CRANFIELD UNIVERSITY

OLINDA CANHOTO

APPLICATIONS OF VOLATILE FINGERPRINT SENSOR ARRAYS FOR RAPID DETECTION OF ENVIRONMENTAL AND MICROBIOLOGICAL CONTAMINANTS

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Applications of Volatile Fingerprint Sensor Arrays for Rapid Detection of Environmental Contaminants

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ABSTRACT

The electronic nose (e-nose) technology has rapidly evolved in the past decade with a range of applications in the food industry, medical diagnosis, and recently environmental monitoring. This is the first time that this technology has been examined in detail for a range of specific environmental applications including: detection of low concentrations of bacterial, fungal and heavy metal contaminants in potable water; analyses of changes in the microbial activity of soil samples amended with heavy metals; and the detection of fungal contaminants in paper samples from library material. In some studies comparisons between different e-nose systems has also been carried out.

The e-nose system based on a conducting polymer (CP) sensor array Bloodhound (BH114) was able to detect different bacterial species (*Escherichia coli*, *Pseudomonas aeruginosa* and *Enterobacter aerogenes)*, initially inoculated in tap, reverse osmosis and bottled water with a concentration of 10^2 cells mL^{-1}, after 24 hrs incubation. In the presence of low concentrations (0.5 ppm) of a mixture of heavy metal ions including cadmium, lead and zinc, the volatile pattern produced by the bacterial species was discriminated from that where no metal was added, probably due to a change in the microbial metabolism.

The Bloodhound e-nose system was also used to detect fungal spores of *Aspergillus fumigatus*, *Fusarium culmorum* and a *Penicillium* species, inoculated in water samples. The initial concentrations were 10^2 – 10^5 spores mL^{-1}. Good discrimination was observed between the control samples after 24 hrs incubation at 25^oC. After 48 hrs incubation, it was possible to differentiate between the various spore concentrations present in water samples. Good reproducibility was achieved as results from different days were consistent and data could be pooled and combined for analysis.

A comparative study was performed with three e-nose instruments, two of them had CP sensor arrays (Bloodhound (BH-114); Neotronics (eNOSE 4000), and the third was a metal oxide (MO) sensor-based system, the NST 3220. The experiments carried out

with the CP based-systems showed similar results when analysing water samples contaminated with 10^4 and 10^2 bacterial cells mL^{-1} after 24 hrs incubation. Both CP and MO based e-nose systems could differentiate control water samples from those contaminated with both bacteria and fungal spores. GC-SPME analyses confirmed the results obtained with the e-nose system of metal ions and bacterial cells in water samples.

At-line studies were performed with the MO array-based system (NST 3220), for the detection of contamination episodes. *E. coli* and *P. aeruginosa* cells were used as contamination agents for tap and reverse osmosis sterile water, in two concentration levels, 10^2 and 10^6 cells mL^{-1}. The samples collected downstream in a simulated watercourse, were analysed by the e-nose over a period of 1-2 hrs. The results suggested the potential of this technique to monitor episodes of bacterial cells at a low concentration in water samples.

Experiments performed in soil samples artificially and naturally contaminated with heavy metal ions were analysed with the MO-based e-nose system. Results indicated that for artificially contaminated soil samples, after 40 days incubation the control samples could be discriminated from those containing 3 and 100 ppm of metal ions. For naturally contaminated soils, the sensor array was only able to separate samples containing a high concentrations of metal ions.

Headspace analysis of cellulose-based agar showed good discrimination between *Aspergillus terreus*, *A. hollandicus* and *Eurotium chevallieri*, after 20 hrs incubation at 25^oC. An increase in the incubation period to 40 hrs resulted in better separation between the control and fungal treatments. *In situ* studies performed on paper samples suggested that the e-nose was able to discriminate between control samples and paper inoculated with 10^3 fungal spores mL^{-1}. The substrate was a determinant factor in the headspace analysis of microbial species. It was shown that the same fungal species produced different volatile profiles according to the growth substrate.

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ABREVIATIONS

AA	-	Atomic Absorption
ANNs	-	Artificial Neural Network
ATP	-	Adenosine Tri-Phosphate
a_w	-	Water Activity
BHI	-	Brain Heart Infusion
BOD	-	Biochemical Oxygen Demand
CA	-	Cluster Analysis
CFU	-	Coliform Forming Unit
COD	-	Chemical Oxygen Demand
CP	-	Conducting Polymer
DFA	-	Discriminant Analysis
DHA	-	Dehydrogenase Activity
FCM	-	Flow Cytometry
FIA	-	Flow Injection Analysis
GC	-	Gas Chromatography
GC/MS	-	Gas Chromatography/ Mass Spectrometry
HPC	-	Heterotrophic Plate Count
HPLC	-	High Pressure Liquid Chromatography
HS-GC	-	Headspace – Gas Chromatography
ICP	-	Inductively Couple Plasma
INF	-	p- iodonitrotetrazolium formazan
INT	-	p-iodonitrotetrazolium chloride
IR	-	Infrared

MEA	-	Malt Extract Agar
MIB	-	Methylisoborneol
MOS	-	Metal Oxide Semiconductor
MTF	-	Multiple Tube Fermentation
NA	-	Nutrient Agar
PC	-	Principal Component
PCA	-	Principal Component Analysis
PCR	-	Polymerase Chain Reaction
PLS	-	Partial Least Squares
PPM	-	Parts Per Million
QCM	-	Quartz Crystal Microbalance
QMS	-	Quadrupole Mass Spectrometry
RH	-	Relative Humidity
RO	-	Reverse Osmosis
SAW	-	Surface Acoustic Wave
SHA	-	Static Headspace Analysis
SPME	-	Solid Phase Microextraction
TOC	-	Total Oxygen Demand
VOCs	-	Volatile Organic Compounds
WHC	-	Water Holding Capacity
WHO	-	World Health Organization
WWTPs	-	Wastewater Treatment Plants

CHAPTER 1.

INTRODUCTION AND LITERATURE REVIEW

1.1 INTRODUCTION

Pollution is a problem that is part of every day life. In the past decades, we learn how it affects Nature and Human health in particular. The World Health Organisation (WHO) together with other national and international organisations, are making a significant effort in order to advise and educate society regarding environmental issues. One of the first steps was to clean the countryside with the introduction of landfill sites; next was to advise consumers for an excessive production of waste, by recycling and reusing primary materials like paper and glass. There is a lot to be done in relation to these first steps, especially in developing countries, but legislative authorities are now focusing on prevention strategies or in the early detection of important pollutants.

The lists of hazardous chemicals and microorganisms are now increasing with more awareness in the scientific community, especially with new and more specialised methods with lower detection limits. The provision of safe drinking water coupled with the rising costs of treating wastewater for discharge is encouraging industry and authorities to examine new ways for water conservation (Dewettinck e*t al*., 2001). Nevertheless, it is important not to forget that pollution follows a cycle, what we find in water may come from the atmosphere or the soils. Wastewater treatment plants are used to clean and reuse drinking water, but what comes out as solid waste or sludge is often heavily contaminated with heavy metals. Currently, sewage sludge because of their potential as fertilisers, are disposed in agricultural land. However, studies in the literature advert for the danger of this amended soils because of their high content in heavy metals (Cambier, 1998).

Drinking water disinfection was a major public health triumph of the 20th century. The use of chemical disinfection in the early 1900s resulted in deaths attributable to these water-borne pathogens virtually ceasing in developed nations. However, recent large outbreaks of waterborne illness (*E. coli* induced gastroenteritis in Canada in 2000, Cryptosporidiosis in the USA in 1993, and cholera in Peru in 1991) serve as dramatic reminders of the need for proper disinfection and control of water-borne pathogens in drinking water. It also suggests that there is a need for continual re-evaluation of

monitoring techniques to ensure effective microbial quality of drinking water (Richardson, 2003). The importance of chemical risk incidents accelerated the practice of environmental risk assessment. The influence of agriculture and municipal wastewater, as well as the ageing of water treatment and distribution systems, continues to raise concerns about the microbiological quality of drinking water (Percival, 2004).

The methods used in certified laboratories that test for water quality are time consuming and very laborious. At present, they cannot be used for on-line monitoring. An essential requirement for rapid methods should be the availability of data in the shortest time. This means that these methods should be faster than the standard methods currently used. Ideally, they should give results within the same sampling day (Banadonna, 2003). These new analytical approaches should also be able to comprise applications for at-line, on-line and auto-sampling/auto-reporting instrumentation for the routine assessment of the quality of incoming raw water being used for drinking water production. There is a potential use for artificial sensing systems to identify and monitor real-life odours in the environment (Bourgeois *et al*., 2003; Canhoto and Magan, 2003).

Throughout modern history, scientists have recognised the power of incorporating biological principles into the design of artificial devices or systems. The human nose is still an important "tool" used in many industries to evaluate the smell of products such as perfumes, and beverage. In microbiology, trained microbiologists can detect or identify a specific microbial species by the characteristic smell of a culture.

A combination of factors such as our understanding of the human olfactory system and the rapid improvements on sensor technology lead to the development of the so-called electronic noses. The concept of an electronic instrument that could mimic the human sense of smell and provide rapid sensory information emerged from publications by Persaud and Dodd in the early 1980's. In this thesis, the definition by Gardner and Bartlett (1994) will be used: "An electronic nose is an instrument, which comprises an array of electronic chemical sensors with partial specificity and an appropriate pattern-recognition system, capable of recognising simple or complex odours".

Advances in technology have been made ever since the early 1980s when researchers at the University of Warwick in Coventry, England, developed sensor arrays for odour detection. Focused primarily on the sensor aspect of the problem, the initial research explored the use of metal oxide devices. Later work at Warwick University explored the use of conducting polymers. In both, sensing was based on conductivity changes (Nagle *et al.*, 1998).

In order to understand the operation of an "Electronic nose" (e-nose), it is helpful first to analyse what is involved in "smelling", and therefore, what constitutes a "smell", i.e., an odour. Odorant molecules have some basic characteristics, the primary ones being that they are light (relative molecular masses up to approximately 300 Da), small and polar and that they are often hydrophobic (Craven *et al.*, 1996). The e-nose mimics the human olfaction system. A sampling unit delivers the odour molecules to a test chamber in which the sensor array is based; the interaction between the sensors and the volatile compounds produce a change in the sensors response; this change is then interpreted by a pattern recognition system. To maximise the use of e-nose technology, a neural network may be installed, which may act like the memory in our brain, creating a library of sensor responses, also known as sensor profiles.

The signals that form the output of a sensor array do not provide a spectrum of odour constituents in the way that, for example, a gas chromatogram do, but rather information relating to the qualities of the odour, which are characterised by particular sensor response signatures. More recently, E-nose-MS combinations are able to provide not only a fingerprint of the odour constituents, but also the mass-charge ratio of its compounds. When looking to a particular compound with a specific mass/charge ratio, it is thus possible to give both qualitative and quantitative information.

Electronic noses do not normally get tired nor do they become sensitised to particular smells: they are not affected by colds, allergies, or spicy food. They do not necessarily require comfortable or safe working conditions. For a production manager then, an instrument that can sample the product flow continuously, or at least frequently, and give a rapid feedback of the results, is desirable even if the accuracy under production

line conditions is not as good as that of the corresponding laboratory instrument in a controlled environment. The situation is less critical for batch production, but here also, rapid results are valuable and can save a batch from having to be reworked or discharged. The methodology normally employed for the detection and identification of microorganisms in water is laborious and time consuming. Figure 1.1 compares the time required to analyse one sample and the time required to obtain a result, using traditional methods used in the detection/quantification of bacteria in water, with e-nose technology. In environmental management, continuous monitoring systems, even if less precise than the laboratory assay, can be invaluable in raising the alarm when a river is subject to pollution, for example from a defective filter in an inflow pipe.

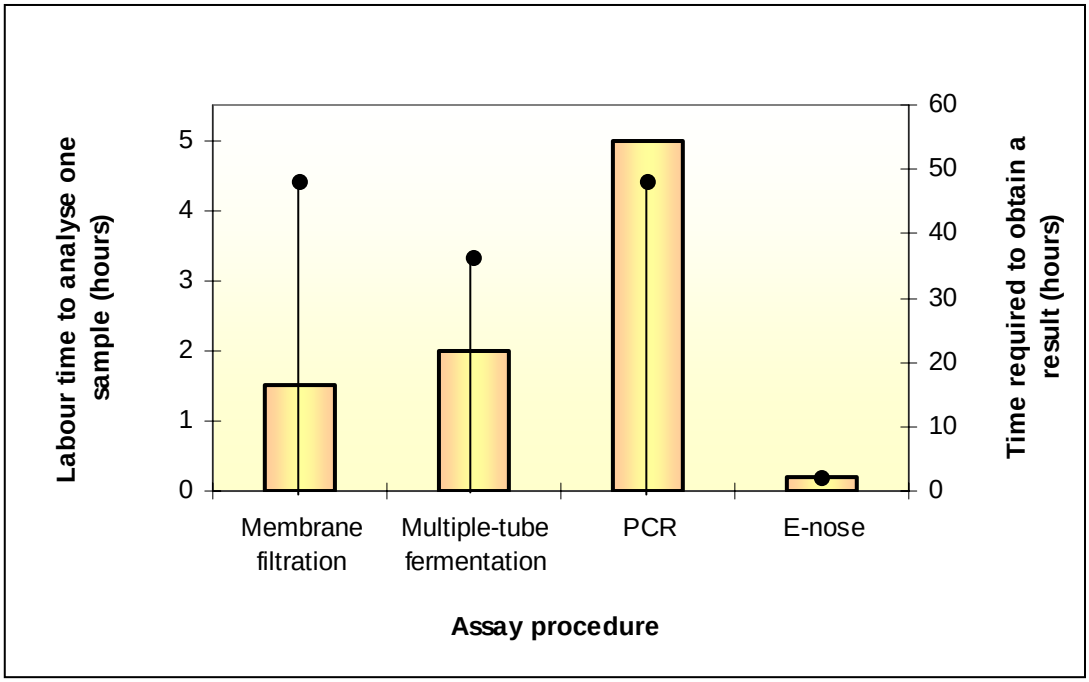


Figure 1.1. Time required to perform the various assays ( ) and to obtain a result ( ) in the detection of bacteria in water samples.

This thesis looks at the application of sensor array technology for the detection of a range of environmental contaminants. The potential for the detection of chemical and microbial contaminants in water; the detection of microbial activity in soil amended

with different concentrations of heavy metals; and the detection of fungal contaminants in library material.

1.2 ELECTRONIC NOSE (E-nose) – The Technology

E-nose systems have advanced rapidly during the past 10 years, the majority of applications being within the food, drink and medical industries. Electronic nose systems comprise sophisticated hardware, with sensors, electronics, pumps, flow controllers, software, data pre-processing and statistical analysis. The sensor array of an electronic nose has large information potential and responds to both odorous and odourless volatile compounds. A series of electronic noses have been produced commercially during the last decades. Figure 1.2 shows a chronological summary of the development of the sensor-based electronic devices. At present, different detection principles (heat generation, conductivity, electrochemical, optical, dielectric and magnetic properties) are used in the basic sensing elements of the e-nose (Ivnitski, 1999).

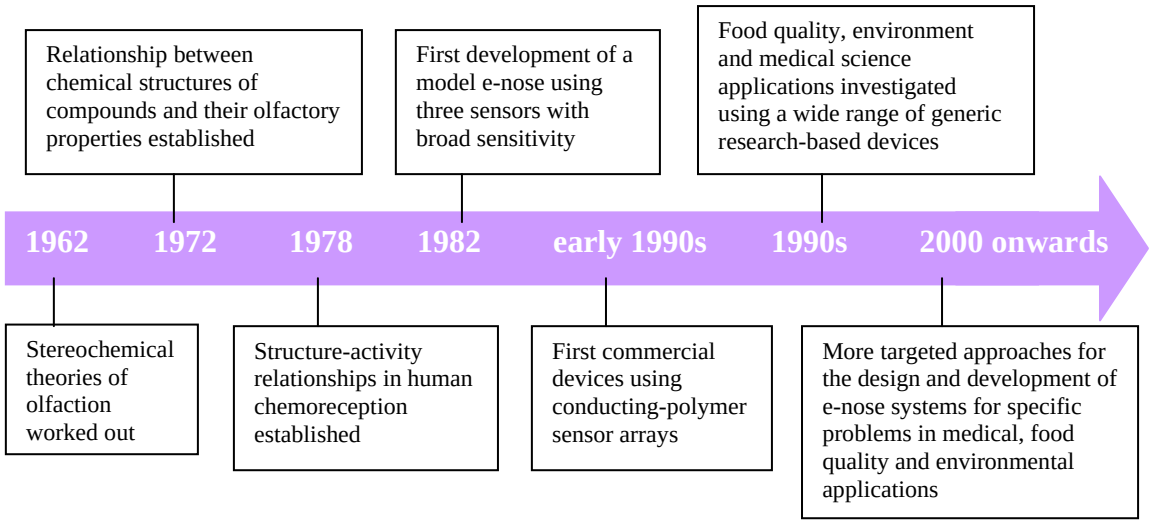


Figure 1.2 Historical perspective of sensor-based electronic devices (*Adapted from* Turner and Magan, 2004).

The mammalian nose can identify and quantify a wide range of volatiles with high sensitivity and recognise substances by combination and relative proportions of compounds. It is thought that this may be achieved by combining a set of sensing elements of broad, overlapping selectivity profiles producing a signal pattern that can be interpreted to identify the compound or set of compounds present. Even today, aroma is still monitored largely by human noses, just as in the herb distillation plant of the middle ages. With e-noses, this situation could change (Breer, 1997). Techniques currently used in the evaluation of the odour and/or flavour of commercial products can be divided into:

- Instrumental Techniques
  - Chromatographic
    - Gas Chromatographic
    - Liquid Chromatographic (HPLC)
  - Mass Spectroscopy
- Sensory evaluation by human panels (group of people with highly trained senses of smell)

The disadvantages of human sensory panels include: subjectivity, poor reproducibility (i.e., results fluctuate depending on time of day, health of the panel members, prior odours analysed and fatigue), time consuming, large labour expense and adaptation (becoming less sensitive during prolonged exposure). In addition, human panels cannot be used to assess hazardous odours (Nagle *et al.*, 1998).

McRae and Falahee (1995) argued that the best way to improve the effectiveness of sensory screening of water at the point of production is to give assessors sensory training. The decision of human panels (assessors) can be influenced by many factors other than the strength of the attribute and the sensitivity of the assessor. They claim that the methods that have proven successful with industrial inspection and military watch keeping is to add artificial “signals” to those being assessed and give the assessors immediate feedback on their success in judging them.

Disadvantages of GC, MS, and GC/MS: these systems are traditionally used to analyse hazardous volatile compounds; GC and GC/MS systems can require a significant amount of time and technical expertise to perform the analysis and then relate the analysis to something useable; and are also very expensive (Keller, 1999).

1.3 E-NOSE *vs* HUMAN OLFACTORY SYSTEM

E-nose research was inspired by the mechanisms involved in human olfaction. Our sense of smell is able to recognise and discriminate extraneous volatile compounds of diverse molecular structure with high sensitivity and accuracy. Certain odourants can be detected and discriminated at ppt levels (Breer, 1997).

It all begins with sniffing, which moves air samples that contain molecules of odours to the thin mucus layer lining the olfactory epithelium in the upper portion of the nasal cavity. Next, the odour molecules interact with the membrane bound receptor proteins of the olfactory cells (Figure 1.3). The number of different types of receptor proteins is the subject of debate but it is thought that there are up to 100 with overlapping selectivities and sensitivities for volatile odour chemicals.

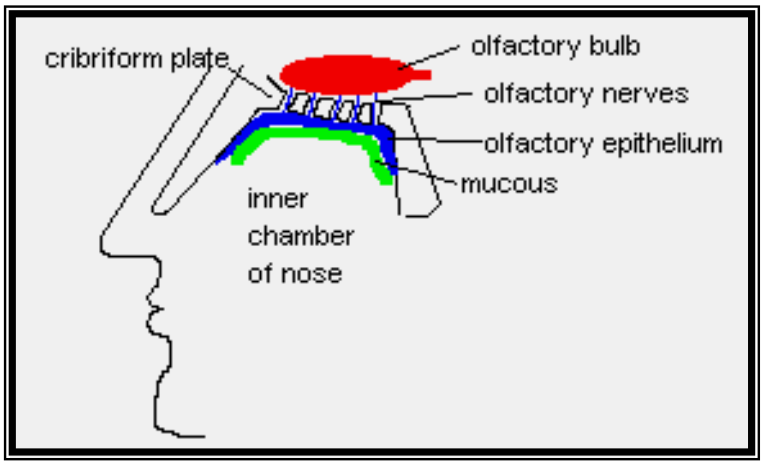


Figure 1.3 Section through human nose representing some components of the olfactory system (adapted from Nagle *et al.*, 1998).

Similarly, an e-nose employs a sensor array where each sensor is non-specific. The structure of the molecule is important in determining the odour. This requires sensors, which are non-specific and responsive to the shapes or structural features of the organic molecules. Ideally, it would be helpful to define what these structural features were and select, or design, our sensors appropriately. At present, a more empirical approach is necessary, making use of available sensor types and attempting to modify sensor designs to meet the requirements of the e-nose (Gardner and Bartlett, 1999).

In the mammalian olfactory system, when odour molecules interact with the different proteins, a series of nerve impulses (electrical stimulus) generated by the olfactory neurons, feed into the olfactory bulb (a structure in the brain located just above the nasal cavity). The overall function of this stage is to reduce the noise by compressing the signals and amplifying the output. This enhances both the sensitivity and selectivity of the olfactory system (Craven *et al.*, 1996). The recognition of odourants and the primary events of olfactory signal transduction occur in the cilia of olfactory receptor neurons; the cilia, extruding from the dendritic knob, are considered scaffolding for the chemosensory membrane, providing a large expansion of the surface area. Upon interaction between odorous ligands and specific receptor proteins a multi-step reaction cascade is initiated, which amplifies the olfactory signal and ultimately leads to the electrical response of the sensory neurons, thus converting the strength, duration, and quality of odorant stimuli into distinct patterns of neuronal signals (Breer, 1997).

The final stage in the human olfactory process is in the brain. The brain receives a set of simplified nerve impulses as patterns of responses and further processes the signals to identify them as particular smells. This process of identification appears to be a learning process, with new smells having to be sampled, recognised and remembered subconsciously in the memory of the individual. The brain that associates the collection of olfactory signals with the odour (Gibson *et al.*, 1997; Keller, 1999).

Similarly, the pre-processing stage in the e-nose processes the signals from the sensor array into a form suitable for input into the PARC (Pattern Recognition) stage. Pattern recognition algorithms are then used to classify and quantify odourants based on the

data stream produced by the detector array (Doleman and Lewis, 2001). Figure 1.4 represents in summary the comparison between artificial and human olfaction.

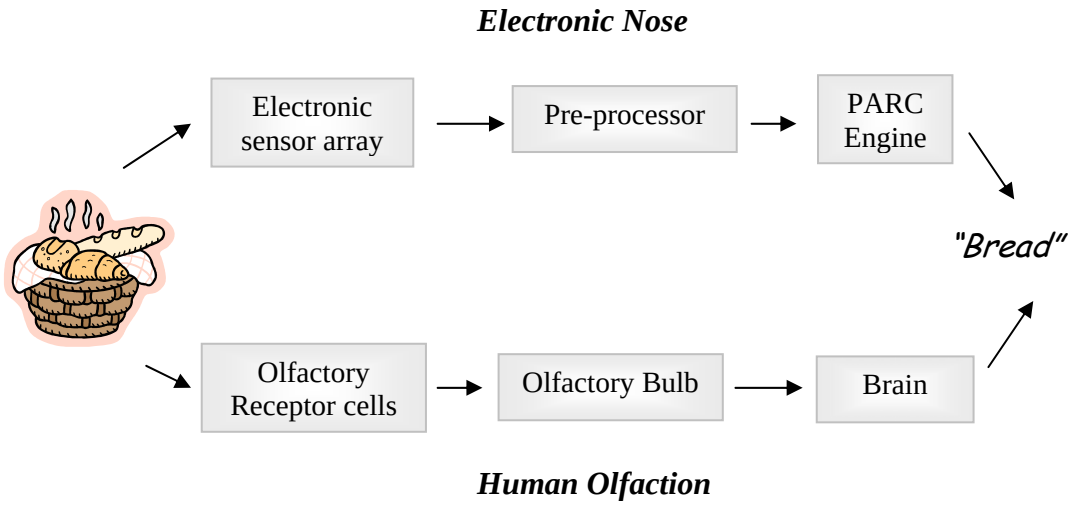


Figure 1.4 Comparison between the three basic elements that comprise and electronic nose and a human nose (adapted from Craven *et al*., 1996).

1.4 SENSOR TECHNOLOGY

The ideal sensor for integration in an e-nose should fulfil the following criteria: high sensitivity; they must respond to different compounds present in the headspace of the sample; high stability and reproducibility; short recovery time; easy calibration; they must also be robust and portable (Ivnitski, 1999). Sensor poisoning is a particular problem that arises when a sensor is exposed, often inadvertently, to a material, which irreversibly binds to, or interacts with, the sensing material leading to a reduction or even total loss of sensitivity. In practice, poisoning is a far greater problem in applications where a wide variety of different sample types and interfering compounds exist (Gardner and Bartlett, 1999).

Various sensor technologies are employed in e-noses (see Table 1.1). The most popular ones that are now used in commercial instruments are: semiconducting metal oxides

(e.g., catalytically doped tin oxide) and electronically conducting polymers. Metal oxides are sensitive to combustible gases, operate at high temperatures (e.g. 400ºC) and use thick-film technology. Conducting polymers respond to polar compounds, operate near room temperature, offer a large choice of types and are manufactured electrochemically (Craven *et al.*, 1996).

Table 1.1 Types of sensors used in electronic nose technology (adapted from Fenner and Stuetz, 1999; Turner and Magan, 2004).

Sensor type	Mode of activity
1. **Piezoelectric crystals**	Sensors containing piezoelectric crystals used in the radio frequency resonance of quartz materials coated with membranes, the adsorption of volatile molecules onto the membrane produces a change in the magnitude of the resonance frequency.
2. **Metal oxide**	The oxide materials in the sensors contain chemically adsorbed oxygen species. When an electrical current passes through the sensors it causes the oxidation of gas molecules via electron transfer from the gas to the metal oxide leading to a change of resistance.
3. **Metal oxide silicon field-effect**	The oxide material in the sensors contains chemically adsorbed oxygen species, which can interact with the volatile molecules, thereby altering the conductivity of the oxide.
4. **Conducting polymer**	In the presence of a gas species, a change in voltage across polymers such as polyaniline, polypyrrole, and polythiopene can be measured. The polymers have different reversible physico-chemical properties and sensitivity to groups of volatile compounds.
5. **Surface acoustic wave**	Similar to quartz crystal microbalance but operate at much higher frequencies.
6. **Optical**	Use fluorescence measurements from photodeposited polymer-fluorescent dyes on bundles of fiber optics.

Some specific sensor types have been used to detect and/or identify particular volatile compounds. Table 1.2 describes some of the most common sensor technologies and the types of volatiles they have been used to detect.

Table 1.2 Sensors for gases and volatiles (Breer, 1997).

Sensing agent	Device example	Analyte examples
Metal oxide semiconductor	Heated resistor	Methane, oxygen, alcohols, aldehydes (broad specificity)
Conducting polymers	Resistor	Alcohols, amines, ethyl acetate, pyridine (broad specificity)
Specifically sorbent polymer film	Optical devices, acoustic devices (SAW, piezocrystal microbalance	Solvent vapors, anesthatic gases (broad specificity)

1.5 SAMPLING AND DATA ANALYSIS

The application of e-nose technology is dependent on a number of key steps. Firstly, the sampling procedure must be consistent, i.e., the same methodology should be the same for each sample. Parameters such as humidity, temperature and sample size must be standardized to ensure that data sets are reliable, reproducible and can be analysed with statistical confidence. Secondly, pattern recognition techniques should be able to analyse large sets of data. The sensor responses generate a significant amount of data and require sometimes complex, multivariable analysis (Turner and Magan, 2004).

1.5.1 Sampling Methods

There are two main odour sampling methods:

- Static Headspace Analysis (SHA)
- Flow Injection Analysis (FIA).

SHA is the more popular and low-cost method and the principle is very simple. The sample is placed on an appropriate container, and left for a period of time so that the headspace becomes saturated with the odours. This headspace is then transferred into the chamber containing the sensor array. FIA is usually computer automated and employs a method where background gas (usually clean air) is constantly being pumped into the sensor chamber. The odour (gas) is injected into the background gas before it reaches the sensor chamber. The ratio of the mixture of background gas to odour-gas can be precisely controlled (Craven *et al*., 1996).

There are several possible methods for the sampling process, which consists of taking up the sample, conditioning it, and transferring it to the analytical equipment. This should be done with maximum efficiency and without altering the composition of the headspace. The most frequent way of introducing a sample to the e-nose is to direct the gaseous compounds into the sensor array chamber by activating a pump, normally located in the rear of the system. The sample uptake requires the control of different parameters like temperature, pressure, volume and time. The performance of the headspace analysis depends on the partition coefficient, the vial volume and the matrix itself (www2.nose-network.org).

Static headspace sampling can be automated with programmed sample conditioning parameters. By taking an aliquot of the gas phase, the volatile components in an essentially non-volatile matrix can be investigated without interference. It can be defined in two main steps. The first is to place the sample in the vial, closing it to concentrate the volatiles in the headspace, letting the sample equilibrate at a desirable temperature and shaking it, if necessary. The second step comprises vial pressurization and subsequent venting so that an aliquot of the vial headspace is introduced into the carrier gas, for example air, helium or nitrogen, and transferred for analysis in the sensor array chamber. With the concentration of the analyte *i* in the gas phase $C_{i(g)}$ and liquid/solid sample phase $C_{i(s)}$, the partition coefficient K is given by equation 1.1.

$$K = \frac{C_{i(g)}}{C_{i(s)}} \qquad \text{(Equation 1.1)}$$

The area of the signal peak Ai for *i* is proportional to the concentration in the gas phase $C_{i(g)}$ and proportional to the original concentration in the sample. Figure 1.5 shows a representation of the signal derived from the sample concentration.

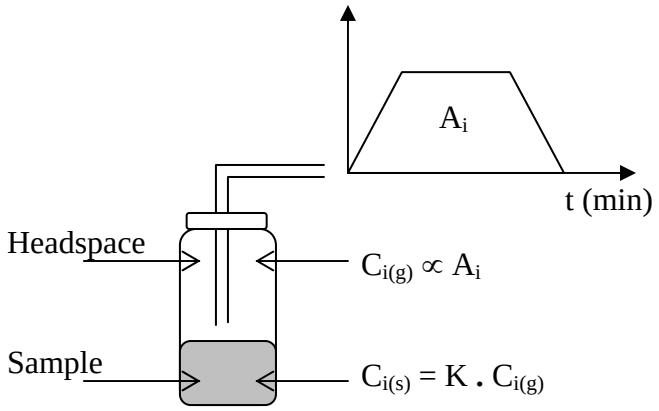


Figure 1.5 Operational principle of a static headspace analysis in a sampling vial and the diagrammatical area (A_i) of a sensor response (adapted from www2.nose-network.org).

1.5.2 Data Analysis

The sensor response to a given sample is composed of three stages, absorbance of the volatiles to the polymer, divergence or maximum response, and desorption of the volatiles from the polymer. Also, the ration between absorption and desorption together with the area bellow the sensor response over time (A_i represented in figure 1.5), comprise the different parameters that fully describe the senor response. Data from the different parameters of a sensor response to a sample are normalised so that concentration dependence is either eliminated or reduced and qualitative information is enhanced (Craven *et al*., 1996).

Electronic noses employ powerful multivariate statistics in order to determine the classification of the samples. Figure 1.6 shows a sumary of the available methods for the analysis of e-nose data. In the present project, the classification methods used were Principal Components Analysis (PCA) as an unsupervised technique and Discriminant Function Analysis (DFA) as a supervised technique.

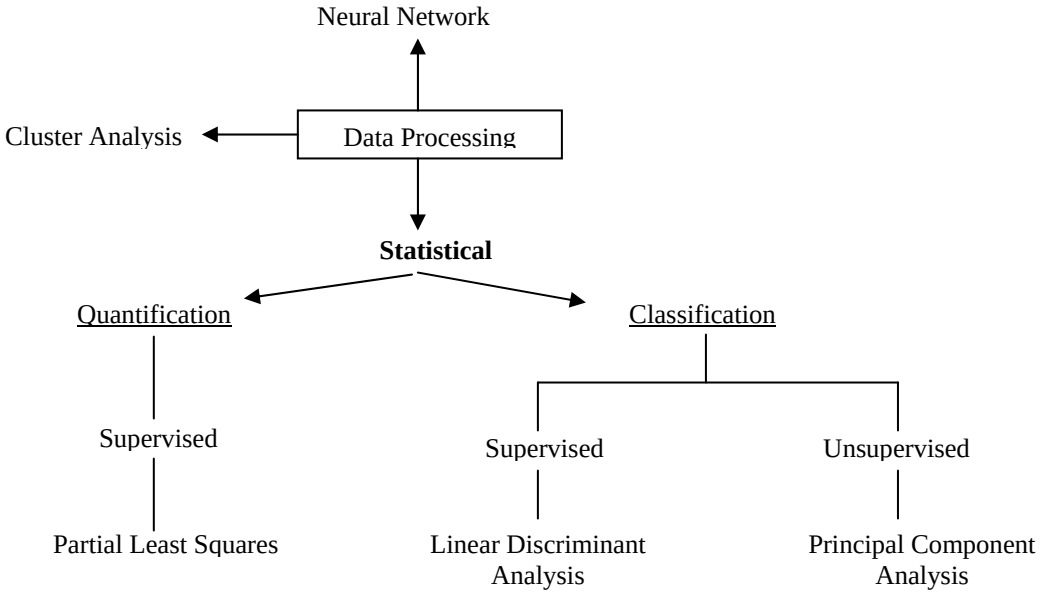


Figure 1.6 Some of the avalilable methods of analysis for data from sensor arrays (adapted from Jurs, 2000).

A more formal method of treating samples is unsupervised pattern recognition which is aimed at detecting similarities. Supervised pattern recognition requires a training set of known groupings to be available in advance, and tries to answer a precise question as to the class of an unknown sample. It is, of course, always necessary to first establish whether chemical measurements are actually good enough to fit into the predetermined groups (Brereton, 2003).

(i) Principal Component Analysis

PCA is probably the most widespread multivariate chemometric technique. Each principal component is characterized by two pieces of information, the scores and the loadings. After PCA, the orginal variables are reduced to a number of significant principal components. PCA can be used as a form of variable reduction, reducing the large original data set to a much smaller more manageable data set which can be interpreted more easily (Figure 1.7). PCA is a method of displaying the data that retains

the most variance between the samples. This is done with no information regarding the sample's classification and is based solely on the variance of the data.

Multivariate analysis is a very powerful tool for analyzing complex data sets; however, with the power comes the chance of obtaining erroneous results. Several of the signs of a possibly inappropriate model are: the PCA does not segregate into groups and if there is a low data point to variable ratio. In general, it is best to use as few variables as possible to develop a model, as this would result in higher data point to variable ratios and result in a simpler, more easily understood model (Goodner, 2001).

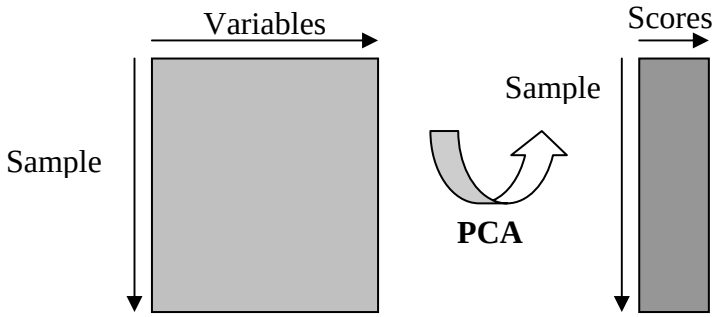


Figure 1.7 Representation of the working principle of PCA (Brereton, 2003).

Cross-validation - A complementary series of methods for determining the number of significant factors is the basis for cross-validation. The residual error will be smaller if 10 rather than nine PCs are calculated. The significance of each PC can be tested out to see how well an "unknown" sample is predicted. In many forms of cross-validation, each sample is removed once from the data set and then the remaining samples are predicted.

(ii) Discriminant Function Analysis

DFA can be used to separate classes of objects or assign new objects to appropriated classes. Discriminant functions are calculated with the objective of maximizing the distance between classes relative to the variation within classes (Jurs, 2000). Most traditional approaches to classification in science are called discriminant analysis. The

majority of statistically-based software refer to DFA by various names such as linear (Fisher) discriminant analysis and canonical variates analysis. Goodner (2001) reviewed a number of studies and demonstrated the over-fitting dangers associated with the use of the large numbers of variables to model sensory and analytical data. The recommendations in statistical literature vary in a minimum ratio of 3:1 and preferably 6:1 ratio of data points to variables. They go on to state that the smallest classification group should contain more observations than the number of variables used in the overall model. DFA uses a priori classifications to maximize the emphasis of those variables that generate the greatest difference between the specified classes (Goodner, 2001).

(iii) Cluster analysis

Explanatory data analysis such as PCA and DFA are used primarily to determine general relationships between data. Sometimes more complex questions need to be answered, such as do the samples fall into groups? Clustering is the operation of determining which objects are most similar to one another and grouping them accordingly (Jurs, 2000). Cluster analysis can also be performed using the Mahalanobis distances to create a dendogram where the sample are clustered in groups.

Mahalanobis distance - This method takes into account that some variables may be correlated and so measures more or less the same properties. However, this method cannot easily be applied where the number of measurements (or variables) exceeds the number of objects, because the variance-covariance matrix would not have an inverse. This method is used to classify samples based on their distance from the group mean. By calculating the group means and then assigning the data points to the closest group mean, a classification matrix can be assembled comparing the predefined groups to the classification assigned by measuring the distances. The higher the percent classified correctly, the better the data has been separated into the desired groups (Goodner, 2001; Brereton, 2003).

1.6 APPLICATIONS

The detection and measurement of chemical compounds in the gas phase is clearly important in most areas of modern life including industrial process control, health and safety and environmental monitoring (Jurs, 2000).

1.6.1 Food Industry

Since the first commercial e-noses were developed a wide range of potential applications have been descibed in the literature. The most common in the early days was for food technology. Work has been done on meat, coffee, fish, beer and other beverages (Schweizer-Berberich, 1994; Pardo, 2000; Lamagna, 2002; Innawong, 2004; Santos, 2004). For odour quality evaluation of food packaging material, Van Deventer and Mallikarjunam (2002), referred to the optimisation of an e-nose for the analysis of inks on food packaging films. The e-nose was also been used to detect fungal activity and the presence of toxins in cereals (Magan and Evans, 2000; Olsson, 2002). Attempts have also been made to differentiate toxic and non-toxic isolates of the same mentioned species (Keshri and Magan, 1998).

Recently, studies in the food industry using conducting polymer sensor arrays have included the early detection and differentiation of spoilage fungi in bread (Keshri, 2002; Needham, 2004), the detection of bacteria and yeasts in milk (Magan *et al*., 2001; Ampuero and Bosset, 2003; Marilley, 2004) and monitoring the quality control of Danish blue cheese (Trihaas, 2002). Ampuero and Bosset (2003) reviewed the applications of the e-nose for dairy products. This review includes examples of the evaluation of cheese aroma (Swiss and Cheddar cheese), the detection of mould in Parmesan cheese and detailed information on how to monitor the quality of milk, including the identification of single strains of disinfectant–resistant bacteria in mixed cultures.

1.6.2 Medical Applications

Monitoring the spoilage of foodstuffs is closely related to the growth of bacteria in a specific growth medium. In other words, an e-nose may be able to recognise characteristic smells from diseases and bacteria cells because cell metabolism is the biological oxidation of organic compounds, such as glucose ($C_6H_{12}O_6$), to yield ATP and secondary metabolites (Figure 1.8).

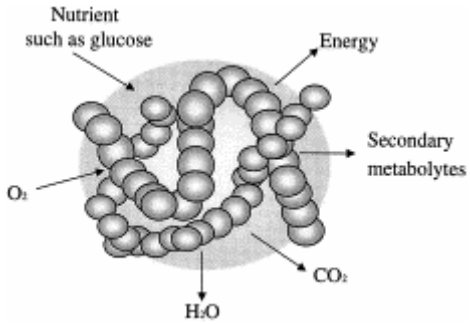


Figure 1.8 Metabolism of a bacterial cell (from Gardner, 2000a)

There is now great interest in the ability of e-nose systems to diagnose illness. It is well known that certain diseases are associated with characteristic smells. For example, diabetes produces the sweet smell of acetone on the breath and stomach ailments are often associated with halitosis. Other diseases, like cancers associated with the lungs, liver and intestine can produce characteristic odours (Gardner, 2000a). Table 1.3 gives an indication of the diversity of volatile compounds originating from different microbial species.

Table 1.3 Volatile chemicals emanating from microorganisms grown in culture (Nilson *et al*., 1996 ; Gibson *et al*., 1997; Scholler *et al*., 1997; Pavlou *et al*., 2000; Wood *et al*., 2001; Scholler *et al*., 2002).

Microorganism	Volatile chemical components
Actinomycetes	Geosmin, isoprene, 2-methylisoborneol, 2-phenylethanol, 1-butanol, acetone, cyclopentanone, sulfur compounds, esters
Pseudomonas aeruginosa	Butanol, methyl ketones, 2-heptanone, 2-aminoaceptophenone, dimethyl trisulphide, isopentanol, isoprene, 1-undecene
Escherichia coli	Ethanol
Enterobacter spp.	2-Methyl-1-propanol, 2-methyl-1-butanol, dimethyl trisulphide
Penicillium sp.	Geosmin, isopentyl alchohol, 1-octene-3-ol, 2-methylisoborneol, 3-octanone

The detection and identification of microorganisms using e-nose technology has been reported on medical diagnostics for diabetes (Wang *et al.*, 1997) and discrimination between *Helicobacter pylori* and other gastroesophageal bacteria (Pavlou *et al.*, 2000). Gardner (2000a) described the work carried out on the use of an e-nose for two medical applications. The identification of pathogens that cause infectious disease of the upper respiratory tract and ears, and diagnosing the presence of sub-clinical or clinical ketosis from the breath of dairy cows. Other applications include the diagnosis of urinary tract infections (Pavlou *et al*. 2003), the identification of lung cancer by the analysis of breath (Di Natale, 2003) and tuberculosis (Pavlou *et al*., 2000). Recently, Fend *et al*. (2004) applied a conducting polymer sensor array based e-nose to monitor haemodialysis in kidney patients.

Apart from medical purposes, the volatile production of microbial cultures has been used for the detection, identification and discrimination of bacteria cultures (Gibson *et al.*, 1997; Holmberg *et al.*, 1998; Linden *et al*., 1998). McEntegart *et al.* (2000)

described the detection and discrimination of coliform bacteria (*E. coli* and *E. aerogenes*) growing in a Brain-Heart Infusion (BHI) medium using an e-nose with interchangeable sensor modules (metal-oxide semiconductor and quartz microbalance sensors). Keshri and Magan (2000) used a conducting polymer sensor based array e-nose to differentiate between mycotoxigenic and non-mycotoxigenic strains of *Fusarium* spp.. In comparison with enzymatic methods, the e-nose proved to be a faster and easier methodology for the early detection of fungal activity prior to visible growth (Keshri, 1998).

1.6.3 Environmental Applications

Classically and routinely, the detection and enumeration of indicator microorganisms of faecal pollution are based on cultural methods. The microorganism was grown on either a solid (agar) or liquid (broth) medium, which supplies the nutritional requirements of the organism. Conventional methods for detecting indicators and pathogenic bacteria in water may indeed underestimate the actual microbial population due to sub-lethal environmental injury, inability of the target organism to take up nutrients and physiological factors, which reduce bacterial culturability (Bonadonna, 2003).

Rompre *et al.* (2002) reviewed the classical and emerging approaches for the detection of coliform bacteria (Table 1.4). Techniques such as multiple-tube fermentation (MTF) and membrane filters are describe in the “Standard Methods for the Examination of Water and Wastewater” for the isolation of members of the coliform group, regarding the metabolic activity of cellular enzymes for the detection of total coliforms, *Escherichia coli* and enterococci. The MTF technique lacks precision in qualitative and quantitative terms.

Table 1.4. Advantages and disadvantages of emerging approaches for the detection of microorganisms in water (adapted from Rompre *et al*., 2000).

New approaches	Advantages	Disadvantages
Biosensors	• Direct detection • Fast response • Simple to use	• Interaction with matrix • Sterilization
Immunological	• Direct detection	• Expensive • Time consuming • Interference with matrix • Low Yield
Flow cytometry	• Fast response	• High level of biomass required • High levels of expertise
Molecular techniques	• High sensitivity	• Interaction with matrix • Time consuming • False positives • High levels of expertise

The application of e-nose technology for environmental monitoring has been examined and looks promising. Several studies report the use of sensor-based systems for on-line monitoring of organic parameters such as the biochemical, chemical and total demand of oxygen (BOD, COD, TOC) and volatile organic compounds (VOCs) in wastewater treatment plants (Fenner and Stuetz, 1999; Stuetz *et al.*, 1999; Bourgeois *et al.*, 2001; Di Francesco *et al.*, 2001; Dewettinck *et al.*, 2001). Gostelow *et al.* (2001) in a review of methods applied to odour measurement in sewage treatment works referred to sensory (olfactometry) and analytical measurements together with the e-nose. Gardner *et al.* (2000b) reported the application of an e-nose to monitor cyanobacterial cultures in

potable water. Apart from cyanobacteria, many other microorganisms could be targets for monitoring to ensure safe and pleasant potable water for consumers.

Non-specific sensor arrays have been used for on-line monitoring of chemical contaminants in wastewater and water samples (Bourgeois *et al*., 2003). Stuetz (2004) recommended the use of the e-nose as a non-invasive technique to provide early warning systems for application at the inlet of wastewater treatment plants (WWTPs). Groschnick (2004) tested a KAMINA micronose model to analyse headspace water samples polluted with chloroform and ammonia, representing chloro-organic solvents and faecal contamination respectively, simulating stagnant and flowing waters.

However, little attempt has been made to examine early detection and discrimination of bacterial and fungal contaminants in potable waters. Furthermore, the presence or absence of heavy metals or pesticides may influence the metabolism of some microorganisms. Previous studies have shown good results when the e-nose was used to detect chemical contaminants in water samples (Canhoto and Magan, 2003; Wright and Higginson, 2004). New knowledge is needed for the rapid detection of water samples contaminated with microorganisms at concentrations in the range $10^1 – 10^3$ cells ml^{-1}. At-line studies would be important to detect contamination episodes of microbial species using e-nose systems, for a fast-response method when water quality is in question. Other applications which have not been previously examined, where the presence of microbial species and changes in their metabolism may be a signal of chemical contamination with heavy metals and pesticides in soil pollution, and indoor air contamination by fungal spores.

In polluted soil, the presence and activity of microorganisms on the surface and subsoil can be detected and measured in many ways, including microbial numbers, microbial biomass, functional activity (such as respiration and N mineralization) and enzyme activities. Several soil bioasays have been developed, such as measurements of total soil biomass, growth, ATP, microbial sensitivity, nitroredutase, esterase and dehydrogenase activity, oxygen uptake rates, and luminescence in pure cultures or degrading consortia. Toxicity tests involve the exposure of the organism or culture cell lines from different

organs and species to toxic compounds under defined experimental conditions (Addondanzi, 2003; Taylor, 2002). However, many of these enzyme-based techniques are time-consuming and require very expensive non-environmentally friendly reagents.

Concerning indoor air pollution, very few methods exist for the detection of fungal contaminants in library materials. Nowadays the conventional method for detecting filamentous fungi that are able to damage and even destroy archival and library materials, is to culture them. This is slow and time-consuming in terms of effective management. Recent studies describe the use of an ATP assay for the detection of viable fungal spores (Rakotonirainy, 2003). Fiedler (2001), used head-space solid-phase microextraction (HS-SPME) to analyse microbial fungal volatiles from indoor air samples. Rapid recognition of the type and intensity of mould infestation in indoor air will efficiently support the prevention of health complaints and diseases. A more refined method for sampling and analyses may allow the detection of the type and intensity of mould contamination for small-contaminated environments.

Recently, studies using sensor arrays have been focused on indoor air pollutants, targeting VOCs and microbial contaminants in library material (Canhoto *et al*., 2004; Zampoli, 2004). Thus, e-nose technology represents an alternative when a fast answer is required regarding the quality of a sample. Several electronic nose instruments have been commercialised using different sensors. Table 1.5 shows some commercial electronic noses currently available.

Table 1.5 Commercially available electronic nose instruments.

Manufacturer	Place of origin	Sensor type	No. of sensors	Size of instrument
Airsense analysis GmbH	Germany	MOS[1]	10	Laptop
Alpha MOS – Multi Organoleptic system	France	CP[2], MOS, QCM[3], SAW[4]	6 - 24	Desktop
AromaScan PLC	UK	CP	32	Desktop
Bloodhound Sensors Ltd.	UK	CP	14	Laptop
Cyrano Science Inc.	USA	CP	32	Palmtop
EEV Ltd. Chemical Sensor Systems	UK	CP, MOS, QCM, SAW	8-28	Desktop
Electronic Sensor technology Inc.	USA	GC[5] column, SAW	1	Desktop
Hewlett-Packard Co.	USA	QMS[6]	-	Desktop
HKR- Sensorsysteme GmbH	Germany	QCM	6	Desktop
Lennartz Electronic GmbH	Germany	MOS, QCM	16-40	Desktop
Nordic Sensor Technologies AB	Sweden	IR[7], MOS, QCM	22	Laptop
Sawtek Inc.	USA	SAW	2	Palmtop

Key to sensor types: 1, Metal oxide semiconductor; 2, Conducting polymer; 3, Quartz crystal microbalance; 4, Surface acoustic wave; 5, Gas Chromatography; 6, Quadrupole mass spectrometry; 7, Infrared.

1.7 OBJECTIVES

This is the first study to examine in detail e-nose systems for detection of threshold concentrations of microbial and chemical contaminants in potable water, to characterize microbial activity in soil and the detection of microbial contaminants in library material. The main objectives of this project were to:

- Detect important water microbial and chemical contaminants, under different environmental conditions;

- Compare the results obtained with different e-nose systems;
- At-line monitoring of contamination episodes in water samples;
- Study the microbial activity in soils contaminated with heavy metal ions;
- Early detection of fungal contaminants in paper samples.

The following studies were carried out to achieve these objectives:

1. Examination of the threshold of detection of heavy metals in different types of water
2. Bacterial and fungal detection and discrimination using different incubation conditions
3. Interaction between bacteria and heavy metals in order to detect any changes in volatile production
4. Comparison between the results obtained with the e-nose system and HS-SPME/GC analysis
5. Comparison between conducting polymer and metal oxide sensor array systems for early detection of bacteria in water
6. Microbial characterization of *in vitro* and *in situ* soil samples contaminated with different heavy metals using a MOSFET-MOS e-nose system
7. Comparison between the results obtained with the MOSFET-MOS in soil assays and enzymatic methods
8. *In vitro* and *in situ* studies on paper samples contaminated with low concentrations of fungal contaminants under different environmental conditions.

A diagram with the different studies for the application of the e-nose system in different environmental spheres is presented in Figure 1.9. Chapters 2 - 6 have integrated materials and methods, results and discussions, and have either been published, or *In Press* or in preparation for publication. A final list of conclusions will be presented.

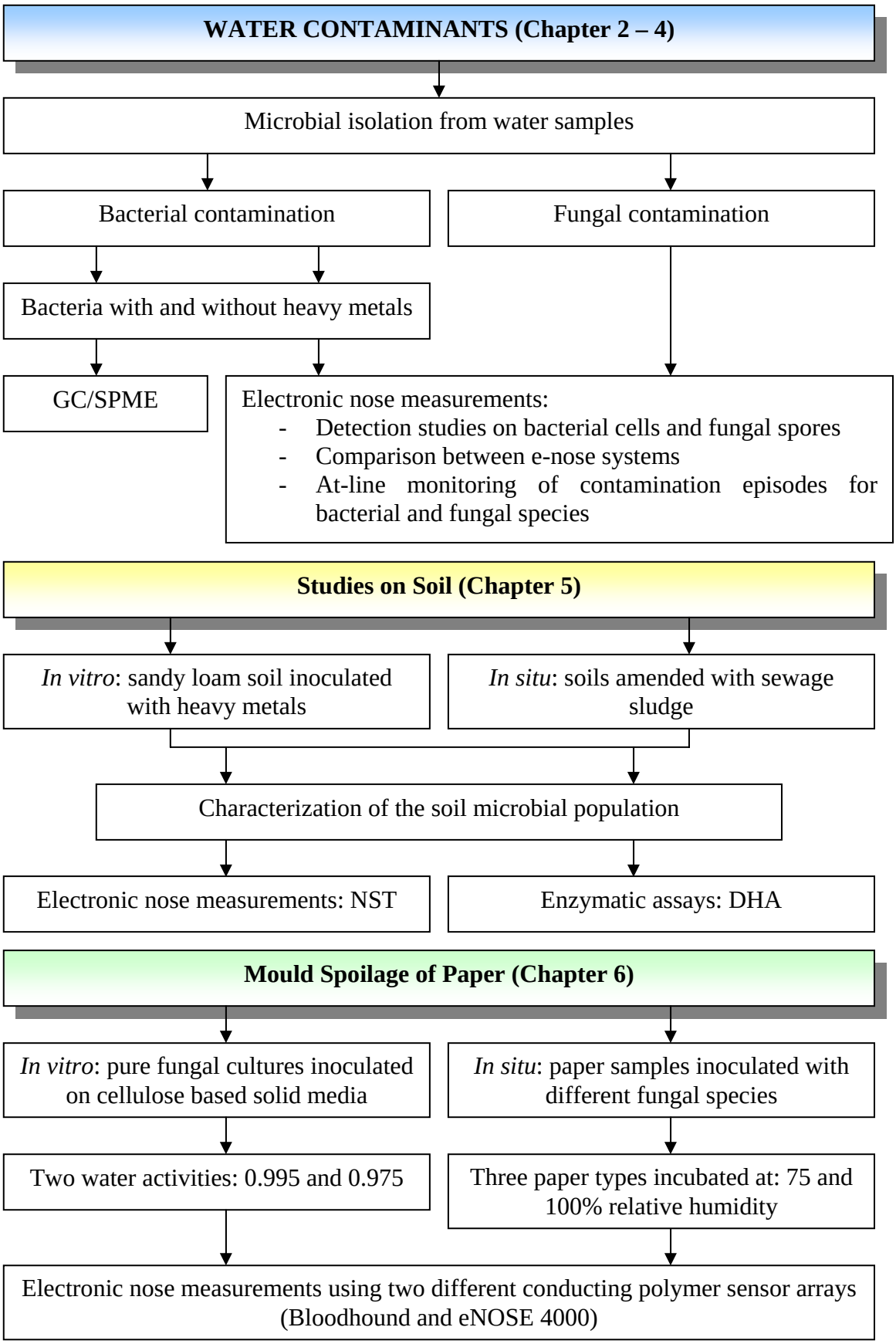


Figure 1.9 Flow chart of different components of studies carried out in this thesis.

CHAPTER 2.

DETECTION OF MICROBIAL AND CHEMICAL CONTAMINANTS IN WATER

2.1 INTRODUCTION

The rising costs of treating water and wastewater and the increasing demand for clean water is prompting industry and authorities to examine new methods for water conservation. Conventional chemical methods used for the analysis of water and wastewater can be accurate, but are mainly based on sample collection and retrospective analysis. The recent availability of commercial sensor arrays, e-noses, for detecting and characterizing headspace odours may offer a more rapid and relatively simple technique for monitoring changes in water and wastewater quality (Gostelow *et al*., 2001).

2.1.2 Microbial Contaminants

It is extremely important to determine the microbiological safety of potable waters because of the risk to public health due to the presence of pathogenic microorganisms. Studies of the microbial flora of raw source waters used in water supply treatment reveals a wide spectrum of diverse organisms. Many of these organisms are of known significance in water supply while others may be pathogens, faecal organisms, and opportunists. It is these latter three groups that are of significance; they cause health problems, interfere with indicator detection, and cause taste and odour problems in potable water (Geldreich, 1996). The pathogenic agents involved include bacteria, viruses, and protozoa, which may cause diseases that vary in severity from mild gastroenteritis to severe and sometimes fatal diarrhoea, dysentery, hepatitis, or typhoid fever (WHO, 1996).

i) Coliforms

Indicator organisms are also called coliforms, they belong to the family Enterobacteriaceae and share similar cultural characteristics. Faecal coliform bacteria (thermotolerant coliforms) possess the characteristics of colifom bacteria but are able to carry out lactose fermentation at 44ºC. The main reason why they have been used as indicators of water quality is that in normal conditions, they do not survive for long time in water. Consequently, their presence is considered as an indication of faecal

contamination. They regarded as an index of theoretical risk for public health and of water quality deterioration (European Agency, 2002).

Escherichia coli bacteria survives in the intestines of man and other warm-blooded animals, and is excreted in the faeces. *E. coli* was introduced into water bacteriology because it was a useful marker of faecal pollution and thus became an important marker in food and water hygiene. All enterovirulent *E. coli* are acquired directly or indirectly from human or animal carriers (Percival, 2004). The monitoring and statutory assessment of the hygienic quality of drinking water is currently based on the determination of bacterial indicators of faecal contamination

Ideally, microbial indicators should provide a measure of health risk associated with the exposure to contaminated water (ingestion or contact). Nevertheless, these groups of microorganisms have many limitations as predictors of risk of waterborne disease Moreover, there are non-faecal sources for these indicator organisms, and in contrast to most enteric pathogens, coliforms may multiply in aquatic environments with sufficient nutrients and optimal temperatures, for example in Tropical regions. Such characteristics may result in false-positive reports of water contamination (Bonadonna, 2003).

The detection and enumeration of all pathogenic bacteria that may potentially be present in water is not only expensive and time consuming but impracticable to perform on a regular basis. In addition, traditional methods used routinely in the control for enteric pathogens are often time-consuming and seldom selective (Csuros and Csuros, 1999; Bonadonna, 2003).

The applications of e-nose technology have been widely reported for early detection and identification of microorganisms and signs of food spoilage (Pavlou *et al*., 2000; Magan *et al*., 2001; Keshri *et al*., 2002). The e-nose has also been used to detect the volatiles emitted by growing bacteria (Gibson *et al*., 1997; McEntegart *et al*., 2000). These studies have generally been done with high concentrations of bacteria, centrifuged cell biomass, or colonies of bacteria grown on an agar surface. In view of these reports, the

key issue with the use of the e-nose in microbial detection is sensitivity rather than selectivity.

ii) Fungi

In comparison to bacteria, fungi have been relatively neglected as inhabitants of the drinking water ecosystem. They occur in a variety of aquatic environments, and it is not surprising that they can be recovered from drinking water distribution systems. Once in the distribution system, fungi may establish themselves on pipe surfaces or in sediment, which can collect in the bottom of pipelines (Nagy and Olson, 1982).

Although filamentous fungi in water are commonly thought to pose no potential public health problems, some of the fungi isolated from mineral water, i.e. *Alternaria alternata* and *Penicillium citrinum*, have some toxigenic potential and could constitute a health risk. It is therefore advisable to count fungal propagules in routine microbiological studies of bottled mineral water and to establish baselines (Cabral and Pinto, 2002). Some pathogenic fungi live in recreational waters, such as pools and beaches; the most common pathogenic fungus is *Aspergillus fumigatus*. It causes pulmonary aspergillosis if breathing contaminated air (Csuros and Csuros, 1999). Tap water is a potential transition route for fungi and may pose a health hazard mainly for the immunocompromised host (Arvanitidou *et al*., 1999).

There is little reliable information on the detection and isolation of fungi from drinking water. It is well known that the enumeration of fungi in environmental samples is problematic both in terms of reproducibility and in terms of what is actually being counted; nonetheless, estimates of fungi in water vary from 0 to 9,000 CFU L^{-1}, with distribution systems often giving figures of 10-50 CFU L^{-1}. Of the isolation methods, membrane filtration has been very widely used although it was developed primarily for bacterial isolations and fungi were (at least) incidental (Kinsey *et al.*, 1999). There are no previous studies using the electronic nose to detect fungal contamination in potable water.

2.1.2 Heavy Metals

"Heavy metals" is an imprecise term that is generally taken to include the metallic elements with an atomic weight greater than 40. The most important heavy metals from the point of view of water pollution are zinc, copper, lead, cadmium, mercury, nickel and chromium. Some of these metals (e.g. copper, zinc) are essential trace elements for living organisms, but become toxic at higher concentrations. Others, such has lead and cadmium, have no known biological function (Abel, 1998). Metals differ from the toxic organic compounds in that they are totally non-degradable, and so they can accumulate in the components of the environment where their toxicity is expressed (Baird, 1998).

Currently pollution by heavy metals is one of our most serious environmental problems. Chemical contaminants in drinking water can be present together with numerous other inorganic and organic constituents. These can include cadmium, lead and zinc, as impurities from the household plumbing systems, in which the pipes, solder or fitting contain some of these metals (WHO, 1996). Metals entering the environment are often in an insoluble form in industrial waste, in discarded manufactured products or as part of naturally occurring mineral deposits (Reeve, 1994). Quite apart from industrial sources, domestic wastes contain substantial quantities of metals because the water has been in prolonged contact with copper, zinc or lead pipework or tanks (Abel, 1998).

Metals may be determined satisfactorily by atomic absorption (AA), inductively coupled plasma (ICP), or, with somewhat less precision and sensitivity, colorimetric methods. The absorption methods include flame and electrothermal techniques. ICP techniques are applicable over a broad linear range and are especially sensitive for refractory elements. In general, detection limits for ICP methods are higher than those used in electrothermal methods. Colorimetric methods are applicable when interferences are known to be within the capacity of the particular method. Preliminary treatment of samples is often required (APHA, 1995). So far, there are no studies using the e-nose technology to detect heavy metals in water.

2.2 MATERIALS AND METHODS

2.2.1 Cultural media and incubation

The following bacterial species were obtained from the UK National Culture Collections were used in this study: *Enterobacter aerogenes* (88), *Escherichia coli* (10000) and *Pseudomonas aeruginosa* (8672).

A microbial screening was performed in Malt Extract Agar (MEA) (Oxoid) using water samples from a water treatment plant. The following species were selected for being the most common amongst the total fungal population: *Aspergillus fumigatus*, *Fusarium culmorum* and *Penicillium* sp..

i) Samples inoculated with bacterial cells

Each bacterial species was grown in nutrient broth (Oxoid), and incubated for 24 and 48 hours at 37^{o}C. These bacterial cultures were used to obtain bacterial cell concentrations of 10^2, 10^4 and 10^8 colony forming units (CFUs) mL^{-1}. The final concentrations were obtained by correlating absorbance (640 nm, visible light) of different concentrations of cells and the actual number of colonies using the spread plate technique on nutrient agar. Three types of water were used to prepare the samples; reverse osmosis (RO), tap and bottled water, in a sampling device with a total volume of 10 mL per sample. Samples were incubated at 25^{o}C for a period of 24 h.

ii) Samples inoculated with fungal spores

Actively growing 7-day-old colonies on MEA (Lab M) at 25^{o}C were used to obtain a spore suspension of 10^2, 10^4 and 10^5 spores mL^{-1}. Fungal spore suspensions were prepared in a 10 mL solution of RO water and a small amount of Tween 80. These concentrations were obtained using a haemocytometer and microscope (Olympus, UK). The samples were incubated in the same conditions as described above

iii) Water samples containing heavy metals

Samples were prepared by adding a mixture of the heavy metals (As, Cd, Pb and Zn) to non-sterile water, to obtain a 10 mL aqueous mixed metal solution in two different concentrations, 0.5 and 2.0 ppm. When bacterial species were mixed with the heavy metals, the final concentrations were adjusted to 10^4 and 10^6 CFUs mL^{-1}. This study was carried out using 4 replicates and the samples were incubated for 24 and 48 hrs at 25^oC.

2.2.2 Gas sampling and sensing procedure

Figure 2.1 illustrates the sampling device used in these experiments. The connection with the e-nose (Bloodhound, BH-114) was made using a specially made air-filter system. It consisted of a pair of 6 cm long Teflon tubing segments (Tygon), a bio-filter (0.45 μm, PTFE Whatman, HepaVent), and an activated carbon filter (Whatman) to ensure clean airflow above the aqueous solution samples headspace. When the e-nose pumps the headspace of the sample, the incoming air is circulated through the sample accelerating the release of volatile compounds in the headspace (Pavlou *et al*., 2000).

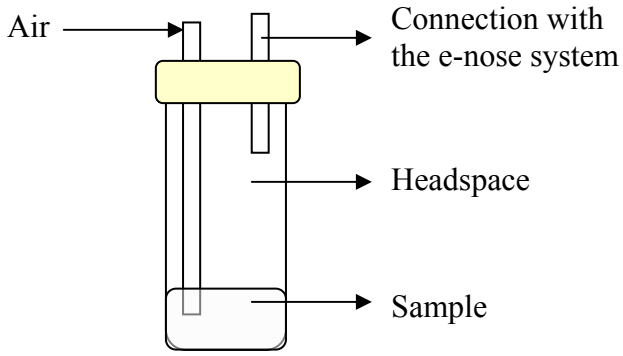


Figure 2.1 Sampling device used to make the connection with the e-nose system.

An electronic nose (model BH-114: Bloodhound Sensors Ltd., Leeds, UK), which employs 14 conducting polymer sensors, was used in this study (Plate 2.1). Activated carbon filtered air is passed over the sensor surface at a flow rate of 4 mL min^{-1} to generate the sensor baseline. The electronic nose was flushed and a one minute interval

was allowed between each sample. Samples were analysed in a random pattern including the controls. Four replicates were used for each treatment and repeated twice.

Plate 2.1 The electronic nose system (model BH-114, Bloddhoud Sensors Ltd., Leeds, UK) used in this study connected to a desktop computer. Key to figure: **1**, E-nose (BH114); **2**, connection to sampling device; **3**, carbon filter.

2.2.3 Data analysis

The software incorporated in the Bloodhound system provides information on the sensor response profile through four sets of data: Adsorption (maximum rate of change of resistance), Desorption (maximum negative rate of change of resistance), Divergence (maximum step response) and Area (area under the actual sensor curve) (Figure 2.2). In this study, the normalised data for divergence and area were analysed using the program xlSTAT (Microsoft Excel add-in) and Statistica (StatSoft). Multivariate techniques such as Principal Components Analysis (PCA), Discriminant Function Analysis (DFA) and Cluster Analysis (CA) were applied to analyse the sensor responses.

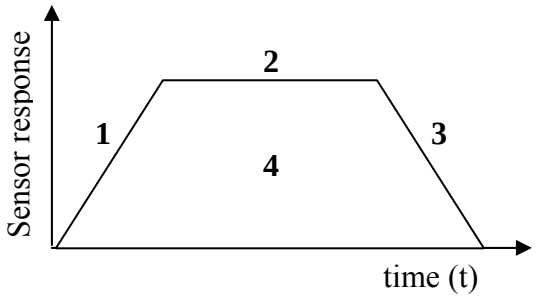


Figure 2.2 Diagrammatic representation of the sensor's response profile over time. *Key to legend*: **1**, Absorbance; **2** Divergence; **3**, Desorbance; **4**, Area.

2.3 RESULTS

Figure 2.3 shows the discrimination for *P. aeruginosa* incubated for 24 hours at 37ºC. The inability to differentiate between 10^2 and 10^4 CFUs ml^{-1} treatments may be due to similar quantities of volatiles being produced in the water at these bacterial concentrations. This suggests that the limit of detection for these bacteria is between 10^2 and 10^4, and this group is clearly separated from the control. The results were consistent, as similar responses were obtained in repeated experiments on different days.

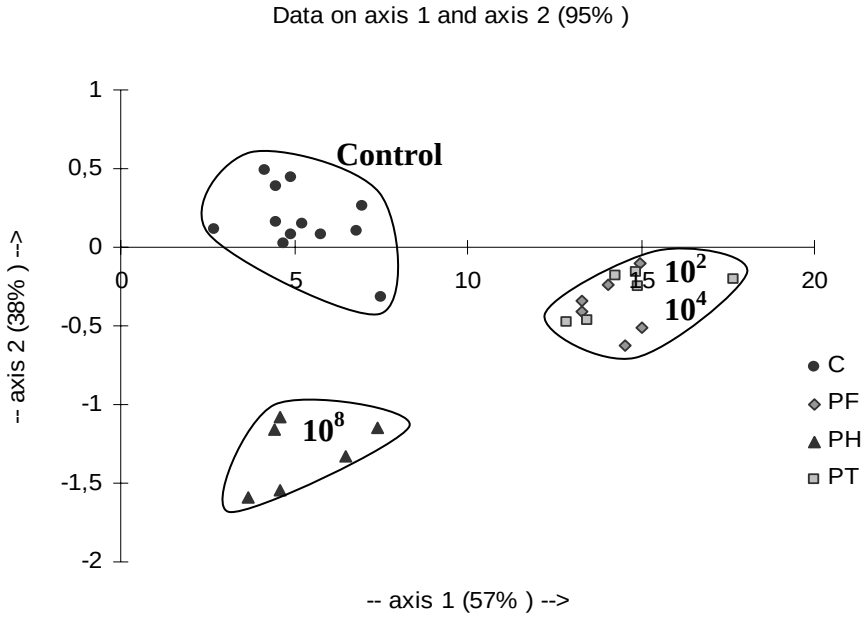


Figure 2.3 DFA of *P. aeruginosa* and control samples, incubated for 24h at 30ºC. Key: **C** - Control; **PT** - 10^2 CFU mL^{-1}; **PF** - 10^4; **PH** - 10^8. The results from different days were analysed combined.

Figure 2.4 represents *E. aerogenes* cells incubated for 48 hours at 25ºC. After 48 h, a clear four-group separation was achieved. The classification matrix for the discriminant analysis that gives the observed versus the predicted is showed in Table 2.1. The different bacteria species were analysed using the same concentration, 10^4 CFU mL^{-1}, and the data from different days were analysed together as one. The results demonstrated that the classification success was 85.71% for the control samples, 83.33% for *E. aerogenes*, 66.67% for *P. aeruginosa*, and 100% for *E. coli* samples.

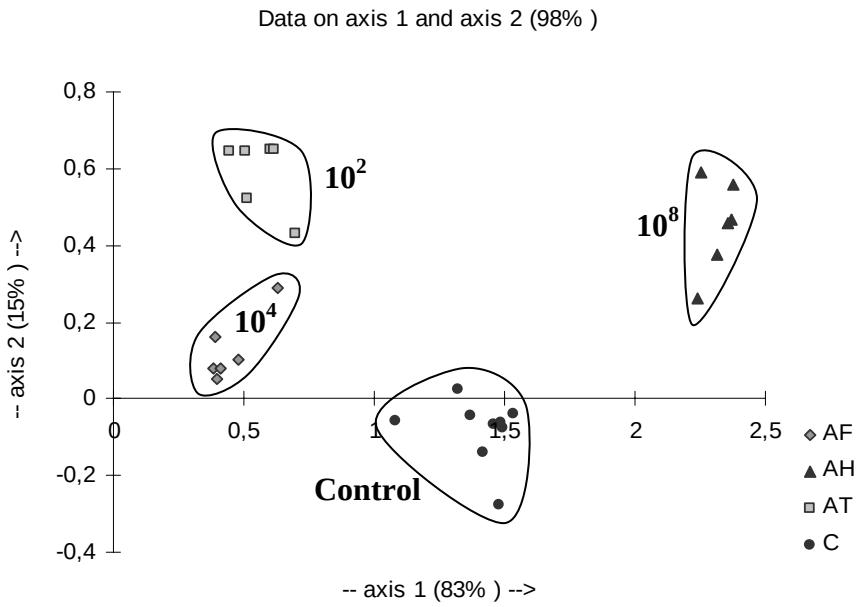


Figure 2.4 DFA of *E. aerogenes* and control samples, incubated for 48h at 30ºC. Key: **C** - Control; **AT** - 10^2 CFU mL^{-1}; **AF** - 10^4; **AH** - 10^8.

Table 2.1 Classification matrix of observed vs predicted of different bacterial species using an initial concentration of 10^4 CFU mL^{-1} and an incubation time of 24 h, and control samples (RO water). Key: **EA** - *Enterobacter aerogenes*; **CO** - Control; **EC** - *Escherichia coli*; **PA** - *Pseudomonas aeruginosa*.

	Classification Matrix (Bac 24 10^4) Rows: Observed classifications Columns: Predicted classifications				
Group	Percent Correct	CO p=.28000	EA p=.24000	PA p=.24000	EC p=.24000
CO	85.7143	6	0	1	0
EA	83.3333	0	5	1	0
PA	66.6667	2	0	4	0
EC	100.0000	0	0	0	6
Total	84.0000	8	5	6	6

- *Fungal Detection- Fungal spores*

Fungal spores were inoculated in RO water for 24 h at 25ºC. Figure 2.5 shows that samples inoculated with 10^5 *Penicillium* spores mL^{-1} could be discriminated from the control samples. Using cluster analysis it was also possible to separate water samples inoculated with *F. culmorum* spores from the control samples (Figure 2.6).

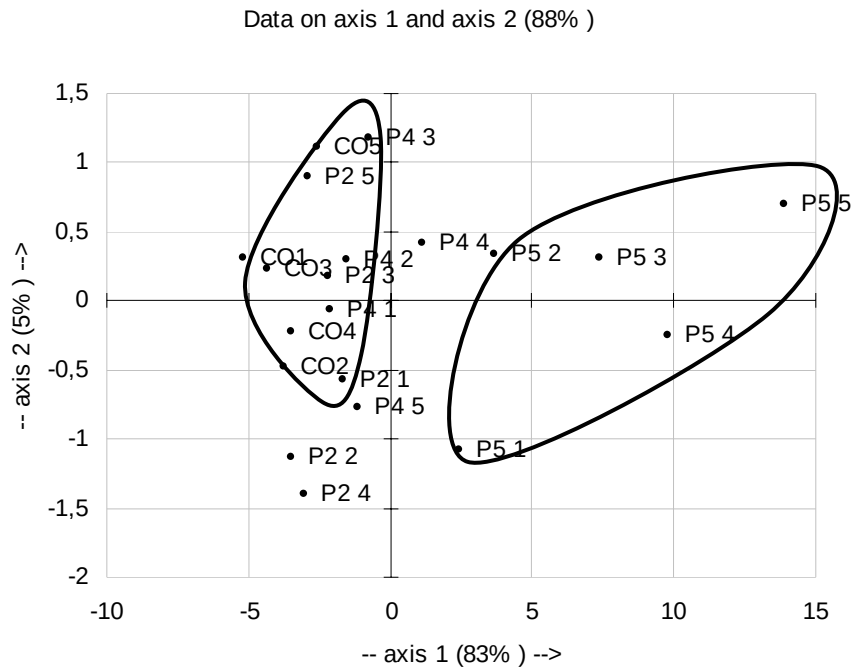


Figure 2.5 PCA map of RO water samples inoculated with *Penicillium* spores, incubated for 24h at 25°C, using five replicates. Key for treatments: **CO** - Control; **P2** – 10^2 spores mL^{-1}; **P4** - 10^4 , **P5** - 10^5 .

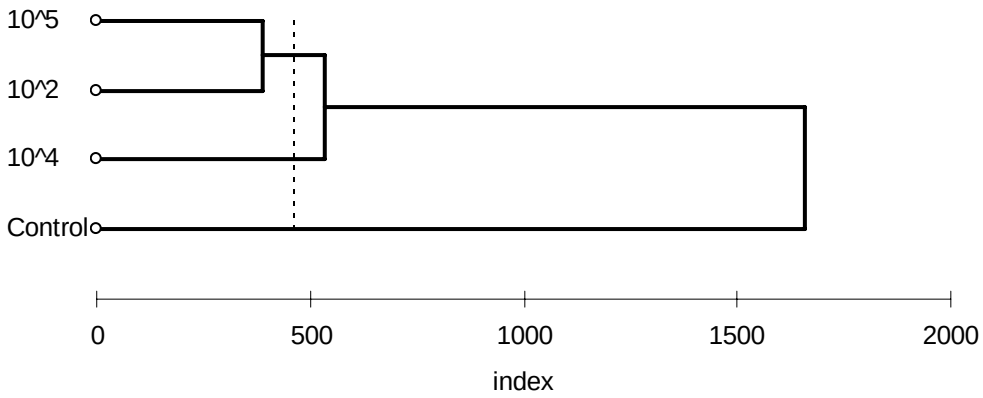


Figure 2.6 Cluster analysis of *F. culmorum* spores inoculated in RO water for 24h at 25°C, using a control sample (RO water) and three spores concentrations 10^2, 10^4 and 10^5 spores mL^{-1}.

Figure 2.7 shows the PCA obtained from normalised divergence and area data of the sensors response to two types of water, bottled and reverse osmosis (RO), treated with 0.5 ppm of a mixed metal solution (As, Cd, Pb and Zn), and RO water (non-sterile) was used as a control sample. PC1 is responsible for 96% of the variability between samples where the control samples are visibly separated from the samples containing 0.5 ppm of the metal solution. Figure 2.8 represents the same treatments using a higher metal concentration, 2.0 ppm. It can be seen that the e-nose was able to distinguish tainted water samples from control ones. As non-sterile water was used as a control, bacterial populations would be present and their activity modified by the presence of the heavy metals. This probably altered the volatile production patterns which were detected qualitatively by the e-nose.

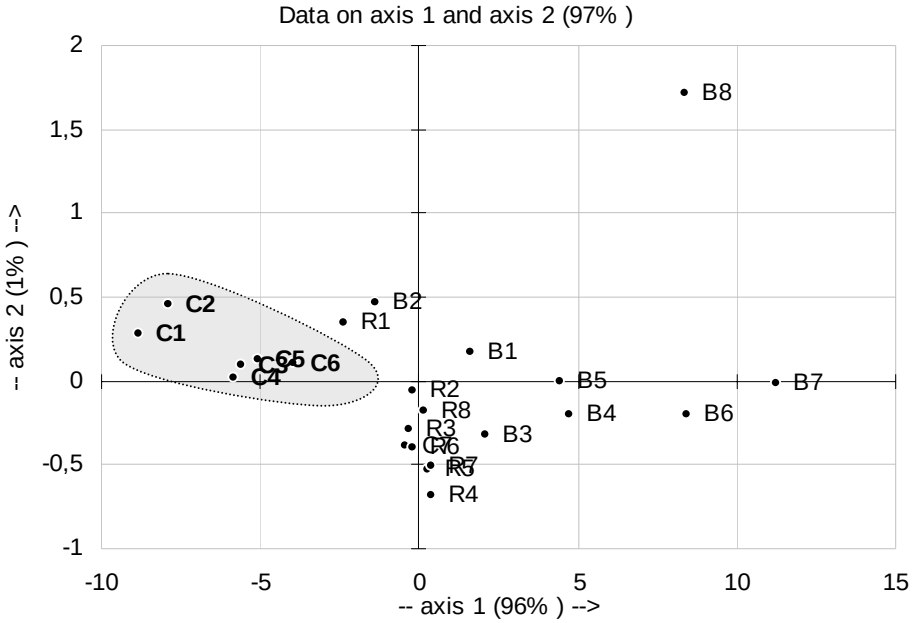


Figure 2.7 PCA map of reverse osmosis (RO) and bottled water with 0.5 ppm of a mixed metal solution (As, Cd, Pb and Zn) discriminated from RO water (shaded area). Key: **C** - RO water; **R** - RO water with metals; **B** - bottled water with metals.

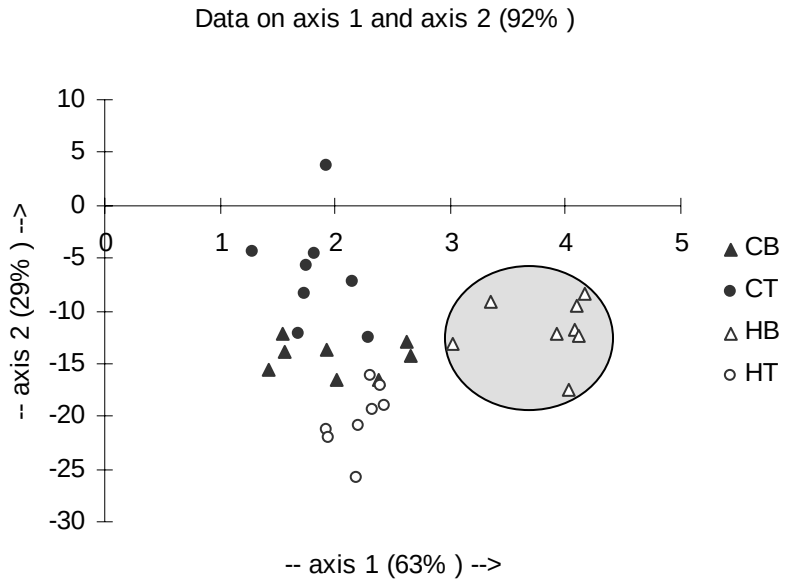


Figure 2.8 DFA of bottled (shaded area) and tap water with 2.0 ppm of a mixed metal solution (As, Cd, Pb and Zn) discriminated from control samples. Key: **CB** - Bottled water; **CT** – Tap water; **HB** - bottled water with metals; **HT** – tap water with metals.

Figure 2.9 represents the sensor responses to controls and that inoculated with *E. coli* cells and a 0.5 ppm of the metal mixture. Across PC1 it is possible to discriminate three groups of samples: control, bacteria and bacteria with metals. The highest level of separation occurs between the control and samples containing bacterial cells inoculated with heavy metals. Figure 2.10 represents a similar experiment using *P. aeruginosa* cells inoculated with the same concentration of metal solution. From the PCA plots on the raw data values, it was possible to differentiate between control samples and those containing bacteria. Although, the discrimination between bacterial cells with and without metals is not as clear as in Figure 2.9, concentrations of 10^8 cells mL^{-1} are visibly separated in the map.

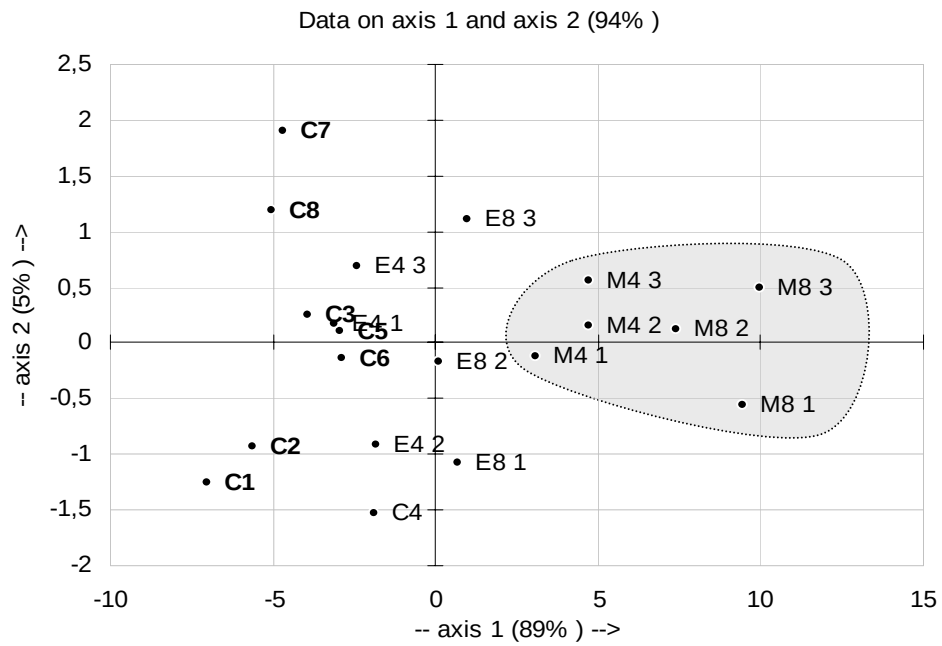


Figure 2.9 PCA of control samples and two cell concentrations of *E. coli* cells inoculated (shaded area) and un-inoculated with 0.5 ppm of a mixed metal solution (As, Cd, Pb and Zn), incubated for 24 h at 30ºC. Key: **C** - Control; **E4** - 10^4 CFU mL^{-1}; **E8** - 10^8; **M4** - 10^4 CFU mL^{-1} with metals; **M8** - 10^8 with metals.

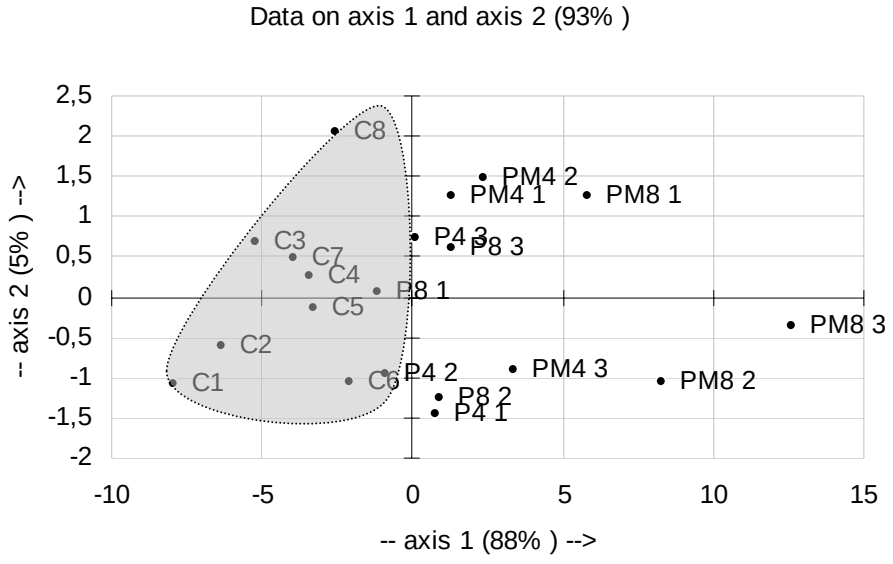


Figure 2.10 PCA of control samples (shaded area) and two cell concentrations of *P. aeruginosa* cells inoculated and un-inoculated with 0.5 ppm of a mixed metal solution (As, Cd, Pb and Zn), incubated for 24 h at 30ºC. *Key to treatments*: **C**, control; **P4**, 10^4 CFU mL^{-1}; **P8**, 10^8; **PM4**, 10^4 CFU mL^{-1} with metals; **PM8**, 10^8 with metals.

2.4 DISCUSSION

This is one of the first studies to examine the potential of using e-nose technology to differentiate water samples on the basis of qualitative volatile production pattern due to microbial or metal interaction. Both bacterial cells and fungal spores could be discriminated from the control samples, at initial concentrations as low as 10^2 cells mL^{-1}. This study shows that potential does exists for detecting the presence of quite low concentrations (0.5 ppm) of important contaminants in water. The use of non-sterile water could be the reason why it was possible to detect the presence of heavy metals in RO and bottled water. Fewtrell *et al*. (1997) carried out a survey on the microbiological quality of still bottled water. They showed that almost 2% of the examined samples failed to meet the required microbiological standards. The presence of low quantities of heavy metals may modify the activity of microorganisms and thus the volatile production patterns enabling further separation to be made. Another explanation might be the formation of volatile metal-organic compounds in natural environment by microbial mediation (Weiner, 2000).

In the last few years, studies have demonstrated that conducting polymer sensors could distinguish between distilled water and distilled water that contains low levels of organic pollutants, suggesting that such a system could be used to screen for tainting compounds in supply waters (Stuetz *et al*., 1998; Dewettinck *et al*., 2001; Ogawa and Sugimoto, 2002). The use of conducting polymer arrays for the discrimination of microorganisms has been successful in other media, e.g. milk-based liquid media where volatiles compounds produced by spoilage bacteria and yeasts using an initial inoculum of $10^3 – 10^4$ CFU mL^{-1} (Magan *et al*., 2001) could be discriminated. McEntegart *et al*. (2000) reported the detection of bacteria in liquid media, of about $5x10^8$ cells mL^{-1}, using a mixture of three sensor types. So far, 10^2 CFUs mL^{-1} is the lowest level at which microbial contaminants can be detected using volatile production patterns. Other studies have tried to use e-nose systems for real time monitoring of waste water streams by measuring changes in general odour production (Bourgeois *et al.*, 2001).

Several studies using drinking water filtered through membrane filters, demonstrate the occurrence of fungi in drinking water. Nagy and Olson (1982) report that the four most frequent genera of filamentous fungi found in chlorinated and unchlorinated drinking water in a distribution system were: *Penicillium*, *Acremoniium*, *Paecilomyces* and *Sporocybe*. Hinzelin and Block (1985), examined the mycoflora of chlorinated drinking water, of 38 samples, and found that 81% were filamentous fungi. They found that three genera represented the majority of the isolates: *Penicillium*, *Aspergillus* and *Rhizopus*. Investigations on the occurrence of fungi in hospitals and community potable waters show that filamentous fungi were isolated in 82.5% of the samples, and the prevailing genera were *Penicillium* and *Aspergillus* (Arvanitidou *et al*., 1999).

Detection of heavy metal contaminants in the environment is critical to ensuring safe drinking water and effective cleanup of human activities that have led to widespread contamination of soil and groundwater. Di Nezio (2004) developed an automated spectrophotometric method for lead determination by flow injection analysis with on-line preconcentration in natural, well and drinking water samples. The method provided a linear range between 25 and 250 μg mL^{-1}, a detection limit of 0.5 ng L^{-1} and a sample throughput of 15 h^{-1}. Xie (2004) development a densely packed indium ultramicroelectrode (UMEAs) for the determination of trace concentration of heavy metals in water. The new UMEAs was fabricated using ion-milling method. Results showed that sensitivities as low as 0.25 μ/L Cd and Pb could be achieved.

All the methods referred to above were based on techniques that are known to be time consuming and requiring sample preparation and highly skilled staff. Until now, there were no studies using e-nose technology for the detection of heavy metals in water samples. It is probably that volatility is not one of the chemical properties of these ions in solution in water. However, to interaction between microbial populations and chemicals ions needs to be taken into account.

The next chapter will evaluate the changes in microbial metabolism in the presence of heavy metal ions, not only with the e-nose systems, but also with a more classical

technique, the headspace-solid phase microextraction / gas chromatography to confirm these responses and changes.

CHAPTER 3.

CROSS-COMPARISON BETWEEN ELECTRONIC NOSE SYSTEMS

3.1 INTRODUCTION

Off-flavour problems are significant for water suppliers, first because tastes and odours are regulated by guidelines for potable water and, secondly, because consumers judge water by what they first perceive. Tastes and odours account for the largest single class of consumer complaints submitted to water utilities (Malleret *et al*., 2003). Perceptions of health risks based on aesthetic properties may result in public demand for greater water treatment, even if actual risks are low (Davis *et al*., 2004).

Odour problems can be related to microbial by-products, disinfectants, and disinfection by-products. Geosmin (earthy-odour), 2-methylisoborneol (MIB, musty-odour), *trans*-2, *cis*-6-nonadienal (nonadienal, cucumber-odour), and *n*-hexanal (lettuce-heart, grassy odour) have been identified in numerous water supplies and tap waters. Geosmin and MIB, which occur in water at concentrations from a few ng L^{-1} to > 800 ng L^{-1}, require ozone or activated carbon and are not readily removed by conventional water treatment processes e.g., chlorination, coagulation, sedimentation and filtration (Whelton *et al*., 2004).

Microorganisms are thought to be one of the most important factors to cause odour and off-taste problems in the water supply industries. To ensure safe recreational water and a continued supply of potable water, frequent monitoring of both raw water sources and finished products for the presence of pathogens is very important. However, the detection and enumeration of all pathogenic bacteria that may potentially be present in water is not only expensive and time consuming but impracticable to perform on a regular basis (Csuros and Csuros, 1999). On the other hand, progressive eutrophication and pollution of surface waters aggravates the problem, causing a steady increase in the number of taste and odour incidents. Most tastes and odours are the result of human influence: indirectly by rapid enhancement of the growth of aquatic organisms under eutrophication or directly by wastewater dumping or accidental spillage as well as by agricultural runoff (Wnorowski, 1992).

A study by Legnani (2000) using the Flavour Profile Analysis showed that the 22% of water in bottles (26/120) had a pronounced odour, earthy and/or mouldy. Although the

bacterial counts were in accordance with the standard limits, on the other hand the fungal contamination was higher in the altered samples, from which various fungal species were isolated, with the prevalence of *Penicillium* spp. The water samples with a strong earthy/mouldy odour showed a high number of isolated strains belonging to the *Penicillium expansum* genus.

A study of 40 water vending machines in Los Angeles (USA), examined water samples for temperature, turbidity, chlorine content, fungal growth, coliform bacteria, fecal coliform bacteria, *Pseudomonas* spp, *Pseudomonas aeruginosa*, and heterotrophic plate counts using plate count agar and R2A agar. The microbial content of the water suggested a need for further research into potential health effects in susceptible populations (Schillinger and Du Vall Knorr, 2004). Gardner *et al.* (2000) reported the application of an e-nose to monitor cyanobacteria cultures in potable water. However, apart from cyanobacteria, many other microorganisms should be monitored to ensure safe and pleasant potable water to the consumers.

3.1.1 Pesticides

Pesticides are a diverse group of xenobiotic compounds of widely variety of chemical affinities, ranging from simple inorganic substances to complex organic molecules (Abel, 1998). Pesticides are introduced into aquatic systems by various means: incidentally in the course of their manufacture, and through discharged consequent upon their use. Surface water runoff from agricultural land and side effects of aerial spaying are especially important, and many serious pollution incidents arise through the accidental or negligent discharge of concentrated pesticide solutions. Additionally, many pesticides are deliberately introduced into water bodies to kill undesirable organisms such as insect or mollusc vectors of human diseases, weeds, fish and algae (Abel, 1998).

Many pesticides are known to be refractory to chemical and biological degradation, and their persistence in the environment has for many years been a cause of concern. Probably the best example of this is the well-known case of DDT, which has been used in such enormous quantities in the last 50 years that no part of the world is now free

from measurable contamination, and its manufacture and use in many countries are now banned or severely restricted (Abel, 1998).

Various analytical techniques such as spectrometry, total halogen methods and biological methods were used in the past. The situation was remarkably improved by the introduction of chromatographic methods in the 1960s. In the early period of environmental pollution control, the organochlorine pesticides were probably the most popular group of compounds and were the focus of research efforts of analytical chemists. Because of the highly hydrophobic character and relatively high thermal stability of organochlorine pesticides, GC soon became the method of choice for their determination. Later, the introduction of capillary columns increased its separation ability. Nowadays, the gas chromatography is still an important and widely used instrument for routine pesticide analysis (Liska and Slobodnik, 1996). Chromatographic methods are sensitive and reproducible but also time-consuming and expensive. In addition, there are growing concerns about the safety of the testing procedures. For chromatographic analysis, the amounts of organic solvents used to extract pesticides are generally 10^8-10^{10} times the amount of pesticide detected (Churchill, 2002).

3.1.2 Electronic Nose Instruments

The appearance of new sensor technologies and the strong technological and scientific foundation motivated existing as well as new companies to enter the e-nose market. The new comers with handheld devices have added a new strength to the market (Vanneste and Geise, 2003). In this chapter samples were analysed with three different instruments from three companies: Bloodhound (BH-114), Marconi Applied Technologies (eNOSE 4000) and Nordic Sensor Technologies (NST 3220)

i) Bloodhound Sensors Ltd.

Conducting polymer (CP) sensor research at Bloodhound Sensors began at the University of Leeds, where the company was based. The rather compact BH114 is a qualitative instrument comprising an array of 14 conducting polymer sensors, a specialised electronic data acquisition system. It operates at room temperature, uses

filtered air that passes through the sensor chamber, and the data processing is performed using Microsoft Excel add-ins and specialized add-ins such as Neuralyst.

ii) Marconi Applied Technologies (now EEV Technologies)

Marconi Applied Technologies, a general designer and manufacturer of electronic components, acquired by EEV Chemical Sensor Systems, was formerly known as Neotronics Scientific (Vanneste and Geise, 2003). The eNOSE range of instruments was originally designed for the laboratory-based profiling of samples through measurements of the characteristic headspace. It comprises a humidity sensor, temperature controlled sampling chamber and a 12 CP sensor array. Nitrogen is used to purge the sensor chamber between sample acquisition.

iii) AppliedSensor Group

On December 2000, Nordic Sensor Technology and MoTech announced the merger between them. The new alliance is called AppliedSensor Group (Vanneste and Geise, 2003). The NST 3220 system includes a carousel with 12 positions, allowing heating (up to 65^{o}C) and cooling of the samples. The system is based on one array of 10 MOSFET sensors and one array of 12 MOS sensors. Optional sensors include a CO_2 IR device. The system was conceived for quality control, process control, environmental analysis and medical diagnosis.

3.1.3 Headspace - Solid-Phase Microextraction (HS-SPME)

The various technique of headspace (HS) sampling comprises static and dynamic HS-GC, and SPME. The technique of SPME is a two step procedure applied to static HS-GC. A fused-silica fibre coated on the surface with a stationary phase and mounted on a modified GC syringe is either emerged in a liquid sample or exposed to the headspace above the sample. After achieving equilibrium, normally between 20 to 30 minutes for HS-GC, the fibre is inserted in the GC injection port and the analytes are thermally desorbed and transferred to the GC column.

The SPME technique is mainly applied to aqueous samples rather than organic solvents. The adsorption-equilibrium phase can take place in a temperature controlled room to increase the release of the volatiles and subsequent adsorption onto the fibre, increasing the sensitivity of the method. The desorption from the fibre takes some time, depending on the analyte volatility, desorption temperature and thickness of the coating, which varies from 7 to 100 μm (Kolb, 1999). After desorption, the process is similar to a simple GC analysis, where a choice of the type of column is imperative.

The objectives of the present study were to investigate (a) compare three different e-nose instruments to sample the headspace volatiles originating from both microbial and chemical aqueous solutions and (b) analyse the profiles of volatile metabolites using HS-SPME combined with GC.

3.2 MATERIALS AND METHODS

3.2.1 Cultural media and incubation

The following microbial species obtained from UK National Culture Collections were used in this study: *Enterobacter aerogenes* (88), *Escherichia coli* (10000) and *Pseudomonas aeruginosa* (8672). The fungal species, *Aspergillus fumigatus* and a *Penicillium* species, were isolated from water samples.

i) Bacteria

RO (reverse osmosis) and tap water sterile samples were inoculated with a concentration of 10^2 – 10^8 cells mL^{-1}. For samples inoculated with heavy metals, a mixture of for arsenic, cadmium, lead and zinc (AS, Cd, Pb and Zn) was prepared for each type of water to obtain final concentrations of 0.5 and 2.0 ppm. Samples were incubated at 37^{o}C for 24h.

ii) Fungi

Actively growing cultures of fungal species were used to inoculate sterile RO and tap water with final concentrations of 10^2, 10^4 and 10^5 spores mL^{-1}. These concentrations

were obtained using a haemocytometer and microscope (Olympus, UK). Samples were incubated at 25^{o}C for 24h.

iii) Pesticides

Pesticides were primarily dissolved in methanol and the samples were prepared by adding the DDT and Dieldrin solutions to 10 mL RO water, to obtain a aqueous solution with two different concentrations for each pesticide (10 and 100 ppm). Samples were kept at a constant temperature (25^{o}C) for 1h to equilibrate the headspace.

3.2.2 Gas sampling and sensing procedure

i) E-nose 1 (model BH-114: Bloodhound)

Each sample was connected with a specially made air-filter system with an activated carbon filter to ensure clean air flow above the aqueous solution samples headspace. The carbon filtered air passes through an array of 14 conducting polymer sensors at a flow rate of 4 mL min^{-1} to generate the sensor baseline. A pump was used to draw the sample's headspace into the sensor chamber. The electronic nose was flushed and a one minute interval was allowed between each sample.

ii) E-nose 2 (eNOSE 4000, Marconi Applied Technology)

Samples were placed in a glass sampling vessel on the sampling stage, and allowed to equilibrate at 30°C for 15 min. The headspace from the sample container was analysed using an eNOSE 4000 sensor array, which employs 12 conducting polymer sensors in a temperature-controlled sensor chamber (30^{o}C). Filtered nitrogen (N_2) was also used to purge the sensors. It uses an automated needle to draw the sample headspace into the sensor chamber. The electronic nose incorporates a non-specific array consisting of twelve polypyrrole conducting polymer sensors and is calibrated against a reference standard (20 g of 1:4, deionised water:1,2-propanediol, 99%) with a filtered nitrogen purge (800 ml/min) (Plate 3.1).

Plate 3.1 Photo of the eNOSE 4000.

iii) E-nose 3 (NST 3220, Nordic Sensor Technologies)

The NST 3220 Lab Emission Analyser collects sample gas and exposes it to electronic chemical sensors (10 MOSFET and 12 MOS). The electronic signal from the sensors is transmitted to the pattern recognition software to process the data. It employs an automated robotic needle to draw sample headspace from a sealed container and expose the sample to the sensor array. It also includes an automated carousel tray where the proper sampling vials are inserted (Plate 3.2).

Plate 3.2 NST 3220 Lab Emission Analyser (Nordic Sensor Technologies).

3.2.3 HS-SPME / GC

RO water samples were inoculated with *Pseudomonas aeruginosa* (10^4 cells mL^{-1}), making a final sample volume of 10 mL. An aqueous mixed solution of heavy metals (As, Cd, Pb and Zn) was added to the bacterial treatments to give a final concentration of 0.5 ppm, and incubated for 24h at 37°C. The control samples for each treatment were RO water and RO water with the metal mixture at 0.5 ppm final concentration. Three replicates were used for each treatment.

Solid-phase microextraction (SPME) was performed using a 50/30 μm PDMS/DVB/Carboxen fibre (Supelco). The fibre was exposed to each sample for 30 minutes (Figure 3.1). After exposure, the fibre was desorbed for 4 min at 60°C in the injection port of a Perkin-Elmer gas-chromatograph (GC) equipped with a flame ionisation detector (FID). SPME-GC was carried out on a 30m, 0.32mm i.d. capillary column with a 25μm film thickness (ZB-Wax, Phenomenex Zebron). Helium (He) was used as a carrier gas at a flow rate of 1.5 mL min^{-1}.

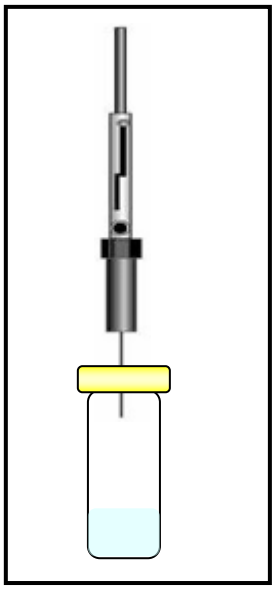


Figure 3.1 Fibre exposed to the sample using a SPME fibre holder (Supelco, Sigma-Aldrich Corporation).

3.2.4 Data analysis

For instrument 1 (Bloodhound BH114), divergence and area were used as normalised data and analysed using the program xlSTAT (Microsoft Excel add-in). For instrument 2 (eNOSE 4000), xlSTAT was also used to analyse the normalised absolute response for the 12 sensors. Principal Components Analysis (PCA), Discriminant Function Analysis (DFA) and Cluster Analysis (CA) techniques were applied to discriminate de contaminants from the control samples. The NST 3220 instrument uses NST Senstool software to acquire data and perform the analysis. The analysis function includes Principal Component Analysis (PCA) and Partial Least Squares (PLS). Data was normalised prior to any analysis.

3.3 RESULTS

From the DFA plots on raw data values based on confidence limit of 95%, it was possible to differentiate between control samples and those containing bacterial cells. Figure 3.2 shows the comparison between the results obtained with two electronic nose systems, the eNOSE 4000 and BH-114, respectively. In both cases the bacterial concentration was 10^4 CFU mL^{-1} with an incubation time of 24 h at 25ºC. A clear separation of the different species was obtained, from the control samples (reverse osmosis water). Figure 3.3 represents the sensor responses of BH-114 to volatiles produced by bacterial cells at a concentration level of 10^2 CFU mL^{-1}, incubated for 24 h at 25ºC. The separation between the different bacterial species is not as clear as when using an initial inoculum of 10^4 mL^{-1}. However, control samples could also be discriminated from those containing bacterial cells.

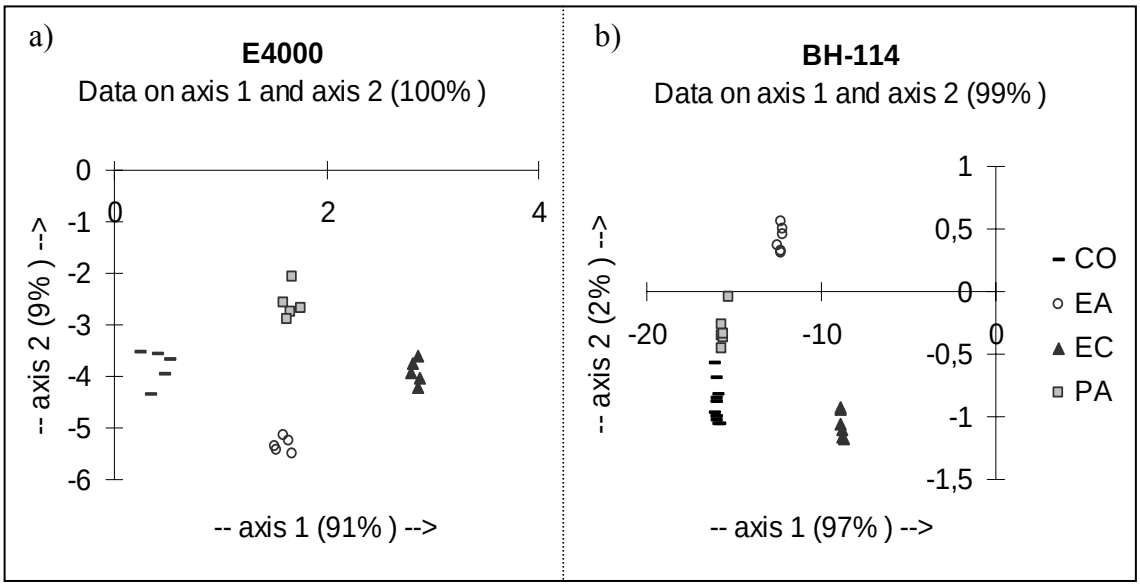


Figure 3.2 DFA based on volatiles produced by 10^4 CFU mL^{-1} bacterial cells in RO water after 24 h growth at 25ºC. (a) Results obtained with the eNOSE 4000 and (b) with the BH-114 electronic nose systems. *Key to treatments*: **CO**, control (RO water); **EA**, *Enterobacter aerogenes*; **EC**, *Escherichia coli*; **PA**, *Pseudomonas aeruginosa*.

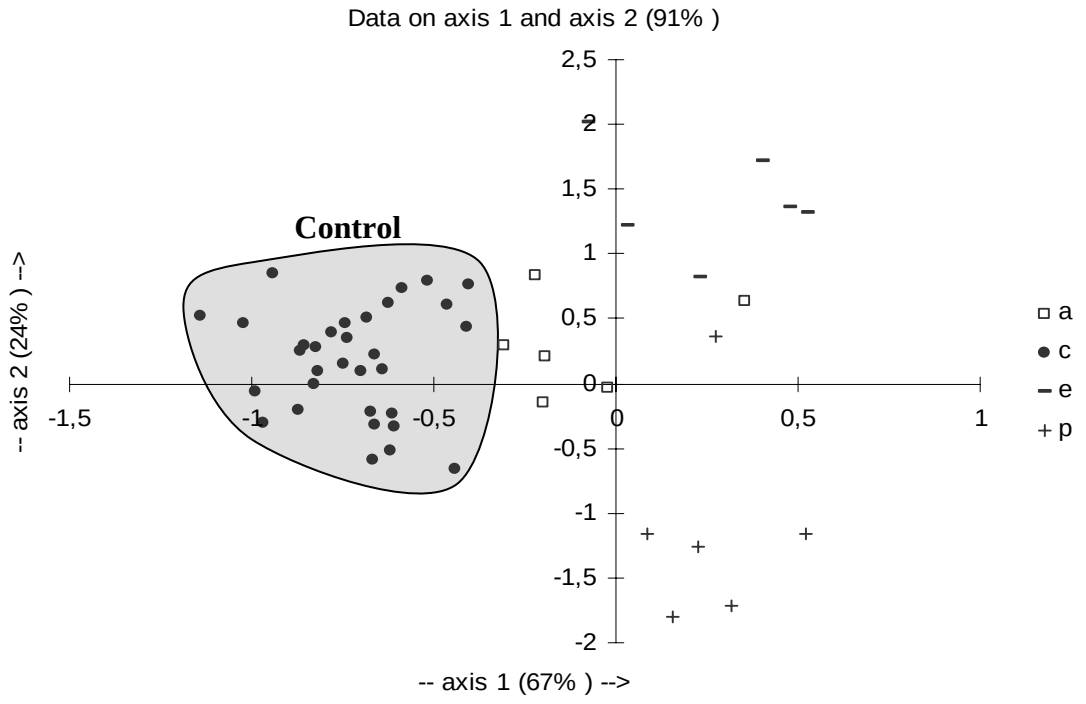


Figure 3.3 DFA of different bacterial species using a concentration of 10^2 CFU mL^{-1} and an incubation time of 24 h, and control samples (RO water). *Key*: **a**, *Enterobacter aerogenes*; **c**, control; **e**, *Escherichia coli*; **p**, *Pseudomonas aeruginosa*.

Figure 3.4 shows the PLS (Partial Least Squares) plot of the MOSFET and MOS sensors profile to *P. aeruginosa* cells (10^2 CFU mL^{-1}) incubated with and without heavy metals for 24 h at 37°C. There is a clear separation between the two sets of data, *P. aeruginosa* with and without metals.

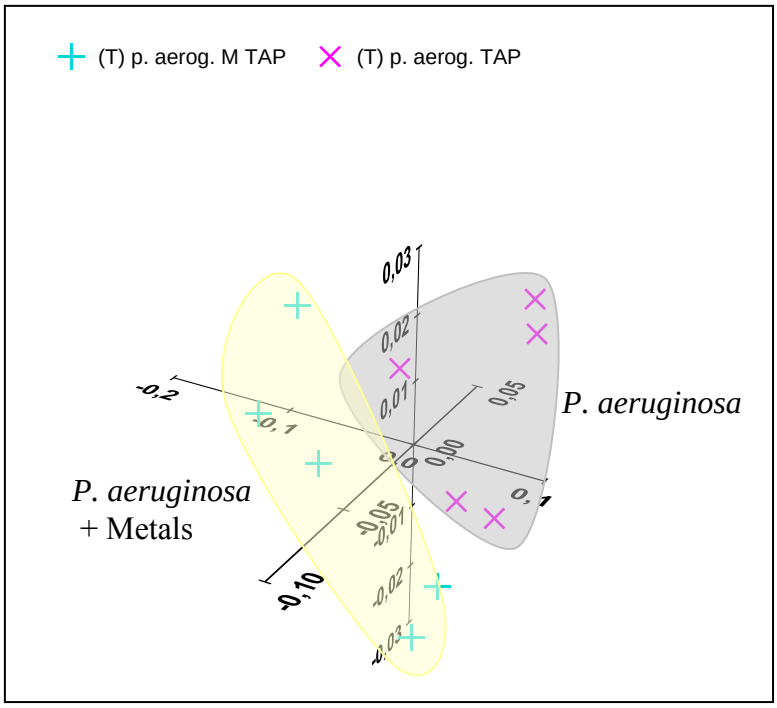


Figure 3.4 PLS of *Pseudomonas aeruginosa* (10^2 cells mL^{-1}) inoculated in tap water with and without metals. The analysis was performed using the e-nose NST 3220.

Figure 3.5 shows the results from experiments done at different times and on different days using the BH-114 e-nose system. Good reproducibility was obtained with *Aspergillus fumigatus* spore loads from the controls. Figure 3.6 is the analysis of the same fungal species (*A. fumigatus*) using a different e-nose instrument, the NST 3220. The plot representing the PLS analysis of MOSFET and MOS sensor profile to tap water samples inoculated with 10^4 spores mL^{-1} shows the separation between the two treatments. Table 3.1 is the confusion matrix of the PLS analysis shown in Figure 3.6. The result indicate that according to the PLS analysis, 40% of samples classified were as the *A. fumigatus* and 60% classified as control were predicted as correct classifications.

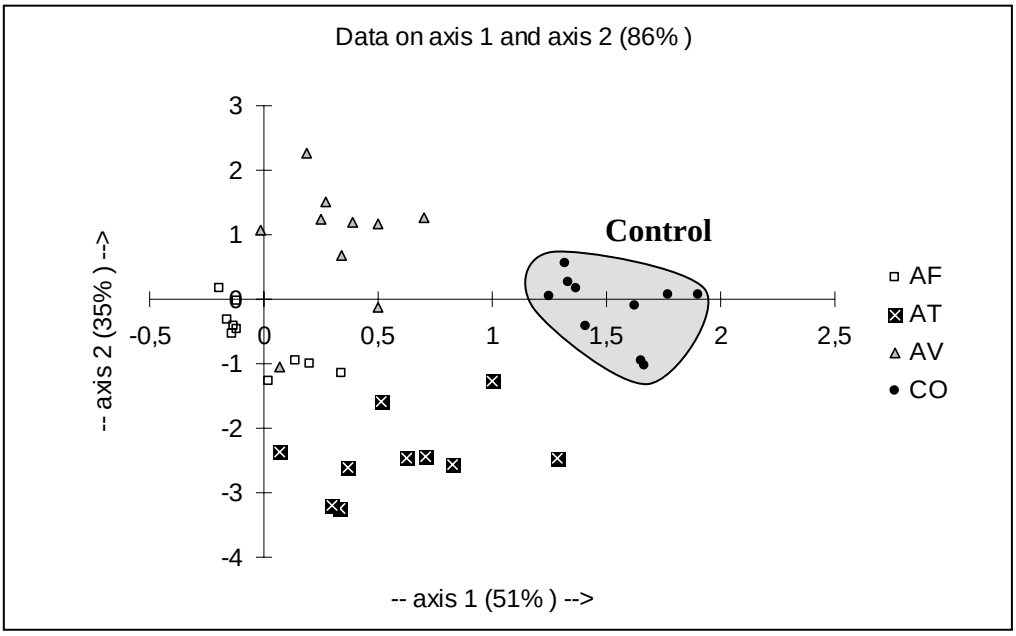


Figure 3.5 DFA of sensor responses to volatiles produced by *Aspergilus fumigatus* spores, and control samples (RO water). *Key to treatments*: **CO**, control; **AT**, 10^2 spores mL^{-1}; **AF**, 10^4; **AV**, 10^5.

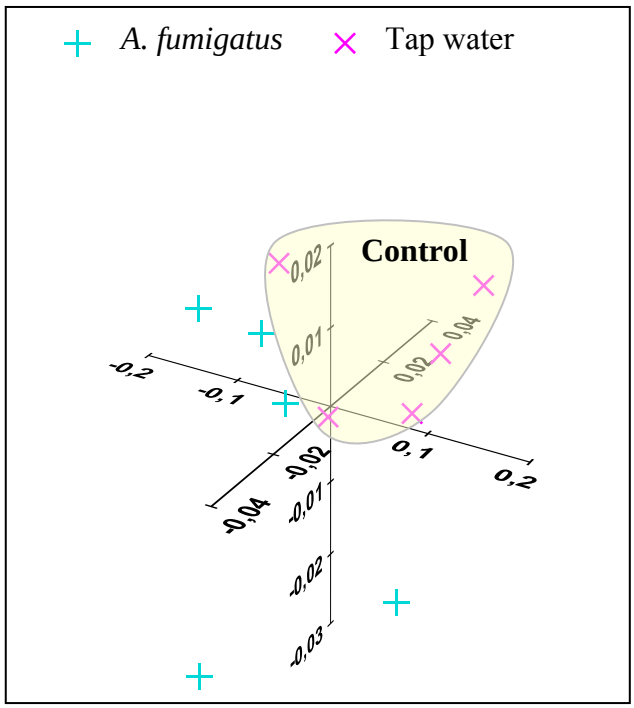


Figure 3.6 PLS analysis of *Aspergilus fumigatus* spores (10^4 spores mL^{-1}) inoculated in tap water and the control samples (tap water). Samples were incubated for 24h at 25°C and analysed using the NST 3220 e-nose system.

Table 3.1 Confusion matrix of PLS analysis observed in Figure 3.6.

	Predicted Class			
Class	*A. fumigatus* (Tap)	Control (Tap)	Undefined	Total
A. fumigatus (Tap)	40% (2)	0% (0)	60% (3)	5
Control (Tap)	0% (0)	60% (3)	40% (2)	5

A good reproducibility is observed in Figure 3.7, where samples of different concentrations of *Pencillium* sp. spores inoculated in RO water were analysed with the BH-114 e-nose system on different days. Discriminant analysis demonstrated that the replicates from different days cluster together according to the respective concentrations, and the control samples are clearly separated from the rest of the samples.

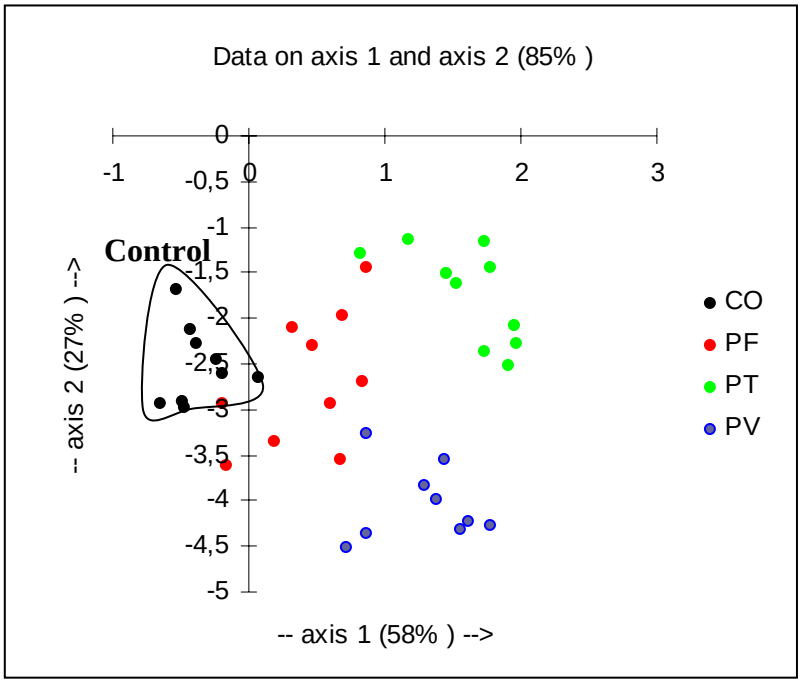


Figure 3.7 DFA plot of *Penicillium* sp. spores inoculated in RO water for 24h at 25oC. The analysis was carry out in different days using the BH-114 e-nose system. *Key to treatments*: **CO**, control; **PT**, 10^2 spores mL^{-1}; **PF**, 10^4; **PV**, 10^5.

Figure 3.8 shows the PCA of two concentrations, 10 and 100 ppm, of DDT and Dieldrin. Regardless of concentrations it was not possible to separate the control samples (RO water) from those containing pesticides.

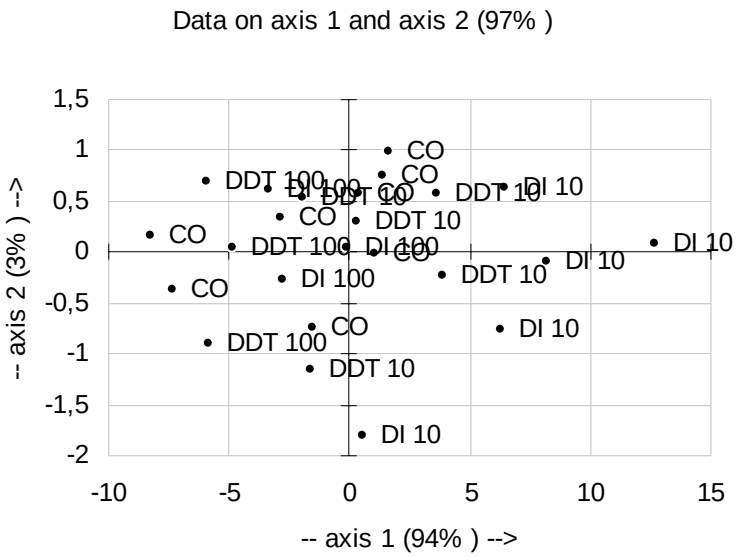


Figure 3.8 PCA plot of two concentrations of DDT and Dieldrin in RO water, and control samples. *Key for treatments*: CO, control; DDT 100, DDT 100 ppm; DDT 10, DDT 10 ppm; DI 100, Dieldrin 100 ppm; DI 10, Dieldrin 10 ppm.

The results obtained with the HS-SPME/GC (Figure 3.9) demonstrated that the volatile profile of each treatment was different according to the different peaks shown in the chromatograph (Appendix A). Peaks highlighted in blue represent samples containing heavy metals, and peaks highlighted in green represent samples containing bacterial cells.

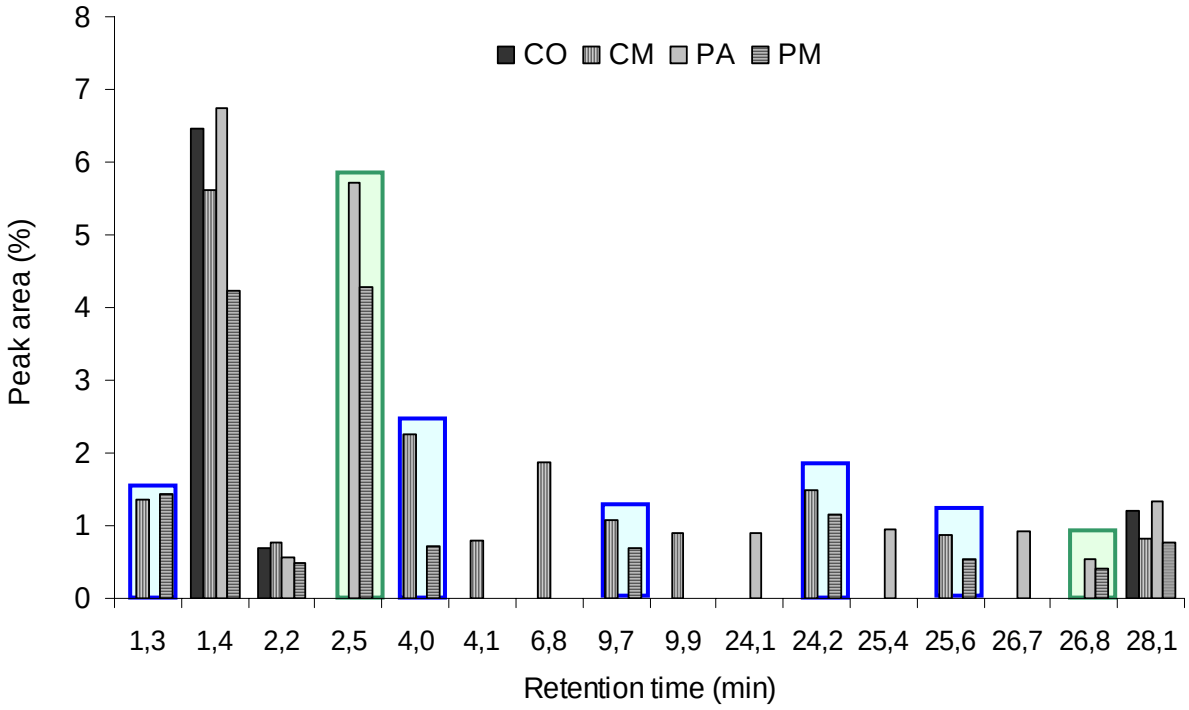


Figure 3.9 HS-SPME/ GC analysis of RO water samples inoculated with *P. aeruginosa* (10^4 cells mL^{-1}) and a mixture of heavy metals (As, Pb and Zn) (0.5 ppm). Samples were incubated for 24h at 37°C. *Key to treatments*: **CO**, control (RO water); **CM**, RO water with metals; **PA**, *P. aeruginosa*; **PM**, *P. aeruginosa* with metals.

3.4 DISCUSSION

In this study, it was possible to compare results from different e-nose systems and different types of sensor arrays. The conducting polymer sensor based ones demonstrated similar results in the analysis of the different bacterial species, at 10^4 and 10^2 cells mL^{-1} incubated for 24h. When water samples inoculated with fungal spores were analysed with both conducting polymer and metal oxide systems, a good separation was observed between the control and the different treatments. This discrimination was confirmed by the different statistical techniques. A good reproducibility was achieved as results from different days were consistent and data could thus be pooled for combined analysis, for bacterial and fungal species.

In the analysis of chemical contaminants, the e-nose was not able to differentiate between pesticide contaminants, regardless of concentration. However, the headspace analysis of samples containing low concentrations of heavy metals using the e-nose system was consistent with the analysis of a more classical technique such as the GC-SPME. In both cases, samples containing the heavy metal mixture could be discriminated from those without heavy metals.

The BH-114 was previously used for the early detection of fungal and bacterial species (Gibson, 1997, Keshri, 2000, Pavlou, 2003, Needham, 2004). The results indicated that the headspace analysis of liquid and solid growth medium inoculated with different microorganisms could be discriminated from the control samples. The other conducting polymer based e-nose instrument was previously used to assess odour from sewage treatment works. The results demonstrated the ability of the e-nose to respond to sewage odours and suggest the potential application of such an instrument to monitor odour emission in a sewage works, as well as assessing the efficiency of odour abatement units (Stuetz *et al*., 1999). Haugen (2001) used an NST 3220 instrument for the discrimination of three different disinfection–resistant bacteria (*Pseudomonas*, *Cedecea* and *Serratia*) as well as a mixture of all three strains cultured in milk. Only the *Pseudomonas* culture showed clear differences from the others.

This is the first time that different benchmark e-nose systems were compared under the same environmental and sampling conditions. Nake (2004) compared two portable e-nose systems, one based on conducting polymer sensors (Cyranose 320, Cyrano Sciences) and one based on metal oxide semiconductor sensors (Pen-2, WMA Airsense Analysentechnik), for outdoor air monitoring of sewage odours directly in the field. The conducting polymer based system was demonstrated to be more dependent on humidity, temperature and day of analysis than the metal oxide system. Overall, the experiments show that the metal oxide semiconductor e-nose instrument appears to be more suitable to outdoor monitoring.

There are no reported studies on the use of the e-nose system for the detection of trace levels of pesticides in water. Alternatively, Mallat (2001) reviewed the development of

immunosensors in the last few years and their practical contribution to environmental analysis with particular emphasis on monitoring of pesticides such as atrazine, simazine, paraquat, alachlor, 2,4-D and isoproturon in water. The immunosensor system detection mechanism is based on a solid-phase fluoroimmunoassay combined with an optical transducer chip chemically modified with an analyte derivative. Quintana (2001) developed a method to monitor pesticides in drinking and related waters in Spain using a multiresidue SPME/ GC-MS. The method was subjected to intra- and inter-laboratory tests and the detection limit was as low as 0.025 μg L^{-1} in water samples.

The solid-phase microextraction technique coupled with different chromatographic techniques has been applied for the detection of water contaminants (Gardner *et al*., 2000; Shang-Da *et al*., 2004; King *et al*., 2004; Monteil-Rivera *et al*., 2004). Centineo (2004) developed a simple and rapid multielemental speciation method to determine simultaneously various organometallic compounds of mercury, lead and tin in natural water samples. The analytical method consists on the ethylation and simultaneous headspace-solid phase microextraction (HS- SPME) of the derivatives and final gas chromatographic-mass spectrometric (GC–MS) analysis. After optimization, detection limits in the low ng l^{-1} level, linearity over three orders of magnitude and repeatability in the range of 3–20% were achieved for all compounds under study. Julák (2003) applied head-space solid phase microextraction combined with gas chromatography for the determination of bacterial volatile fatty acid (VFA) patterns. It was confirmed that VFA production depends on the composition of the cultivation medium, which limits accurate characterisation of particular bacterial species. The described method is suitable for preliminary detection of bacteria, particularly non-sporulating anaerobes, in clinical samples. Analysis can be performed in 30 min without the need for cultivation.

CHAPTER 4.

AT-LINE MEASUREMENTS OF CONTAMINATION EPISODES

4.1 INTRODUCTION

The quality of water depends mainly on its origin. For water analysts, the most important analytical aspect is the source and location of water sampling. Based on this fact, the types of water existing in the environment can be broadly classified into the following categories (Tomar, 1999):

1. Surface water: collected as runoff from rainwater in ponds, rivers, lakes. Another source is the sea. Seawater is referred to as marine water.
2. Ground water: collected underground by seepage of rainwater and surface water through soil.
3. Potable water: water suitable for human consumption.
4. Wastewater: a by-product of various domestic and industrial activities.

Potable water is drinking water and the most important material for human consumption. Hence, it must be free from any sort of contamination, undesirable substances, and pathogenic bacteria hazardous to health and environment (Tomar, 1999). Potable or drinking water can be defined as the water delivered to the consumer that can be safely used for drinking, cooking, and washing. In other words, potable water must meet the physical, chemical, bacteriological, and radiological parameters when supplied by an approved source. It must also be delivered to a treatment and disinfection facility of proper design, construction and operation, and be delivered to the consumer through a protected distribution system (Csuros and Csuros, 1999).

Drinking water contains organic and inorganic matter, which can accumulate to the surface of the drinking water pipelines. The preservation of the biological stability of potable water during its storage in reservoir or its transport through the distribution system can be achieved by (a) the use of chemical disinfectants (in particular by addition of chlorine) which is the widely used technique, or (b) the use of new techniques such as nanofiltration, that can eliminate bacteria and significantly decrease the concentrations of organic matter at the inlet of the distribution network and in the potable water. The formation and quality of these soft deposits mainly depends on the

microbiological and chemical quality of the produced drinking water and on the circumstances prevailing in the distribution system (Sibille, 1998; Zacheus, 2000).

Microbes, such as bacteria, fungi and protozoa, which have survived purification, can easily penetrate into a drinking water distribution network. Some microorganisms, particularly heterotrophic bacteria can adapt to this environment despite the presence of chlorine, and colonise the entire distribution system by the formation of biofilms.

Sibille (1998) estimated that the density of bacterial cells (dead and alive) in suspension in water measured by epifluorescence, varied from 5 $x10^3$ to 10^6 cells ml^{-1}. A wide number of bacterial species were identified, including *Pseudomonas* sp., *Escherichia coli*, *Enterobacter* sp., *Citrobacter* sp. and *Klebsiella*. The presence of organic matter and a low amount of disinfectants results in an increase of bacterial concentrations and subsequently in the manifestation of odours and flavours. The distribution network is not only a water transport system, it is also a bioreactor in which all the parameters including the presence of microorganisms and organic matter need to be considered. In order to evaluate such a complex structure it is essential to implement a monitoring system to test the microbiological quality of potable water (Sibille, 1998).

Monitoring is defined by the International Organisation for Standardization (ISO) as: "the programme process of sampling, measurement and subsequent recording or signalling, or both, of various water characteristics, often with the aim of assessing conformity to specified objectives". This general definition can be differentiated into three types of monitoring activities that distinguish between long-term, short-term and continuous monitoring programmes as follows (Bartram and Balance, 1996):

- Monitoring is the long-term, standardised measurement and observation of the aquatic environment in order to define status and trends.
- Surveys are finite duration, intensive programmes to measure and observe the quality of the aquatic environment for a specific purpose.
- Surveillance is continuous, specific measurement and observation for the purpose of water quality management and operational activities.

The new water-related environmental legislation in Europe clearly defines a need for regular monitoring of a wide, and ever growing, range of organic substances down to low nanogram per litre levels. Since many of the so-called priority substances to be monitored in water are rather difficult to analyse, European expert groups are working on the implementation of the Water framework Directive (2000/60/EC, 2000) to put a considerable effort into proposing a range of techniques and methods for their monitoring. True enforcement demands more frequent monitoring of water catchment areas and also industrial plants need greater control of their wastewater to meet the demands of increased regulation (Tschmelak, *et al*., 2004). The most effective means of assuring drinking water quality and the protection of public health is through the adoption of a preventive approach that includes all steps in water production from catchment to consumer (Sinclair and Rizak, 2004).

Because of the difficulties in isolating and identifying a broad spectrum of organisms with widely differing growth characteristics, little information is available in the literature to identify all heterotrophic organisms found in water supplies. Despite the developments of traditional techniques such as HPLC, GC-MS and the recommended microbiological methodologies, such techniques are still time consuming and require expensive reagents and expert technicians. It is important not to forget the heavy use of solvents involved in appropriate disposal since they are also water pollutants, putting a question mark on their purpose has environmental techniques.

Electronic noses are normally used to give qualitative answers about the sample studied and only in special cases to predict the concentration of individual species in the sample. In terms of the quality of drinking water the electronic nose system could be used to classify it, rather than tell what is making a specific sample undrinkable. The objective of this chapter was to show how the use of the e-nose could be applied for at-line measurements of contamination episodes with bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) in water samples over periods of 1-2 hrs.

4.2 MATERIALS AND METHODS

4.2.1 Microbial species

Actively growing cultures of two bacterial species were used in this study, *Escherichia coli* (10000) and *Pseudomonas aeruginosa* (8672). The bacterial cells were grown in nutrient broth for 24h at 37^{o}C. RO (reverse osmosis) and tap water sterile samples were inoculated with a concentration of 10^6 bacterial cells mL^{-1}. The final concentration was obtained by correlating the absorbance (640 nm, visible light) of different concentrations of cells and the actual number of colonies using the spread plate technique in nutrient agar.

4.2.2 Sampling procedure and Electronic nose analysis

The experiment was set up using a large conical flask where the "affluent" containing the contaminated water was constantly agitated by placing on a magnetic stirrer platform. Plastic tubing conducted the affluent through a peristaltic pump, set to a speed of 2 mL min^{-1}, into the sampling vial (Figure 4.1). Every 5 minutes a sample was collected with approximately 10 mL per vial. At time 0, the affluent contained sterile water or control water as referred in the figures. After 25 minutes or 5 sample collections, the affluent was changed for a container with a final bacterial concentration of 10^6 bacterial cells mL^{-1}. After 45 minutes downstream the affluent was changed again, this time adding more sterile water with an estimated volume so that the final concentration changed from 10^6 to 10^2 bacterial cells mL^{-1}. This period is also detailed as a dilution event in the figures. The monitoring lasted for a total of 70 minutes during which 15 samples were taken and transferred to the e-nose for analysis.

Samples were placed in the NST 3220 Lab Emission Analyser carousel. As mentioned in chapter 3, this electronic nose is equipped with 10 MOSFET sensors, 12 MOS sensors and a CO_2 sensor. It employs an automated robotic needle to draw sample headspace from a sealed container, sampling vial, and expose the sample to the sensor

array. Data were analyzed using principal component analysis (PCA), partial least squares (PLS) and cluster analysis (CA).

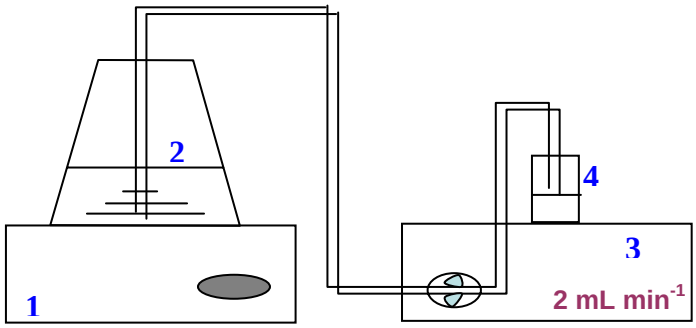


Figure 4.1 Schematic representation of the sampling procedure. *Key*: 1, Stirrer; 2, conical flask with the "affluent"; 3, Peristaltic pump; 4, Sampling vial.

4.3 RESULTS

The results obtained from the analysis of the e-nose demonstrated that it was possible to distinguish between the different phases of the experiment, from a high contamination to a low contamination. Figures 4.2 and 4.3 represent the experiment on RO and Tap water respectively, with *E. coli* cells. The three dimensional graph representation enables the visualization of the three types of axes corresponding to the first 3 principal components. In RO water, there is a good separation of the highest level of contamination, 10^6 cells mL^{-1}, and it is possible to observe that the "clean" water is well separated from the other samples at the top of the figure. In tap water, a similar result was observed, however, the separation between the samples containing bacterial cells and those with "clean" water is more evident compared with the experiment using RO water. In both experiments, it is possible to observe that the *E. coli* cells could be discriminated from the initial first stage of the experiment where only sterile water was sampled.

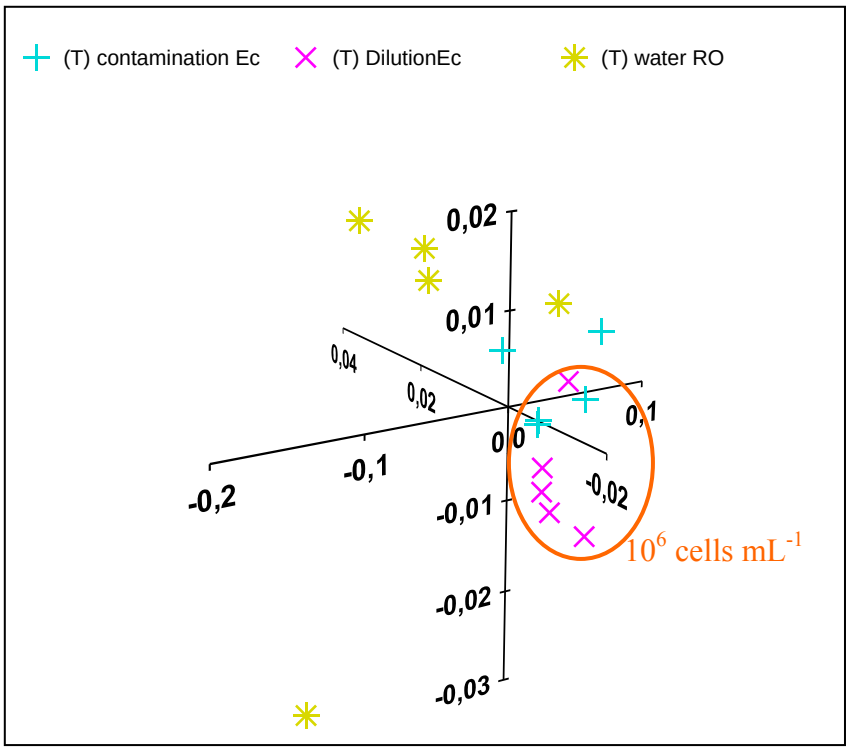


Figure 4.2 PCA map using principal components 1, 2 and 3 of *E. coli* contamination in RO water. *Key*: Contamination Ec, 10^6 cells mL^{-1}; Dilution Ec, 10^2 cells mL^{-1}.

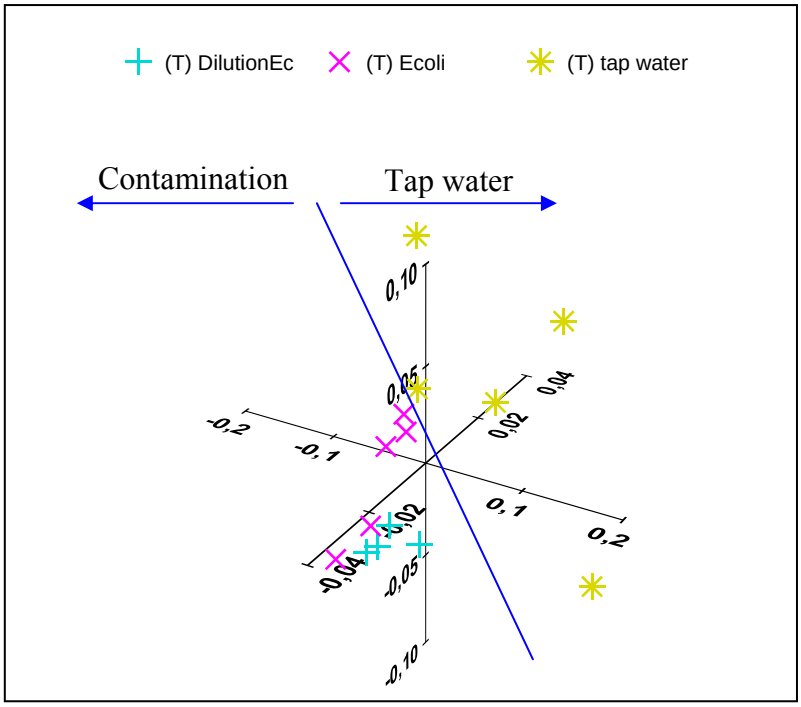


Figure 4.3 PLS map using principal components 1, 2 and 3 of *E. coli* contamination in tap water. *Key*: Ecoli, 10^6 cells mL^{-1}; Dilution Ec, 10^2 cells mL^{-1}.

Figure 4.4 represents the CO_2 sensor response over time of the experiment conducted in tap water using *E. coli* cells. The line indicates the trend of the average CO_2 content of the different samples over time. There was an increase in the CO_2 level when the affluent was changed from sterile tap water to that contaminated with 10^6 cells of *E. coli* mL^{-1}. Another stage is observed when the level of CO_2 decreases after a certain volume of sterile water is added in a second stage and the bacterial contamination is reduced to 10^2 cells mL^{-1}. This indicates that monitoring the CO_2 level is a good indication of the microbial population of a given sample.

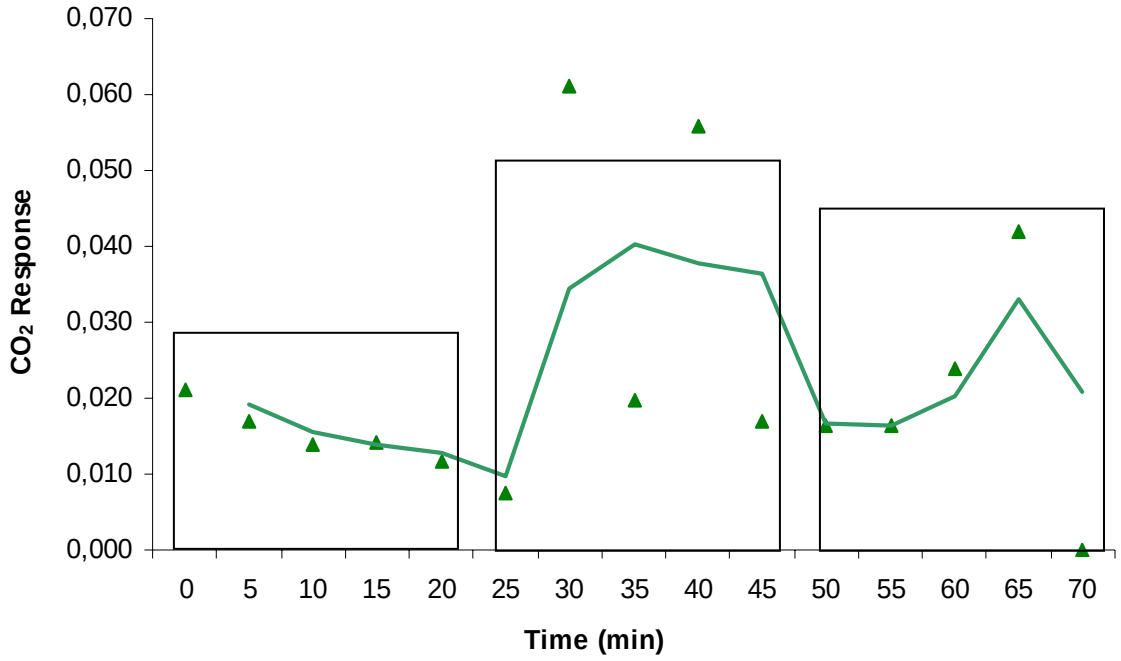


Figure 4.4 CO_2 levels in a sample of tap water inoculated with *E. coli*. The line represents the trend of the mean sensor response.

Figures 4.5 represents the PLS analysis of an at-line monitoring study where sterile tap water was contaminated with *P. aeruginosa*. A good discrimination was observed not only in PC1 but also in PC2. PC1 was responsible for 88.9% of the variability in the data discriminating two different groups of samples, tap water and that contaminated with 10^6 cells of *P. aeruginosa* mL^{-1}. PC2 accounts for 8.5% of the variability, in this case the separation occurred between the diluted samples (10^2 cells mL^{-1}) and the rest of the samples.

Table 4.1 is the confusion matrix of the PLS analysis shown in Figure 4.5. The results according to the PLS analysis, showed that 60% of samples were classified as tap water, 60% as 10^2 cells of *P. aeruginosa* mL^{-1}, and 80% classified as 10^6 cells of *P. aeruginosa* mL^{-1}, were predicted as correct classifications.

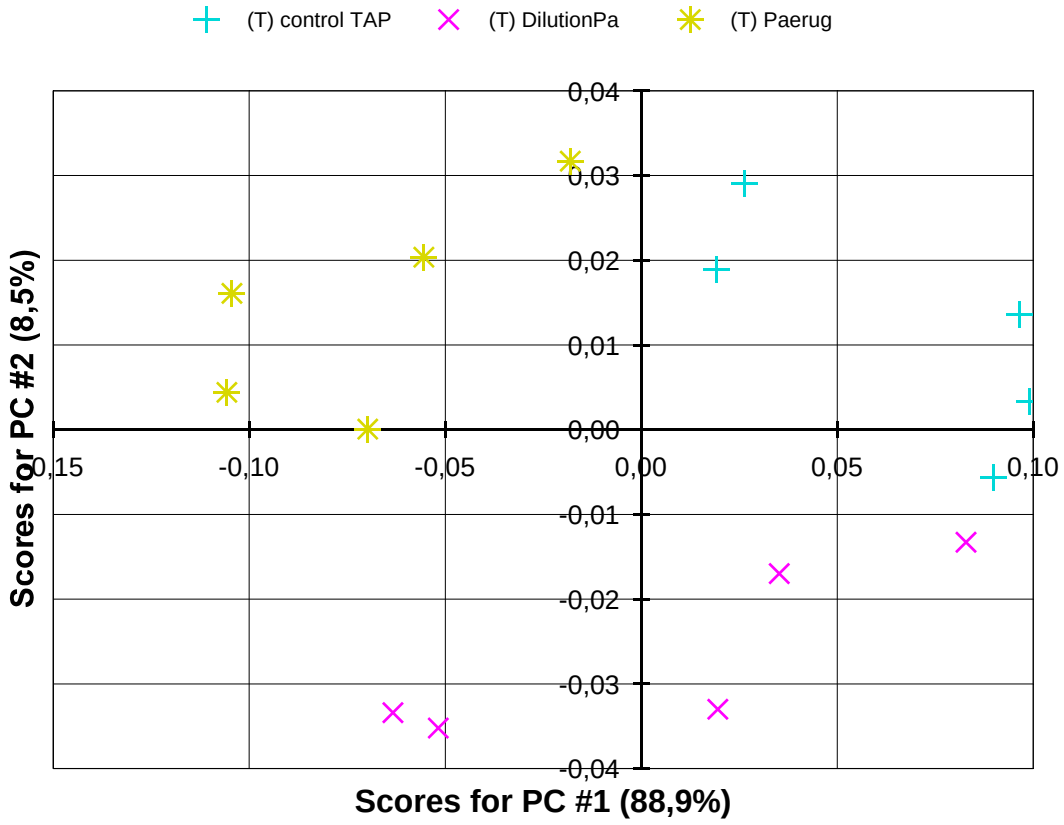


Figure 4.5 PCA map using principal components 1 and 2 of *P. aeruginosa* contamination in tap water. *Key*: Paerug, 10^6 cells mL^{-1}; Dilution Pa, 10^2 cells mL^{-1}; Control TAP, tap water.

Table 4.1 Confusion matrix of PLS analysis observed in Figure 4.5.

	Predicted Class				
Class	Control Tap	Dilution Pa	Paerug	Undefined	Total
Control Tap	60% (3)	0% (0)	0% (0)	40% (2)	5
Dilution Pa	0% (0)	60% (3)	0% (0)	40%(2)	5
Paerug	0% (0)	0% (0)	80% (4)	20% (1)	5

Figure 4.6 shows a three-dimensional representation of the PCA analysis of at-line monitoring study using RO water and *P. aeruginosa* cells, using the three principal components, PC1, PC2 and PC3. Although there was no clear separation between the two groups of samples containing different concentrations of bacterial cells, the control samples, RO water, could be discriminated from those where the bacterial cells were present.

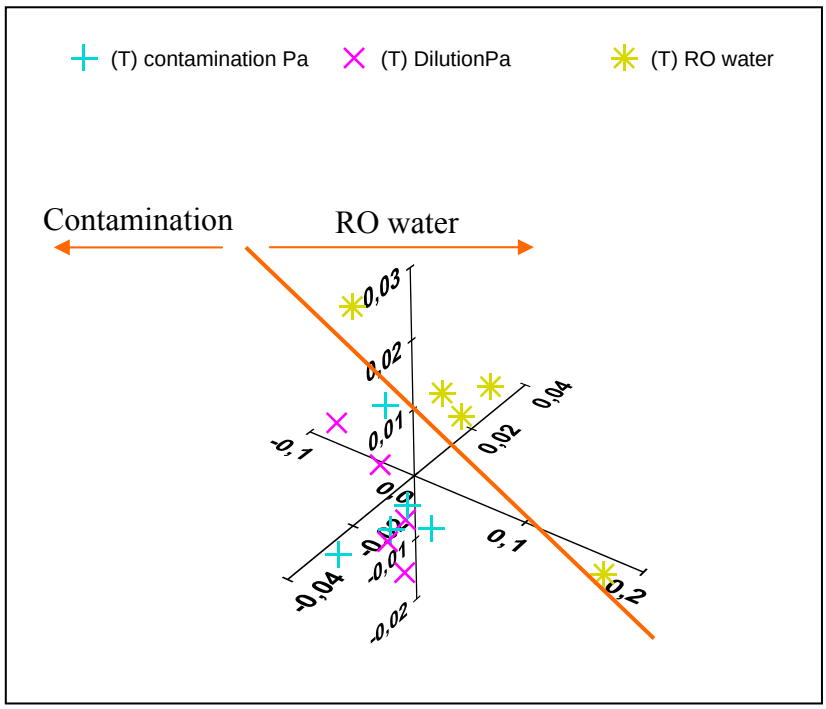


Figure 4.6 PCA map using principal components 1, 2 and 3 of *P. aeruginosa* contamination in RO water. *Key*: Contamination Pa, 10^6 cells mL^{-1}; Dilution Pa, 10^2 cells mL^{-1}.

Figure 4.7, represents the sensor response of one oxide based sensor, MO110, to the changes occurring in an "affluent", for the four types treatments. It is possible to observe that the sensor's response to *P. aeruginosa* inoculated in tap water is higher than the other treatments. The oscillation in the curve is proportional to the concentration of bacterial cells present in the water samples.

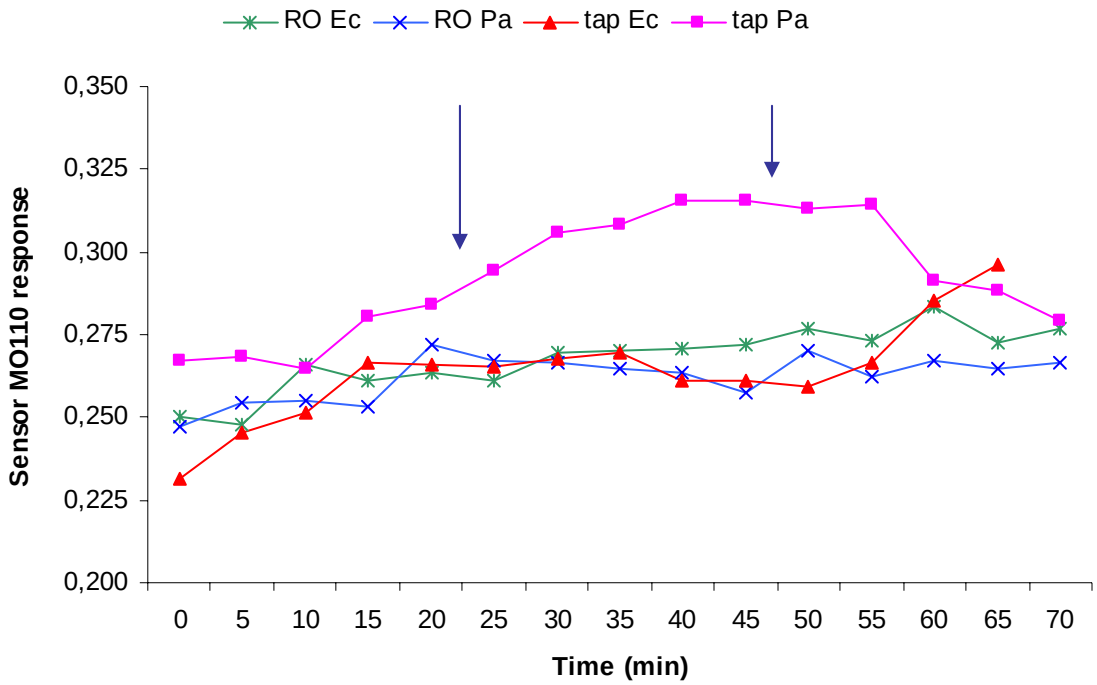


Figure 4.7 Response of a metal oxide sensor, MO110, to the different types of contamination episodes over time. The arrows indicate the time where there was a change in the affluent. *Key*: RO Ec, *E. coli* in RO water; RO Pa, *P. aeruginosa* in RO water; tap Ec, *E. coli* in tap water; tap Pa, *P. aeruginosa* in tap water.

In order to better understand the level of discrimination observed in the four types of at-line monitoring studies in tap water, i.e. two types of contaminants, *E. coli* and *P. aeruginosa*, and two different concentrations, 10^6 and 10^2 cells mL^{-1}, cluster analysis was performed based on the Mahalanobis distance ($p< 0.05$) and represented has a dendogram (Figure 4.8). The cluster analysis representation shows that there was a good separation between the four types of samples, control or tap water, dilution 10^2 bacterial cells mL^{-1}, 10^6 *P. aeruginosa* cells mL^{-1}, and 10^6 *E. coli* cells mL^{-1}. The data used in this analysis was obtained on different days indicating that good reproducibility was achieved using this e-nose instrument.

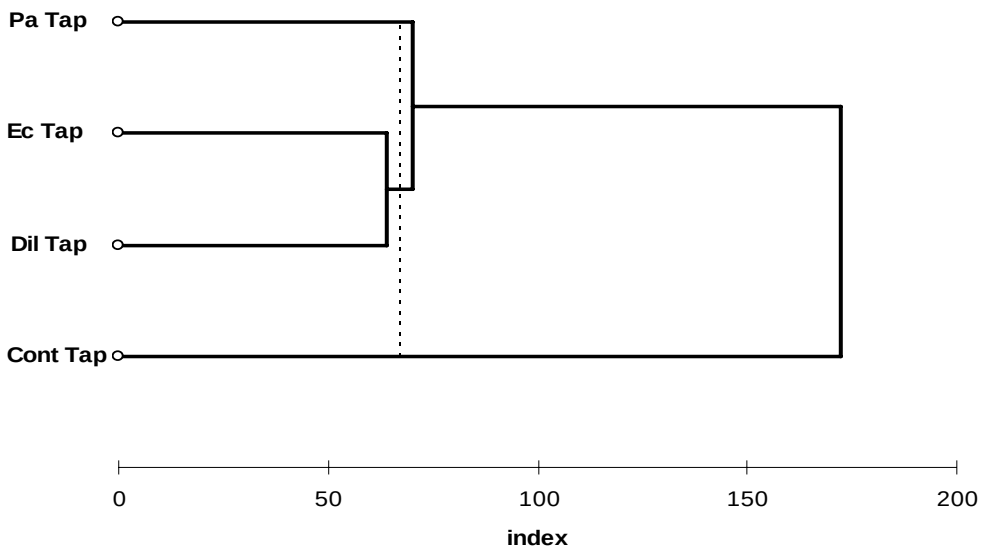


Figure 4.8 Dendogram of the cluster analysis of two types of contamination episodes, one with *E. coli* and the other with *P. aeruginosa*, both using tap water as matrix. *Key*: Pa Tap, 10^6 cells mL^{-1} of *P. aeruginosa*; Ec Tap, 10^6 cells mL^{-1} of *E. coli*; Dil Tap, 10^2 cells mL^{-1} of *P. aeruginosa* and *E. coli*; Cont Tap, sterile tap water.

4.4 DISCUSSION

There is an increasing interest in rapid environmental monitoring of low threshold levels of microbial contaminants, for at- or on-line monitoring of water matrices. Potential exists for using non-specific sensor arrays for detection of contamination episodes. The results presented in this chapter enhances the potential of this technique in the detection of low concentrations of bacterial cells in water samples, including *E. coli*, a member of the coliform group.

The PCA and PLS maps based on the sensor response profiles, indicate a good discrimination between samples containing *E. coli* actively growing cells from non-contaminated samples. CO_2 is also a good indicator of the presence and metabolic rate of microorganisms. Several studies use this technique with a gas chromatograph to evaluate the growth of microorganisms, including fungi and bacteria. Here the results

show a significant increase of the CO_2 level for samples containing 10^6 cells mL^{-1}, and a subsequent decrease when the concentration changed to 10^2 cells mL^{-1}.

For the contamination of both RO and tap water with *P. aeruginosa* actively growing cells, the PCA map of *P. aeruginosa* in tap water reveals that PC1 (88.9%) shows a gradual separation of the different contamination stages. The non-contaminated samples are present in the (x,y) quadrant, the 10^2 cells mL^{-1} are located in the (x,-y) quadrant, and finally the highest contamination level is located in the (-x,-y) quadrant. According to the confusion matrix of the PLS analysis 60 to 80% of the predicted measurements were correctly classified.

Overall, all the data analysis techniques showed a good discrimination between the contaminated and the non-contaminated samples. Differentiation of the different levels of contamination based on the Mahalanobis distances between the former clusters show that it was even possible to differentiate the different levels of contamination. Although the two water matrices were sterile, it was possible to perceive the differences in the headspace volatile profile for each bacterial species in both environments.

The literature shows that the electronic nose systems could be used for the applications in environmental monitoring of wastewater treatment plants, sewage sludge and landfill odours. Bourgeois (2003), developed an on-line measurement system consisting of an array of conducting polymer sensors and a headspace generating flow-cell, tested for continuous analysis of water and wastewater both in a laboratory and in the field at a small wastewater treatment plant. It is also suggested that the sensor array technology could be further developed for non-invasive monitoring above a process stream. To provide an early warning system at the inlet of a treatment works, reducing the chances of pollutants entering the treatment plant preventing the occurrence of tastes and odours in drinking water, or affect the performance of a treatment process.

An e-nose based on a non-specific conducting polymer array was also used to monitor chlorophenols in water samples. The experimental parameters studied were: sample volume, platen temperature, sample equilibration time, loop fill time, sample pressurization time and injection time. Data analysis was carried out using principal

component analysis (PCA) and artificial neural networks (ANNs) to predict the chlorophenols presence in water samples. The obtained results showed that it was possible to differentiate between five chlorophenol groups: monochlorophenol, dichlorophenol, trichlorophenol, tetrachlorophenol and pentachlorophenol (Vasquez, *et al*., 2004). The e-nose system, based on an array of six metal oxide semiconductor (MOS) sensors, has also been used to monitor not only different strains but also the growth phase of cyanobacteria (i.e. blue-green algae) in water over a 40-day period. The results showed that the potential application of neural network based e-noses to test the quality of potable water as an alternative to analytical instruments that are based on chromatographic or microscopic techniques (Gardner, *et al*., 2000).

The development of instruments with the potential to mimic human senses has increased in the last decades. The latest one is the electronic tongue, mainly in the food industry but showing also some interest in areas such as the environmental monitoring. Krantz-Rülcker (2001), reviews the different types of electronic tongues and taste sensors for environmental monitoring purposes, more specifically the performance of multielectrode arrays used for voltammetric analysis of aqueous samples of a drinking water production plant. The main objective of the taste sensors is to detect rather specific chemical compounds that give rise to different taste variables. An electronic tongue is a more general array device describing qualities or quality changes, as well as taste if possible, in foodstuff, raw materials and polluted waters.

Alternatives for environmental monitoring include a novel analytical system AWACSS (Automated Water Analyser Computer Supported System) based on immunochemical technology has been evaluated to measure several organic pollutants at low nanogram per litre level in a single few-minutes analysis without any prior sample pre-concentration or pre-treatment steps. Immunochemically based techniques are a popular alternative to standard methods, however, most of the commercially available techniques suffer from not fully described cross-reactivity of target analytes, matrix effects, limited availability of antibodies and not having capability of multi-analyte analysis (Tschmelak, *et al*., 2004). Meays (2004), reviews some of the molecular and biochemical methods and techniques that are being developed to track sources of bacteria in water and food. Such methodologies are in current use for source tracking

faecal bacteria in the environment, for example: ribotyping, pulse-field gel electrophoresis, denaturing-gradient gel electrophoresis, repetitive DNA sequences (Rep-PCR), host-specific 16S rDNA genetic markers, and antibiotic resistance analysis.

There is a general concern on the use of traditional microbiological techniques, such as membrane filtration and heterotrophic plate count. Most of them based on the fact that these methodologies do not take into account the presence of non-heterotrophic bateria, and are very laborious and are not rapid enough to prevent against an outbreak. A study by Hoefel (2003) reveals that raw and potable water analysis with direct viable count and culture-based techniques reported significantly fewer viable bacteria compared to the number of physiologically active bacteria detected with a new technique using the BacLight™ bacterial viability kit and carboxyfluorescein diacetate (CFDA) coupled with a flow cytometry (FCM). Few bacteria in potable waters examined were culturable by heterotrophic plate count (HPC), even though FCM assays reported between 5.56×10^{2} and 3.94×10^{4} active bacteria ml^{-1}.

The potential for the electronic nose to contribute for an at- or on-line monitoring of water quality is creating new possibilities for a rapid, non-invasive and simple monitoring technique. Further studies are needed to increase the confidence of the application of the e-nose instrument, not only of the legislative authorities but also in the private industry.

CHAPTER 5.

STUDIES ON SOIL

5.1 INTRODUCTION

The increase in regulations and greater awareness of the potential risk caused by soil pollution has highlighted the need for a rapid, reliable and accurate evaluation of the soil environment. Pollution processes may be intentional such as landfill and spray irrigation of sewage, or unintentional, such as spills or leaks (Mirsal, 2004).

5.1.1 Sewage Sludge Amended Soil

Of all urban sources contributing to soil pollution, waste and sewage sludge disposal occupies a central role on this environmental problem. Major urban inputs to sewage water include household effluents, drainage water, business effluents (e.g. car wash, dental uses), atmospheric deposition, and traffic related emissions transported with storm water into the sewage system. Wastewater treatment plants are expected to control the discharge of heavy metals to the environment. However, wastewater treatment systems are designed for removal of organic matter by activated sludge microorganisms, and removal of heavy metals in these systems may be regarded as side-benefits. As a result, effluents from municipal wastewater treatment plants (WWTPs) may contain metals at concentrations above background levels since heavy metals present in the influent wastewater concentrate in sludge (Karvelas, 2003).

The disposal of sewage sludge poses a great problem, since in almost all developed countries the disposal of this sludge by dumping at the sea is being phased out, and the principal method of disposal is now shifting to land use. In fact, the mere use of sludge to amend soils is an “advantageous” process itself. It adds essential organic matter as well as useful nutritive elements like phosphorous and nitrogen to the soil. Wastes such as sewage sludge are not designed for agricultural use, because they contain some level of industrial and commercial discharge and are of variable and unpredictable composition. It is also predictable that they may contain toxic contaminants to soils or the environment in the short or long term. In the case of sewage sludge applied to farmland, research emphasis has been placed on a few heavy metals, those considered

most likely to produce toxic effects on plants, animals and humans (McBride, 2003; Mirsal, 2004).

Heavy metals, which are normally concentrated in the sewage sludge, may accumulate within soil and eventually could be taken up by food crops such as leafy vegetables, which are known to preferentially take up cadmium. Heavy metals in soil can be very toxic at very low concentrations and are in the position to modify the nature of some key biochemical reactions of the soil biota. Contrary to synthesised organic molecules, these elements exist naturally and are permanent, playing a role in the biogeochemical cycles. They represent a component of soil quality because they participate in significant soil functions: ecosystem functioning, substrate of plant production and filters for entry to aquifers. Essential trace elements are also important micronutrients. However, as soon as their concentration in an organism exceeds a certain threshold, they can also become toxic. Considering more particularly soil ecosystems, excessive concentrations of trace elements induce dysfunctions of a biological type (Cambier, 1998).

Environmental impact by heavy metals was earlier mostly connected to industrial sources. In recent years, metal production emissions have decreased in many countries due to legislation, improved cleaning technology and altered industrial activities. Today, major industrial sources include surface treatment processes with elements such as Cu, Zn, Ni and Cr, as well as industrial products that, at the end of their life, are discharged in wastes (Karvelas, 2003).

5.1.2 Microbial Soil Activity

In recent years, the assessment of the effect of soil pollution on indigenous microorganisms has received considerable attention. Even if chemical measurements are more and more versatile, accurate and sensitive, they do not provide direct information regarding the biological effect of toxic compounds, nor about the available concentration for microbial biodegradation (Addondanzi, 2003). Soil monitoring is the regular surveillance and quantification of the amount of pollutants present in a given

location of soil. It should be carried out in a way that enables the detection of spatial as well as time variations in the concentration of pollutants at the site of investigation (Mirsal, 2004). Every plant and every living organism inhabiting a part of the terrestrial environment is, in one way or another, a product of this environment, reflecting the natural conditions prevailing.

Heavy metals in soil exert deleterious effects on the metabolic function of the soil microorganisms, which is one of the most important characteristics in soil. This can either take place via general decomposition activity, whereby organic substrates are broken down into their inorganic constituents, or more specifically, via symbiotic relationships involving the direct exchange of inorganic nutrients for photosynthetic carbohydrates between the microorganism and its host plant, respectively. The addition of heavy metals like Cd, Cr, CU, Ni, Pb and Zn to soil significantly inhibits soil respiration, nitrogen mineralization, and nitrification (Hattori, 1992; Obbard, 2001). However, Moreno (2002) reported that organic amended increased activity and diversity of soil microbial populations, and over time microbial tolerance to heavy metals can be observed.

The presence and activity of the microbial component of surface and subsoil can be detected and measured in many ways, including microbial numbers, microbial biomass, functional activity (such as respiration and N mineralization) and enzyme activities. Several soil bioassays have been developed, such as measurements of total soil biomass, growth, ATP, microbial sensitivity, nitroredutase, esterase, and dehydrogenase activity, oxygen uptake rates, and luminescence in pure cultures or degrading consortia. Toxicity tests involve the exposure of the organism or culture cell lines from different organs and species to toxic compounds under defined experimental conditions (Taylor, 2002; Addondanzi, 2003).

The quantitative and qualitative recovery of microorganisms from environmental samples is essential to understand ecosystem function. A number of binding forces need to be overcome in order to reduce cell-soil associations and allow extraction of microbial cells. Chemical (anionic detergents, ion-exchange resins) and physical

(shaking, blending, ultrasonication) dispersion treatments are often used but even with exhaustive multi-stage extractions, large proportions of bacterial populations remain associated with soil particles. Additionally, some of the dislodged bacteria cannot often be grown on conventional media (Taylor, 2002).

Enzyme activities in soil can be associated with active cells (animal, plant, microbial and entire dead cells). The microbial oxidation of organic substances under aerobic conditions is linked to a membrane-bound electron transfer chain with O_2 as a final electron acceptor. NADH is the form in which electrons are collected from different substrates through the action of NAD-linked dehydrogenases. The electrons are further transferred to the cytochrome system, where they are oxidized by O_2. As dehydrogenases are not active independently of the parent microbial cell as extracellular enzymes in soil, then measurement of the dehydrogenase activity is a good overall indicator of microbial activity (Obbard, 2001). Taylor (2002), reviews a number of methods evaluated to measure and compare microbial presence and activity in surface and subsurface soils. The methods included culturable bacterial and fungal counts, direct counts of total bacteria, DNA extraction and quantification, and arysuphatase, dehydrogenase, fluorescein diacetate hydrolysis, b-glucosidase, phosphomonoesterases and urease activities.

The aim of this chapter was to evaluate, analyse and discriminate between soil samples contaminated with heavy metals, by examining the volatiles produced in soil microcosmos by the microbial biomass. The studies were carried out using enzymatic assays and electronic nose systems.

5.2 MATERIALS AND METHODS

5.2.1 Artificially contaminated soil in a semi-closed static microcosms

Uncontaminated sandy loam soil with no history of heavy metal contamination was used. The soil was sieved to obtain a homogeneous texture. 10g of soil were

moisturized with 1 mL of sterile RO water and incubated over night at 25ºC. Zinc sulphate ($ZnSO_4$) and cadmium sulphate ($CdSO_4$) (Sigma) were added to the soil to obtain concentrations of 3 and 100 ppm. Co-contaminated samples were also tested using a mixture of both heavy metals at the same concentrations. The experiment included three treatments and a control, each in triplicate. To recreate microcosms, the vials had a cap with a septum to allow for gas exchange, and placed in a closed box together with a beaker with water at 25ºC (Figure 5.1). The experiment duration was 40 days; soil samples were taken from each treatment for biological and e-nose analysis after 3 hrs, 12 days and 40 days after initial contamination.

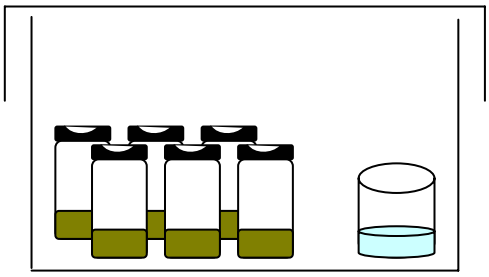


Figure 5.1 Representation of the microcosms kept at 25ºC.

5.2.2 Naturally contaminated soil

This experiment included 9 amended soil types supplied by the Rothamsted Research (Harpenden, UK). Table 5.1 summarizes the characteristics of each sample.

Table 5.1 Description of the characteristics of each soil type used in this study.

Soil No.	Sample ID	Soil pH	Total N (%)	WHC (%)	Total Zn (mg/Kg soil)	Total Cd (mg/Kg soil)
2	Avonmouth 1998 (UK)	4.34	0.316	42	517.92	5.55
3	Brauschweigh 1998 2/26 (Germany)	5.87	0.106	36	180.01	1.19
6	Brauschweigh 1998 2/22 (Germany)	5.13	0.109	34	64.73	0.33
11	BZL	4.43	0.275	28	3540.15	55.86
12	Chinese paddy soil	7.69	0.198	38	91.12	0.11
14	Gleadthorpe treatment 1 (UK)	5.28	0.118	34	59.09	0.23
16	MAAS 1	7.39	0.190	45	618.22	5.16
23	Shipham 1998 (UK)	6.51	0.843	86	27413.39	314.81
24	St. Laurant 98	6.74	0.259	43	27099.75	166.54

5.2.3 Enumeration of viable microorganisms

Heterotrophic soil bacterial and fungal populations were counted on agar-based media. A soil suspension was prepared with 1g of soil and 9 ml of sterile water, and vigorously shaken with a vortex. Ten fold dilutions of the soil suspensions, from 10^{-1} to 10^{-5} were prepared using an aliquot of 200 µl to spread plate. Bacterial colony-forming units (CFU) were grown on nutrient agar (Difco) supplemented with 0.5 g cyclohexamide (Sigma). Fungi were determined on malt extract agar (MEA, Difco), with 0.5 g chloramphenicol (Sigma). Bacterial colonies were counted after 3 days and fungal colonies after 7 days of incubation in the dark at 25^{o}C. All counts were done directly in appropriate dilutions of the extracts and the are expressed in a logarithmic scale per gram of soil.

5.2.4 Total Protein

Protein extraction was performed with the addition of 4 mL of phosphate buffer pH 6.5 (K_2HPO4 and KH_2PO4), to 1g of each soil samples. Samples were placed in a rotary shaker and incubated for 1 h at 4ºC. After incubation, the samples were centrifuged for 6 min at maximum speed and the clear supernatant was collected. For the determination of total protein, Sigma procedure TPRO-562 was used. The supernatant collected from protein extraction was incubated at 40ºC for 30 min with a reagent solution prepared with bicinchoninic acid and copper (II) sulphate (pentahydrate). An albumin solution was used for the calibration curve in a concentration range from 0 to 200 μg mL^{-1}. Absorbance was measured at 530 nm.

5.2.5 Dehydrogenase Activity

The microbial activity of the soil biomass was determined by measuring dehydrogenase activity (DHA) according to the method reported by Von Mersi and Schinner (1991). Using 0.25 g of soil, to determine the reduction of *p*-iodonitrotetrazolium chloride (INT) to *p*-iodonitrotetrazolim formazan (INF). The method was performed in different stages; first, the soil samples are mixed with the substrate (INT) and incubated in a buffer solution. Secondly, the final product is extracted with the addition of N,N-dimethylformamide and finally the product of the reaction (INF) is measured with a colorimetric method. Soil DHA was expressed as gINTF g^{-1} soil $2h^{-1}$.

The INT solution (9.88 mM) was prepared by dissolving 500 mg of INT (Acros) into 2 ml of N,N-dimethylformamide (Fluka), followed by the addition of 50 ml of distilled water. The solution was sonicated for 2 minutes and water was added to bring the volume up to 100 ml. The solution was stored in the dark and always used in the same day. Two batches of samples were prepared, one of them used as a control. For *in vitro* and *in situ* studies, 0.25g of soil of each sample were used and incubated in a solution of 375 μl of Tris-HCl buffer (1M, pH 7.0) (Sigma) and 500 μl of INT at 37^{o}C in the dark, for 2h. For the control batch, the soil was autoclaved for 30 minutes at 121^{o}C before the addition of the buffer and the INT solutions. After the incubation period, samples were vigorously mixed with 2.5 ml of extraction solution ethanol: N,N-dimethylformamide

(50:50 v/v). The samples were kept in the dark and shaken every 20 minutes for 1 h. After filtration, the INF was measured at 434 nm against the control using an UV spectrophotometer.

The calibration curve was prepared using a standard solution of 100 μg INF ml-1. A range of volumes of the standard solution were added to 2.5 ml of extractant solution, 0.25 ml of RO water and 0.375 ml of Tris buffer, in order to obtain a concentration range between 6.25 to 200 μg INF. The absorbance was read at 434 nm.

5.2.6 Headspace Analysis

The NST 33120 instrument was used to analyse the soil samples. After each incubation period, the sample vials were directly placed in the carrousel tray. The e-nose was used not only to detect differences in the sensor responses between treatments and the control, but also to determine the CO_2 response. Data were treated with the incorporated software for PCA and PLS analysis.

5.3. RESULTS

5.3.1 Enumeration of CFUs

Figure 5.2 shows the mean of three sub-cultures of the colony forming units (CFUs) for bacteria and fungi grown in agar-based medium for each soil type. Soil sample number 0 was used as a control and is the same soil type used for artificially contaminated studies. The total bacterial population was higher than the number of fungal species in almost all the soil types, except for the highly contaminated samples (2 and 23) with both Zn and Cd. This may be an indication that fungal species are more resistant to such levels of contaminations.

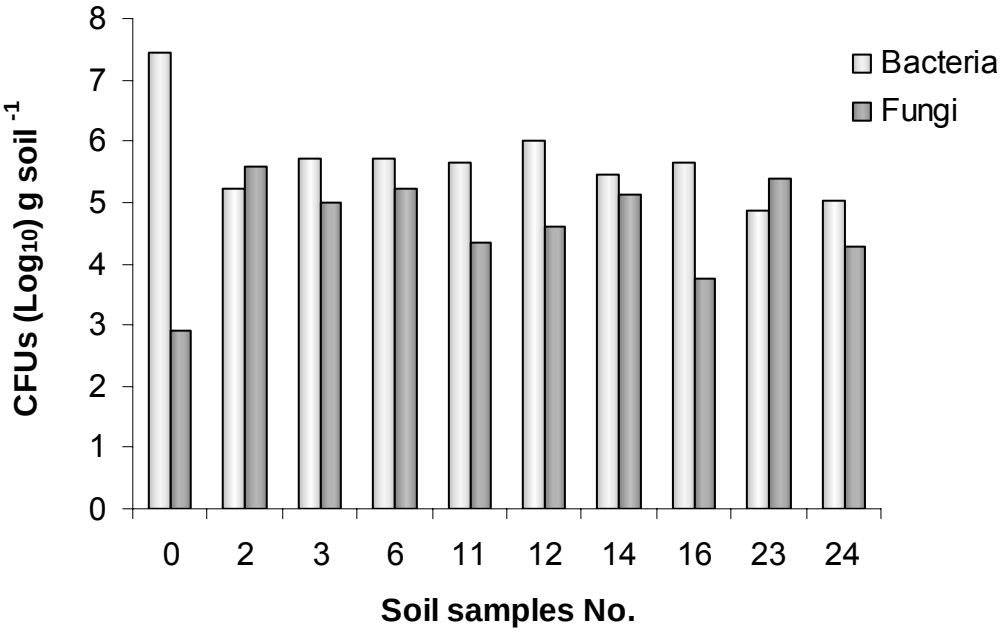


Figure 5.2 Number of CFUs per gram of soil for each soil type, for bacterial and fungal species.

Figure 5.3 shows the interaction between the number of bacteria and fungi and the heavy metals present in the soil. It is clear that in the case of bacterial species, the number of CFUs decreases with an increase in the concentration of metals. On the other hand, for fungal species there was no clear trend on the way that either the concentration of Zn or Cd affects the population.

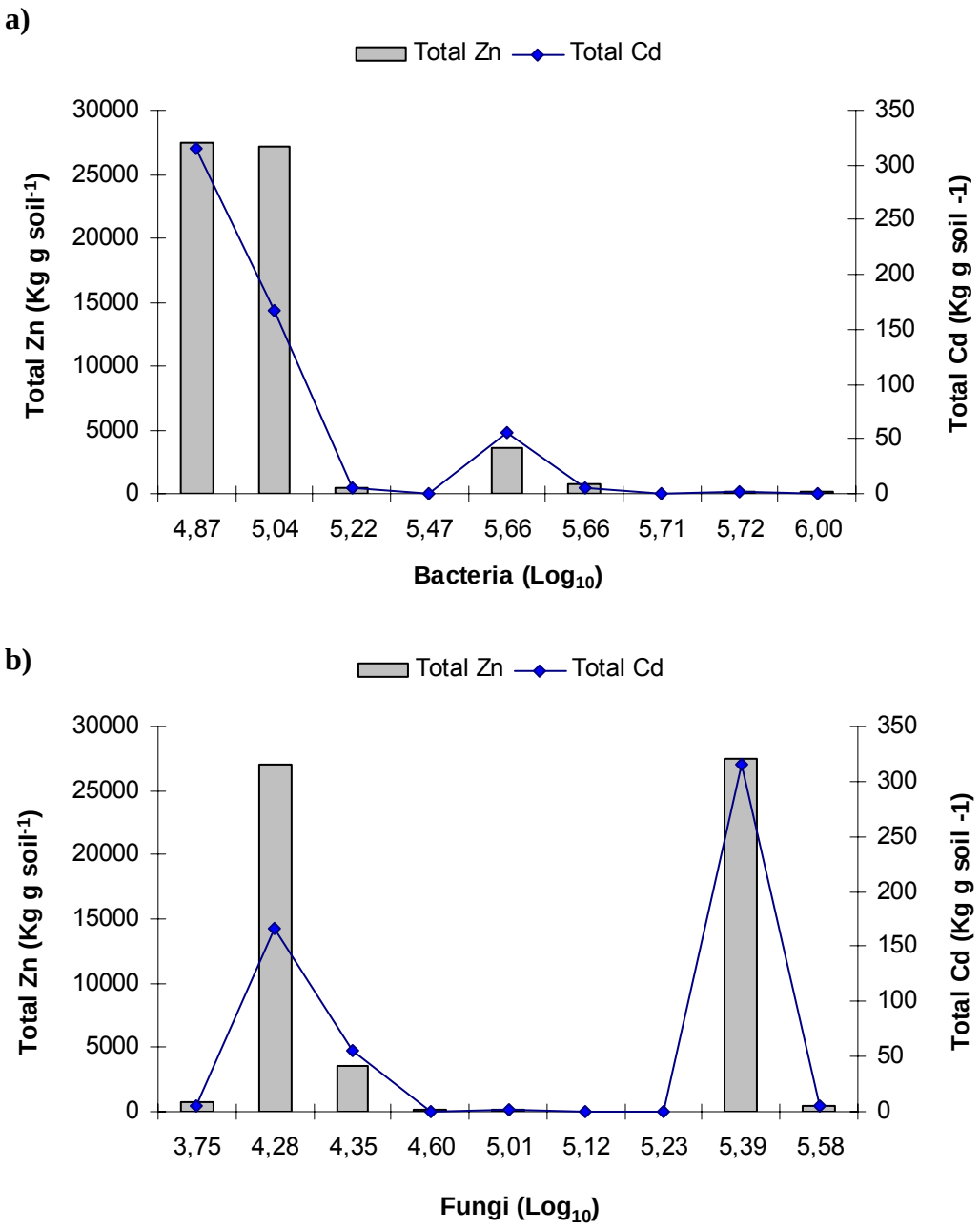


Figure 5.3 The graph represent the microbial population of (a) bacteria (b) fungi, present in the soil samples with the corresponding concentration of Zn and Cd.

5.3.2 Total Protein

Figures 5.4 and 5.5 represent the calibration curve and the concentration of total protein in μg per g of soil sample, respectively.

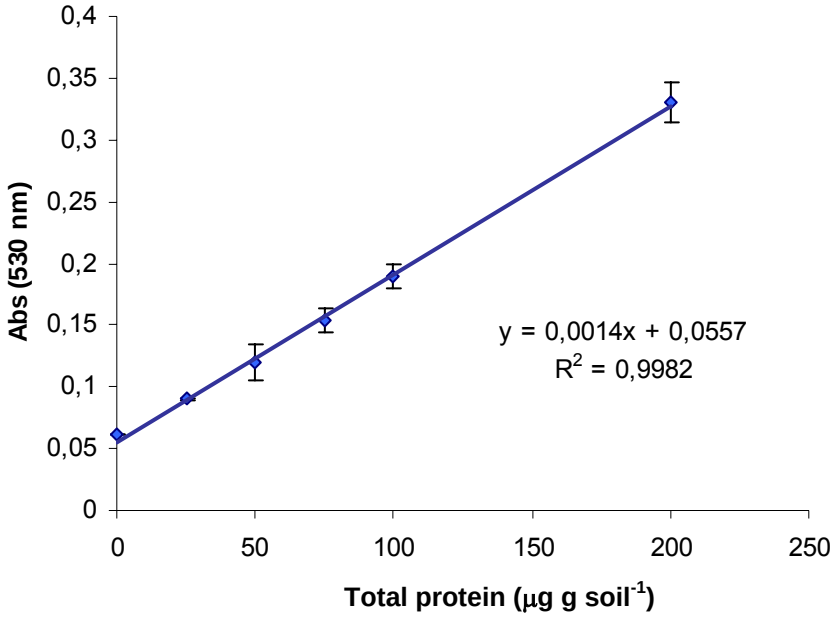


Figure 5.4 Calibration curve obtained from the incubation of an albumin solution with the reagent for 30 minutes at 40°C, the absorbance was measured at 530nm. The vertical bars represent the standard deviation (n=5).

Figure 5.6 shows the relationship between the total protein in each soil type and the population of bacteria and fungi for each soil type. This suggests that there is no direct association between the number of bacterial cells and the total protein. However, the number of fungal species seems to increased together with an increase in the concentration of total protein.

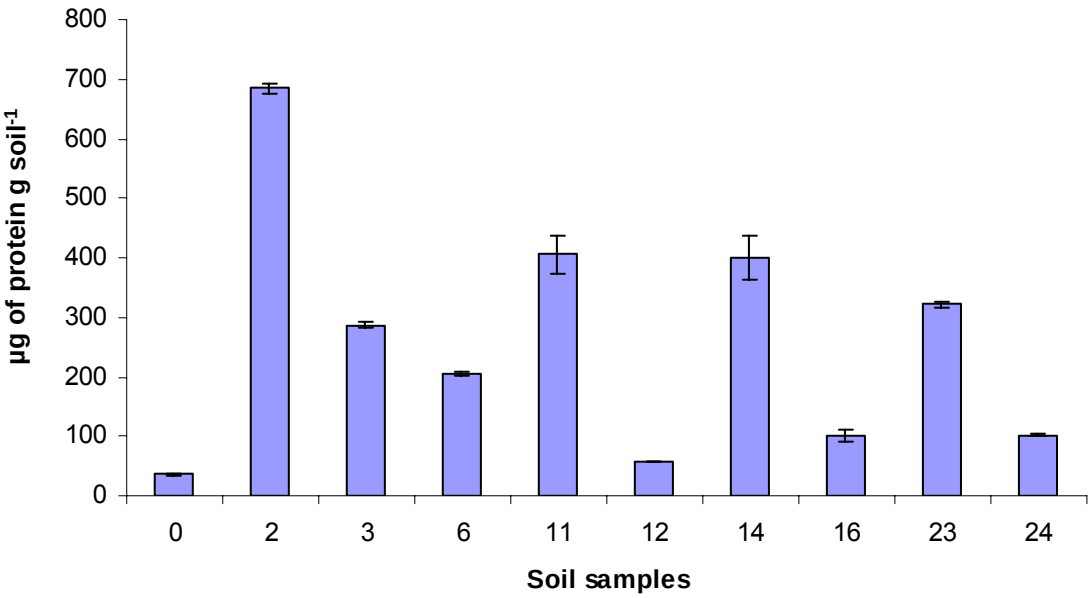


Figure 5.5 Concentration of the total protein extracted from samples of naturally contaminated soil studies. After extraction samples were incubated with the reagent solution for 30 minutes at 40oC and the absorbance measured at 530nm. Vertical bars represent the standard deviation (n=3).

Figure 5.6 Relationship between the total protein and the population of bacteria and fungi per g of soil.

Figure 5.7 relates total protein heavy metal concentration in soils. This pattern is similar to that of the fungal population (see Figure 5.3.)

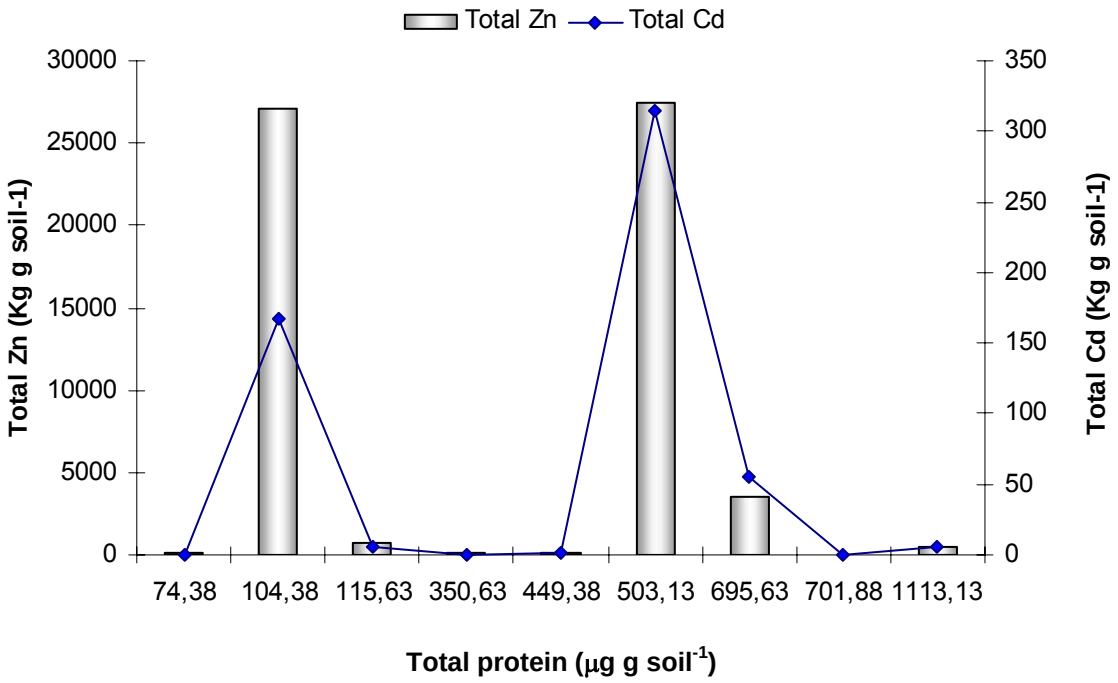


Figure 5.7 Relationship between the total protein and the concentration of heavy metals (Zn and Cd), present in soil samples.

5.3.3 Dehydrogenase Activity

Dehydrogenase activity was assessed using the reduction of INT into INF as an artificial electron acceptor. Figure 5.8 shows the calibration curve used for the determination of the final concentration of INT produced during each assay.

i) Artificially contaminated soil microcosms

The different treatments consisted of two heavy metal, Cd and Zn at two concentrations, 3 and 100 ppm, and co-contamination with a mixture of both metals. Figure 5.9 demonstrates that an increase in metal concentration is related with a decrease in the dehydrogenase activity or, a decrease in microbial activity. Cadmium is the element that most affects DHA in comparison with zinc. However, the co-contaminated samples

seem to have a profile that relies between those of the isolated metals, higher than cadmium and lower than zinc.

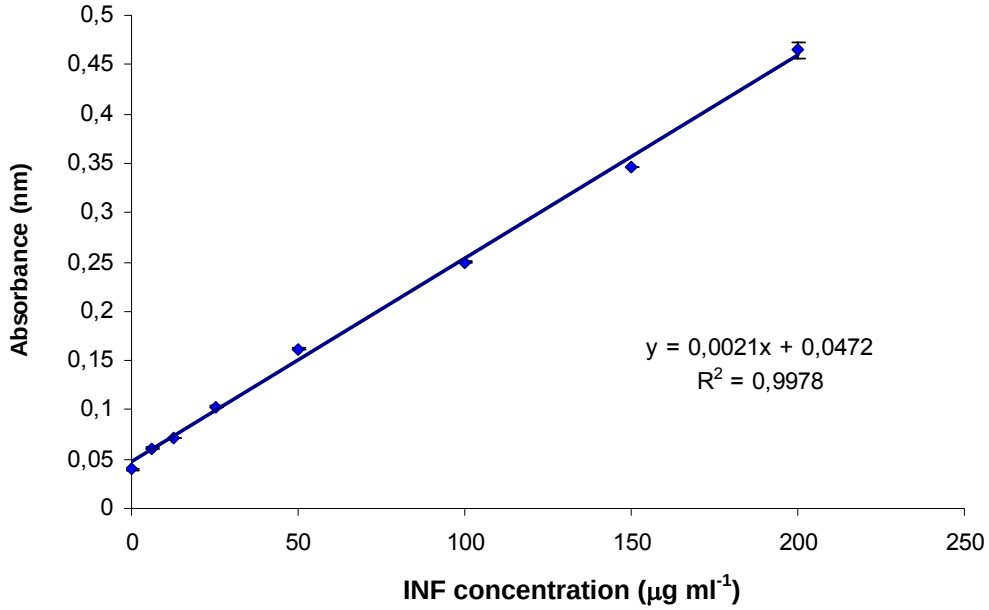


Figure 5.8 Calibration curve obtained with an INT solution with a concentration range from 0 to 200 µg ml^{-1}. The vertical bars represent the standard deviation (n=5).

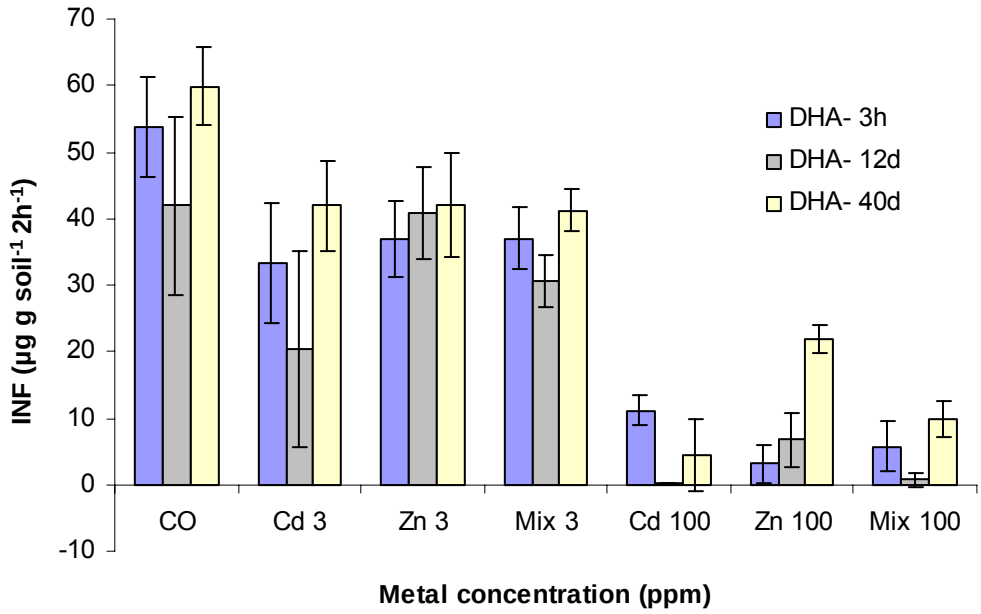


Figure 5.9 Dehydrogenase activity of soil samples incubated with 3 and 100 ppm of heavy metals (Cd, Zn and a mixture of both). The experiment period was 40 days at 25°C and samples were taken after 3h, 12 and 40 days after initial contamination. Bars represent the standard deviation (n=3).

Table 5.2 lists the results of the Fisher test for the different treatments used for the determination of DHA. The control samples are significantly different from those containing heavy metals independently of the element and concentration. Samples inoculated with the same concentration are not significantly different from each other, which means that the concentration is more important than the type of heavy metals present in the soil sample.

Table 5.2 Fisher test scores of the different treatments. Red number indicate a significant difference, with $p < 0.05$.

Fisher test; variable DHA Probabilities for Post Hoc Tests Error: Between MS = 26.206, df = 12.000, α = 0.05

	Sample	{1}	{2}	{3}	{4}	{5}	{6}	{7}
1	CO		0.000449	0.014742	0.002912	0.000000	0.000000	0.000000
2	Cd 3	0.000449		0.077015	0.311334	0.000036	0.000268	0.000039
3	Zn 3	0.014742	0.077015		0.397583	0.000003	0.000014	0.000003
4	Mix 3	0.002912	0.311334	0.397583		0.000008	0.000050	0.000009
5	Cd 100	0.000000	0.000036	0.000003	0.000008		0.225245	0.960450
6	Zn 100	0.000000	0.000268	0.000014	0.000050	0.225245		0.243036
7	Mix 100	0.000000	0.000039	0.000003	0.000009	0.960450	0.243036	

ii) Naturally contaminated soil

The assay for the determination of DHA was performed in moisturised soil samples, after 24h incubation at 25ºC. Figure 5.10 shows the results of the DHA analysis. Figure 5.11 represents the relationship between the DHA of each sample and the corresponding concentration of total protein per g of soil. Since these two parameters are related with the enzymatic activity of the microbial species, there should have the same profile, however, this figure is not conclusive in terms of such relationship.

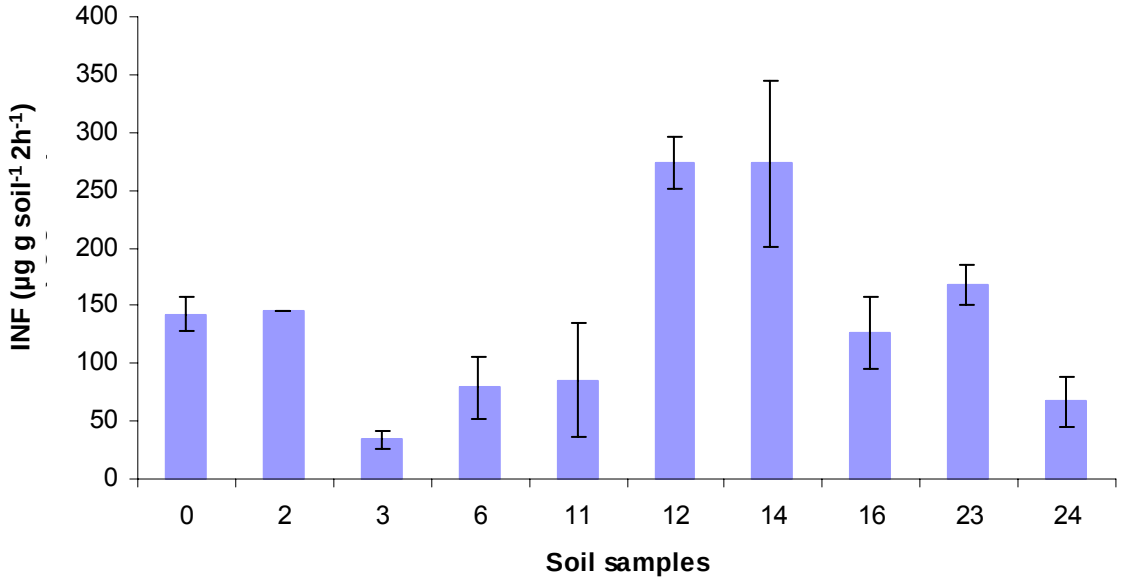


Figure 5.10 Dehydrogenase activity of naturally contaminated soil. The results are presented in µg of INF produced per gram of moisturized soil incubated for 24h at 25°C. Bars represent the standard deviation (n=3).

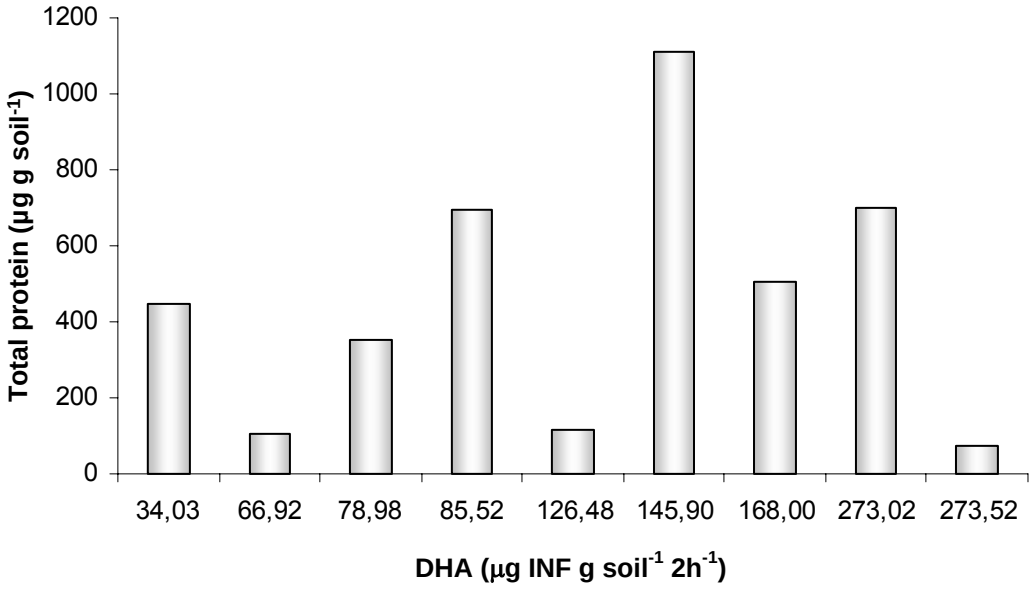


Figure 5.11 Dehydrogenase activity of versus the respective total protein concentration expressed in µg per g of soil.

Figure 5.12 represents the relationship between the DHA and the population of bacteria (a) and fungi (b). There is no statistical correlation (r^2=0.038 and 0.0247, respectively) with a 95% confidence interval, between these two parameters, i.e., an increase in the microbial population is not directly related with an increase in the concentration of INF produced per g of soil per assay.

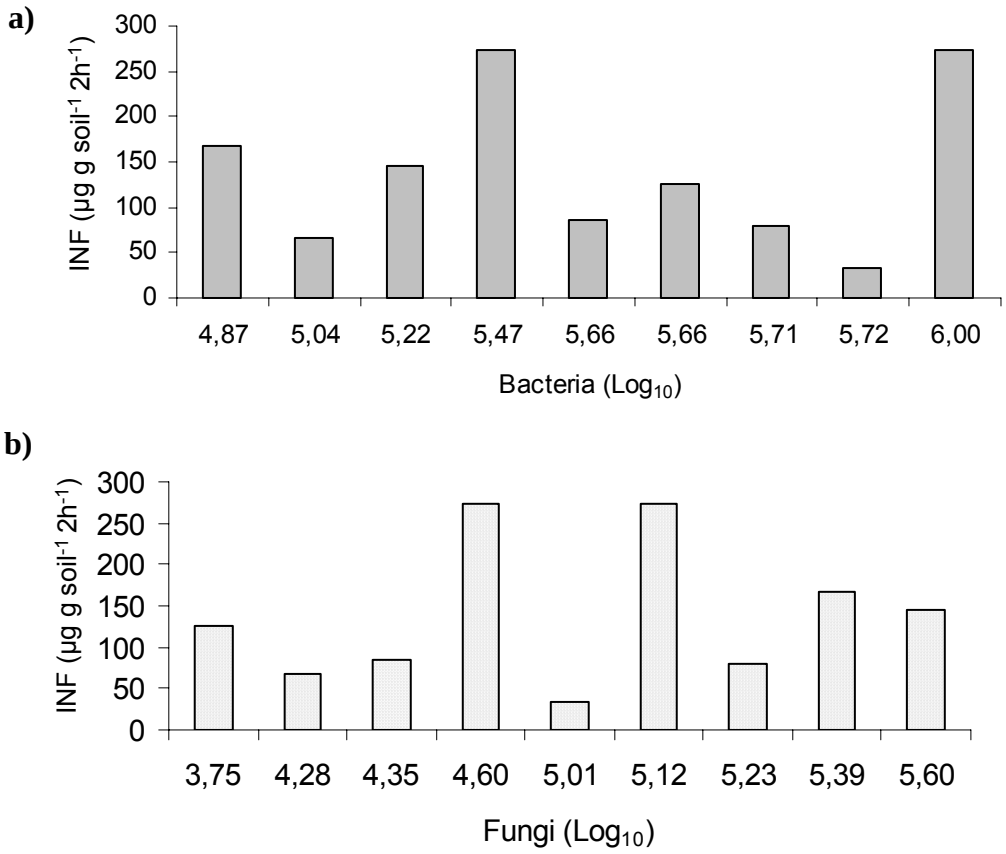


Figure 5.12 Correlation between the population of bacteria (a) and fungi (b) and the corresponding concentration of INF produced per g of soil.

When DHA was plotted against the metal concentration present in each soil sample in figure 5.13, the profile is very similar to that observed for the fungal population plotted against the metal contamination (see Figure 5.3b). Even though figure 5.12 was inconclusive in showing a direct relationship between the number of CFUs and the DHA.

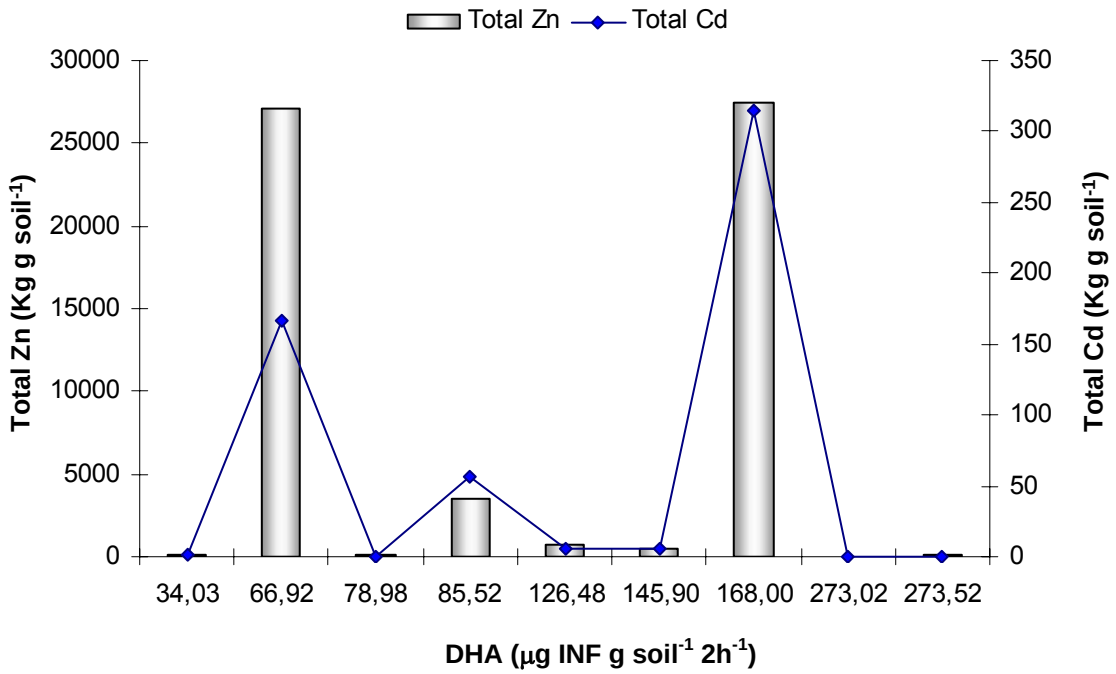


Figure 5.13 Correlation between the DHA and the concentration of heavy metals (Zn and Cd), present in soil samples of naturally contaminated soil.

5.3.4 Headspace analysis

A) CO_2 Sensor Response

i) Artificially contaminated soil microcosms

Figure 5.14 represents the CO_2 sensor response for artificially contaminated soils in comparison with the DHA at each time point of analysis. In the control samples, the CO_2 level increases with time. However, for samples contaminated with heavy metals the CO_2 level after 40 days has a different trend for each level of contamination. For samples containing 100 ppm of heavy metals the CO_2 level overlaps with that of 12 days, which could be an indication of microbial metabolism.

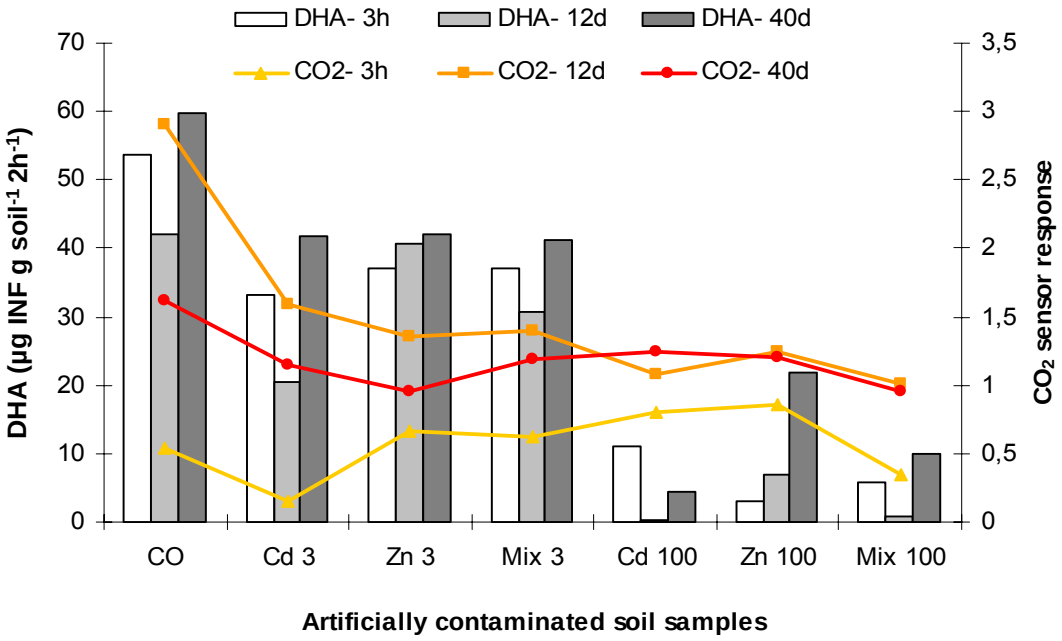


Figure 5.14 Correlation between the DHA and the CO_2 production of the different soil treatments in artificially contaminated soil samples, after 3h, 12 and 40 days of initial contamination.

ii) Naturally contaminated soil

Figure 5.15, represents the CO_2 sensor response to naturally contaminated soil samples, based on the data of table 5.3. An increase in the CO_2 is also directly related with an increase in the production of INF per g of soil per assay.

Table 5.3 Soil sample number and the corresponding CO_2 and DHA results.

Soil No.	**2**	**3**	**6**	**11**	**12**	**14**	**16**	**23**	**24**
CO_2	26.34	5.05	9.31	8.24	53.61	46.96	48.05	53.79	15.66
DHA	145.90	34.03	78.98	85.52	273.52	273.02	126.48	168.00	66.92

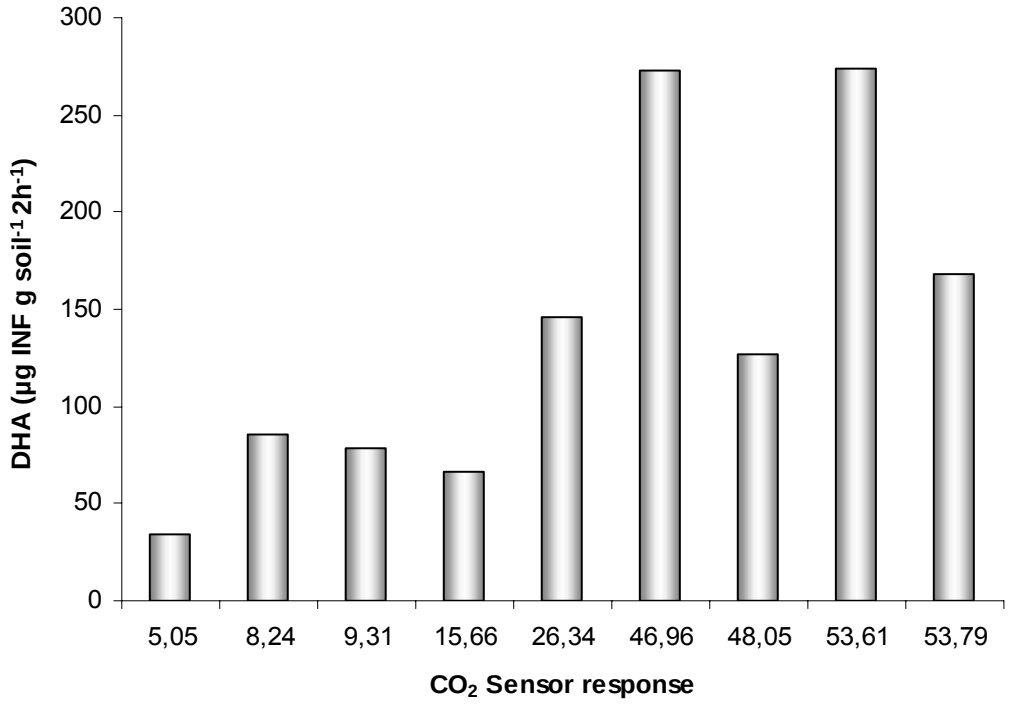


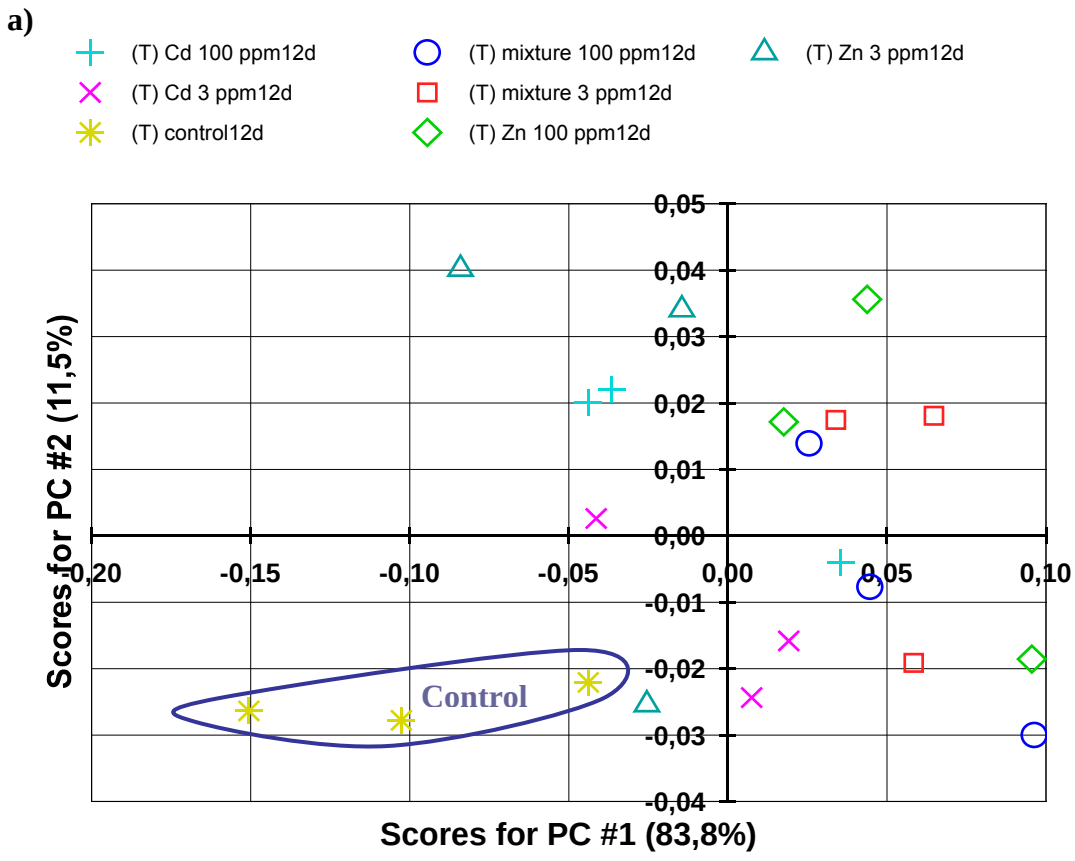
Figure 5.15 Correlation between the DHA and the CO_2 production of naturally contaminated soil samples, after 24h incubation at 25ºC.

B) E-nose sensor profile

The e-nose NST 33120 was previously used to test the contamination of water samples. In this chapter the e-nose was used to investigate whether it could also be applied to detect differences in microbial activity, in soil samples artificially and naturally contaminated with heavy metals.

i) Artificially contaminated soil microcosms

Figures 5.16 (a and b) shows the PCA map of the sensor response to the different soil treatments after 12 and 40 days respectively. After 12 days the more significant discrimination was that of the control samples, although, after 40 days using the first three PCs it was possible to differentiate between the control samples and two concentrations of metals, 3 and 100 ppm. Overall, it was not possible to discriminate between the different heavy metals.



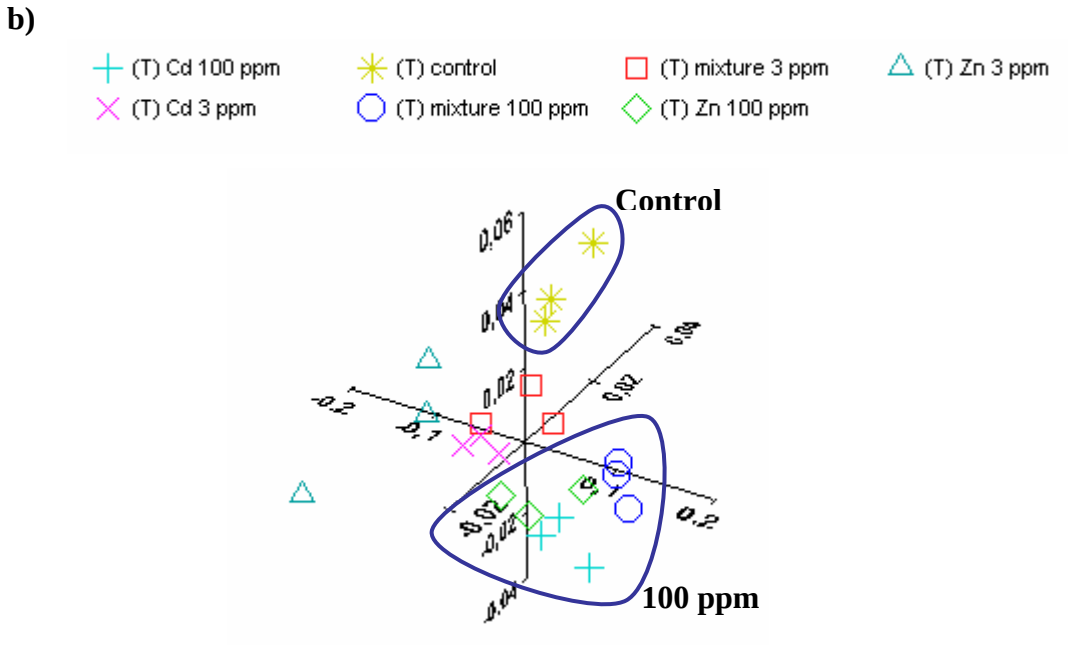


Figure 5.16 PCA map of the first three PCs (1-3) representing the MOS and MOSFET sensor response to artificially contaminated soil samples incubated for (a) 12 days and (b) 40 days at 25ºC.

Figure 5.17 is a PCA map of the sensor response to all the samples contaminated with Cd versus the control, at different sampling times. It is clear that samples are clustered according to the time when the samples were taken, discriminating between 3h, 12 and 40 days.

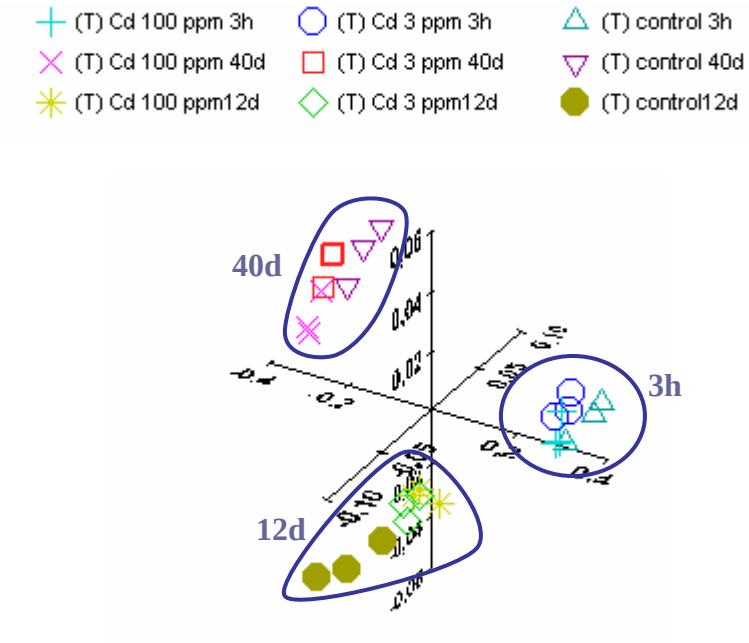


Figure 5.17 PCA map of the three PCs representing the sensor response to the different samples contaminated with Cd, after 3h, 12 and 40 days of initial contamination.

ii) Naturally contaminated soil

The separation between the different soil samples is not has clear as it was observed for artificially contaminated soils. The PCA map shown in figure 5.18 using the first three PCs represents the sensor response to the various soil samples contaminated with different concentrations of heavy metals (Table 5.4). The only group, which, is clearly separated is that of soil number 23, corresponding to the soil with the highest concentrations of Zn and Cd.

Table 5.4 Soil sample number and the corresponding metal concentration.

Soil No.	2	3	6	11	12	14	16	23	24
Total Zn	517.92	180.01	64.73	3540.15	91.12	59.09	618.22	27413.39	27099.75
Total Cd	5.55	1.19	0.33	55.86	0.11	0.23	5.16	314.81	166.54

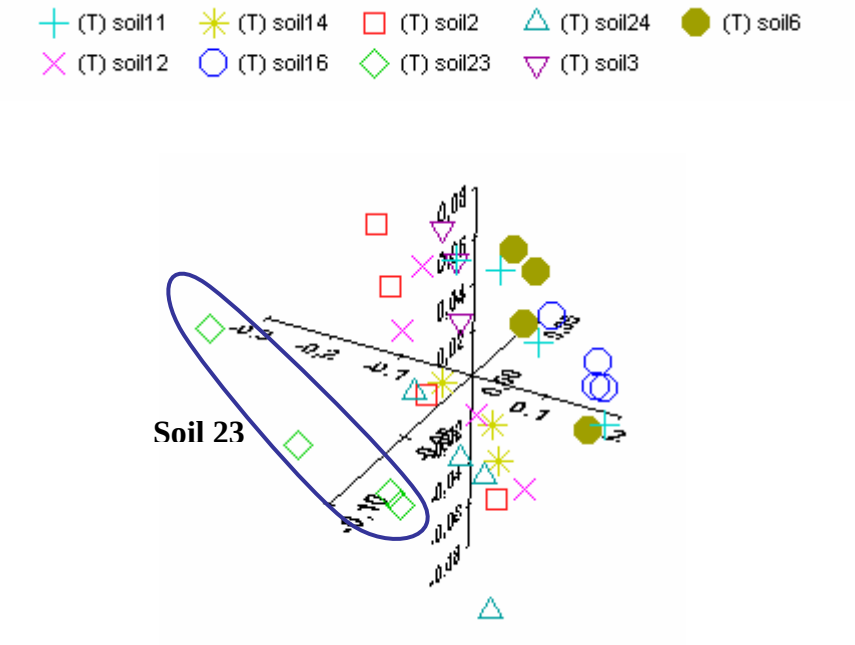


Figure 5.18 PCA map of the first three PCs for the analysis of the sensors response to naturally contaminated soil samples, incubated for 24h at 25ºC.

5.4 DISCUSSION

The results obtain for the determination of the microbial population expressed in CFUs per g of soil indicated that the number of bacterial species were higher than the number of fungal species in almost all the soil samples. However, the relationship between the number of CFUs and the concentration of metals indicated that bacterial species are more sensitive to metal contamination. It was not possible to find a direct relationship

between the microbial population and the total protein produced per gram of naturally contaminated soil.

The analysis of dehydrogenase activity of artificially contaminated soils indicate what looked like an equilibrium stage between 3h and 40 days after initial contamination, since the DHA after 12 days was generally lower than the first and last sampling times. For naturally contaminated soils, it was difficult to find a relationship between the different parameters used to test the microbial biodiversity and metabolic activity. Inconclusive results were observed when the DHA of the different soil samples was plotted against the number of CFUs, and the concentration of heavy metals, however, there seems to be a relationship between the DHA and the total protein. This fact may be related with the available fraction of metals in the soil and the ageing of the soil samples.

The CO_2 analysis has been referred in literature as an important parameter for microbial metabolism. In this study the CO_2 was determined by one sensor incorporated in the e-nose. Results for artificially contaminated soils reveal that the CO_2 increased with time for control and samples containing 3 ppm of contaminant, however, after 40 days the CO_2 of samples inoculated with 100ppm of metals overlapped with that of 12 days, reflecting a decrease in microbial respiration or metabolism. When compared with the DHA of both artificially and naturally contamination, a good relationship was obtained with these two parameters.

In previous chapter the e-nose was applied to detect and discriminate microbial and chemical contaminants in water samples. This is the first time that the e-nose was used to analyse soil samples contaminated with heavy metals. This instrument has the advantage of been a very rapid and non-destructive technique. Differences between contaminated and non-contaminated samples were tested, searching for dissimilarities in the headspace volatiles originated from microbial metabolism.

Results for artificially contaminated soils demonstrated that after 12 days incubation, control samples could be discriminated from those containing 3 and 100 ppm of heavy

metals. After 40 days incubation, it was possible to differentiate between control, samples with 3 ppm and samples with 100 ppm of metals. When the results of the three sampling times were plotted together, a clear separation was observed between the different incubation stages. For naturally contaminated samples, the results were not has clear has the ones described above. The only differentiated sample was soil number 23, the most contaminated one, with high concentrations of cadmium and zinc. As referred previously, ageing may be an important factor to influence how the concentration of metals affects the microbial activity. This factor may suggest that the microbial biodiversity present in such soils is probably resistant to high level of contamination. Overall, the concentrations of the contaminant seem to have a stronger influence in the microbial activity than the type of contaminant.

Moffett (2003) used an amplified ribosomal DNA restriction analysis of the extractable bacterial fraction in soil. The diversity of a zinc-contaminated soil (400 mg Zn Kg^{-1}) compared with that of a control soil (57mg Zn Kg^{-1}) from a long-term sewage sludge experiment (Gleadthorpe, UK), observing a 25% decrease in bacterial biodiversity. Megharaj (2003) in a review of the bioavailability and toxicity of cadmium to microorganisms in soil and the different factors that influence this effect, such as time, soil type, ageing, Cd-source and organisms. He suggests that the toxicity of cadmium is related with the available fraction or soil solution Cd rather than the total Cd concentration. In a study over 180 days with an initial concentration of 3 mg Cd Kg-1 soil, it was observed that the this concentration decreased exponentially with time to <0.0006 mg l^{-1} within 50 days of ageing.

Bacteria seems to be more sensitive to Cd than fungi. Obbar (2001) reported on an ecotoxicological assessment of heavy metals (Zn, Cu, Ni, Cr, Pb, and Cd) in sewage sludge amended soils located in Braunschweig (Germany), including the measurement of dehydrogenase activity. He concluded that selected heavy metals may suppress substrate-induced DHA at high concentrations in sludge amended soils, the level and type of sludge addition has a more significant effect on influencing DHA than the concentration of the toxic elements. Jimenez (2002) evaluated some chemical, physical and biochemical parameters to determine a soil quality index. The results indicate a very

significant correlation between basal respiration and DHA, confirming that intracellular activity could be used as an indicator of microbial activity in soil.

The effects of zinc enriched sewage sludge on microbial activities and biomass in soil were reported by Rost (2001). A closed static microcosms was used to investigate Zn effects on N mineralization in relation to other microbial activities and biomass in a sandy loam soil. Results indicated that all microbial indices were increasingly depressed with increasing Zn concentrations ranging from 50 to 800 μg per g of soil. The reduction of the CO_2 production started at 200 μg Zn g^{-1} of soil, whereas the biomass was only slightly reduced. He suggests that this may be an indication of a change in the microbial community structure towards fungi that are more tolerant to heavy metals than bacteria.

CHAPTER 6.

BIODEGRADATION OF LIBRARY PAPER

6.1 INTRODUCTION

Among the most important factors that influence deterioration of library material are the biological factors, including insects, rodents and microorganisms (Zyska, 1997). Fungal colonisation causes significant disfigurement of library materials, especially books and paper. Apart from the undesirable aesthetic aspects, the presence of some microorganisms can lead to the irreversible degradation of the paper if viable cells are not detected early enough resulting in significant loss in value and quality of such material. Thus early tools for the diagnosis of microbial, and more specifically fungal contamination would be very useful for effective management of such valuable material.

The key environmental factors which determine germination and growth on paper-based material is the prevailing environmental factors of relative humidity, temperature and gas composition (Magan, 1997) and the level of initial contamination of the material. The concentration of viable fungal spores in house dust has been observed to vary from 6 $x10^3$ to 3.2 $x10^6$ CFU (colony forming units) per g of dust (Korpi *et al*., 1997). To prevent fungal growth, books and archives are commonly stored in controlled environments, with temperature and humidity maintained in a defined range, and periodically cleaned of dust (Pinzari *et al.,* 2003).

Microbial contaminants produce a range of volatile compounds including different alcohols, adehydes, ketones, aromatic compounds, amines, terpenes, chlorinated hydrocarbons and sulphuric compounds (Korpi *et al*., 1997). Recently, 1-octen-3-ol was shown to cause genotoxicity effects in the library environment (Fiedler *et al*., 2001). Many contaminant fungi are also known to produce mycotoxins which can also be present in the spores. Many bacteria and moulds produce a characteristic range of volatiles (Magan and Evans, 2001; Tothill and Magan, 2003). This fingerprint could be utilized for early detection of microbial activity on museum materials.

Traditional methods of enumeration of microbial contaminants require a lot of time and labour (Tothill and Magan, 2003). More rapid, early techniques are required for

effective management of paper-based museum materials. Recently, the development and commercialisation of sensor-based devices, e-noses, has provided a possible tool for more effective management. Such instruments provide a rapid, simple and non-invasive sampling technique, for the detection and identification of a range of volatile compounds. Improvements on sensor technology coupled with more accurate protocols are increasing the potential of the e-nose as an analytical tool that does not require highly trained operators.

Due to the growing interest in environmental and work place monitoring, the detection of analytes such as aromatic and halogenated hydrocarbons is of increasing economic importance (Dickert *et al*., 1999). However, no studies have previously attempted to examine the potential of using e-nose technology for the early qualitative detection of microbial contamination of museum materials. This study investigated the potential of using electronic nose technology (12 or 14 conducting polymer sensor-based array systems) for *in vitro* discrimination of three xerophilic *Aspergillus/Eurotium* species on cellulose-based media, and *in situ* detection and differentiation of these species on different types of paper samples.

6.2 MATERIALS AND METHODS

6.2.1 *In vitro* studies

i) Cultural media and incubation

Three fungal strains, *Aspergillus terreus* Thom (ATCC 10690); *Aspergillus hollandicus* (Anam.: *Eurotium amstelodami*. (Mangin) Thom and Church) and *Eurotium chevallieri* L.Mangin, from culture collection of Instituto Centrale di Patologia del Libro, were isolated from deteriorated paper materials (Pinzari *et al*., 2003).

A growth medium of 1% cellulose (Oxoid), 1% malt extract (Lab M) and 2% technical agar (Oxoid), modified to 0.995 and 0.975 water activity (a_w) with glycerol, were inoculated with a 0.2 mL suspension (10^6 spores mL^{-1}) of each species, and spread-

plated over the surface. The plates were enclosed in polyethylene bags and incubated at 25^{o}C. Four replicates per strain were destructively sampled after 20 and 40 h. Un-inoculated medium served as controls at each sampling time (Keshri and Magan, 2000).

ii) Gas sampling and sensing procedure

Single replicate Petri plate cultures were placed in sampling bags (500 mL capacity), with the lid carefully removed, filled with filter-sterilized air and sealed. The bags were incubated for 1 h at 25^{o}C, to equilibrate the headspace. The headspace from each bag was subsequently sampled through an air-filter system, which consisted of a bio-filter (0.45 μm, PTFE Whatman, Hepa-Vent) and an activated carbon filter (Whatman), to ensure clean airflow. An electronic nose system (BH114, Bloodhound Sensors, UK) that uses an array of 14 conducting polymer sensors was used in this study. Samples were analysed randomly, including the controls. The data was collected and analysed by the e-nose software package system.

For each sensor response a set of data includes absorption, desorption, divergence (maximum response), area (of sensor response) and ratio (adsorption/ desorption) is plotted. Normalise data were analysed using xlStat programme (Microsoft Excell add-in). Principal component analysis (PCA), discriminant function analysis (DFA) and cluster analysis (CA) techniques were applied to discriminate between the treatments. PCA is a non-supervised technique which means that the different classes or groups are not defined and the technique finds any hidden relationship between samples. PCA is commonly used to summarise the pattern of correlation among the observed variables (e.g. sensor responses). DFA is a supervised technique, the different classes are defined in the analysis. Linear function analysis involves finding one or more linear combinations that maximises the between-group differences, with 95% confidence. CA uses the Mahalanobi's squared distance between groups from the DFA to construct the dendogram.

6.2.2 *In situ* studies

The same fungal species used for *in vitro* studies were tested *in situ*: *Aspergillus hollandicus*, *Aspergillus terreus* and *Eurotium chevallieri*.

Three types of paper with different compositions, based on the origin of the fibres and the chemical treatments undergone during the manufacturing process, were used in this study. A minimum of three types of paper were required to verify if the sensors could detect differences in fungal growth:

(a) Paper type A (Whatman 1CHR), made of pure cellulose;
(b) Paper type B (produced by Fedrigoni Mills, Italy), called "Freelife vellum", this being a long-life type. It is made with 80% recycled fibres, 15% cellulose chlorine free and 5% cotton fibres;
(c) Paper type C (produced by Fedrigoni Mills, Italy), called "Old mill" ivory, this being uncoated long-life type. It is made with 100% cellulose fibres.

The paper samples were cut into 2 x 6 cm strips. Both sides of the strips were exposed to UV light for 45 minutes, in order to sterilise the surface from airborne fungal and bacterial cells.

i) Inoculation of paper treatments

Mycelial cultures were inoculated on MEA (Malt Extract Agar) and prepared according to Pitt and Hocking (1985). The cultures were kept in the dark at 25^{o}C before their use for the inoculation of paper strips. A spore suspension was prepared in a 0.02% Tween 80 (Merck) and distilled water. Spores were then filtered through a sterile cotton cloth to remove impurities. The spore density was obtained for each strain by spore-counting in a Thoma Chamber and optical microscopy. A defined volume of each spore suspension was diluted with nutritive broth (Sabouraud Broth, DIFCO), in order to inoculate the paper strips.

Each paper strip was inoculated with 100 μL of spore suspension (10^3 spores ml^{-1}). For control samples, paper strips were inoculated with 100 μL of nutritive broth. Four replicates (a-d) for each treatment were used. The paper strips were placed in a 50 mL

polystyrene vial and closed with ventilated caps. Two values of relative humidity (RH) were considered: 100% RH, obtained with distilled water; and 75% RH, obtained with saturated solutions of NaCl. Vials were kept in glass containers in a thermostatically controlled incubator at 27^{o}C for 7 days. A sensor (Hygrolog-D Rotronic AY, Swiss) to register the internal RH, was placed in each container throughout the incubation time. After incubation period, samples were kept in a refrigerator room at 4^{o}C before analysis.

ii) Headspace analyses

Before analysis, samples were placed at 25^{o}C for approximately 30 min to equilibrate volatiles on headspace. Samples were placed in a 50 mL vial and were inserted in a glass sampling vessel on the sampling stage, and allowed to equilibrate at 30^{o}C for 15 min. The headspace from the sampling system was analysed using an eNOSE 4000 sensor array module (Marconi Applied Technologies, UK). This instrument consists of an array of 12 conducting polymer sensors in a temperature-controlled sensor chamber. Filtered nitrogen was also used to purge the sensors between sample acquisition. As previously described for *in vitro* studies, data was analysed using the xlStat program and the same statistical techniques. Even though both e-nose systems operate with the same sensor type (conducting polymers), the sensor arrays are different in their making and number. Apart from that, eNOSE400 flushes the sensor chamber with filtrated nitrogen while BH-114 uses filtrated air. Previous studies have shown that bearing in mind their differences and similarities, results obtained with these systems are similar (Canhoto and Magan, 2003).

6.3 RESULTS

6.3.1 *In vitro* studies

For *in vitro* studies, two aspects were taken into consideration, the water availability (0.995 and 0.975 a_w) and the incubation time (20 and 40 h). Figure 6.1 shows the response of the different sensors in the array to volatiles produced by the fungi on

cellulose agar. This confirms that the fungi produce a slightly different mixture of volatiles in the headspace resulting in significant differences in the sensor responses.

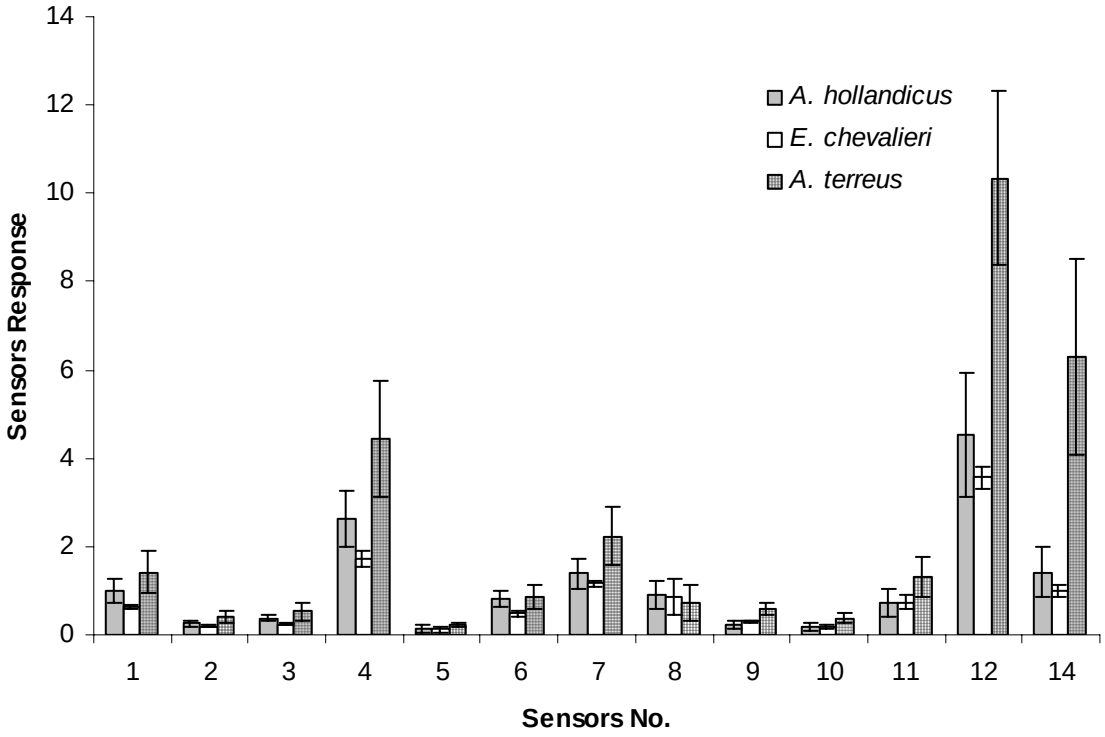


Figure 6.1 Sensor responses to three fungal species (*A. hollandicus, E. chevallieri* and *A. terreus*) inoculated in vitro on a cellulose agar at 0.995 a_w for 20 hrs.

Figure 6.2 shows the principal component analysis (PCA) for the results obtained for the fungal cultures incubated at (a) 0.995 a_w and (b) 0.975 a_w for 20 h at 25^{o}C. In both cases, there was discrimination of the control samples from the other treatments after this period. Discriminant function analysis showed that 98% of the data could be accounted for with clear differentiation between the controls and the spoilage fungi after 20 h (Fig. 6.3). Similar results were obtained at both aw levels.

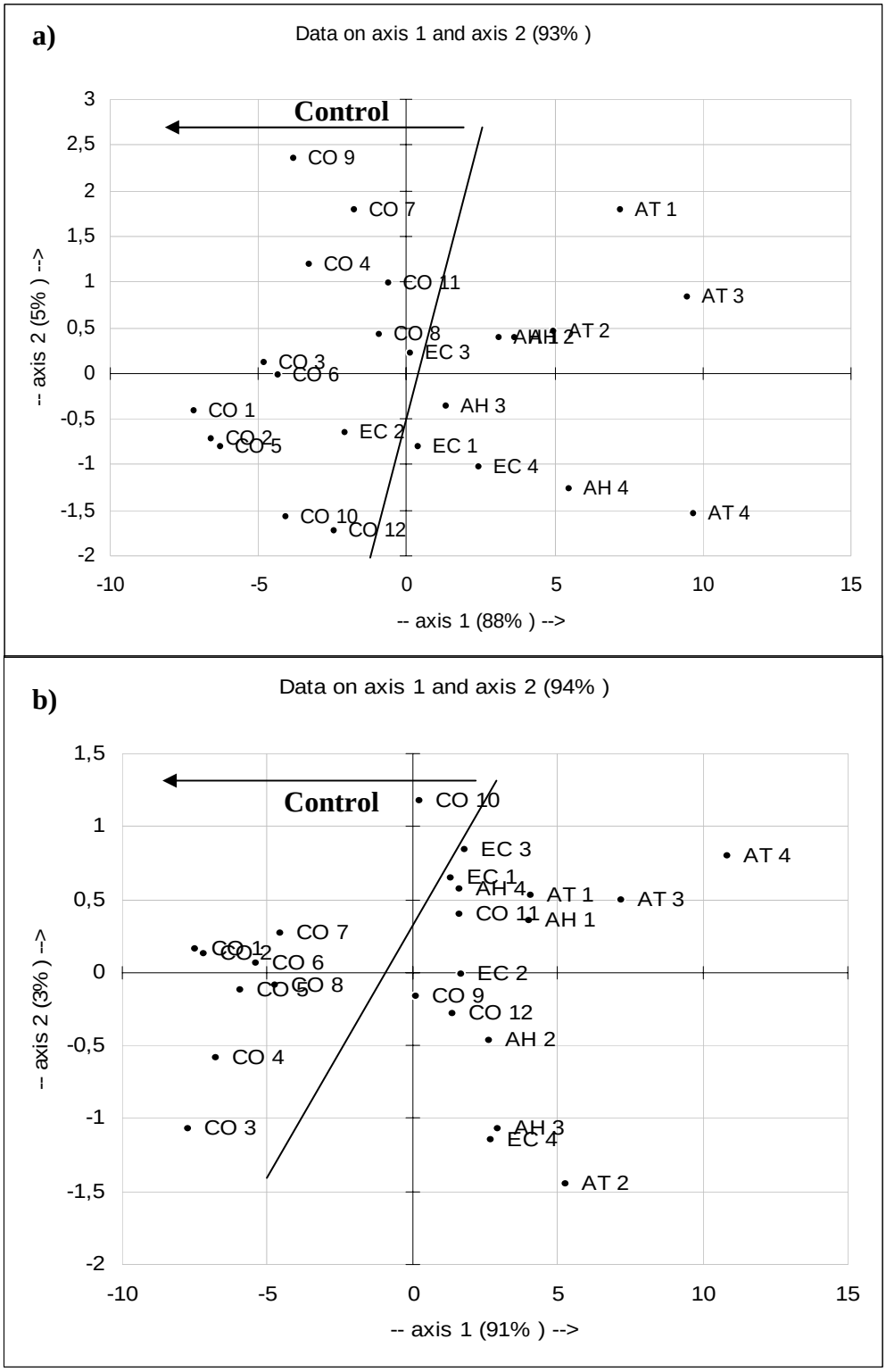


Figure 6.2 PCA map of *in vitro* studies at (a) 0.995 a_w and (b) 0.975 a_w, with 20 h incubation at 25^{o}C. Key for treatments: **CO** (1-12), control; **AT** (1-4), *Aspergillus terreus*; **AH** (1-4), *A. hollandicus*; **EC** (1-4), *Eurotium chevallieri*.

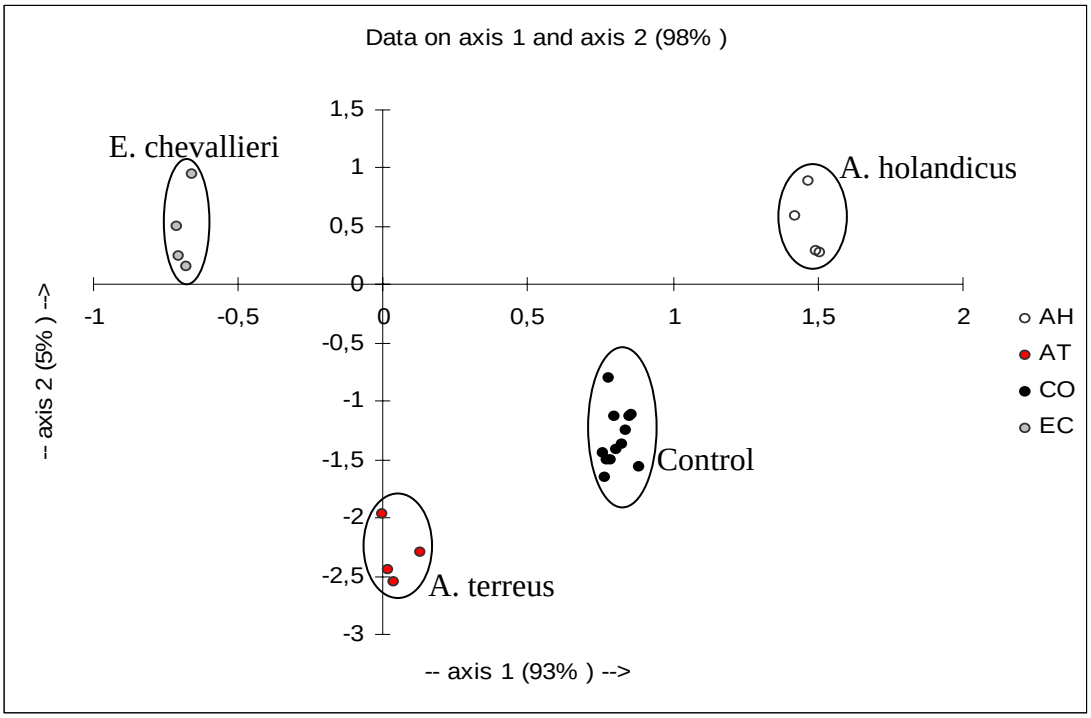


Figure 6.3 DFA of *in vitro* studies at 0.975 a_w incubated for 20 h at 25^{o}C. Key for treatments: **CO**, control; **AT**, *Aspergillus terreus*; **AH**, *A. hollandicus*; **EC**, *Eurotium chevallieri*.

Cluster analysis, based on the Malahanobi's squared distance between groups obtained in the Discriminant Analysis at the 95% confidence limit showed that increasing the incubation time to 40 hrs resulted in a better separation between the control and fungal treatments (Fig. 6.4).

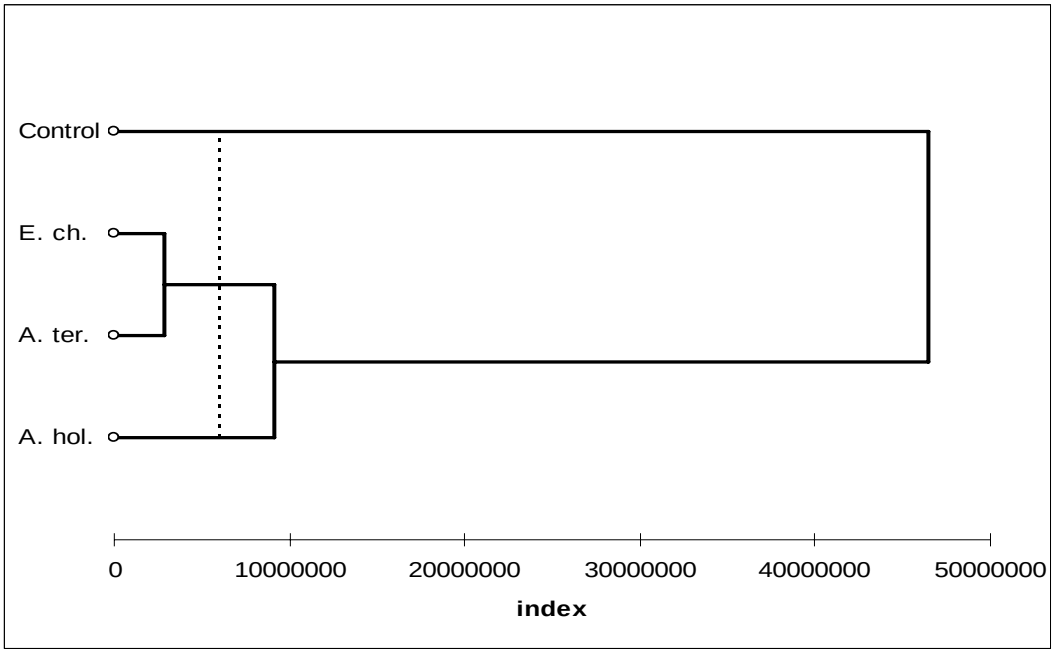


Figure 6.4 Cluster analysis showing the clear separation of the control from *in vitro* samples at 0.995 a_w inoculated with the three types of fungi (**E.ch.**, *Eurotium chevallieri*; **A.ter.**, *Aspergillus terreus*; **A.hol.**, *A. hollandicus*). Samples were incubated for 40 h at 25^{o}C.

6.3.2 *In situ* studies

Figure 6.5 shows a comparison between the response profiles for the 12 conducting polymer sensors of the e-nose employed in this study. This shows that, for each of the treatment fungi, growing on type C paper, each sensor has a different response to the volatiles produced in the headspace.

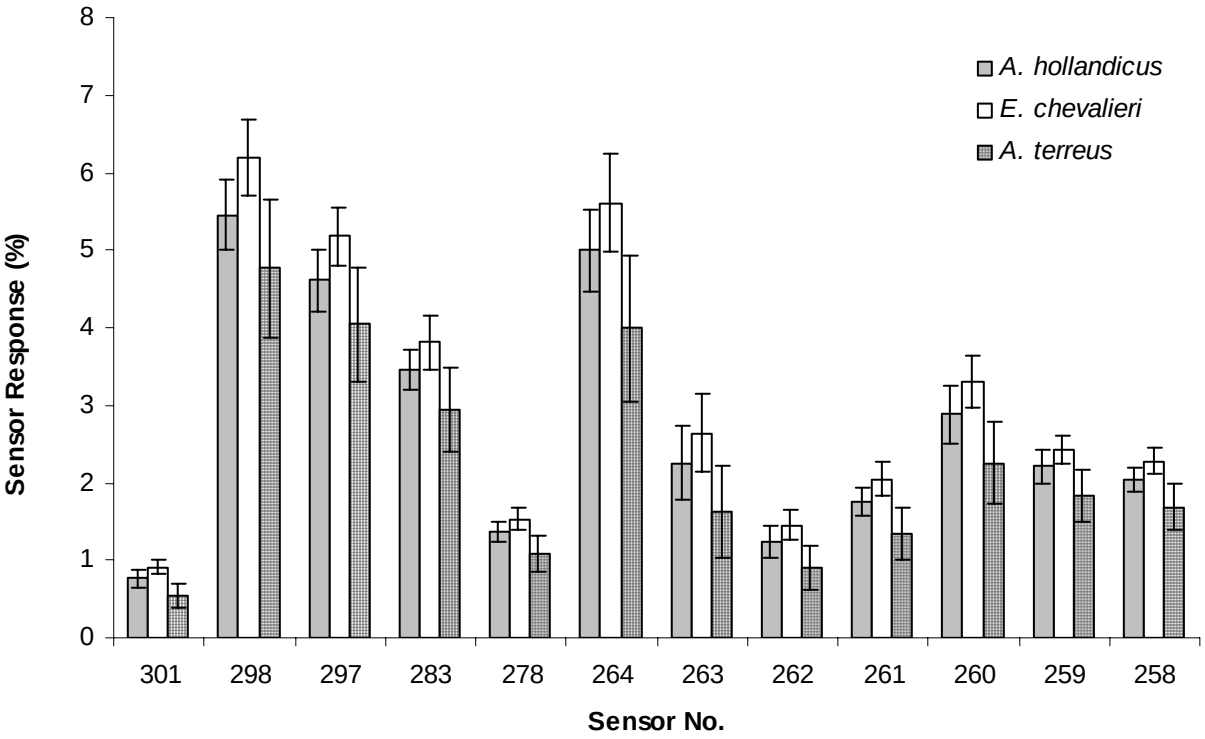


Figure 6.5 Sensor responses to the fungal volatiles produced by the three species on paper C at 100% RH, showing the different responses for each species.

Using the Cluster Analysis to represent the sensor responses (Fig. 6.6), it was possible to obtain a clear separation between the control samples and paper strips (paper type A) inoculated with the fungal species, at two relative humidity levels, (a) 75% and (b) 100%.

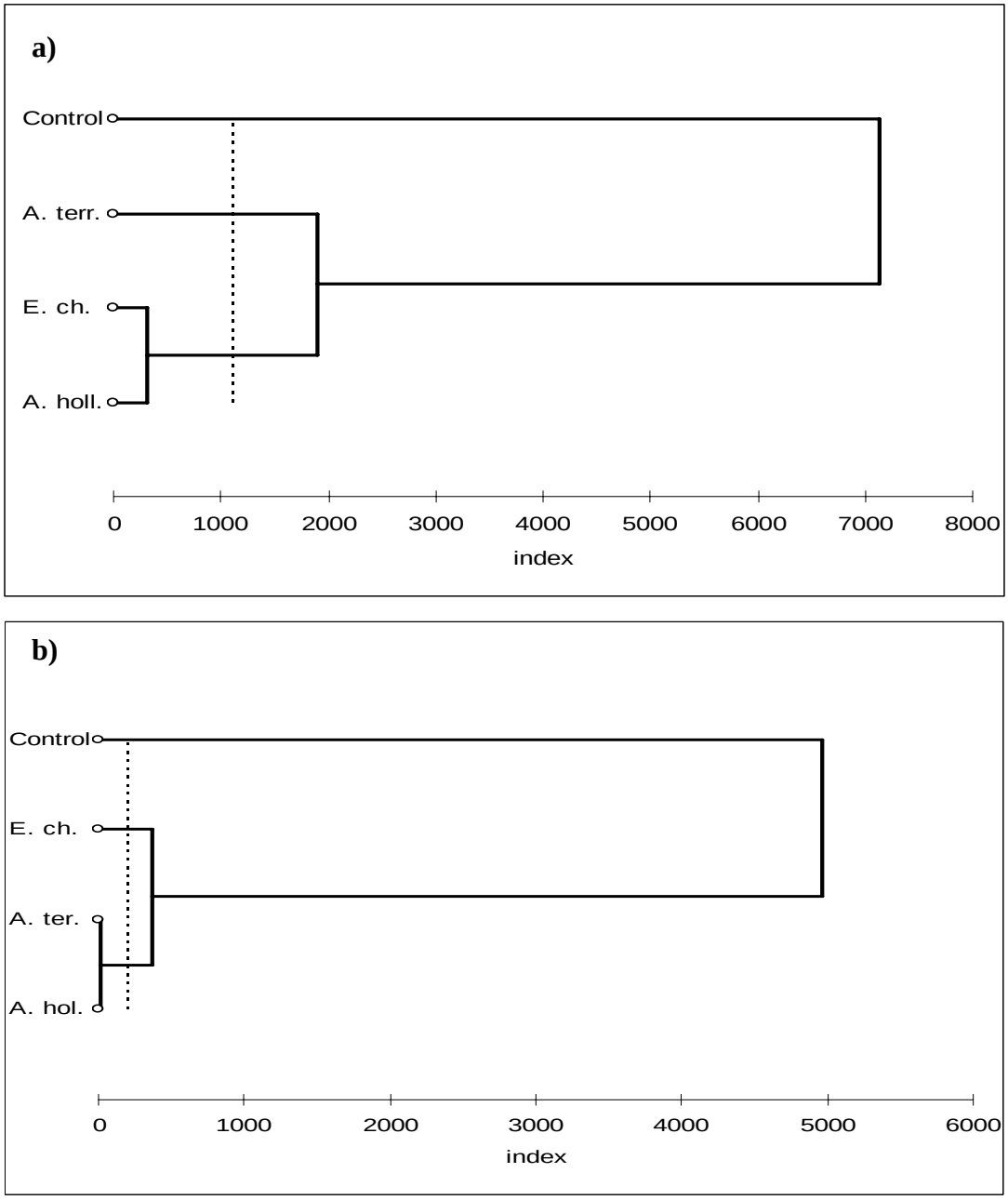


Figure 6.6 Cluster analysis showing the discrimination between the different fungal species inoculated in paper type A (Whatman 1CHR), at (a) 75% RH, and (b) at 100% RH. *Key for treatments*: **E.ch**., *Eurotium chevallieri*; **A.ter**., *Aspergillus terreus*; **A.hol**., *A. hollandicus*.

Figure 6.7 reveal the successful discrimination between the different paper types was possible. However, it was not in this case possible to separate the fungal treatments from the controls within each paper treatment. The e-nose was able to differentiate between the control samples and those inoculated with fungi in the two humidity conditions at 75 RH and 100 RH (Fig. 6.8).

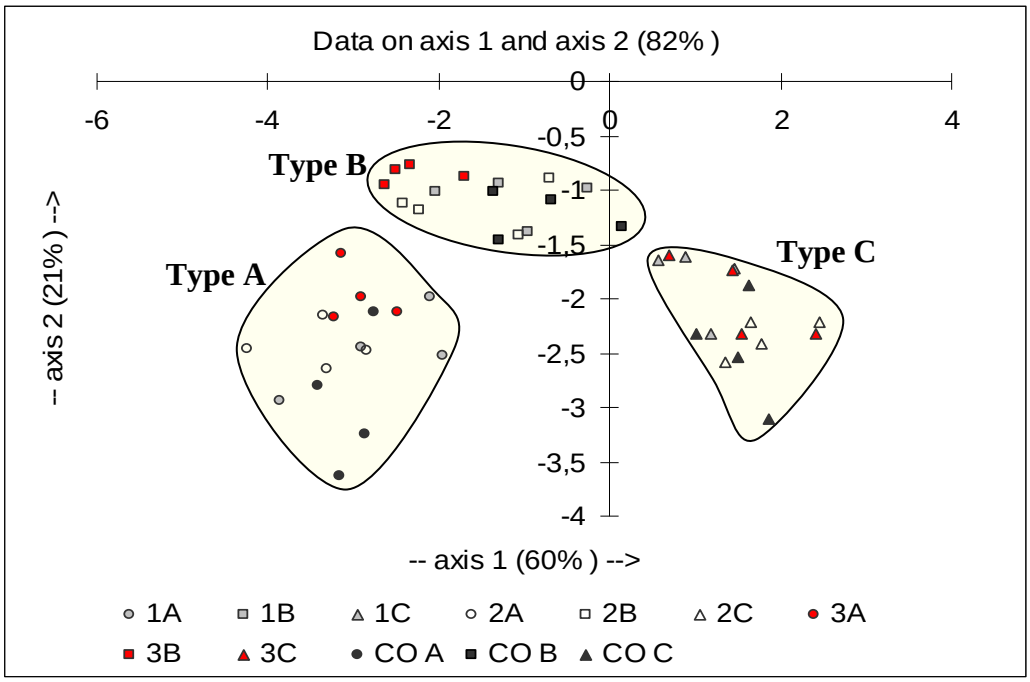


Figure 6.7 DFA of all types of paper (A, B and C), different fungal species (**1**, *Aspergillus hollandicus*; **2**, *Eurotium chevallieri*; and **3**, *A. terreus*), and control samples (CO A, CO B and CO C), at 100% RH.

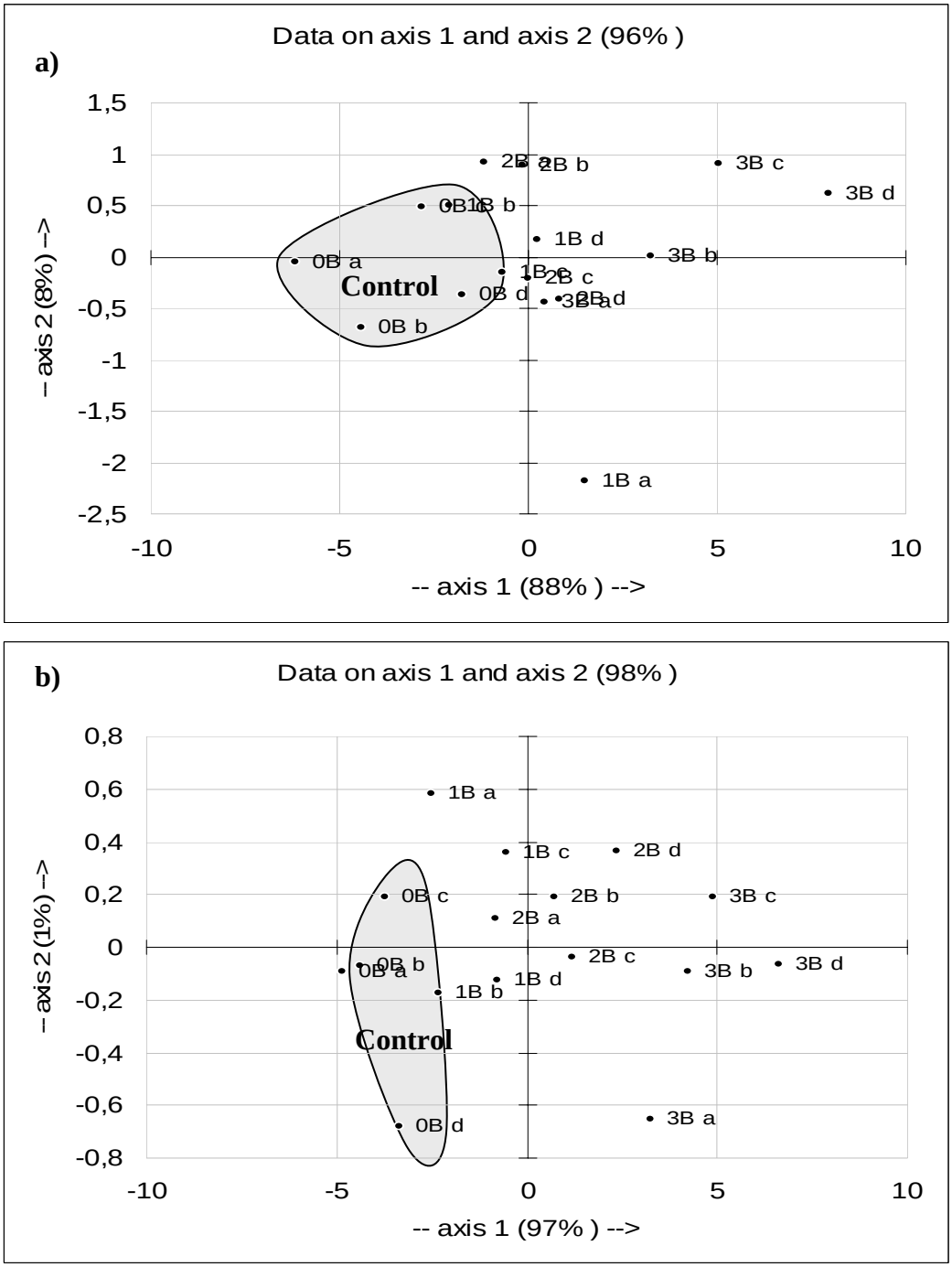


Figure 6.8 PCA map of paper type B inoculated with different fungal species (**1**, *Aspergillus hollandicus*; **2**, *Eurotium chevallieri*; and **3**, *A. terreus*), and control samples (**0**), at (a) 75% RH, and (b) 100% RH. Letters (a - d) stand for replicates.

6.4 DISCUSSION

The response of the sensors to volatiles produced by the spoilage fungi inoculated in cellulose-based medium showed that different types and amounts were slightly different. Since the e-nose is qualitative and not quantitative, it gives a good fingerprint of the differences between control and other treatments.

The *in vitro* studies revealed that successful differentiation could be obtained between spoilage fungi and the controls after 20 h incubation on a cellulose medium at 0.975 a_w. After 40 h the e-nose was able to discriminated between the fungal species. This results are similar to previous studies with food spoilage fungi, where good discrimination was obtained between spoilage fungi grown in agar-based medium and the controls, but where two closely related *Eurotium* species could not be differentiated (Keshri *et al*., 1998). Schiffman *et al*. (2000) found that the e-nose could detect and classify three fungi, *Aspergillus niger*, *A. flavus* and *Penicillium chrysogenum*, incubated in solid agar media after 24 h. Keshri *et al.* (2002) studied the detection of *Eurotium* spp. and *Penicillium chrysogenum* inoculated in food matrices (bread), using a conducting polymer based e-nose and found that successful detection could be made within 36 h, much faster than that which could be obtained using enzyme assays or traditional fungal counts.

In situ studies indicated that the substrate is very important for headspace analysis. Fungal species grown on different paper types produced different volatile profiles. Previous studies have investigated the volatile production of microorganisms in different substrates. For example, Fiedler *et al*. (2001) used GC-MS combined with HS-SPME to explore the volatile metabolites released by different fungal species grown on different substrates. They concluded that more than 150 compounds could be identified but not a single compound was common to all species. Further studies (Claeson *et al*., 2002) investigated the emission of volatile compounds by fungi from building materials and cultures grown in solid media. They found that the production of volatile compounds by fungi varied greatly between different growth media.

Environmental factors such as relative humidity also have an effect on volatile production patterns. Korpi *et al*. (1997) investigated the influence of relative humidity on the growth of microorganisms, including fungi. Their results showed that relative humidity affects the volatile emission and also the growth rate of the different fungal species. More recently, bacterial, yeast and filamentous fungal growth was successfully achieved using a similar e-nose system on food matrices. In this case at 0.95 a_w differentiation was possible after 24 h (Needham *et al*., 2003).

The results obtained with different types of paper and diffirentiation of spoilage fungi, is the first report of this approach for library-based material. The results show that the e-nose was able to discriminate between control samples from paper inoculated with fungal spores, and between some species. The results indicate that the substrate plays an important role when paper samples are being used. This suggests that a different volatile production pattern is probably produced by each fungal species.

CHAPTER 7.

GENERAL DISCUSSION

7.1 GENERAL DISCUSSION

This thesis has examined a range of different environmental applications of e-nose technology for the first time. This technology has broad applications in different environmental spheres for monitoring contaminants rapidly. These applications bring forward a number of general points for discussion.

7.1 Sampling

Sampling regimes are critical for optimising the use of e-nose systems. The use of static or dynamic head space systems have been used depending on the application. The sampling regime including head space volume, equilibrium time, temperature and physical state of the sample all have an impact on the fingerprint produced. Thus optimisation of these parameters is important for obtaining consistent results. Sometimes, solid matrices may require a longer equilibration time that liquid samples where agitation for example can result in a more rapid accumulation of volatiles in the head space. Thus, in all the studies reported in this thesis the same equilibration time and temperature were used for incubating samples prior to analysing for volatiles. This has enabled relatively consistent results to be obtained. Even where two different e-nose systems were used, correlations were good for the detection of microorganisms in water. It is also advantageous to use an external standard solution to account for possible drifts in the instrument.

7.2 Limit of detection

In water samples, it appears that for bacterial species, the e.nose is able to discriminate between non-contaminated samples and those initially contaminated with 10^2 cells mL^{-1}, after 24 hrs incubation. However, if a more detailed discrimination is required such as differentiation between species, a higher initial bacterial concentrations is necessary, for example between 10^4 and 10^6 cells mL^{-1} or a longer incubation time, e.g., 48 hrs. For fungal spores, similar detection limits were observed; initial spores concentrations of 10^2 to 10^5 cells mL^{-1} could be discriminated from control samples after 24hrs

incubation. In solid growth media fungal spores were successfully differentiated from control samples after 20 hrs using an initial concentrations of 10^6 spores mL^{-1}. This suggests that where, for QA purposes, detection of 10-100 spores is required, it would be difficult to achieve using this qualitative approach with e-nose systems.

7.3 Volatile Fingerprints

Since this is a qualitative technique the fingerprint as a whole and the components thereof are both important. Both are promising indicators of the presence of fungal and bacterial species in different matrices. Specific volatile organic compounds (VOCs) have been identified as characteristic of certain species, although it is recognized that the culture medium used affects their metabolism and consequently the type of VOCs produced. In this thesis, the effect of the substrate was clearly demonstrated for bacterial and fungal species. It was shown that a low concentration of heavy metals affected the metabolism of bacterial cells and therefore the volatile fingerprint using both the e-nose and confirmed with GC. Also in studies on paper, the type of paper on which fungal colonisation occurred (e.g. % cellulose present) influenced the volatile profiles produced. While there are now a number of e-nose systems, which are MS-based then quantitative differences, can probably be also used in the analyses. However, sometimes these more sophisticated techniques may give a significant amount of detailed information which may be difficult to interpret.

7.4 Sensor Arrays

The methodology and type of sensor arrays for the detection of a specific contaminant is important and should be chosen according to the application and level of information required. It all depends on the specificity and sensitivity required, i.e., if the target of detection is a specific microorganism in a particular medium, then perhaps a specific single or group of sensors could be utilised. However, if the instrument is to be used for a range of broad applications then this may be more difficult. Often, a more target-optimised system may be better than a generic system based in a non-specific sensor arrays.

Problems of sensor array drift and the efficacy of humidity and analytes on sensor poisoning are two negative aspects of conducting polymer sensors although they operate effectively over a range of ambient temperatures. However, metal oxide sensors require high temperatures to operate and thus require more sophistication in terms of engineering. In this project three different e-nose systems were examined; two based on conducting polymer arrays and one on metal oxide/ion sensors. In this thesis both conducting polymer and metal oxide sensor-based instruments gave good reproducibility, since results obtained on different days were consistent and data could thus be used in combined analysis.

Sensor technology is rapidly evolving so that the number of types, sensitivity and stability of different sensors becoming available is changing regularly. It is possible to find e-nose instruments with highly specific sensors, a mixture of sensor types, and those where a mass spectrometer has replaced the sensor array.

7.5 Artificial Neural Networks (ANN)

There are distinct advantages with using e-noses systems if they are linked to effective software analyses systems. Thus can enable real time analyse by linking to Neural Networks for discriminating at specific levels required. To fully exploit e-nose systems for specific applications, good databases are needed to developed and link to NN programmes which can be used to obtain qualitative answers in a short period of time. This is then a powerful tool to provide an effective early warning signal when conditions may become compromised and effective management could then be employed to prevent undesirable situations. Another possible application is the remote monitoring of distant sampling points connected to a central database. The integration and analysis of this information could easily be done with an ANN operated by a single trained person. Thus, provided clear specific applications of e-nose systems are being examined, an opportunity exists to provide real time answers to important questions being posed in relation to QA.

7.6 Economics

When it comes to water quality, one of the most important factors in analytical methodologies, is how fast a contamination episode can be detected, so that possible odour or taste complains can be avoided. In order to achieve a more efficient and rapid analysis, an at- or on-line monitoring system is probably one of the most desirable solutions. Traditional methods for the detection and isolation of the microorganisms are particularly laborious, time and resource consuming. Therefore, even if the initial costs of buying an e-nose system can be in the order of the £40,000, in the long-term, it requires low maintenance, no sample preparation, a trained technician and a qualitative result is ready in a few hours after sampling.

Nowadays, water companies face more and more strict legislation in terms of drinking water quality, together with an increase in the number of new contaminants. These new contaminants include pharmaceutical residues, such as antibiotics and hormones; disinfectant by-products; and the outbreak of new bacterial species resistant to the conventional disinfection techniques. Non-specific sensor arrays could thus be advantageous since they can be used for different contaminants.

Portable e-nose instruments have and are being developed to facilitate applications to monitor odour emissions, including industrial, agricultural and sewage sludge emissions. In a wastewater treatment plant, portable e-nose instruments can be useful to monitor the quality of the water at different sampling sites during each phase of the water treatment process. Thus, provided that they can be produced at an economic price, probably in the range £7,500-15,000, then a significant number of applications can be envisaged in the environmental sector.

CHAPTER 8.

CONCLUSIONS AND FUTURE WORK

8.1 CONCLUSIONS

i) Bacterial cells inoculated with an initial concentration of 10^2 cells mL^{-1} in different water types, could be discriminated from control samples after 24 hrs of incubation. In the presence of low concentrations of heavy metals (0.5 ppm) the volatile pattern produced by the bacterial cells was different from those where no metal ions were present.

ii) Water samples initially inoculated with $10^2 – 10^5$ fungal spores mL^{-1} were discriminated from control samples after 24 hrs of incubation. After 48 hrs incubation, it was possible to differentiate between the various spore concentrations present in the water samples.

iii) Different e-nose systems were compared with different sensor arrays. The conducting polymer sensor-based systems demonstrated similar results in the analysis of the different bacterial species, at 10^4 and 10^2 cells mL^{-1} incubated for 24h. Water samples inoculated with fungal spores were analysed with both conducting polymer and metal oxide systems and a good separation was observed between the control and fungal treatments. A good reproducibility was achieved as results from different days were consistent and data could thus be pooled for combined analysis, for detection of both bacterial and fungal species.

iv) The e-nose was not able to differentiate between pesticide contaminants, regardless of concentration. However, the headspace analysis of samples containing low concentrations of heavy metals using the e-nose system was consistent with the analysis by GC-SPME. In both cases, samples containing the heavy metal mixture could be discriminated from those without heavy metals.

v) The results from at-line studies using the e-nose for the detection of contamination episodes in water samples suggested the potential of this

technique for the detection of low concentrations of bacterial cells in water, including *E. coli*, a member of the coliform group. Different levels of contamination were detected using a downstream simulation experiment. Results obtained with the CO_2 sensor were coherent with those obtained with the MOS/MOSFET sensor arrays.

vi) Studies in the microbial activity of soil amended with heavy metals indicated that bacterial populations were more susceptible to the metal ions in soil, in comparison with fungal populations. The enzymatic activity expressed by the DHA in artificially contaminated soil decreased with an increase in metal concentration from 3 to 100 ppm. In naturally contaminated soil, the results were not very conclusive in terms of the relationship between the microbial activity and the total concentration of metals present in the soil samples.

vii) The headspace analysis of soil samples using the e-nose system was examined with a CO_2 sensor and the MOS/MOSFET sensor arrays. The CO_2 response suggested that there was a good relationship between basal respiration and DHA. The MOS/MOSFET sensor arrays indicated that for artificially contaminated soil samples, after 40 days incubation the control samples could be discriminated from those containing 3 and 100 ppm of metal ions. For naturally contaminated soils, the sensor array was only able to separate samples containing high concentrations of metal ions. Overall, the e-nose system showed a lower discrimination capacity for soil samples in comparison with the similar analyses performed in water samples.

viii) E-nose studies performed in cellulose-based agar showed a good discrimination between *Aspergillus terreus*, *A. hollandicus* and *Eurotium chevallieri*, after 20 hrs incubation at 25^{o}C. Malahanobi's squared distance between groups with a 95% confidence limit showed that an increase in the incubation period to 40 hrs better separation was obtained between the control and fungal treatments.

ix) Experiments performed in paper samples for the first time suggested that the e-nose was able to discriminate between control samples and paper inoculated with 10^3 fungal spores mL^{-1}. The results indicate that the substrate is very important for headspace analysis of microbial species. It was shown that the same fungal species produced different volatile profiles according to the growth substrate.

8.2 FUTURE WORK

i) Depending on the applications and the type of sample to be analysed, the choice of sensor array can be crucial for the good performance of the instrument. Conducting polymer sensors are particularly affected by humidity, so for many applications metal oxide-based systems are probably more suitable, especially for aqueous samples.

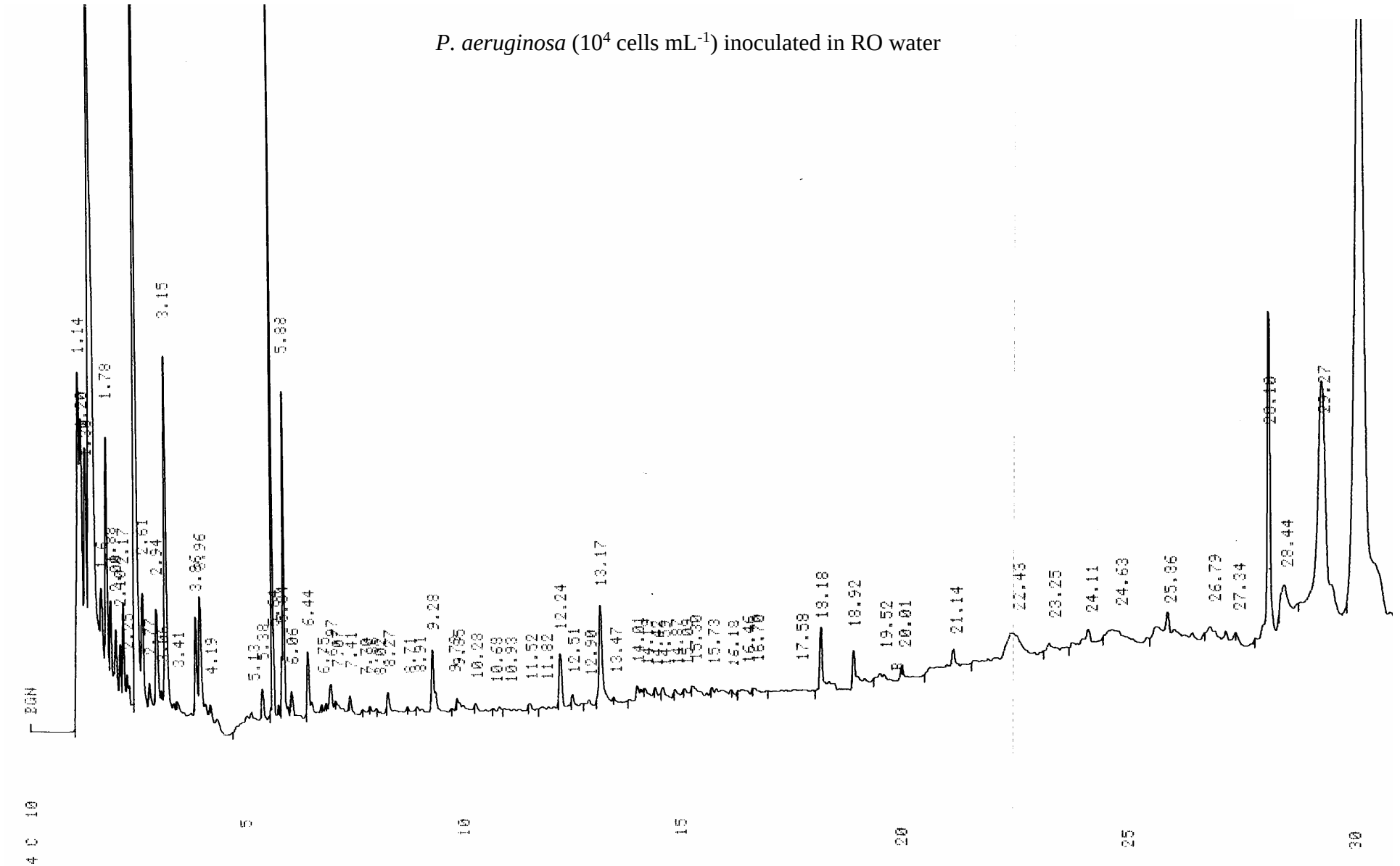
ii) To obtain a good standard operational procedure it is important to overcome the problem caused by sensor drift over time. One of the possible ways of doing so is to run an external standard periodically. The advantage of the standard is to account for changes over time due to the instrument, and create a correction factor to normalize the data.

iii) Apart from the coliform group, it would be interesting to study other microbial groups that cause taste and odour problems in drinking water treatment plants. For example, cyanobacteria are known to produce geosmin and affect watercourses during summer periods. The e-nose system could be used for effective monitoring in areas where these type of microorganisms naturally occur, using its lower detection limit as a preventive technique.

iv) Multivariate statistical analysis is a very reliable way to treat the large data sets produced by the e-nose system. However, the way forward in terms of data analysis is the use of an artificial neural network (ANN). This method is

particularly important when e-nose systems are used for at- or on-line monitoring, so that fresh samples can be rapidly compared with those stored in the database. Significant opportunities exist for application of these techniques to data obtained with e-nose systems.

v) For environmental applications more detailed knowledge is needed of the potential for use in predicting water and soil quality. Where clean up technologies have been employed or soil enrichment systems have been implemented then could e-nose systems be used for qualitative measurement of such improvements based on volatile finger prints.


P. aeruginosa (10^4 cells mL^{-1}) inoculated in RO water
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6.44
6.75
7.41
8.27
8.71
9.28
9.79
10.08
10.68
10.93
11.52
11.82
12.24
12.51
12.90
13.17
13.47
15.73
15.19
16.46
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22.45
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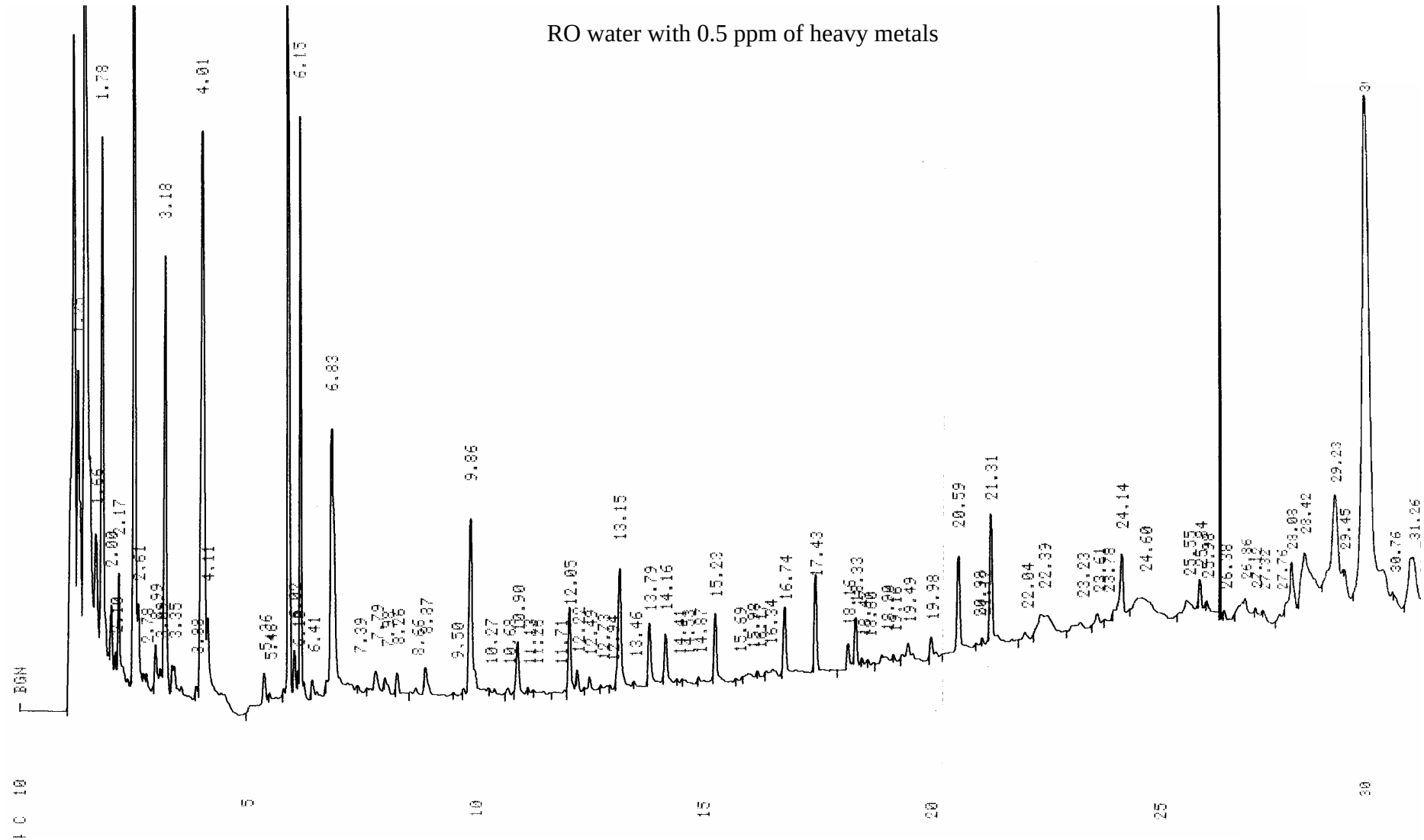
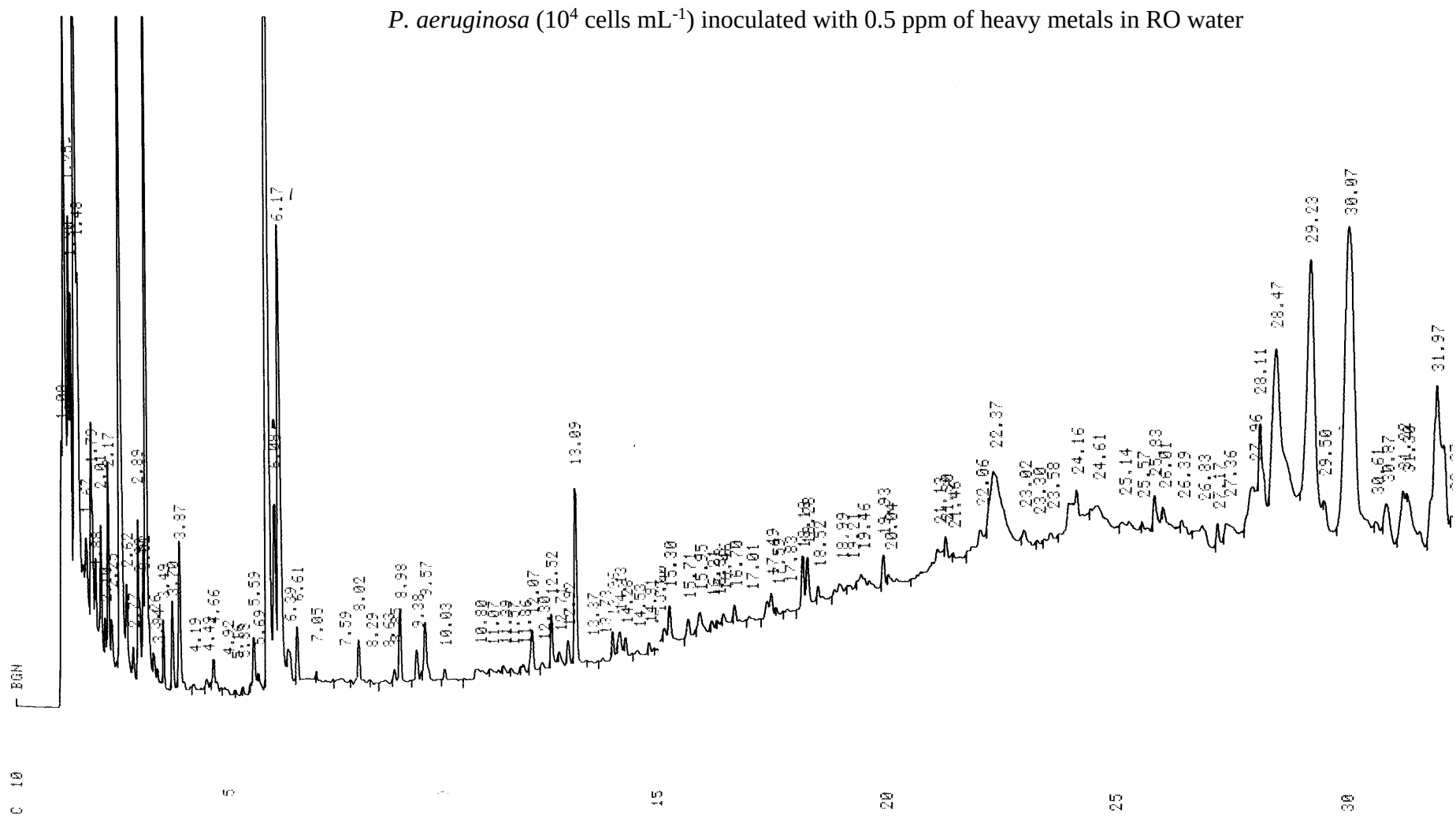
RO water with 0.5 ppm of heavy metals




P. aeruginosa (10^4 cells mL^{-1}) inoculated with 0.5 ppm of heavy metals in RO water
BGN
C 10
5
15
20
25
30
6.17
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22.37
22.06
24.16
24.61
28.47
29.03
30.07
31.97