processed by a data base capture software system [8,9,13]. One important feature of these sensors is that they are not specific to any individual volatile but rather, they respond to a wide range of volatile compounds [12]. Therefore, the use of an array of sensors with broad and overlapping sensitivities to different groups of volatiles (e.g. alcohols, ketones, aldehydes) is a key for enabling a unique and characteristic volatile profile (or fingerprint) of each sample/treatment to be obtained [8,13–15].

So far, E-noses have been employed mainly for disease diagnostics [13,16,17], as well as for evaluation of quality and early detection of spoilage in food products [9,18–21]. In recent years, a growing interest in environmental diagnostics has led to an increase in E-nose applications for evaluating and monitoring the quality of potable water, wastewater and air [22–25]. At present, however, very few attempts have been made to determine E-nose potential for soil applications [26].

Recently, two studies have reported the use of sensor arrays for the detection of pollutants in contaminated soil [15–26], but so far, none as focused on their application for examining the impact of temperature, moisture and organic matter inputs on soil volatile fingerprints. Arnold et al. [26] employed a metal-oxide sensor based micro-nose (KAMINA) integrated into a soil gas percussion probe system for on-site detection of 11 typical volatile soil pollutants (such as chlorinated, aromatic and aliphatic hydrocarbons) in contaminated soil. Authors were able to discriminate between different pollutants and predict their concentration in soil based on concentration versus signal calibration curves [26]. Another report by Kurup et al. [15] described the use of an array of metal oxide sensors coupled to a membrane interface probe (MIP) for rapid on- and off-site screening of gasoline contaminated sites. These studies demonstrate that potential exists for qualitative soil volatile fingerprint analyses using sensor arrays to be used for rapidly characterising soil status.

In this study, an E-nose system comprising of an array of 14 conducting-polymer sensors was employed for sampling the headspace volatiles of three soil types (a sandy loam, a calcareous clay soil and a volcanic ash) incubated under different temperature (16, 25 or 35 °C) and water potential (–0.7 or –2.8 MPa) conditions and with different nutrient (glucose or wheat straw) contents. Based on qualitative soil volatile fingerprint analysis, the aim was to evaluate the potential of using the E-nose for a rapid and non-invasive characterisation of soil microbial activity, as influenced by soil environmental conditions and nutrient inputs. This was done by testing the ability of the E-nose for (a) discriminating between soil types and different soil environmental conditions, and (b) detecting and monitoring the impact of nutrient addition into soil. The selection of soil temperature and water potential values to use was based on the range of these parameters for which soil microbial activity is generally the highest, i.e. between 20 and 30 °C, and 0 and –1.5 MPa, respectively.

2. Materials and methods

2.1. Soil preparation, conditions and treatments

Samples of three soil types (a sandy loam, a calcareous clay soil and a volcanic ash) were obtained from distinct environments with no soil management in the last 4 years. Table 1 summarizes the main properties of each soil and its collection site. Plant residues and stones were removed manually at the time of collection and soils were sieved (2 mm) and air-dried at 20 °C for 7 days prior to use. Air-dried soil samples (10 g) were weighed into Universal (25 ml) bottles and target soil water potentials of –0.7 and –2.8 MPa were set by reference to a soil adsorption curve and the addition of sterile reverse osmosis (RO) water. In this way, all soils were under the same environmental conditions, i.e. same soil temperature and water potential, at the time of analysis.

For the treatments involving nutrient addition into soil, glucose or wheat straw were added to air-dried soil, both at a rate of 5 or 10 mg g⁻¹ soil, contained within Universal bottles. Glucose was previously dissolved in RO water and then added to soil in the form of a solution for allowing better homogenisation. The volume of water used for dissolving it was the same as that required for setting the target soil water potential. The wheat straw was kept at 0 °C for two weeks prior to use in order to inactivate mites, after which it was ground and mixed thoroughly with the air-dried soil. Setting the appropriate soil water potential was done by adding RO water and by reference to a straw-amended soil adsorption curve. Universal bottles containing the soil treatments were placed open inside polyethylene boxes with 400 ml of a glycerol/water solution, in order to maintain the equilibrium relative humidity the same as that of the soil sample. Five replicates of each treatment were incubated in controlled temperature chambers at 16, 25 or 37 °C for 1, 2 or 10 days. All treatments which received glucose or wheat straw were under the same soil environmental conditions. This allowed the impact of nutrient addition, as well as nutrient type, on soil volatile production patterns to be studied.

Table 1

Summary of the main properties of the three soil types used in this study and the main characteristics of the sampling site

	Sandy loam	Clay-based	Volcanic ash
SOC content (mg) ^a	23.8	12.1	4.00
Water content (mg) ^a	690	353	282
Sand content (mg) ^a	578	320	841
Clay content (mg) ^a	181	470	13.0
Silt content (mg) ^a	241	210	146
pH	6.5	7.1	5.9
Sampling site	Bedfordshire, UK	Algarve, Portugal	Mauna Kea, Hawaii
Precipitation (mm) and temperature (°C) on site ^b	560; 12	300; 17	400; 15

^a Values are g⁻¹ oven dry soil.

^b Precipitation and temperature on site are average values y⁻¹.

2.2. Sensor technology

The E-nose used in this study is the commercial sensor array Bloodhound BH-114 developed by Scensive Technologies Ltd., Leeds, UK. It employs 14 conducting-polymer sensors, comprised by 16 different materials chosen from doped polyaniline, polypyrrole and polythiophene with a variety of substitute groups bound to the polymeric matrix. Polymers were produced by electropolymerisation (cycling the potential between -0.1 and $+0.8$ V, except for thiophenes for which $+1.7$ V was used) of the corresponding monomers directly onto clean gold interdigitated transducers [27]. The sensor materials themselves as well as their exact production method remain the subject of confidentiality and copyright of the company. A general description of the production of conducting-polymer sensors is given by [8] and examples of typical production of non-patented polyaniline-, polypyrrole- and polythiophene-based sensors have been described elsewhere [9,11,28,29]. Nonetheless, what is important is that sensors obtained using this approach are non-specific and also that sensors based on different polymer characteristics vary in terms of sensitivity once in contact with the same volatile mixture. Regarding sensor reproducibility, this is assured during manufacture by an accurate control of the production conditions and passing them through a series of quality control assays [27].

2.3. Volatile sampling and sensing procedures

Static headspace analysis (SHA) was the sampling technique used. Following incubation, Universal bottles containing the sample were sealed with parafilm and left at room temperature for 3 h, ensuring an adequate accumulation and distribution of volatiles in the headspace above the soil sample. Five replicates of each treatment were analysed twice in a random pattern using the Bloodhound BH-114 operating at ambient temperature.

Soil headspace was sampled by a vacuum pump through a needle at a rate of 150 ml min^{-1} into a specially prepared air filter system, which consisted of a 6 cm long Teflon tubing segment (Tygon) equipped with a bio-filter ($0.45 \mu\text{m}$, PTFE Whatman, Hepa Vent). Different E-nose settings were tested at the beginning of the experiment in order to find those producing the optimum sensor output. Those selected for subsequent analyses were the following: absorption 5 s, desorption 10 s, flush 5 s and baseline 5 s. The overall sampling time for each replicate was less than 1 min. When using the SHA method, the volatiles are quickly delivered to the sensor array and almost immediately removed from it, allowing a quick recovery of the baseline and reduction of the overall sampling time [9].

Air was sampled three times at the beginning and at the end of every sampling occasion and after each set of 10 samples, aiming to reduce carry over effects from one sample to the next. It also helped defining a standard reference point and monitoring sensor drift. The sensory unit used two calibration points, a baseline and the headspace of a RO water sample. Activated carbon filtered air was set to pass over the sensor surface at a flow rate of 4 ml min^{-1} for generating the baseline and preparing

the array for the next sampling cycle. One minute was allowed between consecutive samplings in order to ensure sensors had returned to baseline values. The E-nose was left switched on for over 1 h before sampling in order to allow the sensor array to stabilise.

2.4. Interpretation and statistical analysis of sensor response

Sensor output was analysed by a built-in statistical software package (xlStat, Microsoft Excel add-in). For each of the sensors comprising the array, a typical response over time (response curve) generated data corresponding to different sensor parameters: (a) absorption of the volatiles to the polymer (highest rate of change in resistance); (b) saturation or equilibrium between the volatiles and the polymer, which produces the maximal response (divergence); (c) desorption of the volatiles from the polymer (highest negative rate of change in resistance); (d) area under the response curve (directly proportional to volatile concentration) and (e) ratio (absorption/desorption) [16,27]. In this study, data for divergence was used for characterising sensor response, taking into consideration that the reproducibility of the system was the highest for this parameter.

Before any statistical method was employed, sensor output was normalised, ensuring that data obtained is independent (or less dependent) on volatile concentration, so that only qualitative information is taken into account [30]. PCA was the multivariate classification method employed for data reduction and outlier detection, while retaining most of the variance within the data set [30,31]. As an exploratory technique, PCA allows identifying the relationships between samples/treatments, highlighting their similarities and differences [17,30,31].

At the end of each experiment, the loading plots were examined and those sensors contributing the most to the overall variability within the data set were selected (never less than 10). Those whose response contributed the least to the information explained by either PC1 or PC2 had their output removed from the data set in order to reduce noise. PCA was performed based on the responses of the selected subset of sensors. Sensor 13 was found to be a considerable source of noise, presenting the most variable responses to replicates of the same treatment both in preliminary reproducibility tests and throughout the study. This probably reflects damage or a defect in this sensor and therefore, its response was not included in the results presented.

3. Results

3.1. Replication of sensor array

Replication of the sensor array was firstly examined within the range of temperature and water potentials used. Fig. 1 shows an example of the low variability obtained between sensor responses to five replicates of the same treatment, in this case for sandy loam incubated at 25°C and -0.7 MPa for 1 day. Similar results were obtained irrespective of soil treatment.

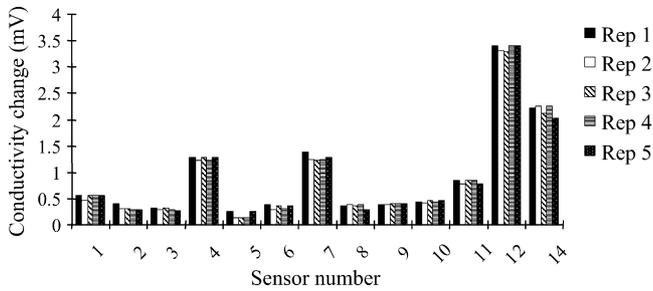


Fig. 1. Sensor relative responses expressed as changes in conductivity (mV) showing low variability between five replicates (1–5) of the sandy soil.

3.2. Discrimination between soil types

Fig. 2 shows the PCA plot for the three soils incubated at 25 °C and -0.7 MPa for 1 day. It shows that differences in volatile production patterns clearly discriminated between the different soil types, where principal components (PCs) 1 and 2 accounted for 93% of the variance within the data set. The highest level of differentiation was obtained along PC2 between clusters of the clay soil and sandy loam, whereas that and volcanic ash were placed in clusters closer together, mainly along PC1. Similar results were obtained independently of soil water potential and temperature.

3.3. Temperature and water potential effects on volatile fingerprints

Soil water potential and temperature had a combined impact on microbial volatile production patterns. For better interpretation of the results and underline the combined impact of the two factors, the PCA was performed using all the treatments involved in this particular experiment. As an example, Fig. 3 shows the PCA plot of data for replicates of the sandy loam incubated for 2 days at three temperatures (16, 25 and 37 °C) and both water potentials (-0.7 and -2.8 MPa). A clear differentiation between samples at different temperatures was obtained at both soil water potential treatments (separated in the plot by the dashed line) accounting for 92% of the variance within the data. Interestingly, this separation was more pronounced under the drier soil conditions (-2.8 MPa). Similar results were obtained for the calcareous clay soil and volcanic ash.

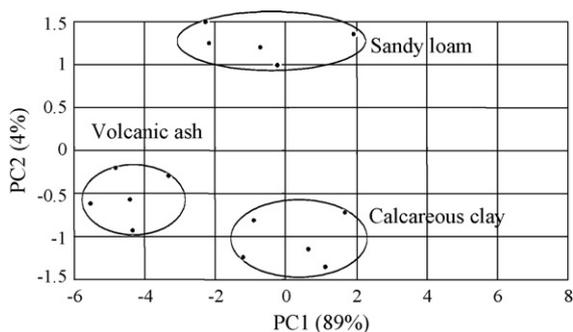


Fig. 2. PCA plot showing five replicates of each soil type incubated for 1 day at 25 °C, -0.7 MPa. Five replicates of each treatment were used.

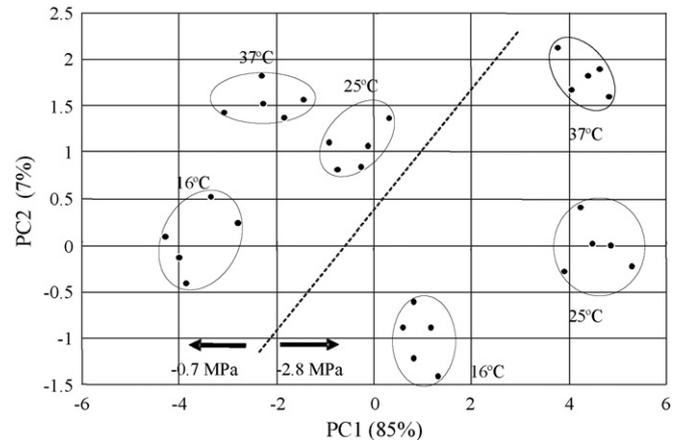


Fig. 3. PCA map for replicates of the sandy soil incubated for 1 day at three temperatures (16, 25 and 37 °C) under two moisture regimes (-0.7 and -2.8 MPa) separated in the plot by the dashed line. Five replicates of each treatment were used.

3.4. Impact of nutrient inputs over time

Fig. 4 shows the PCA plot of data for samples of calcareous clay soil amended with glucose and wheat straw at two different concentrations and incubated at 25 °C for (A) 1 and (B) 10 days. After 1 day of incubation the PCA plot shows two main clusters, corresponding to the glucose-amended samples and the remaining treatments, discriminated along PC1 (Fig. 4A). On this plot, straw treatments and the untreated controls were grouped together within the same cluster, suggesting similarities between their volatile patterns. However, following a 10-day incubation period, three clusters were attained, indicating that volatile fingerprints of straw-treated soil were now distinctively different from that of the controls (Fig. 4B). Interestingly, in both cases there was little discrimination between concentrations of the same amendment type, even after a 10-day incubation period.

4. Discussion

This study examined the potential of using an array of non-specific conducting polymer sensors for discriminating between soil types and different soil environmental conditions and treatments. Results indicate that qualitative soil fingerprint analyses using E-nose technology can be employed as a rapid, sensitive and non-invasive tool for characterising soil status, detecting changes in soil conditions and monitor processes such as organic matter degradation.

The clear discrimination between different soil types by PCA is most likely to reflect differences between their physical and chemical properties. In fact, soil properties determine not only the characteristics of native microbial communities (in terms of community size, diversity and metabolic activity) but also that of native soil organic matter [32–37], which consequently may influence the type and amount of volatiles produced by microbial activity. In the same way, soil temperature and water potential, as well as nutrient inputs into the soil, were shown to have a considerable impact on soil volatile patterns, irrespective of the soil matrix.

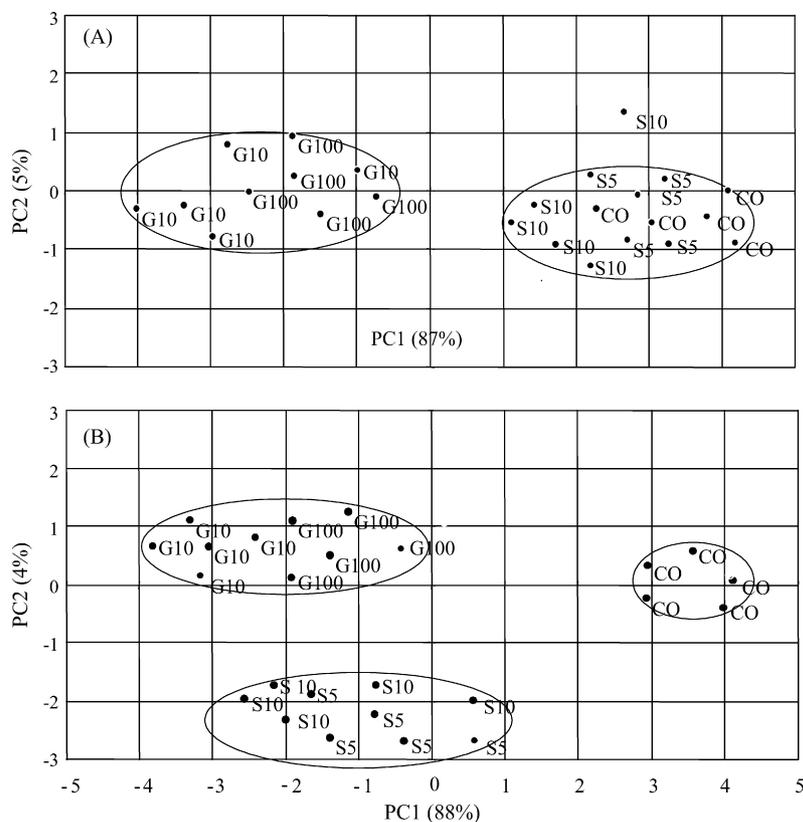


Fig. 4. PCA map for replicates of calcareous clay soil amended with glucose (G) and wheat straw (S) at 5 and 10 mg g⁻¹ soil, incubated for (A) 1 day and (B) 10 days at 25 °C. Five replicates of each treatment were used.

Additionally, nutrient type rather than concentration was found to influence soil volatile fingerprints the most. The PCA results showed a temporal discrimination between glucose and wheat straw treatments, which might be due to differences between their nutritional composition and subsequently, between their assimilation rates by soil microbial communities. Glucose is a readily utilisable carbon source, whereas straw consists mainly of hemicellulose and lignin [38], breaking down very slowly. Clearly, the separation between glucose and straw-treated samples after an incubation period of 10 days implies substantial differences between volatiles produced in both treatments.

Methodology regarding sample preparation and sensing procedure was consistent throughout this study and for each set of soil temperature and water potential conditions, reproducibility of the sensor array was analysed. For most of the sensors comprising the array, there was low variability between responses to replicates of the same treatment independently of soil type, temperature and moisture. Although conducting-polymer sensors are known for presenting a high sensitivity to humidity [9,23,27], the reproducibility of this sensor array was not affected by water potentials of -0.7 and -2.8 MPa, and supports previous studies performed using similar conducting polymer sensor arrays [20].

The SHA method was adequate for the aims of this work, allowing a clear discrimination between volatile fingerprints of the several soil treatments to be obtained. This supports previous studies where the same sampling method was employed for sensing the headspace of a diverse range of substrates

[19,20,24,25,39], despite contrary suggestions that it may promote overlapping between volatile profiles of different treatments [9].

As previously mentioned, the use of a non-specific array of polymer sensors does not allow identification of specific volatile compounds, but rather to produce a volatile fingerprint which is then used for characterising a given sample [13–15]. Conducting polymer sensors, like those employed in this study, respond to both odorous and odourless volatiles, with the highest sensitivity towards high and low weight hydrophilic (polar) compounds. Their limit of detection is within the ppm concentration range [9,12,30,40]. Groups such as alcohols, ketones, fatty acids, esters, amines, as well as those containing sulphur produce particularly strong sensor responses, whereas fully oxidised volatile species (e.g. CO₂, NO₂ and H₂O) lead to lower responses [10].

Based on rapid and non-invasive soil volatile fingerprint analyses, the application of this technology in soil science and agriculture looks promising. Data from this study strongly suggest that soil volatile patterns are likely to be affected by the same key environmental factors that influence soil microbial activity. Also, the temporal discrimination that was obtained by PCA between soil treatments following nutrient addition is highly pertinent. It indicates that there is potential for the use of this approach to complement other methods, such as respiration measurements and enzymatic activities, for monitoring the impact of organic matter inputs and management practices on soil microbial activity.

The possible link between soil volatile profiles and the characteristics of the underlying microbial populations may also be relevant. It suggests that this approach could be a valuable contribution for complementing studies on soil microbial community structure and functional diversity, as well as on examining their role in the overall health of agricultural soils.

E-nose technology may, additionally, offer a way of comparing and characterising different soil systems, sites or agricultural areas and perhaps their responses to environmental fluxes (such as management and restoration practices or the presence of pollutants). For instance, when qualitative geo-environmental site comparison is required, a non-specific array of sensors similar to that applied in this study could complement or even replace conventional time-consuming, laborious and expensive methods (such as drilling, sampling and lab-based GC, GC/MS or laser-induced fluorescence analyses) [15]. Versatility, simplicity of use and the potential for automation and portability for on-site applications are additional qualities that make this technology even more appealing, especially when taking into consideration the low sampling cost associated with these features [15,23].

5. Conclusions

Soil microbial volatile patterns were found to be influenced by key factors influencing soil microbial activity, such as temperature, moisture and nutrient addition. A non-specific array of conducting polymer sensors was able to discriminate between different soil environmental conditions and treatments based on volatile production patterns in a quick, reproducible and non-invasive way. It indicates potential for complementing other methods, such as respiration and soil microbial community structure and functional diversity analyses, for assessing and characterising soil microbial activity, as influenced by environmental factors and organic matter inputs.

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