Master in Chemical Engineering

Study on Biofilms formed by herbicide degrading microorganisms from agricultural soils

Master Thesis

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Abstract

The main objective of this study is to evaluate the microbial degradation of an herbicide, bromoxynil, used in the massive production of corn, by the microorganisms present in an agricultural soil. The work was designed to try to identify the main members of the bacterial community with a potential role on the degradation of the herbicide

For this purpose it was prepared an experiment in reactors for the microbial colonization of sand treated with bromoxynil by microorganisms in agricultural soil. The microbial colonization of the sand was analysed using culture-dependent and molecular methods and the degradation of bromoxynil was assessed. The results were compared with those from control reactors (sterile or free of the herbicide). In addition, the ability of the microbial communities established on the sand treated with bromoxynil for removing the herbicide form aqueous solutions was also analysed.

The results indicate that microorganisms are essential for the degradation of bromoxynil and suggest that *Ralstonia* sp. may be among the bacteria involved in the degradation of this herbicide. Other organisms detected or isolated from the microbial communities in the sand treated with bromoxynil were *Alcaligenes sp., Ochromobactrum* sp. and *Achromobacter* sp. The accumulation of the bromoxynil metabolite 3,5-dibromo-4-hydroxy-benzoic acid was not detected in the samples analysed. The elimination of bromoxynil in aqueous solutions by the microorganisms established on the sand was also proved.

Keywords: herbicide, microbial degradation, soil

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Notation and Glossary

List of Acronyms

AM	Ammonifiers
APS	Ammonium persulphate
BAM	2,6-dichlorobenzamide
Br	Bromoxynil
BrAC	3,5-dibromo-4-hydroxybenzoic acid
BrAM	3,5-dibromo-4-hydroxybenzamide
C	Carbon
CFU	Colony Forming Units
DAPI	4'-6-Diamidino-2-phenylindole
DGGE	Denaturing Gradient Gel Electrophoresis
E	Sterile
FISH	Fluorescent In Situ Hybridization
HBr	Hydrogen Bromide
MNP	Most Probable Number
MSM	Minimal Salts Media
N	Nitrogen
NE	Not Sterile
NE+Br	Not Sterile with Bromoxynil
PH	Heterotrophic Population
ppm	Parts Per Million (mg/L)
SM	Amylolytics
TEMED	N,N,N,N-Tetramethyl-Ethylenediamine
TFA	Trifluoroacetic acid
TV	2,5-Diphenyl-3-(α-naphthyl)tetrazolium chloride
WHC	Water Holding Capacity

1 Introduction

1.1 Theme Introduction and Presentation of the Project

Numerous pests can affect the growth of crops, which form a part of our daily diet, and pesticides are used to prevent the proliferation in crop fields of those pests, which include insects, bacteria, fungi, snails, viruses, rodents and weeds. The ideal pesticide should only be toxic to the target organisms, should be biodegradable, and should not leach into the groundwater. Unfortunately, this is rarely the case therefore the widespread use of pesticides in modern agriculture is of increasing concern (Johnsen *et al*, 2001; Wollansky and Harrill, 2007).

Potential toxicity of pesticides to non target organisms is the main hazard associated with their agricultural use. Human population can be exposed to these toxic chemicals via the gastrointestinal tract, respiratory system, as well as through the skin (Juraske *et al*, 2007; Lee *et al*, 2007; Yehia *et al*, 2007; Sallam and Morshedy, 2008). Occupation or accidental exposure to high doses of pesticides can result in life-threatening poisoning. The use of improper equipment for handling and bad cleaning practices, are particularly dangerous. However, long-term environmental exposure also has a negative impact on health. This is particularly important when human populations are exposed to pesticides through drinking contaminated water (Smith *et al*, 1999; Harman *et al*, 2004). There are several routes by which drinking water can be contaminated by pesticides. The main source is pesticide leaching to ground water following application on agricultural fields. Bromoxynil can be transported from treated agricultural land in irrigation or rainwater, affecting the quality of receiving streams (Baxter and Cummings, 2006).

Another important source of contamination by pesticides is the incorrect storage and disposal of expired pesticides in unsealed pits (Badach *et al*, 2007). It is obvious that the persistence of agricultural pesticides in soil and plants contributes to an increased risk of contact with these compounds and their potentially harmful effect on different compartments of the ecosystems. Selection of resistant species is also a serious problem.

The effects of pesticides on human and animal health depends on the nature of the compound although several disorders have been mentioned for many of the compounds used, among those cancer due to the induced mutagenicity and carcinogenicity (Narayana *et al*, 2004) or infertility (Saradha *et al*, 2008).

Although low-dose pesticides have been produced so that lower concentrations of a potentially toxic compound is introduced in the environment , in many cases there is still great concern about their influence on soil microbial community (Crecchio *et al*, 2001).

Herbicides are a type of pesticide used to prevent weeds from affecting the growth of crops. The focus of this study is the herbicide bromoxynil. The common use of bromoxynil begun after growing concerns in the European Union included atrazine in the EC Draft List of Highest Priority Pesticides (Category 1) because of its persistence and ability to disrupt the endocrine system. Bromoxynil replaced atrazine to some extent because they can both be used in corn fields and have the same path of action (Li *et al*, 2007; Singh *et al*, 2008).

Bromoxynil (3,5-dibromo-4-hydroxybenzonitrile) (Figure 1) is an halogenated aromatic nitrile herbicide employed for the control of annual broad-leaved weeds in grain crops (Health Canada, 2007). This compound acts as a, selective, contact, post-emergent herbicide which inhibits photosynthesis (Washington State Department of Transportation, 2006).

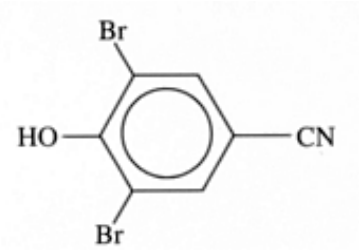

Figure 1: Molecular structure of Bromoxynil

For technical and practical reasons bromoxynil is generally applied as its octanoate ester (Figure 2) from which bromoxynil is released after the hydrolysis of the ester bond. Bromoxynil is especially effective in the control of weeds in cereal, corn, sorghum, onions, flax, mint, turf and on non-cropland (Extoxnet, 1996). Although it is used as a substitute of a highly toxic herbicide, bromoxynil has been shown to have prejudicial ecological effects on birds and aquatic organisms (Extoxnet, 1996).

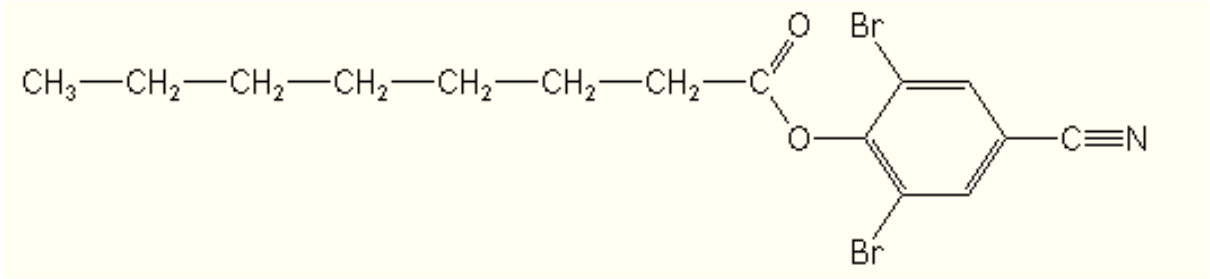

Figure 2: Molecular structure of Bromoxynil Octanoate

The half-life of bromoxynil in soil varies in literature from a few hours to several months (Hsu and Camper, 1975; Topp *et al*, 1992; Millet *et al*, 1998; Semchuck *et al*, 2003; Pampulha and Oliveira, 2006) depending on the soil characteristics and environmental conditions. Biodegradation is the primary mechanism of dissipation (Topp *et al*, 1992) although photolytic degradation has also been reported. Potential leaching and surface runoff of bromoxynil to groundwater are low although its potential loss on eroded soil is intermediate. Bromoxynil has moderate volatility and potential loss via evaporation to the atmosphere is moderate. Bromoxynil is primarily absorbed through leaves with limited translocation to other plant parts (Washington State Department of Transportation, 2006).

Some of the pathways studied to degrade Bromoxynil include the use of different enzymes (Holtze *et al*, 2006), different microorganisms (Pampulha and Oliveira, 2006; Baxter and Cummings, 2006; Dubey and Holmes, 1995) including bacteria (Topp *et al*, 1992; Baxter and Cummings, 2006; O'Reilly *et* Turner, 2003). A possible pathway for the degradation of bromoxynil is the hydrolysis of the nitrile group (Martinkova, 1993; Banerjee *et al*, 2002) since nitriles are highly toxic, mutagenic and carcinogenic in nature (Pollak *et al*, 1991). Another important pathway is the degradation of the cyanide group (Topp *et al*, 1992; Baxter and Cummings, 2006; Dubey and Holmes, 1995). Nitrile-metabolizing enzymes efficiently degrade these cyano group-containing herbicides and prevent them from entering the food chain. *Agrobacterium radiobacter*, a bromoxynil-degrading soil bacterium, is used for the degradation of the herbicide (Banerjee *et al*, 2002).

Biological remediation of soils is the most logical solution for pesticide contamination since soils are a natural haven of microorganisms. The ideal remediation strategy for persistent pesticides is a bacterium or consortium of bacteria present in, or added to, the soil and which are able to use the pesticide as a nutritional source, thereby reducing its concentration and preventing leaching to underground waters. In the case of bromoxynil, this compound may be a good carbon or nitrogen source for bacteria which can then contribute to diminish its toxicity.

This sounds very simple but the persistence, degradation and mobility of pesticides and their metabolites in soil depends upon many factors, of which biological factors are among the most important. Microorganisms, particularly bacteria, because of their enormous metabolic versatility and their ability to develop new metabolic routes for the degradation of xenobiotics, are essential agents for the elimination of pesticides and of other compounds introduced by humans in the environment. On the other hand, some metabolites produced as a result of this microbial activity are in themselves more mobile and toxic than the original compound. In the case of relatively new compounds introduced into the environment such as bromoxynil, it is important to study the potential accumulation of toxic metabolites and the

microbial communities involved in their degradation. The information obtained about soil microbial degradation of pesticides is also essential for the exploitation of these decontaminating abilities and the development of cost-effective and environmentally friendly bioremediation technologies.

The present work was designed to analyse the colonisation of sand contaminated with bromoxynil by microorganisms present in agricultural soil with a history of bromoxynil applications. The main focus of the study was on bacteria growing on the contaminated sand. Analysis of the degradation of this herbicide after microbial colonization, including the measurement of potential metabolites, was also done.

1.2 Work Contribution

Bromoxynil is an herbicide that has been synthesized since 1976 (Poignant *et al*, 1976) but that only became popular and of extensive use with the recent ban of atrazine.

Published data on the microbial degradation of bromoxynil is scarce, and the present work aims to provide new information on the degradation of this compound by soil microorganisms paying special attention to bacterial activity.

1.3 Thesis Organization

The present work was planned for studying the colonisation of sand treated with the herbicide bromoxynil by microorganisms from an agricultural soil and for analysing the effect of this colonisation on the bromoxynil concentration.

The first step was to carry out a chemical and microbiological characterization of the soil used. In a second step, experiments for the microbial colonisation of sand treated with bromoxynil were established. Control experiments with sterile sand and sand non-treated with bromoxynil were also prepared. Finally the sand samples (treated and non-treated with bromoxynil, sterile and non-sterile) were chemically and microbiologically analysed.

The report is organised in chapters as follows.

The first chapter describes the setup of the experiments done and their main purpose.

The second chapter presents the methods used including microbiological and chemical analysis carried out on sand and soil samples before and after carrying out the experiments of colonisation. More specifically the determinations of bacterial densities by plate count and most probable number (MPN), the analysis using molecular methods applied to both total DNA extracted from colonised sand and to bacterial isolates, the general physicochemical

characteristics of the sand and soil used (pH, humidity, water holding capacity), the bromoxynil concentration in the reactors before and after incubation. This was carried out to evaluate how quickly and extensively bromoxynil is degraded and by which microbial colonisers.

The third chapter presents the results and discussion of the methods applied.

2 State of the Art

Despite the widespread use of herbicides containing nitrile groups, such as dichlobenil (2,6-dichlorobenzonitrile), bromoxynil (3,5-dibromo-4-hydroxybenzonitrile), ioxynil (4-hydroxy-3,5-diiodobenzonitrile), and buctril (2,6-dibromo-4-cyanophenyl octanate), comparatively little is known about the microbial metabolism of nitriles and, in particular, the mechanism of cleavage of the C-N bond by microorganisms. Harper (1976, 1977) showed that a number of microorganisms isolated from soil were capable of using benzonitrile or 4-hydroxybenzonitrile as a sole carbon and nitrogen source.
A bacterial strain was isolated from a soil sample which could use acetonitrile as a sole source of carbon and nitrogen (Bandyopadhyay *et al*, 1986). The strain was classified into the genus *Arthrobacter* and designated *Arthrobacter* sp. Strain J-1 (Yamada *et al*, 1979). Subsequently, it was found that this strain could also grow on benzonitrile as a sole carbon and nitrogen source. In the course of an investigation on the microbial degradation of benzonitrile by this organism, two very similar enzymes with only a few differences in their properties were found. This organism also grew on acetonitrile as a sole carbon and nitrogen source with the production of nitrile hydratase (Asano *et al*, 1982) and amidase (Winter *et* Karlson, 1976). Thus, it was found that there were two different types of nitrile-degrading enzymes operative in this organism.

Nitrile hydratase transforms aliphatic nitriles to the corresponding amides only, without the formation of carboxylic acids and ammonia. Therefore, there are at least two distinct pathways for nitrile hydrolysis in this microorganism. One is a pathway in which both nitrile hydratase and amidase are involved, and the other is one in which only nitrilase is involved. The chemical hydrolysis of acetonitrile, propionitrile, and benzonitrile proceeds exclusively via amides (Rabinovitch *et al*, 1942; Rabinovitch *et al*, 1942; Rabinovitch *et al*, 1942).(Bandyopadhyay *et al*, 1986)
Other biodegradation experiments showed a rapid disappearance of bromoxynil (Smith, 1971). In a biofilm reactor *Agrobacterium radiobacter* was able to degrade bromoxynil within a short time (Müller *et* Gabriel, 1999). In soil and in cultures of *Pseudomonas putida 13XF*, various metabolites resulted from stepwise hydrolysis of the cyano group (formation of the corresponding benzamide and benzoic acid), from partial debromination, and from hydroxyl methylation (Golovleva *et al*, 1988). Vokounová *et al* (1992) and Gabriel *et al* (1996) also found *Pseudomonas putida 13XF* to be able to convert bromoxynil to 3,5-dibromo-4-hydroxybenzamide and 3,5-dibromo-4-hydroxybenzoic acid in the presence of a carbon and energy source, indicating that this strain possesses nitrile hydratase activity (formation of the

benzamide) as well as amidase activity (formation of the benzoic acid). *Klebsiella pneumonia* subsp. *ozaenae* uses bromoxynil as nitrogen source by direct formation of 3,5-dibromo-4-hydroxybenzoic acid and NH_4^+ with the enzyme nitrilase (McBride *et al*, 1986). Smith and Cullimore (1974) observed a bromoxynil degradation to 3,5-dibromo-4-hydroxybenzamide and 3,5-dibromo-4-hydroxybenzoic acid and a further nonidentified metabolite by a strain of *Flexibacterium*. An investigation was made of the possible formation of degradation products using the bacterium *Variovorax* sp. DSM11402, as this bacterium has previously shown degradation potential towards dichlobenil and demonstrated that bromoxynil was fully transformed into its corresponding amide in 2–5 days (Holtze *et al*, 2004, Nielsen *et al*, 2007). This amide was not further degraded within 18 days, and formation of other degradation products was not observed (Nielsen *et al*, 2007).

In contrast, a *Flavobacterium* strain degrades bromoxynil by cleavage of the nitrile group and formation of cyanide and 2,6- dibromohydrochinone (Topp *et al*, 1992). The responsible enzyme is a hydroxylase capable to transform pentachlorophenol. In model aquifers two main pathways of bromoxynil metabolism were found: hydrolysis of the nitrile group and replacement of bromine by chlorine when chloride was present in the matrix (Graß *et al*, 2000). *Desulfitobacterium chlororespirans* is another bacteria that has been shown to degrade bromoxynil but this time in anaerobiosis or transient aerobiosis by debromination in soils (Cupples *et al* 2005). This bacteria uses bromoxynil as an electron acceptor for growth.

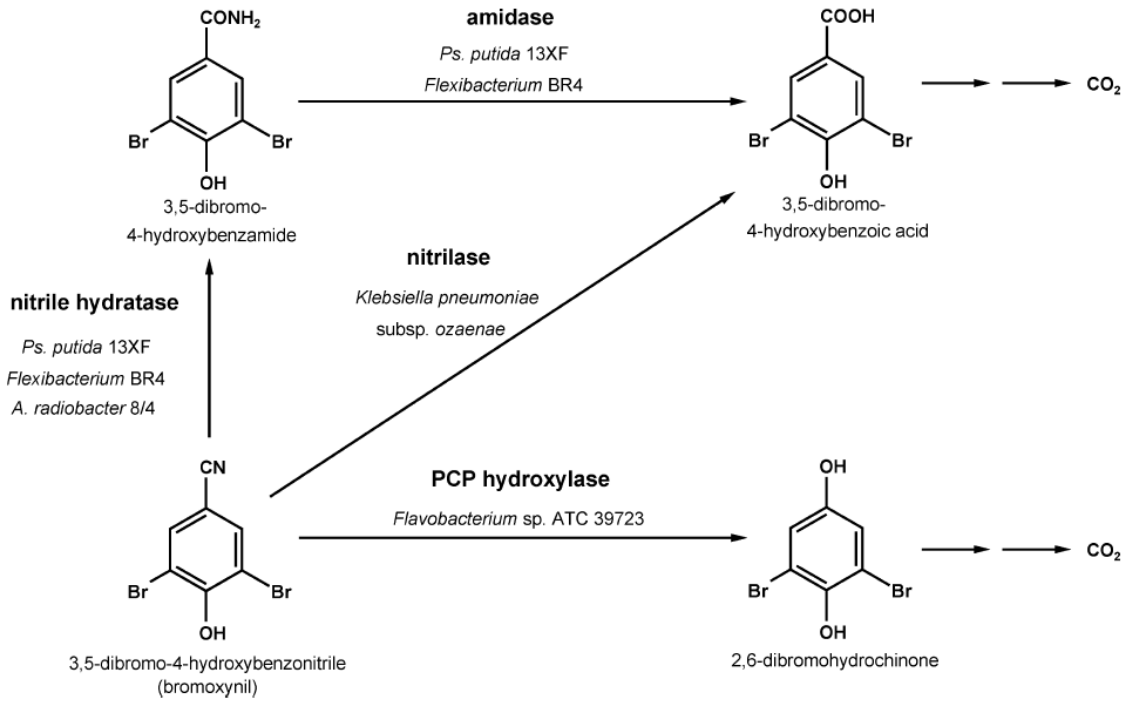

Figure 3: Initial degradation reactions of bromoxynil and some enzymes and microorganisms involved (Smith *and* Cullimore, 1974; McBride *et al*, 1986; Topp *et al*, 1992; Vokounová *et al*, 1992; Gabriel *et al*, 1996).(Rosenbrock et al, 2003)

Abiotic degradation of bromoxynil by photolysis is also reported (Millet *et al*, 1998; Texier *et al*, 1999). Two polar metabolites were observed as degradation products of bromoxynil. One metabolite could be identified as 3,5-dibromo-4-hydroxybenzoic acid and the other was characterized as 3,5-dibromo-4-hydroxybenzamide.

3 Technical Description and Discussion of Results

The detailed description of the methods and techniques as well as preparation of solutions and media is presented in appendix 1 and additional results, images and tables can be consulted in appendix 2.

3.1 Experiments

3.1.1 Experiment for the microbial colonization of sand treated with bromoxynil

The experiment for obtaining sand impregnated with bromoxynil colonised by soil microorganisms was carried out in glass reactors.

The reactors were cylindrical and 16cm high. The six reactors had a 5.5cm internal diameter. The bottom of the reactors was connected to a polypropylene tube closed with a metallic clip.

The reactors were assembled by placing layer of glass wool at the bottom; over this layer, the reactors were filled with 200g of autoclaved sand treated with bromoxynil as described below (3 reactors) or not treated with the herbicide (3 control reactors). The sand was covered by another layer of glass wool and finally by a layer of 30 g of soil. The soil used in the reactors came from a land in Spain in a place called Cabanas in A Baña (NW Spain) where corn is grown and the sand made of quartz and came from de outskirts of Santiago de Compostela.

The moisture of the sand was adjusted to the field capacity by adding mineral medium (see Appendix I). Mineral medium was used instead of water to provide enough inorganic nutrients to microorganisms colonising the sand, because a deficiency of these elements could restrict microbial growth and degradation of bromoxynil. The glass wool was also wetted with mineral medium to allow the movement of microorganisms from the soil layer to the sand. The soil humidity was equivalent to the field capacity at the moment of the assembly, therefore there was no need to add mineral medium.

Similar systems containing sterile sand treated with bromoxynil but without a layer of soil were prepared.

The reactors were covered with polypropylene film (Parafilm®) and wrapped in aluminium foil. The polypropylene film was used to prevent evaporation and the aluminium foil was used to prevent the incidence of light and consequent growth of photosynthetic organisms since

this study's focus was on the degradation of bromoxynil by non-photosynthetic soil microorganisms, the main members of the edaphic microbial community.

The reactors were then incubated in a chamber at 25ºC for one month. The polypropylene film and the aluminium foil were renewed when necessary (Figure 4).

Figure 4: The six reactors with the soil, three of which containing sand impregnated with bromoxynil.

The sand used for these experiments was quartz sand sieved at 0.5 mm, washed twice with HCl (24%) and rinsed with abundant deionised water and dried at 50ºC. After these treatments the sand was sterilised by applying 3 consecutive cycles of 30 min at 121ºC in an autoclave. Between each autoclaving cycle the sand was incubated at room temperature for 24h-72h. The repeated autoclaving and incubation at room temperature was done to ensure the germination and death of microbial spores.

A solution of 1% bromoxynil in acetone sterilised by filtration (polyester syringe filter with 0.20μm of pore diameter (CHROMAFIL, PET-20/25, Macherey-Nagel)) with pH 9.5 was prepared and diluted by mixing 125μl of this solution in 50ml of pure acetone. This diluted solution was used to apply the bromoxynil to the sand by mixing thoroughly 33.0 ml of the diluted solution with 600g of sand. The sand used for preparing the control reactors without bromoxynil was treated in the same way with pure acetone. The acetone of both, sand with and without bromoxynil, was allowed to evaporate before assembling the reactors. All these manipulations of the sand, acetone and bromoxynil solutions were done under sterile conditions.

The concentration of Bromoxynil used to impregnate the sand was around 5 fold superior to the concentration recommended by the manufacturer for agricultural soils. The objective was to induce the establishment of a microbial community tolerant and/or with a role on the

degradation of bromoxynil on the sand. At the same time, it is highly probable that microorganisms on the soil surface are exposed to this level of the herbicide after its immediate application in agricultural lands, therefore the amount used artificially is not higher than present in the field.

In summary, the experiment contained three reactors with sand treated with bromoxynil and in contact with soil (source of microorganisms) henceforth referred to as NE+Br and two type of control reactors: three reactors with sand not treated with the herbicide and in contact with soil (NE) and other three reactors with sand treated with bromoxynil that were kept sterile along the experiment (without soil) (E).

The three reactors without bromoxynil (NE) were used to monitor other components eventually present in the sand and which might be extracted and appear when the sand extracts are analysed for the bromoxynil content by HPLC (High Pressure Liquid Chromatography) (explained below). The reactors were also used to study the microorganisms from the soil which colonise the sand and to compare these microorganisms with those established in the sand with bromoxynil.

The sterile reactors (E) were used as a reference of the potential degradation of bromoxynil in abiotic conditions.

After one month of incubation, the reactors were disassembled. The sand and soil were separated and divided in portions for the different analysis.

Several analyses of the soil and sand were carried out before and after the incubation, following the methods described in section 3.2 and in Appendix 1:

Before the incubation:

- Analysis of the pH of the sand;
- Analysis of the moisture and water holding capacity of the soil and sand;
- Analysis of the bromoxynil concentration in the sand treated and non treated with bromoxynil;
- Determination by most probable number of the heterotrophic population, starch mineralisers, ammonifying microorganisms and microorganisms able to use different C substrates in the soil;

After the incubation:

- Analysis of the pH of the sand;
- Analysis of the moisture of the soil and sand;

- Analysis of the bromoxynil concentration in the sand treated and non treated with bromoxynil;
- Determination by most probable number of the heterotrophic population, starch mineralisers, ammonifying microorganisms and microorganisms able to use different C substrates in the soil and sand;
- Plate count determinations of microorganisms able to grow on a medium with bromoxynil as the sole C and/or N source in the soil and sand;
- Extraction of the DNA present in the sand from the colonising microorganisms.

3.1.2 Experiment for testing the elimination of bromoxynil from aqueous solutions by soil microorganisms established on sand treated with bromoxynil

The experiment was setup to test the ability of the microorganisms that were able to colonise the sand treated with bromoxynil to eliminate the herbicide from aqueous solutions by preparing new glass reactors. The reactors used for this experiment were the same described in 3.1.1.

Three reactors were filled with 100g of sterile sand not treated with bromoxynil, which was previously mixed with 5g of sand treated with bromoxynil and colonised by soil microorganisms (from the previous experiment). The sand was placed in the reactors between two layers of glass wool. After assembling the reactors, 70mL of mineral medium containing 3.5 mg $_{bromoxynil}.L^{-1}$ were added and the system was incubated 5 days in a chamber at 25ºC.

Four sterile reactors were also assembled. The reactors contained 50g of autoclaved sand and 2.5g of sand used in the reactors from the previous experiment. After assembling the reactors 35mL of mineral medium containing 3.5 mg $_{bromoxynil}.L^{-1}$ were added and then incubated 5 days in a chamber at 25ºC.

After this period the mineral medium was removed by opening the clip that closed the polypropylene tube at the bottom of the reactors. The content of the reactor was washed by passing deionised water (once) and mineral medium without bromoxynil (twice) through the sand. After the washing steps, mineral medium with bromoxynil was applied again and the system incubated for 20 days. The concentration of bromoxynil was measured after 12h, 10 and 20 days of incubation.

3.2 Methods

This section presents the different determinations carried out. The detailed description of the media used for cultivating microorganisms and other details of the techniques applied are explained in the Appendix 1.

3.2.1 Determination of humidity

The humidity of the soil and the sand was calculated. Samples were dried to constant weight at 105ºC.

3.2.2 Determination of water holding capacity

The water holding capacity (WHC) is the measure of the quantity of water that that soil or sand are capable of retaining. It's the humidity of a soil after a long period of rain and posterior gravitational drainage. It depends on the amount of organic matter in the soil as well as on soil particle size (Fuller, 2007). The experiments were carried out at 100% of the WHC, which ensures good aeration and provides enough water for microbial development

The WHC was measured as the humidity remaining in the soil or sand (previously saturated with water by capillarity) after applying a pressure equivalent to pF=2 (pressure of a column of water of 100 cm on a section of 1 cm^2). The determinations were made in Richard membrane apparatus.

3.2.3 pH determination

The microbial degradation of bromoxynil could lead to the production of HBr which would result in reduction of pH in the soil and/or sand. Therefore pH in the sand before assembling and after disassembling the reactors was measured under agitation with a ratio sand:water 1:2.5 using a calomel electrode.

3.2.4 Determination of culturable microorganisms

3.2.4.1 Preparation of soil inocula for microbial counts

5g of soil or sand were suspended in tubes with 45mL of sodium hexametaphosphate solution (1%) and were shaken for about 1 hour in an end-over-end shaker so as to detach microbial cells from the solid particles. The suspensions were diluted in 10 fold series from 10^{-1} up to 10^{-8}.

3.2.4.2 Microbial Determinations by Most Probable Number (MPN)

Culturable heterotrophic bacteria, the ammonifying and the amylolytic populations, as well as the ability of soil/sand microbial communities to utilize different C substrates were

determined by the most probable number (MPN) technique. The MPN of bacteria able to grow in media with bromoxynil as carbon and nitrogen source, bromoxynil as ~~a~~ carbon source, bromoxynil as nitrogen source and in a rich medium supplemented with bromoxynil were also estimated.

The MPN Technique is used to estimate microbial population sizes when quantitative assessment of individual cells is not possible. Exact cell numbers of an individual organism frequently cannot be measured in soils due to heterogeneous populations or unavailability of a suitable diagnostic media. The MNP technique relies on the detection of specific qualitative attributes of the microorganism of interest (Woomer, 1994).

The pattern of carbon substrate utilization by the microbial communities was analyzed by selecting 31 carbon sources from those included in Ecoplate™ from Biolog Inc. or recommended by Kennedy (1994).

The list of substrates used in the microtiter plates is shown in Appendix 1.

The substrates used belong to different chemical groups: carbohydrates, carboxylic acids, amino acids and polymers.

The redox dye, tetrazolium violet (2,5-Diphenyl-3-(α-naphthyl)tetrazolium chloride (TV), 15mM) was used to indicate growth (dehydrogenase activity), and was added to the different media using the ratio 1:100 (v/v, TV:media).

Microtiter plates were prepared as described in Appendix 1 and were inoculated with 50μL of the different soil or sand dilutions. In the case of the heterotrophic population, ammonifiers, amylolytics and the media containing bromoxynil 4 wells per dilution level (10^{-1} to 10^{-8}) of each soil/sand sample were inoculated (each microtiter plate contained samples from three reactors). In the case of media with different C substrates one well per dilution level (10^{-2} to 10^{-7}) of each soil/sand sample was prepared and the MPN was estimated taking into account results of the three replicate reactors.

Growth/substrate utilization was indicated by violet colour development of the tetrazolium redox dye and results were recorded after 1 and 3 weeks incubation at 28 ºC before incubation for the soil samples and after incubation for the soil and sand samples.

3.2.4.3 Plate Count Determinations

The plate count technique was used to determine the culturable bacteria able to growth in four different solid media containing: I) bromoxynil as sole carbon and nitrogen source, II) bromoxynil as carbon source and an added nitrogen source, III) bromoxynil as nitrogen source and an added carbon sources and IV) bromoxynil and added carbon and nitrogen sources. As

indicated in the previous section this technique was applied for soil samples before incubation as well as for soil and sand samples after incubation.

50μL of the different soil/sand dilutions were spread on the surface of Petri dishes prepared with these media. The plates were incubated at 28 ºC and the number of colonies formed was recorded after 1 and 3 weeks of incubation. Only the plates with 30-300 colonies were taken into account for the calculations.

This technique allows to count the number of microorganisms in a sample of soil and at the same time to observe the different microbial morphologies present in the soil sample so it provides information on the number as well as on the diversity of the microorganisms growing on the media used.

3.2.5 Denaturing Gradient Gel Electrophoresis

Genetic fingerprinting of microbial communities provides banding patterns or profiles that reflect the genetic diversity of the community. Denaturing Gel Electrophoresis (DGGE) of PCR-amplified gene fragments is one of the genetic fingerprinting techniques used in microbial ecology. In DGGE, similar sized DNA fragments are separated in a gradient of DNA denaturants according to differences in sequence (Schäfer *et* Muyzer, 2001).

Amplification of DNA extracted from mixed microbial communities with primers specific for 16S rRNA gene fragments of bacteria result in mixtures of PCR products. Because these products all have the same size, they cannot be separated from each other by agarose gel electrophoresis. However, sequence variations between different bacterial rRNAs bring about different melting properties of these DNA molecules, and separation can be achieved in polyacrylamide gels containing a gradient of DNA denaturants, such as urea and formamide. PCR products enter the gel as double-stranded molecules. As they proceed through the gel the denaturing conditions gradually become stronger and PCR products with different sequences start melting at different positions (Schafer *et* Gerard, 2001).

DNA from the different samples of sand treated and not treated with bromoxynil was extracted after incubation of the reactors. 16S rDNA fragments were successfully amplified. The details of DNA extraction and PCR amplification and concentration of PCR products are included in Appendix 1.

The instruction manual of the IngenyphorU-2 Electrophoresis system (Figure 5) was followed for the gel preparation as well as for the Electrophoresis itself.

Figure 5: Electrophoresis cassette assembled in the buffer reservoir.

Approximately 500ng of DNA were loaded from each sample in the gel.

The electrophoresis was done for 16h at 100V in buffer TAE 0.5x.

3.2.6 Measure of concentration of Bromoxynil and metabolites

50g of soil or sand were air dried and mixed with 100 ml of dichloromethane:methanol 90:10 acidified with 1% trifluoroacetic acid and shaken overnight in a glass bottle in an end-over-end shaker.

The content of the glass bottle was vacuum filtered, the soil or sand discarded and the filtrate evaporated in a rotavapour.

After the evaporation the residue was redissolved in 5mL of acetonitrile and then filtered with a polyester syringe filter with 0.20μm of pore diameter (CHROMAFIL, PET-20/25, Macherey-Nagel). These extracts obtained are kept at 4ºC until analysis.

The analysis of bromoxynil in aqueous solutions was carried out directly, without extraction with organic solvents, after filtering the samples through the filters detailed above.

The amount of bromoxynil and of its known metabolite 3,5-dibromo-hydroxy-benzoic acid (DBHB) were analysed in the sand extracts by HPLC (High Performance Liquid Chromatography).

HPLC analysis was carried out using a Waters equipment.

The mobile phase used was composed of acetonitrile and water in a ratio of 50:50 acidified to pH 2.3 with TFA (Trifluoroacetic acid). The flow of the mobile phase was 1.4 mL/min. The UV detector was set at 214 nm.

The column is manufactured by Hypersil Gold aQ manufactured by Thermo, and was 25 cm long and 4.6mm wide with particle size of 5μm. Hypersil GOLD aQ is a polar endcapped C18 phase, it also offers superior retention of polar compounds. Dispersive interactions are the primary mechanism of retention with alkyl chain bonded phases.

3.3 Results and Discussion

3.3.1 Determination of humidity

The percentage of humidity determined for the soil was 30.4% and for the sand 3.4%.

There wasn't any difference in the humidity of the soil or the sand before and after incubation.

3.3.2 Determination of water holding capacity

The water holding capacity of the soil was determined to be 47.11mL.100g$^{-1}_{\text{dry soil}}$ and of the sand 4.5mL.100g$^{-1}_{\text{dry sand}}$.

3.3.3 pH determination

The result of the determination of pH for the sand from the reactors with bromoxynil before incubation was 7.04 and after incubation was 6.84.

Although the difference wasn't very large, this could be indicative of bromoxynil degradation.

3.3.4 Determination of culturable microorganisms

The results presented in this section focus mostly on the microorganisms in the sand specially on those that colonised the reactors with bromoxynil although the results for the soil before and after incubation are also presented.

3.3.4.1 Microbial Determinations by Most Probable Number (MPN)

The experiment carried out allowed the colonisation of the sand treated with bromoxynil by microorganisms from the agricultural soil with history of application of this herbicide. The $\log_{10}$ of MPN per gram of sand of heterotrophic microorganisms after the incubation was 7.1±0.7. The sand not treated with bromoxynil was also colonised by microorganisms, reaching similar densities to the ones in the sand treated with the herbicide (Figure 6). The abundance of amonifying microorganisms was also high in both types of samples. Nevertheless the density of starch mineralisers was lower in the sand treated with bromoxynil than in the control. This was more evident after 1 week of incubation of the inoculated media, which suggests that the herbicide alters the community of microorganisms able to degrade starch

thus, in the bromoxynil treated reactors, the microorganisms with fast growth on this C source were less abundant.

The $\log_{10}$ MPN per gram of soil of heterotrophic microorganisms in the soil at the beginning of the experiments was 6.5±0.2, the values of $\log_{10}$ MPN for the amonifying and amylolytic densities were 6.0±0.3 and 6.1±0.5, respectively. The density of these microorganisms tended to increase slightly at the end of the experiment, but it did not change significantly (Figure 6). The optimal conditions of moisture and temperature during the experiment can explain the change observed.

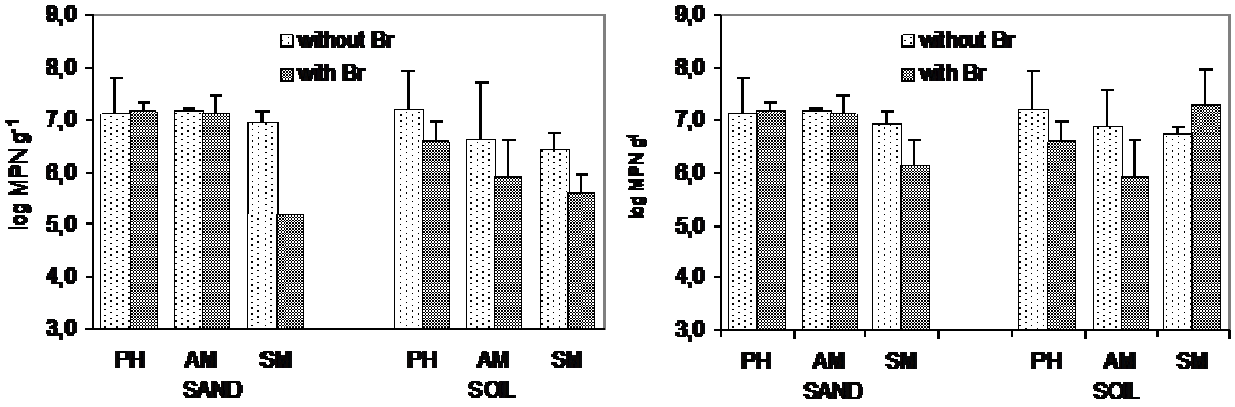

Figure 6: Estimates of the heterotrophic population (PH), ammonifiers (AM) and starch mineralizers (SM) by Most Probable Number in the sand and soil samples at the end of the experiment of colonization after 1 week (left) and after 3 weeks (right).

Surprisingly the densities of different microorganisms in the sand samples were as high as in the soil. The bromoxynil applied, little amounts of the acetone used, organic compounds remaining in the sand after the acid washing or lixiviated from the underlaying soil can be some of the possible C sources necessary for sustaining these populations. In water samples treated with low amounts (1ppm) of the herbicide acetochlor other authors found that the log of the densities of total and viable bacteria can reach values higher than 6 (Foley *et al*, 2007), which is in agreement with the results obtained.

In regard to the ability of the microbial communities established on the sand samples for degrading several C sources, Table 6 (Appendix 2) shows the $\log_{10}$ MPN per gram of soil or sand estimates 1 and 3 weeks after the inoculation of the medium with different substrates. Part of the table is shown bellow.

The incubation time had little or no influence on the profiles of C degradation by the microbial communities of both, the soil and the sand samples. For most of the substrates the variation of the MPN estimates between 1 and 3 weeks of incubation was lower than one logaritmic unit.

The communities in both the sand treated and not treated with bromoxynil as well as in the soil showed high metabolic diversity as analysed by the profiles of degradation of different C substrates. In the case of soil samples the abundance of organisms able to degrade different substrates is similar, regardless of the presence of bromoxynil in the underlying sand. In the case of the sand samples it can be pointed out that the MPN of microorganisms able to degrade lactose, the organic acids galacturonic and malonic the δ–lactone of galactonic acid and the amines β-phenyl ethylamine and putrescine tended to be more abundant in the reactors with bromoxynil than in the non-treated ones.

Table 1: List of substrates where the MPN of microorganisms was larger in the reactors with bromoxynil.

	Reactors			
	without Br		with Br	
Substrate	**1 week**	**3 weeks**	**1 week**	**3 weeks**
(6)α-lactose	5.1	6.3	7.0	7.0
(14)D-galactonic acid δ-lactone	3.0	BDL	5.2	5.2
(15)galacturonic acid	5.9	5.9	8.1	8.1
(18)malonic acid	8.4	8.4	ADL	ADL
(25)β-phenyl ethylamine	5.9	5.9	4.8	4.8
(26)putrescine	4.4	4.8	5.1	5.6

On the contrary the MPN of degraders of glucose1-phosphate, o-hydroxybenzoic acid and the aminoacids phenylalanine and threonine were lower in the bromoxynil treated sand than in the control.

Table 1: List of substrates where the MPN of microorganisms was larger in the reactors without bromoxynil.

	Reactors			
	without Br		with Br	
Substrate	**1 week**	**3 weeks**	**1 week**	**3 weeks**
(12)α-D- glucose 1-phosphate	6.6	7.1	5.7	6.3
(16)o-hydroxibenzoic acid	7.8	7.8	6.5	6.8
(21)L-phenylalanine	8.4	8.4	5.6	6.0
(23)L-threonine	6.3	ADL	4.3	4.3

3.3.4.2 Plate Count Determinations

The colony forming units (cfu) on the plates with media containing C and N sources and supplemented with bromoxynil reached similar average values in the sand treated or not treated with bromoxynil, Log (cfu) g^{-1} of 5,2±0,1 g^{-1} and 5,0±0,2 g^{-1}, respectively. Surprisingly these values were very similar to the counts on the plates with bromoxynil as sole C and/or N source (Table 3 in the Appendix 2). Moreover the counts on these plates were also very similar in the sand treated with bromoxynil or not treated with the herbicide. It has to be pointed out that most of the colonies on these plates with bromoxynil as sole C and/or N source were very small and their size did not increase during the three weeks of incubation which suggests that are oligotrophic organisms able to grow in trace impurities of the agar.

In the case of MPN estimates, the number of microorganisms using bromoxynil as C and/or N source was below the detection limit both after 1 or 3 weeks incubation of the inoculated microplates. These results are not in agreement with the plate count technique and seem to confirm that most of the organisms forming colonies on the plates with bromoxynil as sole C and/or N source do not grow on the herbicide but on other impurities in the chemical used for preparing the plates. On the other hand, it has been shown that some organic compounds can support growth of microorganisms but do not provide enough flow of electrons for the efficient reduction of redox dyes as TV used (Johnsen *et al*, 2002).

As stated before most of the colonies on the plates with bromoxynil as sole C and/or N source were very small (around 1 mm). Nevertheless some bigger colonies were observed: In the plates with bromoxynil as sole C and N source two main types of colonies were found: one colony morphology was bright white with a smooth edge and the other was greyish with several inner circles and a rugged edge. In the case of the plates with bromoxynil as sole N source inoculated with microorganisms from the reactors not treated with the herbicide, two colony morphologies were observed while in plates with inocula from the reactors with bromoxynil the diversity of colony morphologies was greater.

Some of the colonies growing on the plates with bromoxynil as sole C and/or N source and inoculated with the dilution 10^{-4} were recultivated until pure isolates were obtained (in general 1-3 representatives of each morphotype were recultivated). Attention was also paid to a small colony white, opaque that appeared in the plates with Bromoxynil as sole N source inoculated with bacteria from the reactors treated with the herbicide but not in those plates prepared with inocula from the non treated reactors. 18 of those colonies have been recultivated and one is shown in the figure bellow.

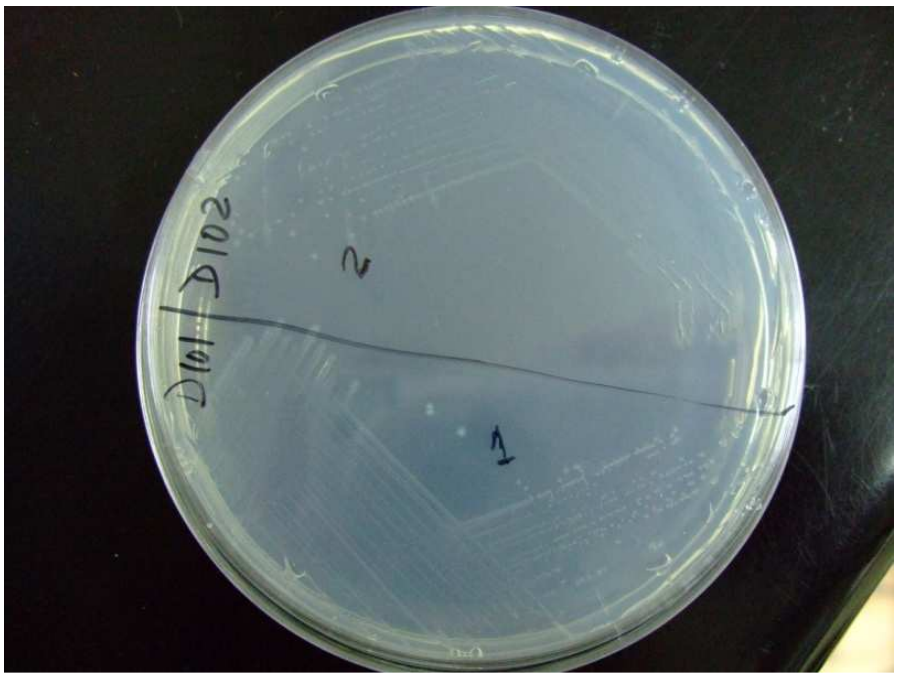

Figure 7: Recultivated colonies from one of the reactors with bromoxynil.

Fragments of the gene coding the 16S rRNA of few of the isolates from the reactors with bromoxynil obtained by recultivation in media with bromoxynil were analysed by DGGE together with the amplicons obtained from the DNA from the sand samples with the mixture of microorganisms. The isolates originating a band on the same position as the main bands of the profiles of the bacterial communities from the sand samples were selected. A long fragment of the 16S rDNA of this isolates (positions 27-1492 of the Escherichia coli gene numbering) (Appendix 1) was amplified and sequenced. The results are presented and discussed in the next section.

3.3.5 Bacterial Community analysis by DGGE and identification of some relevant members

The DGGE profiles of fragments of the gene coding bacterial 16S rRNA amplified from the total DNA extracted from sand treated and non treated with bromoxynil are shown in Figure 8.

The profiles from different replicate extractions of the same reactor were very similar and some even identical, which supports the reliability of the technique used. On the other hand there were not relevant differences in the profiles of bacterial communities from the samples treated or not treated with bromoxynil. Nevertheless few bands were absent or weaker in the samples not treated with the herbicide compared with the ones treated (Figure 8).

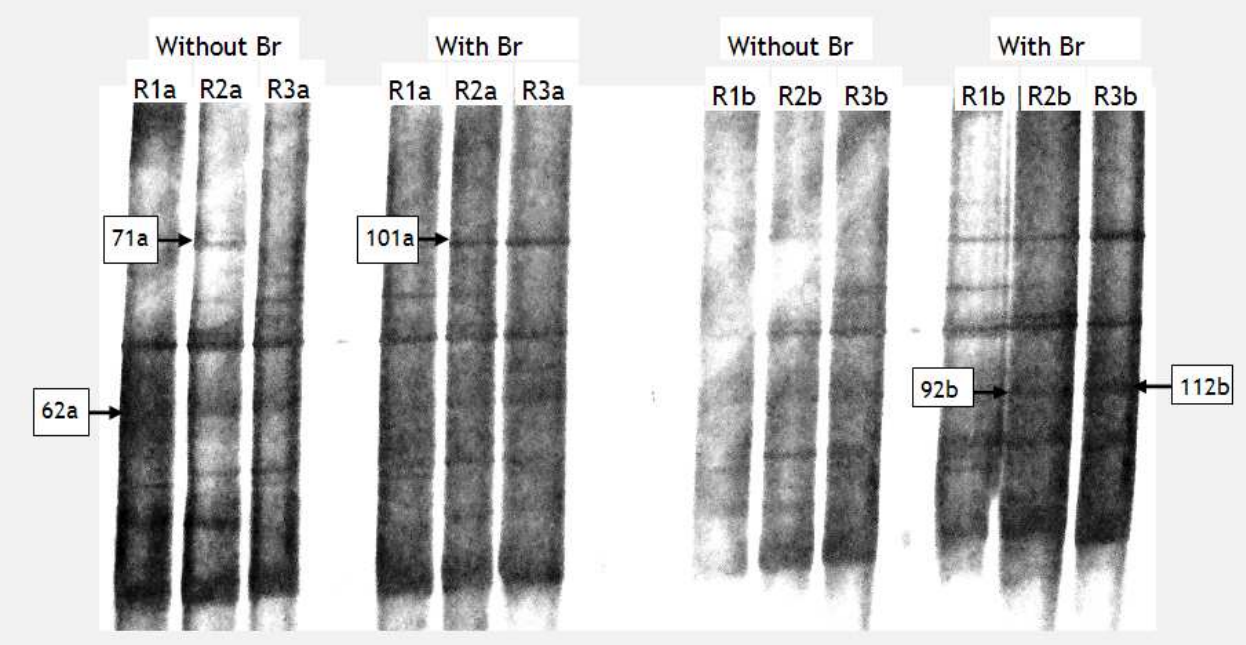

Figure 8: DGGE profiles of the bacterial communities on the sand treated and not treated with bromoxynil. Br= bromoxynil; R1, R2, R3: replicate reactors; a, b: replicate DNA extractions. Arrows with label point the bands that were excised and sequenced.

The bands identified in the figure above were excised and sequenced and the sequences compared with those in public databases lead us to the following results:

The sequence of the band 71a showed a similarity of 100% with the 16S rRNA sequence of several *Pandoraea* isolates (Osaki *et al*, 2006). These organisms have been associated with the degradation of aromatic hydrocarbons and chlorine compounds in bioremediation of contaminated soils when in consortium with other bacteria. In our case the band with the sequence related with these type of bacteria was in the profile from a reactor without bromoxynil, therefore we cannot discuss about its possible role on the degradation of the herbicide.

Surprisingly the band 101a that appeared in a position very similar to band 71a has a sequence related with *Ralstonia* (similarity 97.8%). It is noteworthy that this sequence matched perfectly that of one of the isolates sequenced (isolate D107). These type of bacteria was isolated from plates with bromoxynil as sole N source and corresponded to one of the colony morphologies that was much more abundant in the reactors treated with the herbicide than in the ones not treated. Moreover, another isolate (D91) with the same characteristics than 101a but cultivated from a different reactor with bromoxynil was also identified as *Ralstonia* and had a sequence with only few mismatches with isolate D107. Among the closest relatives in the database to these two isolates (similarities 98.2%-99%) were organisms able to degrade herbicides and other compounds with chemical structures related with bromoxynil like phenol, 4-nitrophenol, p-aminobenzoic acid or 2,4-

dichrolophenoxyacetic acid (Tomei *et al*, 2006; Kulakov *et al*, 2002; Macur *et al*, 2007; Hong *et al*, unpublished). Therefore the results suggest that a strain of *Ralstonia* could be one of the organisms in the bacterial communities analysed that is involved in the degradation of bromoxynil.

The sequence of the band 62a was related with sequences of *Alcaligenes,* as was the sequence of band 92b. The closest relatives showed a similarity lower than in the case of other bands (92.7-93.9%), which can be attributed to the poorer quality of these sequences. Among the closest relatives could be found phenol degraders (Zhang *et al*, 2004; Caiyin, unpublished) or degraders of the pesticide methyl parathione (Jiang *et* Yan, unpublished) or clones from reactors degrading chlorobenzene or monochlorobenzene (Alfreirer *et al,* 2002). The sequences of these bands were also related with those of the two isolates obtained from one reactor treated with bromoxynil and cultivated on plates with this herbicide as sole C and N source (isolates A10C3 and A10C7a). These two isolates had identical 16SrRNA sequences and among their closest relatives (99.4% similarity) was *Achromobacter insolitus* (Coenye *et al*, 2003) and another strain of *Alcaligenes* (Zou *et al*, 2007). *Alcaligenes* are a gram-negative, pathogenic, opportunistic bacteria that can be found in soil and water that was identified as a degrader of nitrophenol and phenol which are widely used in the pharmaceutical, agricultural and chemical industries (Xiao *et al*, 2007) and phenylacetic acid which is an intermediate compound of the degradation of a lot of pollutants including aromatic compounds (Abe-Yoshizumi et al, 2004). In our case, the role of these organisms in the degradation of bromoxynil cannot be assumed without further studies because *Alcaligenes* was detected in both reactors with and without bromoxynil.

In the case of the sequence from band 112b the closest relative (95.9 % similarity) in the database was *Ochrobactrum anthropi* (Lebuhn *et al*, 2000; Qiu *et al*, 2006). The sequence of this band also matched one of the isolates obtained (A9C6) cultivated from one reactor without bromoxynil. In the case of the isolate its sequence matched perfectly (100% similarity) with that in the database (Lebuhn *et al*, 2000). *Ochrobactrum* are gram negative coco bacillus bacteria that is frequently found in soil (Lebuhn *et al*, 2000) as well as environmental and hospital water sources and is associated with the degradation of methyl parathion, a commonly used insecticide which is extremely toxic (Qiu *et al*, 2006). Given that the band sequence related with this organism was from the community of a reactor treated with bromoxynil and the isolate from a reactor without bromoxynil, it is not possible to relate its presence with the degradation of this compound without further studies.

3.3.6 Measure of concentration of Bromoxynil and metabolites

The results presented on this section focus mostly on the sand impregnated with bromoxynil since it was where the changes were expected and relevant to the objectives of this experiment.

The determination of bromoxynil by HPLC was possible using the method described. The retention time for the peak that corresponds to Bromoxynil was between 5 and 5.6 minutes (see Figure 13 in Appendix 2). This variation in the retention time was observed probably due to changes in room temperature as the equipment used does not allow the control of the temperature of the column.

The figure bellow shows the initial concentrations of Bromoxynil in the non sterile and in the sterile reactors before and after microbial colonisation is shown on Figure 9. It can be observed that there is no difference in the initial and final concentrations in the sterile reactors which indicates that abiotic degradation of this compound did not take place. In the non sterile reactors degradation of bromoxynil took place although it was more evident and significant in the two of the replicate reactors. The third reactor had already shown some differences in the type and number of microorganisms that grew in the plates as well as in the estimates for the MPN. This leads to believe that the colonisation of the sand in this reactor was different for some reason. Some possible explanation could be that the movement of microorganisms from the soil to the sand was hindered because of a lack of humidity on the layer of glass wool placed between the soil and the sand or occurrence of anaerobic conditions at some moment.

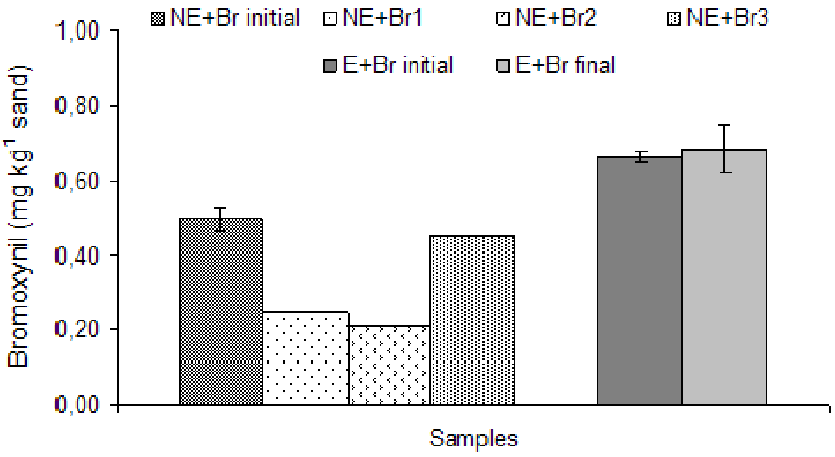

Figure 9: Concentration of bromoxynil in the sand before and after the colonization by soil microorganisms.

Standard solutions of 3,5-dibromo-hydroxy-benzoic acid (DBHB) were analysed by HPLC and a calibration curve obtained. Nevertheless this compound was not detected in any of the samples analysed, which indicates that if this metabolite was originated it was further degraded.

As indicated in the methods section, a second experiment was carried out to test the ability of the microbial colonisers of the sand treated with bromoxynil to eliminate this herbicide from aqueous solutions (Figure 10). After the first addition of a solution of bromoxynil, the change in the concentration of the herbicide was very small. This could be due to the short period of contact with microorganisms and also to a lag time needed for the proliferation of degraders in the reactors. In agreement with this, after the second addition, the concentration of bromoxynil decreased, while in the sterile reactors it remained constant. As in the case of the first experiment, the presence of the metabolite DBHB was not detected.

These results confirm the ability of the soil microorganisms that colonise the sand treated with bromoxynil to degrade this compound and emphasises the importance of microbial degradation for the removal of this compound from the environment. Future work is needed for the identification of the members of the microbial community essential for the elimination of this compound as well as for optimising the degradative capacity of isolates or microbial consortia.

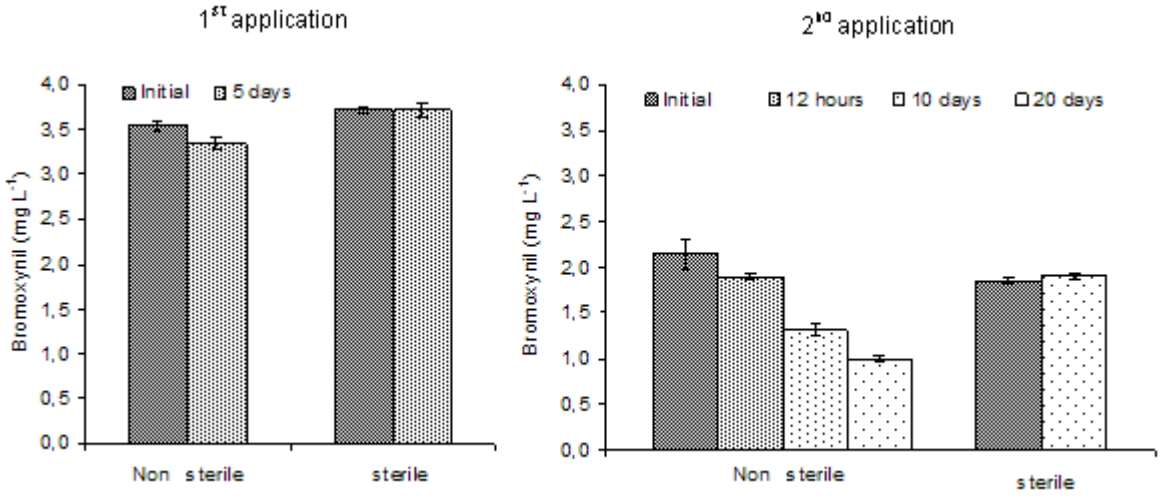

Figure 10: Concentration of bromoxynil in aqueous solutions from reactors used to test the degradation activity of the microorganisms that colonised sand impregnated with this herbicide. Hours and days correspond to the time that the contaminated water was in contact with microorganisms.

4 Conclusions

Bromoxynil degradation was observed in reactors filled with sand impregnated with the herbicide and in contact with soil, while in sterile reactors the amount of bromoxynil did not change. This indicates the paramount importance of the microbial activity for the degradation of bromoxynil and the essential role of microorganisms for avoiding the accumulation of this herbicide.

Differences in the microbial communities growing in the reactors with bromoxynil and without bromoxynil were also observed though the effect of the herbicide was not intense. Among the microorganisms studied, a negative effect of bromoxynil on amylolytics and on the microorganisms able to degrade some aminoacids, salicilic acid and glusose-1-phosphate was observed at least in a short period of time.

Ralstonia sp., *Alcaligenes* sp., *Ochrobactrum* sp. were among the main members detected or isolated from microbial community in the sand treated with bromoxynil.

The plate count observations and the molecular data suggest that *Ralstonia* sp. seems to play a role on the degradation of bromoxynil.

The microorganisms established in the sand impregnated with bromoxynil were able to remove this herbicide from aqueous solutions reducing its concentration from 2 mg L^{-1} to 1 mg L^{-1} in 20 days.

The bromoxynil metabolite 3,5 dibromo-4-hydroxy-benzoic acid was not detected in any of the samples analysed which doesn't mean that no metabolites were formed.

5 Evaluation of the work done

5.1 Accomplished Objectives

The work done allowed to obtain information on microbial degradation of the herbicide bromoxynil, proving that this biotic process is essential for the removal of this herbicide from soil. Some bacteria potentially involved in the degradation of this herbicide were also identified and a collection of bacterial isolates has been obtained. Future work is needed to shed light on the ability of the isolates of transforming bromoxynil: i) alone or in consortia, ii) using the herbicide as sole C source or with the help of other C sources for supporting growth.

5.2 Other Experimental Work

Other experimental work done for this study was **F**luorescent **I**n **S**itu **H**ybridization (FISH) but the protocol and the equipment used was meant for cell size and density a little larger than the ones observed in the sand samples from the reactors. This protocol (detailed in appendix 1) was tried nevertheless but the results were inconclusive as observation of cells was difficult and there were a lot of other particles that weren't cells that fluoresced so that counting and identifying cells was unreliable. To obtain conclusive results a lot of optimization would have been needed (different filters, different protocol used more specifically for environmental samples, optimization of the sample size).

5.3 Limitations and Future Work

One of the main limitations was the unexpected density of microorganisms present in the sand not treated with bromoxynil, which made difficult to find differences in the microbial communities attributable to the herbicide.

As to future work, there are a lot of herbicides used in crop fields and in the environment. Although some are obviously more dangerous than others, it is important to monitor the impact that they have in the environment, in the food and in the water and to make sure that they serve their purpose without harm to its surroundings. In regard to bromoxynil and the continuation of the work done it would be interesting to further analyse the isolates obtained paying attention to their ability for transforming bromoxynil. The optimization of the removal of bromoxynil from aqueous solutions by the communities established on the sand would also be of interest as they could be applied to the treatment of water contaminants.

5.4 Final Appraisal

Overall it was a short experimental work where I learned a lot from experienced researchers, applying new techniques and actually obtaining some interesting conclusions.

6 Bibliographic References

- Abe-Yoshizumi, R.; Kamei, U.; Yamada, A.; Kimura, M.; Ichihara, S.; 2004; "The Evolution of the phenylacetic acid degradation pathway in bacteria"; Bioscience, Biotechnology and Biochemistry; Volume 68; Issue 3; pages 746-748.

- Alfreider, A.; Vogt, C.; Babel, W.; 2002; "Microbial diversity in an in situ reactor system treating monochlorobenzene contaminated groundwater as revealed by 16S ribosomal DNA analysis"; Systematic and Applied Microbiology; Volume 25; Number 2; pages 232-240.

- Asano, Y.; Fujishiro, K.; Tani, Y.; Yamada, H.; 1982; "Aliphatic nitrile hydratase from *Athrobacter* sp. J-1: purification and characterization"; Agricultural and Biological Chemistry; Volume 46; pages 1165-1174.

- Bandyopadhyay, Amal Kumar; Nagasawa, Toru; Asano, Yasuhisa; Fujishiro, Kinya; Tani, Yoshiki; Yamada, Hideaki; 1986; "Purification and Characterization of Benzonitrilases from *Athrobacter* sp. Strain J-1"; Applied and Environmental Microbiology; Volume 51; Number 2; pages 302-306.

- Banerjee, A.; Sharma, R.; Banerjee, U. C.; 2002; "The nitrile-degrading enzymes: current status and future prospects"; Applied Microbiology and Biotechnology; Volume 60; Issue 1-2; pages 33-44.

- Baxter, J.; Cummings, S. P.; 2006; "The application of the herbicide bromoxynil to a model soil-derived bacterial community: impact on degradation and community structure"; Letters in Applied Microbiology; pages 659-665.

- Baxter, Joanne; Cummings, Stephen P.; 2006; "The future and current applications of microorganism in bioremediation of cyanide contamination"; Springer; Antonie van Leeuwenhoek; Volume 90; pages 1-17.

- Cocolin, Luca; Aggio, Daniele; Manzano, Marisa; Cantoni, Carlo; Comi, Giuseppe; 2002; "An application of PCR-DGGE analysis to profile the yeast populations in raw milk"; International Dairy Journal; Volume 12; Issue 5; pages 407-411.

- Coenye, T.; Vancanneyt, M.; Falsen, E.; Swings, J.; Vandamme, P.; 2003; "*Achromobacter insolitus* sp. nov. and *Achromobacter spanius* sp. nov., from human clinical samples"; International Journal of Systematic and Evolutionary Microbiology; 53; pages 1819-1824.

- Crecchio, Carmine; Curci, Maddalena; Pizzigallo, Rosa; Ricciuti, Patrizia; Ruggiero, Pacifico; June 2001; "Molecular approaches to investigate herbicide-induced bacterial community changes in soil microcosms"; Biology and Fertility of Soils; Volume 33; Issue 6; pages 460-466.

- Cupples, Alison M.; Sanford, Robert A.; Sims, Gerald K.; 2005; "Dehalogenation of the herbicides Bromoxynil (3,5-Dibromo-4-Hydroxybenzonitrile) and Ioxynil (3,5-Diiodino-4-Hydroxybenzonitrile) by *Desulfitobacterium chlororespirans*"; Applied and Environmental Microbiology; Volume 71; Number 7; pages 3741-3746.

- Dubey, S. K.; Holmes, D. S.; 1995; "Biological Cyanide Destruction mediated by microorganisms"; World Journal of Microbiology & Biotechnology; Volume 11; Issue 3; pages 257-265.

- European Bioinformatics Institute; 2006-2008; Fasta Nucleotide Database; European Molecular Biology Laboratory
  http://www.ebi.ac.uk/fasta33/nucleotide.html

- Extoxnet, Oregon State University, 1996
  http://extoxnet.orst.edu/pips/bromoxyn.htm

- Figueira, M. M.; Ciminelli, V. S. T.; Linardi, V. R.; 1995; "Bacterial degradation of metal cyanide complexes."; In: Jerez; C. A.; Vargas, T.; Toledo, H.; Wiertz, J. V.; Biohydrometallurgical Processing; University of Chile; Chile; pages 333–339.

- Freeman, Jennifer L.; Beccue, Nathan; Rayburn, A. Lane; 2005; "Differential Metamorphosis alters the endocrine response in anuran larvae exposed to T3 and atrazine"; Aquatic Toxicology; Volume 75; pages 263-276.

- Fuller, Stephen; 2007
  http://www.geocities.com/CapeCanaveral/Hall/1410/lab-Soil-06.html

- Gabriel, J.; Veková, J.; Vosáhlo, J.; 1996; "High Performance Liquid Chromatographic Study of the aromatic nitrile metabolism in soil bacteria"; Journal of Chromatography B.; Volume 681; pages 191-195.

- Gershater, Markus; Sharples, Kate; Edwards, Robert; 2006; "Carboxylesterase activities toward pesticide esters in crops and weeds"; Phytochemistry; Volume 67; Issue 23; pages 2561-2567.

- Girvan. M. S.; Bullimore. J.; Pretty. J. N.; Osborn. A. M.; Ball. A. S.; 2003; "Soil type is the primary determinant of the composition of the total and active bacterial communities in arable soils"; Applied and Environmental Microbiology; Volume 69; pages 1800–1809.

- Golovleva, L. A.; Pertsova, R. N.; Kunc, F.; Vokounová, M.; 1988; "Decomposition of the herbicide Bromoxynil in soil and in bacterial cultures"; Folia Microbiology; Volume 33; pages 491-499.

- Graß, B.; Mayer, H.; Nolte, J.; Preuß, G.; Zullei-Seibert, N.; 2000; "Studies on the metabolism of hydroxybenzonitrile herbicides: I. mass spectrometric identification"; Pest Management Science; Volume 56; pages 49-59.

- Hall, Tom; 1997-2007; BioEdit; Ibis Biosciences; Carlsbad; California. http://www.mbio.ncsu.edu/BioEdit/bioedit.html

- Harman, W. L.; Wang, E.; Williams, J. R.; 2004; "Reducing atrazine losses: water quality implications of alternative runoff control practices"; Volume 33; Issue 1; pages 7-12.

- Harper, D. B.; 1976; "Purification and properties of an unusual nitrilase from *Nocardia* N. C. B. I. 11216"; Biochemical Society Transactions; Volume 4; pages 502-504.

- Harper, D. B.; 1977; "Microbial metabolism of aromatic nitriles"; Biochemical Journal; Volume 165; pages 309-319.

- Health Canada, Environmental and Workplace Health, 2007 http://www.hc-sc.gc.ca/ewh-semt/pubs/water-eau/bromoxynil/index_e.html

- Holtze, M. S.; Juhler, R. K.; Aamand, J.; Hansen, H. C. B.; 2004; "Transformation of dichlobenil to 2,6-dichlorobenzamide (BAM) by soil bacteria harbouring nitrile hydratases and amidases or nitrilases"; Proceedings of the European Symposium on Environmental Biotechnology; ed. by Verstraete W. AA Balkema Publishers, Leiden; pages 779–783.

- Holtze, Maria Sommer; Sørensen, Jan; Hansen, Hans Christian B.; Aamand, Jens; 2006; "Transformation of the herbicide 2,6-dichlorobenzonitrile to the persistent metabolite 2,6-dichlorobenzamide (BAM) by soil bacteria known to harbour nitrile hydratase or nitrilase"; Biodegradation; Springer; 17; pages 503-510.

- Holtze, Maria S.; Hansen, Hans Christian B.; Juhler, René K.; Sørensen, Jan; Aamand, Jens; 2007; "Microbial degradation pathways of the herbicide dichlobenil in soils with

different history of dichlobenil exposure"; Environmental Pollution; Volume 148; pages 343-351.

- Hsu, J. C.; Camper, N. D.; 1975; "Degradation of ioxynil and bromoxynil as measured by a modified spectrophotometric method"; Canadian Journal of Microbiology; Volume 21; Issue 12; pages 2008-2012.

- Johnsen, Anders R.; Bendixen, Karen; Karlson, Ulrich; 2002; "Detection of Microbial Growth on Polycyclic Aromatic Hydrocarbons in Microtiter Plates by Using the Respiration Indicator WST-1"; Applied and Environmental Microbiology; Volume 68; Number 6; pages 2683–2689.

- Johnsen, Kaare; Jacobsen, Carsten S.; Torsvik, Vigdis; Sorensen, Jan; 2001; "Pesticide effects on bacterial diversity in agricultural soils-a review"; Biology and Fertility of Soils; Volume 33; Issue 6; pages 443-453.

- Jørgensen, Kirsten S.; 2007; "*In situ* Bioremediation"; Finnish Environment Institute; pages 285-301.

- Juraske, R.; Antón, A.; Castells, F.; Huijbregts, M. A. J.; 2007; "Human intake fractions of pesticides via greenhouse tomato consumption: Comparing model estimates with measurements for Captan"; Chemosphere; Elsevier; Volume 67; pages 1102-1107.

- Kennedy, A. C.; 1994; "Carbon utilization and fatty acid profiles for characterization of bacteria"; In Waver, R. W.; Angle, S.; Bottomley, P.; Bezdicek, D.; Smith, S.; Tabatabai, A.; Wollum, A. (eds); Methods of Soil Analysis: Part 2. Microbiological and biochemical properties; Soil Science Society of America, Wisconsin, USA; pages 546-556.

- Kjær, J.; Ullum, M.; Olsen, P.; Sjelborg, P.; Helweg, A.; Mogensen, B. B.; 2001; "The Danish Pesticide Leaching Assessment Programme, Monitoring Results May 1999-June 2000"; Geological Survey of Denmark and Greenland, Copenhagen, Denmark.

- Köhne, J. Maximilian; Köhne, Sigrid; Šimůnek, Jirka; 2006; "Multi-process herbicide transport in structured soil columns: Experiments and model analysis"; Journal of Contaminant Hydrology; Volume 85; pages 1-32.

- Kulakov, L. A.; McAlister, M. B.; Ogden, K. L.; Larkin, M. J.; O'Hanlon, J. K.; 2002; "Analysis of bacteria contaminating ultrapure water in industrial systems"; Applied Environmental Microbiology; Volume 68; Issue 4; pages 1548-1555.

- Lebuhn, M.; Achouak, W.; Schloter, M.; Berge, O.; Meier, H.; Barakat, M.; Hartmann, A.; Heulin, T.; 2000; "Taxonomic characterization of *Ochrobactrum* sp. Isolates from soil samples and wheat roots, and description of *Ochrobactrum triciti* sp. nov. and *Ochrobactrum grignonense* sp. nov."; International Journal of Systematic and Evolutionary Microbiology; Volume 50; pages 2207-2223.

- Lee, Martijn K. van der; Weg, Guido van der; Traag, Wim A.; Mol, Hans G.J.; 2007; "Qualitative screening and quantitative determination of pesticides and contaminants in animal feed using comprehensive two-dimensional gas chromatography with time-of-flight mass spectrometry"; Journal of Chromatography A; Elsevier; pages 1-15.

- Li, Q.; Luo, Y.; Song, J.; Wu, L.; 2007; "Risk assessment of atrazine polluted farmland and drinking water: a case study"; Bulletin of Environmental contamination and toxicology; Volume 78; pages 187-190.

- Macur, R. E.; Wheeler, J. T.; Burr, M. D.; Inskeep, W. P.; 2007; "Impacts of 2,4-D application on soil microbial community structure and on populations associated with 2,4-D degradation"; Microbiological Research; Volume 162; Issue 1; pages 37-45.

- Martinkova, L.; 1993; "Microbial Transformation of Nitriles"; Chemicke Listy; Volume 87; Issue 3; pages 187-197.

- Mathew, C.; Nagasawa, T.; Kobayashi, M.; Yamada, H.; 1988; "Nitrilase catalyzed production of nicotinic acid from 3-cyanopyridine in *Rhodococcus rhodochrous* J1"; Applied Environmental Microbiology; Volume 54; pages 1030–1032.

- McBride, Kevin E.; Kenny, James W.; Stalker, David M.; 1986; "Metabolism of the herbicide Bromoxynil by *Klebsiella pneumonia* subsp. *ozaenae*"; Applied and Environmental Microbiology; Volume 52; Number 2; pages 325-330.

- McCarthy, Conor; 1996-1998; Chromas; School of Health Science; Griffin University; Southport, Queensland; Australia
  http://www.technelysium.com.au/chromas.html

- Millet, Maurice; Palm, Wolf-Ulrich; Zetzsch, Cornelius; 1998; "Abiotic Degradation of Halobenzonitriles: Investigation of the Photolysis in Solution"; Ecotoxicology and Environmental Safety; Environmental Research, Section B; Volume 41; pages 44-50.

- Müller, D.; Gabriel, J.; 1999; "Bacterial degradation of the herbicide Bromoxynil by *Agrobacterium radiobacter* in biofilm"; Folia Microbiology; Volume 44; pages 377-379.

- Muyzer, G; Brinkhoff, T.; Nubel, U.; Santegoeds, C.; Schafer, H.; Wawer, C.; 1998; In Molecular Microbial Ecology Manual (ed A. D. L. Akkermans, J. D. van Elsas and F. J. de Bruijn, "Denaturing gradient gel electrophoresis (DGGE) in microbial ecology"; Kluwer Academic Publishers, Dordrecht; pages 1-27.

- Muyzer, G; Waal, E. D.; Uitterlinden, A. G.; 1993; "Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA"; Applied Environmental Microbiology; vol 59; pages 695-700.

- Narayana, K.; Narayan, P.; D'Sousa, U. J.; 2004; "Is our water polluted?"; Indian Journal of Medical Sciences; Volume 58; Issue 12; pages 528-530.

- Nielsen, M. K. K.; Holtze, M. S.; Svensmark, B.; Juhler, R. K.; 2007; "Demonstrating formation of potentially persistent transformation products from the herbicides bromoxynil and ioxynil using liquid chromatography-tandem mass spectrometry (LC-MS/MS)"; Pest Management Science; Volume 63; pages 141–149.

- Nogales, Balbina; Moore, Edward R. B.; Llobet-Brossa, Enrique; Rossello-Mora, Ramon; Amann, Rudolf; Timmis, Kenneth N.; 2001; "Combined use of 16S ribosomal DNA and 16S rRNA to study the bacterial community of polychlorinated biphenyl-polluted soil"; Applied Environmental Microbiology; Volume 67; Number 4; pages 1874-1884.

- O´Reilly, C.; Turner, P. D.; 2003; "The nitrilase family of CN hydrolysing enzymes – a comparative study"; Journal of Applied Microbiology; 95; pages 1161-1174.

- Ormad, M. P.; Miguel, N.; Claver, A.; Matesanz, J. M.; Ovelleiro, J. L.; 2008; "Pesticides removal in the process of drinking water production"; Chemosphere; Volume 71;pages 97-106.

- Osaki, S.; Kishimoto, N.; Fujita, T.; 2006; "Isolation and Phylogenetic Characterization of Microbial Consortia able to Degrade Aromatic Hydrocarbons at High Rates"; Microbes and Environments; Volume 21; pages 44-52.

- Pampulha, M. E.; Oliveira, A.; 2006; "Impact of an Herbicide Combination of Bromoxynil and Prosulforon on Soil Microorganisms"; Current Microbiology; Volume 53; pages 248-253.

- Poignant, Pierre; Thellot, Bernard; Rognon, Jacques; 1976; France; United States patent. http://v3.espacenet.com/textdoc?DB=EPODOC&IDX=GB1429102&F=1

- Pollak, P.; Romender, G.; Hagedorn, F.; Gelbke, H-P.; 1991; In: Elvers, B.; Hawkins, S.; Schulz, G.; Ullman's encyclopedia of industrial chemistry; 5th edition; Volume A17; Wiley-VCH; Weinheim; pages 363–376.

- Qiu, X.; Bai, W.; Zhong, Q.; Li, M.; He, F.; Li, B.; 2006; "Isolation and characterization of a bacterial strain of the genus Ochrobactrum with methyl parathion mineralizing activity"; Journal of Applied Microbiology; Volume 101; Issue 5; pages 986-994.

- Rabinovitch, B. S.; Winkler, C. A.; 1942; "The hydrolysis of aliphatic nitriles in concentrated hydrochloric acid solutions"; Canadian Journal of Research-Section B.; Volume 20; pages 221-230.

- Rabinovitch, B. S.; Winkler, C. A.; Stewart, A. R. P.; 1942; "The hydrolysis of propionitrile in concentrated hydrochloric acid solutions"; Canadian Journal of Research-Section B.; Volume 20; pages 121-132.

- Rabinovitch, B. S.; Winkler, C. A.; Stewart, A. R. P.; 1942; "Kinetics of the alkaline hydrolysis of propionitrile"; Canadian Journal of Research-Section B.; Volume 20; pages 185-188.

- Sakai, Y.; Ogawa, N.; Fujji, T.; Sugahara, K.; Miyashita, K.; Hasebe, A.; 2007; "2,4-Dichlorophenoxyacetic acid degrading genes from bacteria isolated from a soil in Japan"; Microbes and Environments; Volume 22; pages 145-156.

- Sallam, Khalid Ibrahim; Morshedy, Alaa Eldin Mohammed; 2008; "Organochlorine pesticide residues in camel, cattle and sheep carcasses slaughtered in Sharkia Province, Egypt"; Food Chemistry; Elsevier; Volume 108; pages 154-164.

- Saradha, B.; Vaithinathan, S.; Mathur, P. P.; 2008; "Single exposure to low dose of lindane causes transient decrease in testicular steroidogenesis in adult male Wistar rats"; Toxicology; Elsevier; Volume 244; pages 190-197.

- Schäfer, Hendrik; Muyzer, Gerard; 2001; "Denaturing Gradient Gel Electrophoresis in Marine Microbial Ecology"; Methods of Microbiology. Chapter 2; John Paul (Ed); Academic Press; London; Volume 30; pages 425–468.

- Semchuk, K. M.; McDuffie, H. H.; Senthilselvan, A.; Dosman, J. A.; Cessna, A. J.; Irvine, D. G.; 2003; "Factors associated with detection of bromoxynil in a sample of rural residents"; Journal of Toxicology and Environmental Health A; Volume 66; Issue 2; pages 103-132.

- Singh, Shashi B.; Lal, Shashi B.; Pant, Shashi; Kulshrestha, Gita; 2008; "Degradation of atrazine by an acclimatized soil fungal isolate"; Journal of Environmental Science and Heath-Part B; Volume 43; pages 27-33.

- Smejkal, Christopher W.; Vallaeys, Tatiana; Seymour, Fabian A.; Burton, Sara K.; Lappin-Scott, Hilary M.; 2001; "Characterization of (R/S)-mecoprop [2-(2-methyl-4-chlorophenoxy) propionic acid]-degrading Alcaligenes sp.CS1 and Ralstonia sp. CS2 isolated from agricultural soils"; Environmental Microbiology; Volume 3; Number 4; pages 288-293.

- Smith, A. E.; 1971; "Degradation of Bromoxynil in Regina Heavy Clay"; Weed Research Journal; Volume 11; pages 276-282.

- Smith, A. E.; Cullimore, D. R.; 1974; "The in vitro degradation of the herbicide bromoxynil"; Canadian Journal of Microbiology; Volume 20; pages 773-776.

- Smith, Charles N.; Payne Jr., William R.; Pope Jr., John D.; Winkie, Jonathan H.; Parrish, Rudolph S.; 1999; "A field study to compare performance in stainless steel research monitoring wells with existing on-farm drinking wells in measuring pesticide and nitrate concentrations"; Chemosphere; Volume 38; Number 4; pages 875-889.

- Tarnowsky, B. I.; Spinale, F. G.; Nicholson, J. H.; 1991; "DAPI as a useful stain for nuclear quantitation"; Biotechnic Histochemistry; Volume 66; Issue 6; page 297.

- Texier, I.; Giannotti, C.; Malato, S.; Richter, C.; Delaire, J.; 1999; "Solar photodegradation of pesticides in water by sodium decatungstate"; Catal. Today; Volume 54; pages 297-307.

- Tomei, M. C.; Rossetti, S.; Annesini, M. C.; 2006; "Microbial and kinetic characterization of pure and mixed cultures aerobically degrading 4-nitrophenol"; Chemosphere; Volume 63; Issue 10; pages 1801-1808.

- Topp, Edward; Xun, Luying; Orser, Cindy S.; 1992; "Biodegradation of the Herbicide Bromoxynil (3,5-Dibromo-4-Hydroxybenzonitrile) by Purified Pentachlorophenol Hydroxylase and Whole Cells of *Flavobacterium* sp. Strain ATCC 39723 Is Accompanied by Cyanogenesis"; Applied and Environmental Microbiology; Volume 58; Number 2; pages 502-506.

- Van Dillewijn, Pieter; Villadas, Pablo J.; Toro, Nicolás; 2002; "Effect of a *Sinorhizobium meliloti* Strain with a Modified *putA* Gene on the Rhizosphere Microbial Community of Alfalfa"; Applied and Environmental Microbiology; Volume 68; pages 4201-4208.

- Vokounová, M.; Vacek, O.; Kunc, F.; 1992; "Effect of glucose and ribose on microbial degradation of the herbicide bromoxynil continuously added to soil"; Folia Microbiology; Volume 37; pages 128-132.

- Vosáhlová, Jolana; Pavlů, Lenka; Vosáhlo, Jan; Brenner, Vladimir; 1997; "Degradation of Bromoxynil, Ioxynil, Dichlobenil and their mixtures by *Agrobacterium radiobacter*"; Pesticide Science; Volume 49; pages 303-306.

- Washington State Department of Transportation, 2006 http://www.wsdot.wa.gov/maintenance/pdf/Bromoxynil.pdf

- Weisburg, William G.; Barns, Susan M.; Pelletier, Dale A.; Lane, David J.; 1991; "16S Ribosomal DNA Amplification for Phylogenetic Study"; Journal of Bacteriology; Volume 173; Number 2; pages 697-703.

- Wiedmann-al-Ahmad, M.; Tichy, H. V.; Schön, G.; 1994; "Characterization of Acinetobacter type strains and isolates obtained from wastewater treatment plants by PCR fingerprinting"; Applied Environmental Microbiology; Volume 60; pages 4066-4071.

- Winter, A.; Karlson, C.; 1976; "LKB application note 219"; LKB Produktor A. B.; Bromma, Sweden.

- Wolansky, M. J.; Harrill, J. A.; 2007; "Neurobehavioral toxicology of pyrethroid insecticides in adult animals: A critical review"; Neurotoxicology and Teratology; Elsevier; pages 1-24.

- Woomer, Paul L.; 1994; "Most Probable Number Counts"; Tropical Soil Biology and Fertility Programme, Nairobi, Kenya; Methods of Soil Analysis-Part 2. Microbiological and Biochemical Properties-SSSA Book Series, nº 5; Chapter 5; Soil Science Society of America; pages 59-79.

- Xiao, Y.; Zhang, J. J.; Liu, H.; Zhou, N. Y.; 2007; "Molecular characterization of a novel ortho-nitrophenol catabolic gene cluster in Alcaligenes sp. Strain NyZ215"; The Journal of Bacteriology; Volume 189; Issue 18; pages 6587-6593.

- Yamada, H.; Asano, Y.; Hino, T.; Tani, Y.; 1979; "Microbial utilization of acetonitrile"; Journal of Fermentation Technology; Volume 57; pages 8-14.

- Yehia; Mona A. H.; El-Banna, Sabah G.; Okab, Aly B.; 2007; "Diazinon toxicity affects histophysiological and biochemical parameters in rabbits"; Experimental and Toxicologic Pathology; Elsevier; Volume 59; pages 215-225.

- Zhang, X.; Gao, P.; Chao, Q.; Wang, L.; Senior, E.; Zhao, L.; 2004; "Microdiversity of phenol hydroxylase genes among phenol-degrading isolates of *Alcaligenes* sp. From an activated sludge system"; FEMS Microbiology Letters; Volume 237; Issue 2; pages 369-375.

- Zou, C. S.; Mo, M. H.; Gu, Y. Q.; Zhou, J. P.; Zhang, K. Q.; 2007; "Possible contributions of volatile-producing bacteria to soil fungistasis"; Soil Biology and Biochemistry; Volume 39; Number 9; pages 2371-2379.

Appendix 1 Detailed Material and Methods

1. Bromoxynil solution

This is a 1% bromoxynil solution in acetone used to impregnate the sand in the reactors.

1g of bromoxynil (98%, Aldrich) was weighed and dissolved in 100mL of acetone. The pH was then adjusted to pH 9.5 with sodium hydroxide 0.1N. The solution was then filtered with a polyester syringe filter with 0.20μm of pore diameter (CHROMAFIL, PET-20/25, Macherey-Nagel).

2. Mineral Medium or MSM (Minimal Salts Media)

This is the media added to the reactors to provide inorganic nutrients for the growth and proliferation of microorganisms on the sand treated with Bromoxynil.

To prepare 1L of the media, it was necessary to add 100mL of phosphate buffer (10X), 10mL of a solution with salts and 1mL of ferric ammonium citrate solution.

Phosphate buffer (10X) was prepared by dissolving 140g of sodium phosphate ($Na_2HPO_4.12H_2O$) and 20g of monopotassium phosphate (KH_2PO_4) to 1L of water.

50mL of the ferric ammonium citrate solution were prepared by dissolving 0.68g of ferric ammonium citrate in 50mL of water.

The solution of salts (100x) consists of a mixture of 600mL, 300mL and 100mL of the following three solutions respectively.

The first solution was prepared by adding 100g of $(NH_4)_2SO_4$ to 20g of $MgSO_4.7H_2O$ to 600mL of water.

The second solution was prepared by adding 4.62g of $Ca(NO_3)_2.3H_2O$ to 300mL of water.

The third solution, an oligoelements solution (1000x), was prepared by adding 1.3mL of HCl (37%), 0.07mL of $ZnCl_2$, 0.1mL of $MnCl_2.4H_2O$, 0.0062mL of H_3BO_4, 0.19mL $CoCl_2.6H_2O$, 0.017mL of $CuCl_2$, 0.024mL of $NiCl_2.6H_2O$ and 0.036mL of $Na_2MoO_4.2H_2O$.

3. Plate Count Determinations

For the preparation of these solid media the following solutions were added to 600mL of 1.75% agar in water:

- 50mL of 1% bromoxynil solution at pH 9.5 (filtered sterilised).
- 100mL of 10x Winogradsky solution without N (containing 5g K_2HPO_4, 5.1g $MgSO_4.7H_2O$, 1.0g NH_4NO_3, 2.5g NaCl, 0.04g $Fe_2(SO_4)_3.7H_2O$, 0.05g $MnSO_4.H_2O$ in 1L deionised water) plus soluble starch (1.5g.L^{-1}) and oligolelements (as described above).
- 100mL of 10x phosphate buffer at pH 7.
- 10mL of 10mg/mL cicloheximide.

The media supplemented with N source also contained, 50mL of 10g.L^{-1} NH_4CL and 5g.L^{-1} $NaNO_3$ solution.

The media supplemented with C sources also contained, 2.7mL of 20% fructose, 1.3mL of 40% glucose, 2.2mL of sodium gluconate,1.4mL of sodium lactate and 6mL of 5M sodium succinate.

4. Microbial Determinations by Most Probable Number

Heterotrophic population was estimated in yeast-extract medium (1.0g yeast extract, 1.0g glucose, 0.5g KNO_3, 0.2g $MgSO_4.7H_2O$, 0.5g K_2HPO_4, 0.1g $CaCl_2$, 0.1g NaCl, 0.01g $FeCl_3$ in 1L deionised water) plus oligoelements (1.5mg $FeSO_4.7H_2O$, 0.3mg H_3BO_4, 0.19mg $CoCl_2.H_2O$, 0.1mg $MnCl_2.4H_2O$, 0.08mg $ZnSO_4.7H_2O$, 0.02mg $CuSO_4.5H_2O$, 0.036mg $Na_2MoO_4.2H_2O$,0.024mg $NiCl_2.6H_2O$). Amylase-producers were cultured in Winogradsky's saline medium. Ammonifiers were evaluated in Winogradsky's saline solution and L-aspargine (0.2g.L^{-1}) as the only nitrogen and carbon source plus oligoelements (as described above). The media containing bromoxynil were prepared as described above for the petri plates.

The pattern of carbon substrate utilization was analyzed by selecting 31 carbon sources from those included in Ecoplate™ from Biolog Inc. or recommended by Kennedy (1994) as referred in Table 3. Individual carbon source stock solutions were prepared at a concentration of 10% (p/v), filter-sterilized and added to saline medium (1.75g K_2HPO_4, 0.5g KH_2PO_4, 0.582g NH_4Cl and 0.25g $MgSO_4.7H_2O$ in 1L deionized water, adjusted to pH 7) using the ration 1:100 (v/v, C source:saline medium).

Table 3: List of substrates used. (continued)

nº	Substrate
1	Tween 40 (polyoxyethylene sorbitan monopalmitate)
2	Tween 60 (polyoxyethylene sorbitan monooleate)
3	α-ciclodextrin
4	Glycogen
5	D (+) cellobiose
6	α-lactose
7	β-metil D-glucoside

Table 3: List of substrates used. (continuation)

nº	Substrate
8	D (+) xylose
9	i-erythritol
10	Maltose
11	N-acetyl-D-glucosamine
12	α-D- glucose 1-phosphate
13	Glyceraldehyde
14	D-galactonic acid δ-lactone
15	galacturonic acid
16	o-hydroxibenzoic acid
17	p-hydroxibenzoic acid
18	malonic acid
19	L-arginine
20	L-asparagine
21	L-phenylalanine
22	L-serine
23	L-threonine
24	L- glutamic acid
25	β-phenyl ethylamine
26	Putrescine
27	α-keto butyric acid
28	malic acid
29	Mannitol
30	α-glycerolphosphate
31	Pyrocatechol
32	Water

The media were dispensed in 96 wells microtiter (150μL per well). In the case of the media with different C substrates, each microplate contained 8 different substrates (one different substrate in each row of the microplate). Wells containing saline medium with TV without C substrates were also prepared as controls.

Each well of microtiter plates was filled by dispensing 150μL of C substrate:saline medium with TV, and each set of carbon sources was replicated three times in a single 96 well MT plate. A tenfold dilution of the first soil suspension (10^{-2}) was used to inoculate the MT plates (sand and soil belonging to the 6 reactors).

5. DNA soil extraction

The PowerSoil™ DNA Isolation Kit (MO BIO Laboratories, Inc) was used to extract DNA from the sand after the disassembly of the reactors in order to recover the DNA present in the sand and proceed to amplification and posterior analysis and identification of microorganisms. The kit is intended for use with environmental samples.

To the PowerBead tubes provided, 0.5g of sand was added and vortexed gently for rapid and thorough homogenization. Then 60μL of the provided solution designated as solution C1 was added to help cell and the tubes inverted several times before vortexing them horizontally for 10 minutes at maximum speed. This is done since the collision of the beads with the microbial cells will cause the cells to break open. The tubes were then centrifuged at room temperature for 30 seconds at 10000xg and the supernatant transferred to a clean 2mL collection tube provided.

To these tubes 250μL of the supplied solution C2 was added. The tubes were vortexed for 5 seconds and incubated at 4ºC for 5 minutes allowing the precipitation of non-DNA to occur as it would probably inhibit posterior DNA amplification. The tubes were centrifuged at room temperature for 1 minute at 10000xg and 600μL of supernatant were transferred to a clean 2mL collection tube.

Then 200μL of the supplied solution C3 were added and the tubes were incubated at 4ºC for 5 minutes. The tubes were then centrifuged at room temperature for 1 minute at 10000xg and 750μL of supernatant transferred to a clean 2mL collection tube.

1.2mL of the supplied solution C4 was added and the tubes vortexed for 5 seconds. 675μL of the volume in the tube was loaded onto a spin filter and centrifuged for 1 minute at 10000xg at room temperature. The flow through was discarded and the same process repeat another 2 times. The DNA binds to the silica membrane in the spin filter and contaminants pass through.

500μL of solution C5 was added to the tubes and they were centrifuged at room temperature for 30 seconds at 10000xg. Solution C5 is an ethanol based wash solution used to further clean the DNA that is bound to the silica filter membrane in the spin filter to wash contaminants that remain in the DNA. The flow through was discarded and the tubes were centrifuged again at room temperature for 1 minute at 10000xg. The spin filter was then placed in a clean 2mL collection tube and 100μL of solution C6, which is a sterile elution buffer added to the centre of the white filter membrane.

The tubes were centrifuged again at room temperature for 30 seconds at 10000g and the spin filter discarded. The DNA was then stored frozen at -20ºC.

6. DNA biomass extraction (Wiedmann-al-Ahmad et al, 1994)

One or two isolated colonies were placed in tubes with 50μL of autoclaved water. The tubes were vortexed, incubated at 95ºC for 10 min, chilled in ice for 5 min and centrifuged at 16000xg. The supernatant was transferred to a new tube and stored at -20ºC.

7. PCR amplification

The polymerase chain reaction was used to amplify fragments of the gene coding 16S ribosomal RNA. Some of the amplified fragments were used to analyse and compare by molecular methods (DGGE) the bacterial communities that colonised the sand treated and not treated with bromoxynil and to sequence and identify some of the main bacterial members of those communities. Amplification of 16S rDNA was also carried out in genomic DNA of some of the bacterial isolates obtained for posterior sequencing.

For DGGE analysis the fragment of bacterial 16S rDNA between position 341 and 907 (*Escherichia coli* 16S rRNA gene sequence numbering) was amplified using the forward primer 16S-341F with the sequence 5' CGC CCG CCG CGC CCC GCG CCC GTC CCG CCG CCC CCG CCC GCC TAC GGG AGG CAG CAG 3' (Muyzer *et al*, 1993) and the reverse primer M16S-907R with the sequence 5' CCG TCA ATT CAT TTG AGT TT 3' (Muyzer *et al*, 1998). Primers were synthesised by Invitrogen.

The PCR mixtures (20μL) contained the following reagents:

- 2μL of 10x PCR Buffer (Invitrogen);
- 2μL of 1mM dNTPs (Invitrogen);
- 1μL of 50mM Cl_2Mg (Invitrogen);
- 0.8μL of 10μM of each of the primers mentioned above;
- 0.14μL of 5U/μL Taq DNA polymerase Recombinant (Invitrogen);
- 2μL of DNA.

The PCR cycling conditions were the following:

- 5mins at 94ºC
- 1min at 94ºC
- 1min at 65ºC (touchdown -0.5ºC per cycle) } 20 cycles
- 3min at 72ºC
- 1min at 94ºC
- 1min at 55ºC } 15 cycles
- 3min at 72ºC
- 7min at 72ºC

For the sequencing of the gene coding 16S rRNA of bacterial isolates the fragment was amplified between position 27 and 1492 was amplified using the primers 16S-27F with a sequence of 5' AGA GTT TGA TCM TGG CTC AG 3' (Weisburg *et al*, 1991) and 16S-1492R for reverse with a sequence of 5' TAC GGY TAC CTT GTT ACG ACT T 3' (Weisburg *et al*, 1991). Primers were synthesised by Invitrogen.

The PCR mixtures (20μL) contained the following reagents:

- 2μL of 10x PCR Buffer (Invitrogen);
- 2μL of 1mM dNTPs (Invitrogen);
- 1μL of 50mM Cl_2Mg (Invitrogen);
- 0.8μL of 10μM of each of the primers mentioned above;
- 0.14μL of 5U/μL Taq DNA polymerase Recombinant (Invitrogen);
- 2μL of DNA.

The PCR cycling conditions were the following:

- 2mins at 94ºC
- 1min at 94ºC
- 1min at 55ºC
- 2min at 72ºC

} 30 cycles

- 10min at 72ºC

8. Preparation, electrophoresis and staining of agarose gels

Routine agarose gels were prepared in TAE 1x and the electrophoresis run at 100V. After electrophoresis the gels were stained in a solution of Ethidium bromide (1-2 mg L^{-1}) for about 30 minutes, washed in water for about 15 minutes and observed under UV light.

9. TAE buffer preparation

TAE buffer 50x (with a concentration 50 fold the standard running concentration) was prepared by dissolving 242g of Tris base, 57.1mL of glacial acetic acid and 100mL of 0.5M EDTA in 800mL of distilled water, adjusting the pH to 7.8 with acetic acid and completing 1L with distilled water. From this stock solution running TAE solutions with concentration 1X and 0.5X were prepared by dilution in deionised water.

10. DGGE analysis

The technique was applied according to the instruction manual of the equipment used (INGENYphorU-2x2).

The buffer system was filled up to the minimum level (17L) with buffer (TAE 0.5X). The temperature was set at 60ºC.

The gel prepared was constituted of a 6% of polyacrylamide and had a denaturing gradient between 35% and 60% of urea-formamide. The concentrations of urea and formamide used to prepare a 100% denaturing gradient solution are 7M and 9M respectively.

The electrophoresis was done for 16h at 100V in buffer TAE 0.5x.

11. Silver Staining of polyacrylamide gel

The polyacrylamide gel was then stained with a silver solution according to the following protocol:

- The gel was fixed for 30 minutes in an aqueous solution with 12% methanol and 10% acetic acid
- The gel was then washed with water three times for two minutes (Van Dillewijn et al, 2002).
- The gel was then stained for 20 minutes with a silver solution containing 0.2g of silver nitrate in 200mL of water.
- After the staining step the gel was washed with water and treated with developing solution until bands began to be visible. The developing solution contained 0.02g of NaBH4 (sodium borohydride), 0.8mL of formaldehyde, 3g of sodium hydroxide in 200mL of water.
- The gel was washed again and the developing solution neutralised with an aqueous solution with 0.75% sodium carbonate for 10 minutes.

Finally the gel was covered with a preservative solution prepared with 25% absolute ethanol and 10% glycerol in water (Girvan et al, 2003).

12. Purification of PCR products previous to sequencing

The Montage® PCR Centrifugal Filter Devices (Millipore) were used to purify the PCR product from the 16S amplifying reactions so that the clean DNA could be sent for sequencing. The protocol followed was according to the manufacturer instructions.

13. Fluorescent In Situ Hybridization (FISH)

This technique was used with the objective of identifying groups of bacteria present in the reactors. The initial results indicated that extensive optimization would have been necessary to get good results so. As time for this project was short, the experimental work was focused on other techniques

13.1. Reagents for cell fixation

- PBS (3X): 390mM NaCl in 30mM phosphate buffer (pH 7.2): 0.49g KH_2PO_4 dissolved in 80mL, 2.3g NaCl added and pH adjusted to 7.2. The volume is then made up to 100mL.
- PBS (1x): 130mM NaCl in 10mM phospate buffer (pH 7.2): 33mL of PBS (3x) made up to 100mL with distilled water.
- 4% Paraformaldehyde in PBS
- 98% Ethanol at –20ºC
- 50%,80% and 98% ethanol
- MiliQ water at 4ºC
- 1M NaOH: 4g of NaOH dissolved in 80mL of distilled water and made up to 100mL
- 1M HCl

13.2. Reagents for hybridization

- 10% KOH in 95% ethanol: 10g KOH dissolved in 95% ethanol
- 5M NaCl: 29.2g NaCl dissolved in 80mL milliQ and made up to 100mL
- 1M Tris/HCl (pH 8.0): 12.1g Tris base dissolved in water milliQ, pH adjusted to 8.0 with HCl and the volume made up to 100mL.
- Formamide
- 0.5M Na_2EDTA (pH 8.0): 18.1g Na_2EDTA dissolved in 80mL, pH adjusted to 8.0 and volume made up to 100mL.
- 10% (v/v) SDS: 2g of sodium dodecyl sulphate dissolved in 20mL of milliQ water.

13.3. Preparation of the fixative

- 6.5mL milliQ water was heated to 60 ºC, under running hot tap water.
- 0.4 g of paraformaldehyde was added.

  Caution was taken with paraformaldehyde as it is very toxic.
- One drop of 1 M NaOH was added and the solution was shaken vigorously until it clarified. It took between 1 and 2 minutes.
- The solution was removed from the heat source and 3.3mL of 3x PBS was added.

- The pH was adjusted to 7.2 with the same amount of drops of NaOH used to clarify the solution before but this time of HCl.
- The solution was filtered through a 0.2µm membrane filter.
- The solution was kept on ice until used.

For the fixation of the samples, freshly prepared fixative was used to ensure better results.

13.4. Fixation of cells

Two different methods were used for the fixation of the cells, the first one as done for granules of microorganisms in slurry reactors (Method I) and the second one as done for soil samples (Method II) (Nogales et al, 2001).

13.4.1. Method I

Sand samples were suspended in 1XPBS and sonicated to disaggregate cells from the sand and disperse them in the liquid. Thirty seconds of ultrasounds were applied at a cycle of 0.5 and amplitude of 60. The cells were harvested by centrifugation and washed and resuspended in 1xPBS. For the fixation, three volumes of fixative were added to one volume of suspension and the mixture was kept on ice for 3h. Afterwards the cells were washed once more resuspended in 1xPBS, mixed with 1.25 volume of 98% ethanol (-20ºC) and stored at –20ºC.

13.4.2. Method II

- PBS 4% was added to the sand in a ratio of 2:1 and the samples were stored at 4ºC for 16h.
- The samples were then washed in 1XPBS three times and stored in a mixture of ethanol and PBS in a ratio of 1:1 (v/v) at -20ºC.

As fixation constitutes a method to preserve the samples we used both fixation methods but in fact only applied immobilization and hybridization to the first method samples. The other samples were kept as backup samples in case the fixation done by the first method proved not to be adequate.

13.5. Immobilization of cell on microscope slides

11µL of cell suspension were spread in each well of the 8 wells gelatine coated Teflon/glass microscope slides (or 7µL in the case of 10 wells Teflon/glass microscope slides) (Figure 11) and were dried at 46ºC for 10 min. The cells were dehydrated by successive 3 minute passage through 50%, 80% and 98% ethanol and dried at room temperature. After immobilization the slides were stored at 4ºC for up to 1 week.

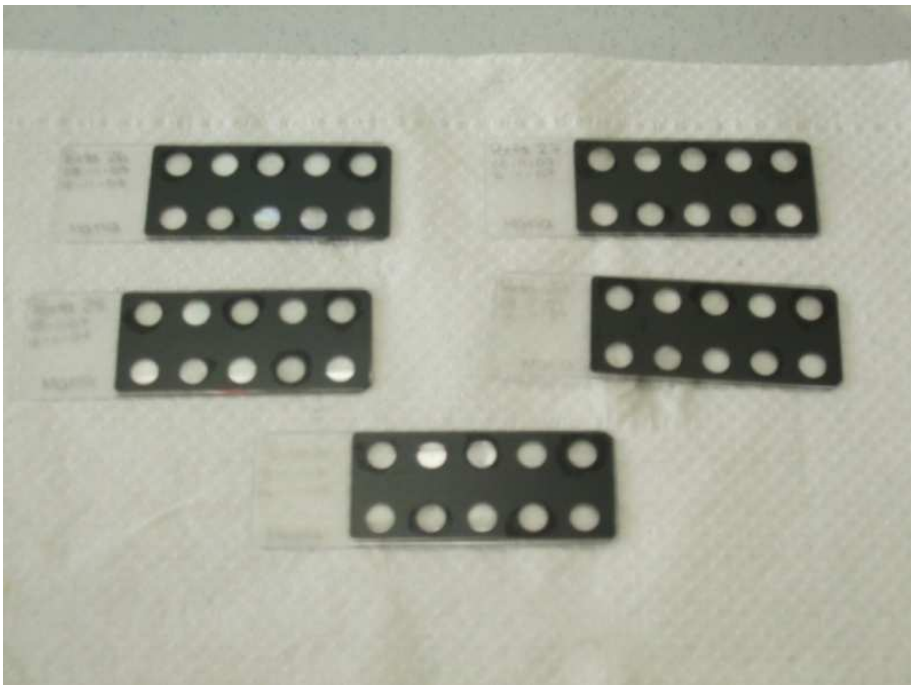

Figure 11: Special Teflon slides with a hydrophobic covering used for FISH.

The microscope slides used for immobilization were Teflon coated. This hydrophobic coating separates 8 or 10 wells, depending on the microscope slide used. These slides allow the application of probes preventing mixing of probe in adjacent wells. Even so, only one probe per slide was used to be absolutely certain that they would not be mixed and to have 3 or 4 replicates of the samples.

13.6. Hybridization

The hybridization buffers contained 360µL of 5M NaCl, 40µL of 1M Tris/HCl (pH 8.0), 4µL of 10%(w/v) SDS, the amount of formamide adequate for the different probes (Table 4) and were made up to 2000 µL with milliQ water.

- 10 µL of hybridisation buffer per well per microscope were pipetted into an eppendorf tube.
- 1 µL of probe stock solution per well per microscope slide was added to each eppendorf tube.
- This mixture was then evenly divided between the 3 or 4 wells of the microscope slide in question.
- A hybridization tube was prepared by folding a tissue, putting it into the 50mL Falcon tube and pouring the rest of the hybridization buffer onto the tissue.
- The slide was then immediately transferred into the hybridization tube and incubated for 1.5 h at 46ºC.
- In this interval of time the washing buffer was prepared according to a table given and preheated in a water bath at 48 ºC.

Table 4: Probes used for the analysis of the sand samples collected from the six non sterile reactors (continued)

	Probe	Citoc	Probe sequence (5'▫3')	% F	*Target organisms*
1	**EUB338I**	Fluos	GCT GCC TCC CGT AGG AGT	**20**	*Bacteria domain*
3	**EUB338II**	Fluos	GCA GCC ACC CGT AGG TGT	**60**	*Bacterial lineages not covered by probe EUB338. Planctomycetales*
16	**NONEUB**	Cy3	ACT CCT ACG GGA GGC AGC	**20**	*Control probe complementary to EUB338*
22	**ALF1B**	Fluos	CGT TCG TTC TGA GCC AG	**20**	*Alphaproteobacteria, some Deltaproteobacteria, Spirochaetes*
23	**BET42a***	Cy3	GCC TTC CCA CTT CGT TT	**35**	*Betaproteobacteria*
24	**BETComp**	-	GCC TTC CCA CAT CGT TT		
25	**GAM42a***	Cy3	GCC TTC CCA CAT CGT TT	**35**	*Gammaproteobacteria*
26	**GAMComp**	-	GCC TTC CCA CTT CGT TT		
33	**ARC915**	cy3	GTG CTC CCC CGC CAA TTC CT	**35**	*Archaea*
39	**PAE997**	Cy3	TCT GGA AAG TTC TCA GCA	**0**	*Pseudomonas spp.*
7	**PLA46**	Cy3	GAC TTG CAT GCC TAA TCC	**30**	*Planctomycetales*

Different probes can be used in the same hybridization reaction provided that the fluorochromes have compatible spectra (fluos fluoresces in the green area and cy3 fluoresces red) and that the percentage of formamide doesn't differ more than 15%.

For all the samples, microscope slides were prepared using probes eub338I and noneub together, alf1, gam42a* and gamcomp, bet42a* and betcomp, arc915, eub338I and eub338II and pae997 and pla46 separately.

13.7. Preparation of washing buffer for in situ hybridization at 46ºC (washing at 48 ºC, 20 min)

- Pipet into a 50 mL Falcon tube and mix:
  - Tris/HCl (pH 8.0): 1mL.
  - 5 M NaCl and 0.5 M EDTA (pH 8.0) were added according to the percentage of formamide used for each probe.
- The Falcon tube was then filled up to 50mL with milliQ water.
- The washing buffer was preheated at 48 ºC prior to use as was mentioned above.
- After being at 46ºC for 1.5h, the hybridisation buffer was quickly rinsed from the microscope slides with the washing buffer.

- The slide was then transferred in the rest of the wash buffer and incubated for 15min at 48ºC in the water bath.
- Then, the washing buffer was removed by rinsing the slide with milliQ water and the slides were left to dry at 46ºC.
- After being dry, the wells were embedded with Vectashield which amplifies fluorescence and avoids fading and a cover slip was put on the microscope slide. 4μL were used on the microscope slides with 8 wells and 3μL on the microscope slides with 10 wells.
- Specimens were then kept at 4ºC until analysed with fluorescence microscope which was no more than a week later.

14. DAPI Staining

4'-6-Diamidino-2-phenylindole (DAPI) is known to form fluorescent complexes with natural double-stranded DNA, showing fluorescence specificity for AT, AU and IC clusters. This is useful to visualize nuclear DNA in both living and fixed cells. DAPI staining is used to determine the number of cells and assess gross cell morphology (Tarnowski et al, 1991).

Appendix 2 Complementary Results

1. Plate Count Determination

The colony forming units from the soil samples before incubation in the plates with bromoxynil were 3.6 $\log_{10}.g^{-1}$ of dry soil and in the plates with bromoxynil and nitrogen were 4.08 $\log_{10}.g^{-1}$ of dry soil.

Table 5: Colony forming units from the soil and sand in the reactors ($\log_{10}$), growing in plates with bromoxynil as C and N source (Br), Bromoxynil as C source (Br+N), Bromoxynil as N source (Br+C) and in a rich media supplemented with bromoxynil (Br+C+N).

		Reactors					
		without Bromoxynil			with Bromoxynil		
		1	2	3	1	2	3
Br	sand	6.52	6.47	6.42	6.30	6.63	6.21
	soil	4.72	ADL	4.48	4.64	4.72	ADL
Br + N	sand	6.39	6.38	6.14	6.46	6.53	6.11
	soil	ND	ND	ND	ND	ND	ND
Br + C	sand	6.27	6.02	6.05	6.36	6.41	6.23
	soil	5.32	5.45	5.43	ADL	5.25	5.30
Br + C + N	sand	6.12	5.97	6.26	6.28	6.28	6.17
	soil	5.26	5.20	5.08	5.08	4.78	5.15

ADL stands for “Above Detection Limit” referring to the plates which presented more than 300 colonies. ND stands for “Not Defined” referring to the plates which became contaminated.

Reactors 6, 7 and 8 had sand without bromoxynil and reactors 9, 10 and 11 had sand with bromoxynil.

2. Microbial Determinations by Most Probable Number (MPN)

Table 6: Most probable number of microorganisms in soil and in sand growing in different substrates after an incubation period of 1 week and 3 weeks. Results are expressed in Log_{10}. (continuation)

	Reactors							
	without Br				with Br			
	soil		sand		soil		Sand	
Substrate	1 week	3 weeks	1 week	3 weeks	1 week	3 weeks	1 week	3 weeks
(1)Tween 40	ADL	ADL	ADL	ADL	ADL	ADL	ADL	ADL
(2)Tween 60	5.8	6.4	6.6	6.6	5.1	5.4	6.8	6.8
(3)α-ciclodextrin	4.5	4.5	4.1	5.1	4.3	4.3	4.3	4.6
(4)glycogen	4.3	4.3	3.8	3.8	4.4	4.4	3.8	3.8
(5)D (+) cellobiose	7.6	8.1	7.8	7.8	8.4	8.4	7.5	7.5
(6)α-lactose	ADL	7.0	5.1	6.3	5.0	5.0	7.0	7.0
(7)β-metil D-glucoside	7.0	ADL	7.0	ADL	ADL	ADL	ADL	ADL
(8)D (+) xylose	7.6	8.1	8.1	8.1	8.1	8.1	8.4	8.4
(9)i-erythritol	6.8	6.8	7.8	7.8	6.2	7.8	8.1	8.4
(10)maltose	7.3	7.3	7.8	8.1	7.4	7.4	7.6	7.6
(11)N-acetyl-D-glucosamine	7.8	7.8	7.8	7.8	ADL	ADL	7.6	7.8
(12)α-D- glucose 1-phosphate	4.0	5.1	6.6	7.1	4.8	4.8	5.7	6.3
(13)glyceraldehyde	5.9	5.9	7.4	8.1	5.5	5.5	7.3	7.3
(14)D-galactonic acid δ-lactone	5.5	5.5	BDL	BDL	5.1	5.1	5.2	5.2
(15)galacturonic acid	5.8	5.8	5.9	5.9	5.1	5.1	8.1	8.1
(16)o-hydroxibenzoic acid	7.0	7.0	7.8	7.8	6.4	7.3	6.5	6.8
(17)p-hydroxibenzoic acid	6.0	6.8	7.6	7.6	4.3	4.8	7.0	8.4
(18)malonic acid	7.5	7.5	8.4	8.4	7.0	7.0	ADL	ADL
(19)L-arginine	7.8	ADL	8.4	8.4	ADL	ADL	8.4	8.4
(20)L-asparagine	7.5	7.5	7.8	7.8	ADL	ADL	7.6	7.6
(21)L-phenylalanine	ADL	ADL	8.4	8.4	ADL	ADL	5.6	6.0
(22)L-serine	7.8	7.8	7.8	7.8	ADL	ADL	7.3	7.3
(23)L-threonine	5.1	5.4	6.3	ADL	5.1	5.1	4.3	4.3
(24)L- glutamic acid	7.8	7.8	8.1	8.1	ADL	ADL	8.1	8.1
(25)β-phenyl ethylamine	3.5	5.3	5.9	5.9	3.6	3.6	4.8	4.8
(26)putrescine	4.6	5.1	4.4	4.8	4.1	4.8	5.1	5.6
(27)α-keto butyric acid	5.8	5.8	5.7	6.8	5.3	5.6	6.8	7.1
(28)malic acid	7.8	7.8	8.1	8.1	7.5	7.5	7.4	7.8
(29)Mannitol	7.3	7.3	8.1	8.1	6.1	7.3	7.4	7.8
(30)α-glycerolphosphate	4.8	7.0	5.8	7.8	4.4	7.3	5.6	5.8
(31)Pyrocatechol	5.8	5.8	7.8	7.8	5.7	6.1	7.8	7.8
(32)Water	0.0	0.0	0.0	0.0	0.0	0	0.0	0.0

Table 7: Estimates of the heterotrophic population (PH), ammonifiers (AM) starch mineralizers (SM) by Most Probable Number for sand and soil samples in the reactors after 1 and 3 weeks (wk) incubation.

		Reactors											
		without Br 1		without Br 2		without Br 3		with Br 1		with Br 2		with Br 3	
		1 wk	3 wks	1 wk	3 wks	1 wk	3 wks	1 wk	3 wks	1 wk	3 wks	1 wk	3 wks
PH	**sand**	6.3	6.3	7.6	7.6	7.4	7.4	7.3	7.3	7.2	7.2	7.0	7.0
	soil	8.0	8.0	6.6	6.6	7.0	7.0	7.0	7.0	6.5	6.5	6.2	6.2
AM	**sand**	7.1	7.1	7.2	7.2	7.2	7.2	6.8	6.8	7.5	7.5	7.0	7.0
	soil	7.6	7.6	5.4	6.2	6.8	6.8	5.2	5.2	5.9	5.9	6.6	6.6
SM	**sand**	7.2	7.2	6.8	6.8	6.8	6.8	5.2	5.8	5.2	5.9	5.2	6.7
	soil	6.2	6.8	6.2	6.6	6.8	6.8	5.4	8.0	6.0	6.7	5.4	7.2

3. Denaturing Gradient Gel Electrophoresis

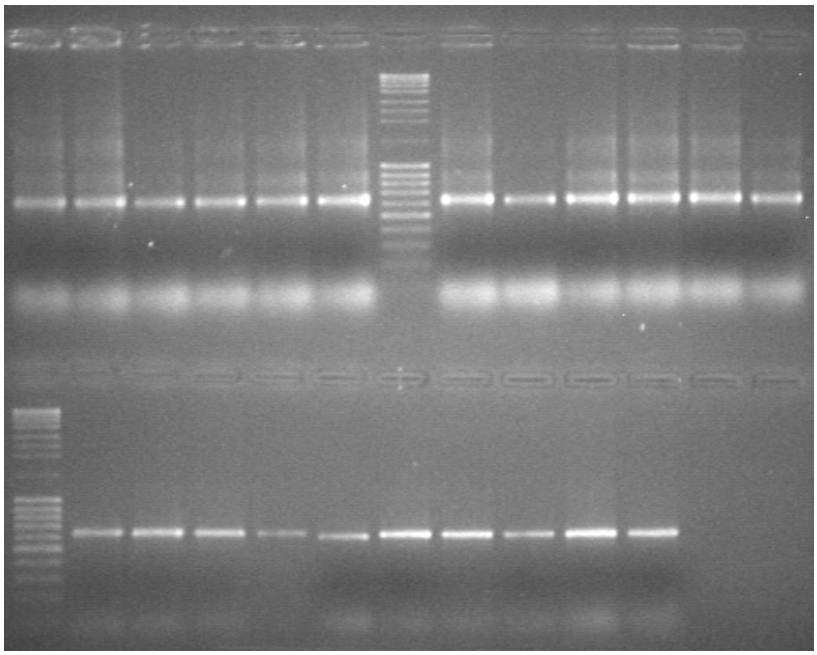

Figure 12: Image of an agarose gel used to quantify the mass of DNA present in the concentrated PCR products.

4. Measure of concentration of Bromoxynil and metabolites

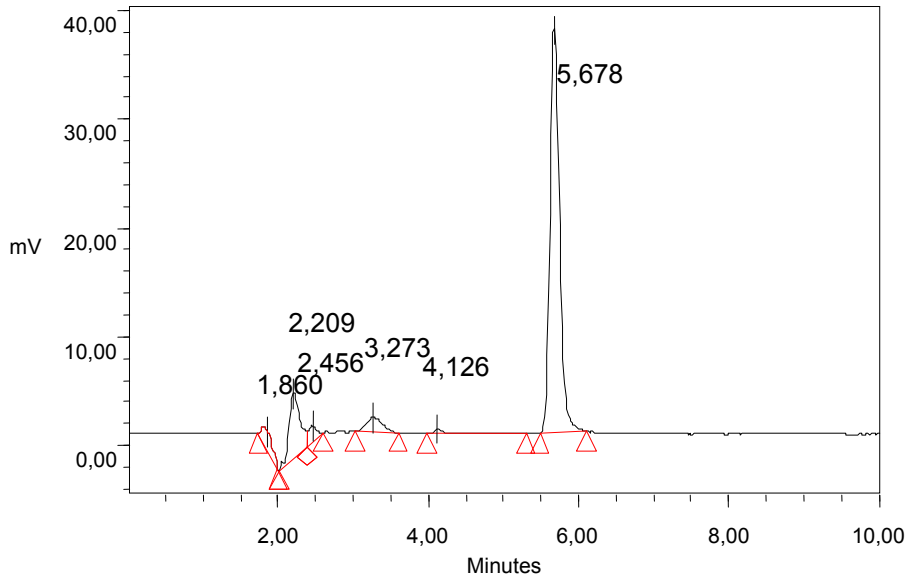

Figure 13: Chromatogram of a standard solution of Bromoxynil with a 3ppm concentration.

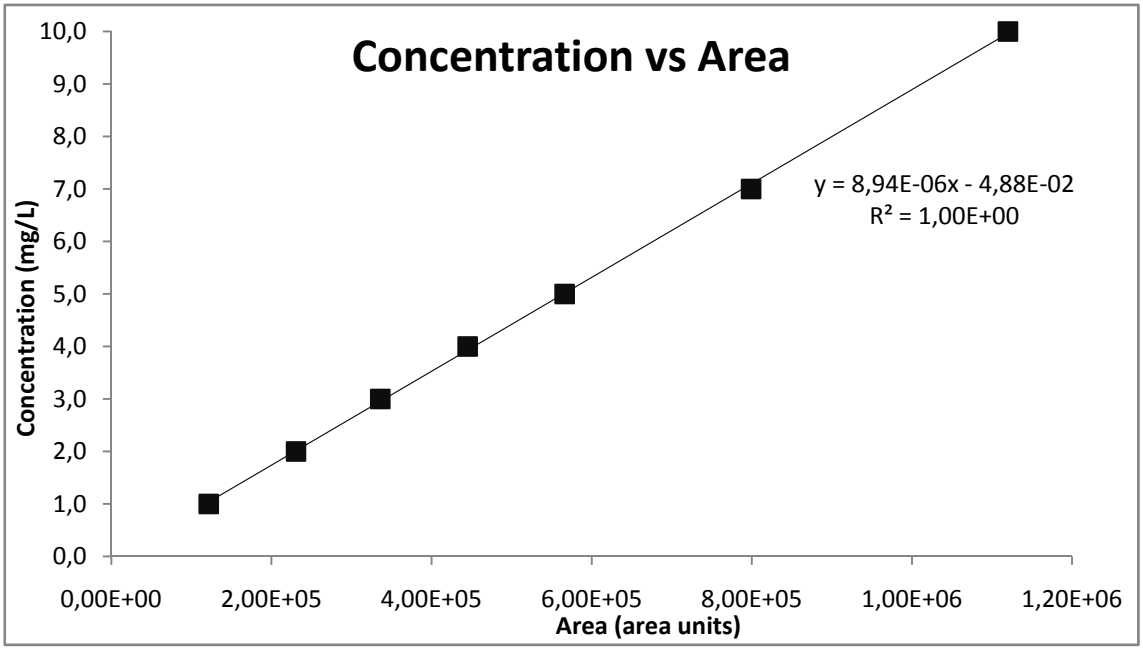

Figure 14: Graph showing the calibration curve and equation of concentration vs area obtained measuring the standard solutions of bromoxynil in the HPLC.