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Evaluation of pesticides degradation by microorganisms in the soil of a new cropping system : Isolation and characterization of pesticide-degrading strains of soil microorganisms

BENJAMIN GUILLAUME

TRAVAIL DE FIN D'ETUDES PRESENTE EN VUE DE L'OBTENTION DU DIPLOME DE MASTER BIOINGENIEUR EN SCIENCES ET TECHNOLOGIES DE L'ENVIRONNEMENT

ANNEE ACADEMIQUE 2020-2021

CO-PROMOTEURS:
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Abstract

Among the process that determine the fate of pesticides in soils, microbial degradation is recognized as one of the most important. Biodegradation of pesticides seems to be achieved through the contribution of a few key species that exhibit degradation capabilities. However, in field conditions the real effect of these key species on the fate of pesticides is still poorly understood. We attempted to isolate and identify some of them capable of degrading metamitron, metazachlor, bentazon, s-metolachlor and chlortoluron after selective enrichmeent cultures in minimal medium. Their degradation capacities were evaluated in a minimal liquid medium with pesticide as the only source of carbon and nitrogen. The feasibility of assessing the degradation behaviour of degrading strains on undisturbed soil columns was performed. A special focus was made on the effect of autoclaving on soil properties that might control the fate of pesticides. In total, 85 pesticide-resistant cultures were isolated. 27 of them showed significant enzyme capacity with ABTS as substrate which may indicate their ability to degrade phenolic compounds. Three isolated strains (SM 12, SM 2 and SM 14) were suggested to be able to degrade metolachlor. Three other strains (MET 8, MET 17 and MET 12) were proposed to be able to degrade metamitron. One strain (MZ 3) was potentially able to degrade metazachlor. The soil column study revealed for the first time that autoclaving may influence the soil hydraulic conductivity by affecting the soil structure. This can lead to inaccurate assessments of pesticide fate in experiments conducted under abiotic soil columns and inoculated with degrading strains.

Résumé

Parmi les processus qui déterminent le devenir des pesticides dans les sols, la dégradation par les micro-organismes est considérée comme l'un des plus importants. La biodégradation des pesticides semble être réalisée par quelques microorganismes clés qui présentent des capacités de dégradation. Cependant, dans les conditions réelles sur champs, l'effet de ces organismes clés sur le devenir des pesticides est encore peu connu. Nous avons tenté d'isoler et d'identifier certaines de ces espèces clés capables de dégrader le métamitron, le métazachlore, le bentazone, le s-métolachlore et le chlortoluron. Celles ci ont été sélectionnées après des cultures d'enrichissement sélectif en milieu minimal. Leurs capacités de dégradation ont été évaluées dans un milieu liquide minimal avec le pesticide comme seule source de carbone et d'azote. La faisabilité de l'évaluation du potentiel de dégradation des micro-organismes dégradants sur des colonnes de sol en structure non perturbées a été réalisée. Une attention particulière a été portée sur l'effet de l'autoclavage sur les propriétés du sol qui pourraient impacter le devenir des pesticides. Au total, 85 cultures résistantes aux pesticides ont été isolées. 27 d'entre elles ont montré une capacité enzymatique significative avec l'ABTS comme substrat, ce qui pourrait indiquer leur capacité à dégrader les composés phénoliques. Trois souches isolées (SM 12, SM 2 et SM 14) ont été suggérées comme étant capables de dégrader le métolachlore. Trois autres souches (MET 8, MET 17 et MET 12) ont été proposées comme étant capables de dégrader le métamitron. Une souche (MZ 3) était potentiellement capable de dégrader le métazachlore. L'étude sur colonne de sol a révélé pour la première fois que l'autoclavage pouvait influencer la conductivité hydraulique du sol en affectant sa structure. Cela peut conduire à des évaluations inexactes du devenir des pesticides dans les expériences menées sous des colonnes de sol abiotiques et inoculées avec des souches dégradantes.

1 State of the art

1.1 Introduction to pesticides

According to the European Commission, pesticides and plant protection products are defined as substances used for the protection of crops. They are mainly used in agriculture, forestry or horticulture. They contain at least one active substance which aims to protect plants against pests and diseases. They destroy and prevent the appearance of unwanted plants, insects and fungi by influencing their vital processes (European Commission, 2021). In this work, the focus is on herbicides (specifically on metamitron, metazachlor, bentazone, s-metolachlor and chlortoluron) which are pesticides acting on weed control (Heap, 2014). Herbicides control weeds by disrupting their life processes. Most herbicides act either on the process of photosynthesis or pigment synthesis, or on the metabolic pathways responsible for the biosynthesis of molecules essential for plant survival. To a lesser extent, some act on respiration or as simple growth regulators (Casida, 2010).

The use of pest control measures dates back thousands of years. The Romans already used amurca, an effluent from the extraction of olive oil rich in phenolic compounds, to fight against diseases, insects and weeds (Smith and Secoy, 1976; Janakat *et al.*, 2015). The modern synthetic pesticides as we know today were developed in the 1930s (Zimdahl, 2018). After the Second World War, the use of pesticides became widespread and contributed greatly to increasing yields (Singh, 2017). Since then, the global pesticide market has grown steadily to reach USD 31,756 million in 2001 (Zimdahl, 2018). However, since the 2010s, the evolution of global pesticide use seems to have stabilised, but with large variations between developed and developing countries (FAO, 2021). At the same time, there has been a decrease in the use of pesticides in Belgium certainly due to more rational use, the increase in organic farming and the general decrease in agricultural surfaces.

While herbicides have improved weed control and reduced the cost and labour required to control weeds, they have limitations that humanity must address (Gianessi, 2013). Herbicides used to control certain pests can have effects on non-target organisms, including humans (Özkara *et al.*, 2016). Exposure to some pesticides can cause cancer, chronic kidney disease, infertility, immune system suppression, endocrine disruption and neurological disorders (Gupta, 2004). Pesticides can cause both diffuse and concentrated pollution. Depending on the properties of each pesticide, they can end up in water bodies once they have been applied to crops (Özkara *et al.*, 2016). Pesticides can then deteriorate the structure and functions of aquatic ecosystems or even end up in drinking water (Schäfer *et al.*, 2007; Syafrudin *et al.*, 2021). In addition, herbicide resistances have emerged in recent years and can compromise the effectiveness and profitability of some herbicides (Heap, 2014).

1.2 Fate of pesticides in the environment

Once applied, pesticides are subject to immobilisation, transport and degradation processes that determine their persistence and mobility in soils (Figure 1.1). These dynamic processes are largely influenced by the physical, chemical and biological properties of the soil (Andreu and Picó, 2004), but also by the

intrinsic properties of the pesticide such as water solubility and adsorption proprieties (Fenner *et al.*, 2013) (ICSS, 2006).

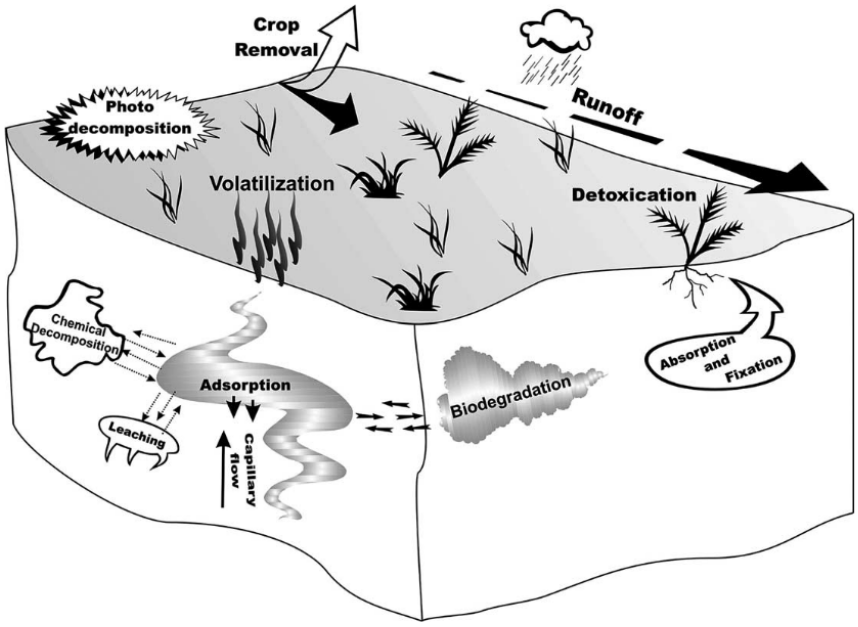

Figure 1.1: Fate of pesticides in the environment : Figure from Andreu and Picó (2004).

1.2.1 Immobilisation processes

1.2.1.1 Adsorption/dessorption

A key process controlling the fate of pesticides in soil is the importance of their sorption to soil constituents (Ghafoor *et al.*, 2013). In soils, adsorption involves the extraction of a solute from a solution to a solid surface until an equilibrium between the concentration in solution and at the surface is reached. Desorption is the inverse process. Adsorption may involve ion exchanges, metal cations interactions, polar interactions, hydrogen bonds, charge transfers and London-Vander Waals dispersion forces/hydrophobic effects. The rate and the strength of adsorption depends on both proprieties of the absorbed molecule and the absorbent such as the electronic structure, the size of the molecule, the solubility, the water-octanol partition coefficient, the clay and organic matter content (Calvet, 1989).

The sorption behavior of a pesticide is predicted by its organic carbon partition coefficient k_{oc} which corresponds to the sorption constant k_d divided by the soil organic matter content. Indeed, there is a strong correlation between the organic matter content and the sorption behavior of pesticides in a soil. This is explained by the fact that non-polar organic matter in the soil attracts pesticides to its surface because they are generally also non-polar. The k_{oc} is widely accepted as a measure of pesticide mobility in soils and is a tool for assessing the leaching behavior of pesticides (Wauchope *et al.*, 2002). However, k_{oc} may be a poor predictor of pesticides adsorption for soils with low organic matter content, which may lead to incomplete pesticide risk assessments, in subsoils for example (Ghafoor *et al.*, 2013).

1.2.1.2 Non-extractable and bound residues

Non-extractable residues and bound residues are often considered synonymous, although they are not exactly the same, which leads to misconceptions (Karasali and Pavlidis, 2021). Non-Extractable residues (NER) are described as pesticide residues that can not be extracted from the soil with " soft " extraction methods while Extracable Residues (ER) can be. The difference between NER and bound residues (BR) is the reversibility of their binding to the soil matrix. NER are strongly bound to the matrix but can potentially be reversible, whereas BR are indistinguishable from the matrix or soil organic matter as they

often share covalent bonds. NER and BR are then unlikely to be available to organisms and are not taken into account in pesticide risk assessments (ECETOC, 2013).

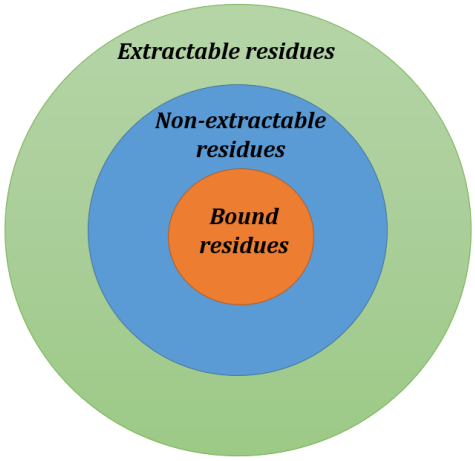

Figure 1.2: Representation of extractable, non-extractable and bound residues proposed by the ECETOC (2013)

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1.2.2 Degradation

Degradation can be defined as the transformation of pesticide molecules into simpler molecules. The new compound is generally less toxic than the parent compound, according to the specific reaction (Gavrilescu, 2005). Degradation is an important process that govern the fate of pesticides and occurs through chemical degradation, photodegradation or microbial degradation (Arya *et al.*, 2017).

1.2.2.1 Chemical degradation

Chemical degradation of pesticides might occur by hydrolysis, redox and ionization reactions. Hydrolysis consist in the breakdown of a molecule thanks to a reaction with a water molecule or a hydroxyl group. The reaction rate is influenced by the pH of the solution. Redox reactions result in the transfer of electrons to an oxidised species from the reduced one. Redox reactions can be enhanced by metal ions and also depends on redox conditions (Gavrilescu, 2005).

1.2.2.2 Photodegradation

Photodegradation can be an important part of the degradation of pesticides in the aquatic environment (Fenner *et al.*, 2013). There is a distinction between direct and indirect photolysis. Direct photolysis occurs when the pesticide reaches an excited state after direct absorption of solar radiation in the UV-visible range. The excited pesticide is then degraded by homolysis, heterolysis or photoionisation. This type of degradation is however not the most important in nature because of the small solar absorption range of pesticides (Burrows *et al.*, 2002). Then there is indirect photolysis, which leads to the formation of reactive species by absorption of photons by other compounds such as dissolved organic matter (Fenner *et al.*, 2013). These reactive species transfer their energy to the pesticides, which in turn become excited and follow the same reactions as in direct photolysis; this is called photosensitised degradation. In other cases, certain compounds (such as hydrogen peroxide or ozone) absorb photons and form hydroxyl radicals (OH.) that react directly with the pesticides (Burrows *et al.*, 2002).

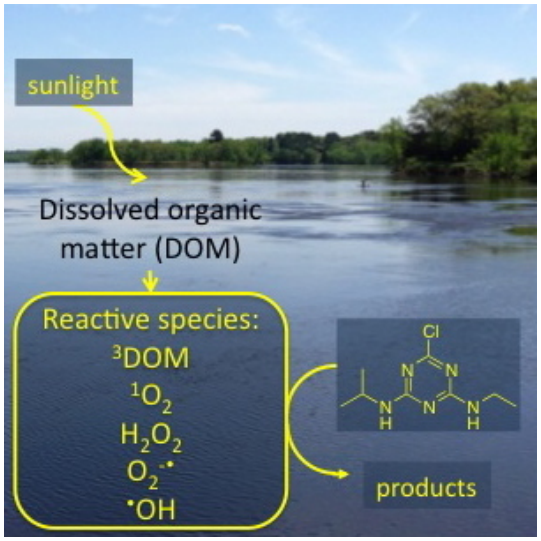

Figure 1.3: Indirect pesticides photolysis : Figure from Remucal (2014).

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1.2.2.3 Microbial degradation

Microbial degradation is recognized as the most important pesticide degradation process (Fenner *et al.*, 2013). Contaminants in soils and aquifers are mainly degraded by both bacteria and fungi (ICSS, 2006), (Ye *et al.*, 2018). However, bacteria dominate the enzymatic degradation of pesticides. This is due to the ability of bacteria to reproduce and evolve rapidly, but also to carry out horizontal transfers of genes coding for the degradation of pesticides (Fenner *et al.*, 2013)(See Chapter 1.3).

1.2.3 Transfer routes

1.2.3.1 Volatilization

After application of a pesticide, it may volatilize from the soil or plants surface into the atmosphere by evaporation or sublimation. The volatile behavior of a pesticide depends on its physicochemical properties and is represented by its Henry's law constant (H) (Bedos *et al.*, 2002). Volatilization rates might vary from 2.4% of the applied dose in 24 day for atrazine (H = 2.87×10^{-4} Pa.m^3.mol) to 90% in 7 days for trifluralin (H=4.03 Pa.m^3.mol) which is banned today (Taylor and Spencer, 1990). Volatilization rates can also be affected by environmental conditions and agricultural practices (temperatures, wind, application rate, mode of application, ...) (Bedos *et al.*, 2002). Pesticide volatilization is an important transfer route that should be taken into account in pesticides risk assessment, as it constitutes direct exposure for workers, bystanders and residents (European Food Safety Authority, 2014).

1.2.3.2 Leaching and runoff

Leaching is an important process of solute transport from the topsoil to the groundwater. If the infiltration capacity of the soil is exceeded, pesticides can be transported to surface water by runoff (Carter, 2000). The most commonly occurring pesticides in surface water and groundwater are those that are used the most and have the greatest mobility and persistence patterns in the hydrological system (Syafrudin *et al.*, 2021).

Pesticides can enter the water system through diffuse sources (spray drift, volatilization/precipitation and field runoff or leaching) or point sources (farm tank filling, washing, spills, leaks, overspray or permitted discharge) (Carter, 2000). Point source releases should not be underestimated as they mostly occur in cleaning areas of farms, where the soil is covered with concrete or gravel. In these areas, the

absorption/degradation capacity of the soil is low, creating a high risk of groundwater being contaminated (De Wilde *et al.*, 2009).

Pesticides are more commonly detected in surface water than in ground water. The presence of pesticides in surface waters is indeed more common due to the direct and rapid transport of pesticides by surface runoff. However, groundwater is much slower to recover once it is polluted (Gilliom *et al.*, 2006).

1.3 Degradation of pesticides by micro-organisms in soils

1.3.1 Soil microorganisms

1.3.1.1 Definition

The soil-plant system is coloniZed by an enormous diversity and abundance of micro-organisms which makes soils the largest reservoir of biodiversity on earth (Lukac, 2017). It is estimated that in 1 cm^3 of surface soil there are 4-20 x 10^9 of bacteria, including 100-9000 different species. In a single gram of soil, 150-1200 species of protists and 200-235 species of fungi forming 100 metres of fungal hyphae (including micorhyzae) can be found (Bardgett and Van Der Putten, 2014). Within the rhizosphere, which is the region of the soil where micro-organisms and plant roots interact, micro-organisms shape the major ecosystem functions and services provided by soils (Cardinale *et al.*, 2012; Reinhold-Hurek *et al.*, 2015). They support production, regulation and support services. Soil micro-organisms regulate diseases, can decontaminate and sanitize soils and water. They are also essential for soils formation, transformation of organic matter, regulation of biogeochemical cycles and provision of essential nutrients to plants (Saccá *et al.*, 2017). On the other hand, soil microorganisms benefit from the plant system to support their growth through the uptake of root exudates and decaying plant tissue (Philippot *et al.*, 2013). This is because soil micro-organisms tend to attach themselves along the roots of plants and decaying material (Marilley *et al.*, 1998). It is also estimated that the abundance of micro-organisms in the soil-plant complex is three times higher than in bare soil (Reinhold-Hurek *et al.*, 2015).

1.3.1.2 Spatial variability of soil micro-organisms distribution

As soil micro-organisms are heterogeneously distributed in space, the ecosystem services and functions provided by soils, such as pesticide degradation, are also spatially heterogeneous (Verhagen *et al.*, 2015 ; Pinheiro *et al.*, 2015).

Soil micro-organisms are distributed over the whole planet in a non-random way. This non-random dispersion is caused by selection pressures applied by the environment (Tecon and Or, 2017). At the continental scale, microbial abundance and activity are determined by soil and climatic conditions, such as water, nutrients and soil organic carbon availability (Serna-Chavez *et al.*, 2013). At the field scale, a pH gradient can explain variations in bacterial populations and abundance (Rousk *et al.*, 2010). At the meter scale, a patchy distribution of microbial biomass and diversity can be detected. This patchy distribution of micro-organisms can be related to a patchy distribution of nutrient sources such as available soil organic carbon. Therefore, micro-organisms "hot spots" are usually found beside decaying materials or roots of plants that release exudates. The microbial community is then associated with a single plant or plant population (Tecon and Or, 2017). Thus, operations on cultivated fields influence the communities and the diversity of micro-organisms (de Graaff *et al.*, 2019).

The biodiversity of soil micro-organisms is very heterogeneous at the profile level, with great vertical variability. Indeed, there are more differences (in diversity) between a few centimetres of depth within the same soil profile than between surface soils located hundred kilometres apart. The diversity, biomass and

activity of micro-organisms decreases exponentially with depth, from the aerated and rich topsoil to the nutrient-poor, water-saturated subsoil (Eilers *et al.*, 2012). Indeed, a vertical gradient in the distribution of nutrients, oxygen, water, pH and temperatures creates distinct conditions for microbial life (Tecon and Or, 2017). Therefore, this vertical gradient of micro-organism activity is directly related to a vertical gradient of pesticides degradation activity, where the majority of pesticides biodegradation occurs in the upper soil layer (Verhagen *et al.*, 2015; Rodríguez Cruz *et al.*, 2008).

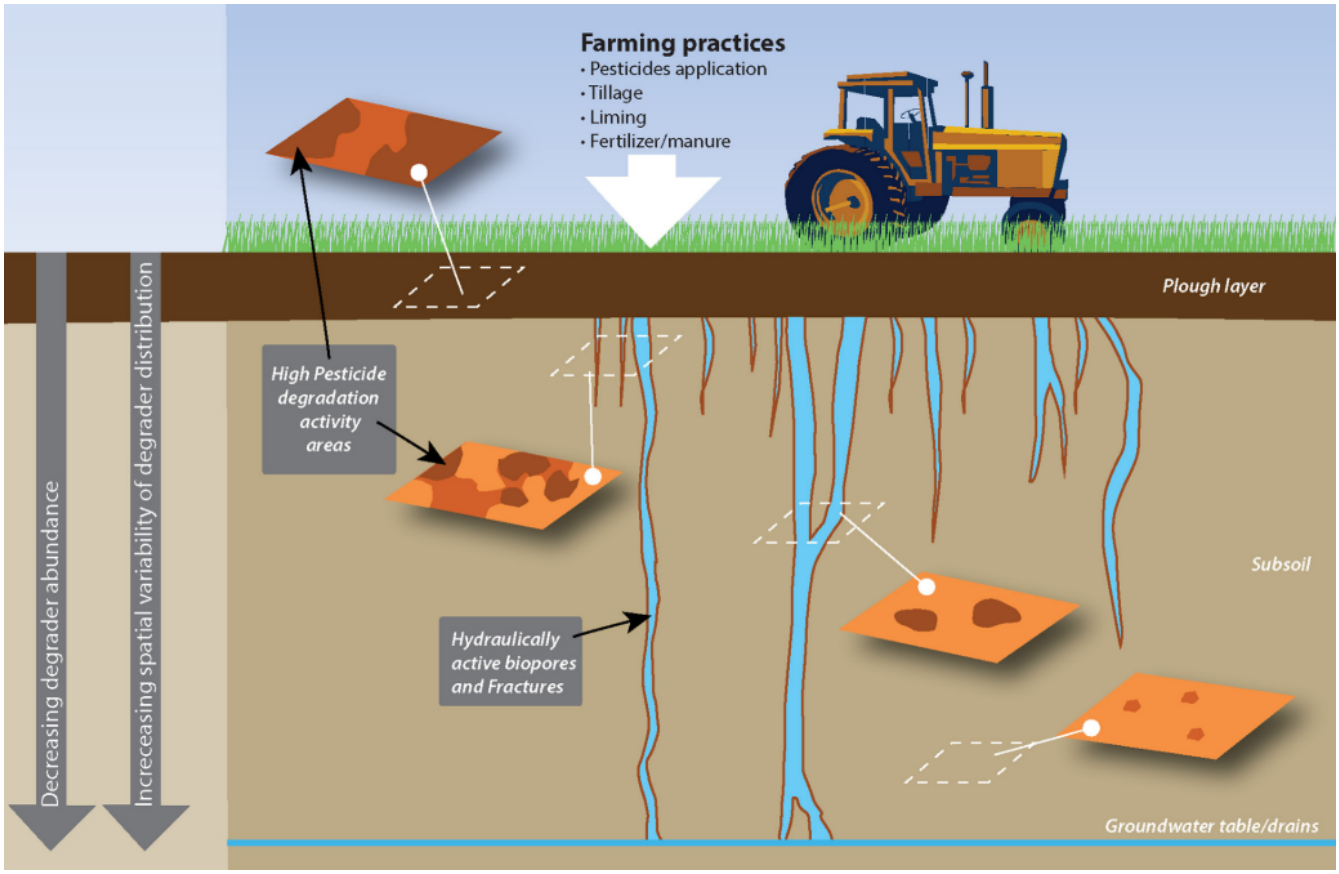


Figure 1.4: Horizontal and vertical spatial distribution of pesticide degradation affected by soil structure and biota : Figure from Dechesne *et al.* (2014).

From the centimeter to the nanometer scale, soil is best described as a complex structure of pores and aggregates, where biota is strongly involved in the formation (Oades, 1993). The wide variety of pore and aggregates sizes creates many different niches for soil micro-organisms (Young *et al.*, 2008). Large pores are more likely to be colonized by aerobic microbes while anaerobic microorganisms dominate small pores and center of aggregates (Ranjard and Richaume, 2001). At this scale, bacteria in the topsoil are found in patches and their distribution is linked to the local substrate deposition (Nunan *et al.*, 2003). Moreover, these patches are often spatially isolated due to low connectivity of the soil network or due to aggregate encapsulation. Colonies separated by small distances, actually, may not interact because of unconnected pores (Tecon and Or, 2017). This consideration of the soil network connectivity and the heterogeneous distribution of micro-organisms on sub-cm scales is really important in the context of the degradation of pesticides by degrading micro-organisms. Indeed, the accessibility of pesticides (by diffusion) to degrading micro-organisms is a major factor that drives pesticide degradation and fate in soils. Thus, the heterogeneous distribution of degrading micro-organisms and the heterogeneous diffusion processes in soils lead to a heterogeneous degradation of pesticides on a sub-cm scale (Monard *et al.*, 2012b; Pinheiro *et al.*, 2015). In subsoils, pesticide degradation occurs even more heterogeneously because degrading communities are less numerous and more randomly distributed in patches (Figure 1.4) (Badawi *et al.*, 2013b). However, subsoil vertical preferential flow paths, where downward transport of pesticide may occur, constitute hotspots for bacteria and degraders (Badawi *et al.*, 2013a). In these macropores,

oxygen, moisture and solutes (eg : pesticides) are more readily available for bacterial growth and activity (Bundt *et al.*, 2001).

1.3.2 Factors affecting microbial degradation of pesticides in soils

The degradation of pesticides in soils involves different mechanisms that depend on factors intrinsic to the pesticides, soil characteristics and climatic conditions.

1.3.2.1 Pesticide composition

The molecular structure of a pesticide determines its physical and chemical properties and its intrinsic biodegradability (Figure 1.5). Slight changes in pesticide structure may result in a dramatic impact on the susceptibility to biotransform (Pal *et al.*, 2006). As an example, minor structural differences between phenylurea herbicides may influence the rate of degradation and the persistence in soils (Hussain *et al.*, 2015). Polar groups such as OH and COOH and in pesticides can provide an attack site for microbes (Pal *et al.*, 2006). Inversely, halogen or alkyl substitutions on aromatic rings have shown to increase the resistance of pesticides to biodegradation (Bollag, 1974).

1.3.2.2 Pesticide solubility

In general, microbial degradation is more limited with compounds with low water solubility than with compounds with higher water solubility (Figure 1.5) (Cork and Krueger, 1991). Indeed, only the dissolved part of pesticides can be degraded by micro-organisms (Jaiswal *et al.*, 2017). Pesticides water solubility depends on temperature, pH, polarity, hydrogen bonding, size of the molecule (Zacharia and Tano, 2011).

Property	Degradability	
	More easily	Less easily
Solubility in water	Soluble in water	Insoluble in water
Size	Relatively small	Relatively large
Functional group substitutions	Fewer functional groups	Many functional groups
Compound more oxidized	In reduced environment	In oxidized environment
Compound more reduced	In oxidized environment	In reduced environment
Created	biologically	chemically by man
Aliphatics	Aliphatic up to 10 C-chains Straight chains Aromatic compounds with one or two nuclei	High molecular weight alkanes Branched chains Polyaromatic hydrocarbons
Substitution on aromatic rings	-OH, -COOH, -CHO, -CO $-OCH_3$, $-CH_3$	-F, -Cl, $-NO_2$ $-CF_3$, $-SO_3H$, $-NH_2$
Substitutions on organic molecules	Alcohols, aldehydes, acids, esters, amides, amino acids	Alkanes, olefins, ethers, ketones, dicarboxylic acids, nitriles, amines, chloroalkanes
Substitution position	*p*-Position *o*- or *p*-Disubstituted phenols	*m*- or *o*-Position *m*-Disubstituted phenols

Figure 1.5: Influence of intrinsic factors on pesticides degradability : Figure from Gavrilescu (2005).

1.3.2.3 Pesticide adsorption/desorption proprieties

The sorption characteristics of chemicals in soil may influence their availability to degrading organisms (Cork and Krueger, 1991). In soils, pesticides are reversibly found in a free state in the soil solution or adsorbed to the soil particles, the aggregates and the organic matter by physical, electrostatic and chemical bonds (Katagi, 2013). Thus, the sorption properties of pesticides may determine the amount of pesticides solubilized in the soil solution available for microbial degradation (Torstensson, 1988).

1.3.2.4 Soil water content

A low water content decreases the probability of encounter between degraders and the substrate by limiting the diffusion of the substrate (Monard *et al.*, 2012b). Dry conditions can lead to a drop in microbial activity, a reduction in the number of micro-organisms and a loss of mobility of mobile degraders (Han and New, 1994). As a result, higher soil moisture contents, by enhancing the growth, the activity, the spread of degraders and mass transfer, may lead to higher mineralization rates (Monard *et al.*, 2012b). However, at high water contents (close to the water holding capacity of the soil) microbial growth is limited by oxygen transfer rates which leads to a lower mineralization rates. An optimum of mineralization has been found at -0.015 MPa (Schroll *et al.*, 2006).

1.3.2.5 Soil organic matter

Soil organic matter has two antagonistic effects on the degradation of pesticides. On one hand, the adsorption of pesticides to soils is strongly dependent on the organic matter content, which leads to a decrease in the amount of pesticides available in the soil solution (Li *et al.*, 2003; Rodríguez-Cruz *et al.*, 2006). On the other hand, a high turnover of soil organic matter promotes microbial growth and activity. (Pagel *et al.*, 2014). It also leads to greater degradation of pesticides by cometabolism [1] (Pal *et al.*, 2006).

1.3.2.6 Soil pH

The relationship between the rate of pesticide degradation and pH has been demonstrated in field-scale studies (Bending *et al.*, 2003; Rodríguez-Cruz *et al.*, 2006). It has been shown that pH has a strong selective influence on soil microbial communities and affects the soil sorption of pesticides (Lauber *et al.*, 2009; Franco *et al.*, 2009).

1.3.2.7 Soil biota

Soil degradation of pesticides is related with the presence of few key degrading species. High rates of pesticide degradation have been observed following repeated applications, due to the building up of degrading communities (Bazhanov *et al.*, 2016). Actually, (Monard *et al.*, 2012b) found that total community diversity and abundance is a bad predictor for pesticides degradation rates. This is due to the fact that degrading populations are few in number compared to the total community (Dechesne *et al.*, 2014).

1.3.3 Molecular approaches for pesticides biodegradation

The degradation of pesticides by micro-organisms is driven by the need for nutrients, energy or detoxification. Sometimes the microbial degradation of pesticides is altruistic and occurs cometabolically (Laura *et al.*, 2013).

[1] Cometabolism : "process by which a contaminant is fortuitously degraded by an enzyme or cofactor produced during microbial metabolism of another compound" (Hazen, 2010).

1.3.3.1 Enzymatic degradation of pesticides by micro-organisms

Enzymatic transformation is by far the most important route of pesticides detoxification in soils (Van Eerd *et al.*, 2003). Fungi and bacteria produce extra- and intracellular enzymes for the degradation of pesticides (Jaiswal *et al.*, 2017). Some white-rot fungi have the ability to produce extracellular enzymes that act on various aromatic organic compounds, including some pesticides. These extracellular enzymes such as lignin peroxidase, manganese peroxidase, laccase and oxidases are also involved in lignin degradation (Laura *et al.*, 2013).

Even though the degradation pathways of pesticides are specific to each pesticide and the degrading degrading microorganisms associated with, the metabolism of pesticides by micro-organisms can be summarized by three successive phases where several types of enzymes are involved in turn. The first phase consist in an oxidation, reduction or hydrolysis of the parent compound (Van Eerd *et al.*, 2003). Hydrolases, esterases and mixed function oxidases are the main enzymes families involved in this phases (Laura *et al.*, 2013). This phase leads to a more soluble and usually a less toxic product. The second phase is the conjugation of a parent compound or a pesticide metabolite with a glucose, a glutathione or an amino acid. This phase is carried out intra- or extracellularly by micro-organisms involving enzymes such as manganese-lignin peroxidas or Glutathione-S-transferases (Van Eerd *et al.*, 2003). The third phase consist in the conversion of the second phase metabolites into non-toxic secondary conjugates (Jaiswal *et al.*, 2017).

1.3.3.2 Genetics

Pesticide degradation genes of soil bacteria are found in catabolic plasmids, transposons or chromosomes. These genes encode for degrading enzymes (Laemmli *et al.*, 2000). The molecular aspect of pesticide degradation by fungi remains more unknown. However, it is known that *Phanerochaete chrysosporium* can release cytochrome P450 enzymes involved in the breakdown of xenobiotics. These enzymes are encoded in the fungal genome by two tandem P450 monooxygenases genes (Harshavardhan and Jagjit S, 2004).

Bacteria are subject to horizontal transfers of degrading genes within a population trough the conjugation of self-transmissible plasmid genes (De Souza *et al.*, 1998; Devers *et al.*, 2005). In addition, degrading genes are more frequently found after prolonged exposure to pesticides, as they give better fitness to individuals which spread to the whole population under the selection pressure applied by the pesticide (Devers *et al.*, 2008). Some studies try to assess the biodegradation function of pesticide in soils by enumerating pesticides degrading genes by reverse transcription quantitative PCR (RT-qPCR) (Monard *et al.*, 2012a).

1.3.4 Effect of pesticides on soil micro-organism communities

Herbicides can have impacts on different members of microorganism communities in soils. These impacts can be both positives and negatives (Rose *et al.*, 2016).

1.3.4.1 Effect on microbial and enzymatic activity

The effects of pesticides on soil enzymatic activity depend on the type of enzyme activity, the type of pesticide, the inoculation rate, the soil properties and the experimental conditions. Generally, the effects of pesticides on soil enzyme activity are indirect and are due to changes in the size, structure and functionality of microbial communities (Gianfreda and Ruggiero, 2006). Indeed, a first effect of pesticides is the reduction of the biomass of microorganisms in the soil due to its toxicity. This reduction in biomass can lead to a general slowdown in heterotrophic respiration, degradation of organic matter and microbial activity involved in nutrient cycling (e.g. nitrogen fixation) (Rose *et al.*, 2016).

A second effect of pesticides on soil microorganisms is the increase in the degrading activity of the same pesticide due to the increase in degrading populations (Bælum *et al.*, 2008). In most cases, the application of pesticides reduces the fitness of some micro-organisms, allowing other, more adapted, to occupy these empty niches (Wołejko *et al.*, 2020).

In addition, the effects of herbicides on plants can have an indirect effect on underneath soil microorganisms. A change in the composition of above-ground plants caused by herbicide application can lead to a change in the diversity and activity of soil microorganisms. Herbicides are expected to cause a general decrease in exudate production in the rhizosphere, which may affect the micro-organisms that depend on it (Rose *et al.*, 2016). Changes in functional diversity following the application of pesticides may result in changes in enzyme activity within the soil.

Some activities may be inhibited while others are stimulated following the addition of pesticides to the soil. Soil phenol oxidase activity, assayed on ABTS substrate, is often stimulated by the addition of 10 pesticides and can be a good indicator of pesticide pollution in soils. In contrast, arylsulphatase activity is generally inhibited by the same 10 pesticides. This fluctuates depending on the pesticide and when the activity is measured after the pesticide inoculation. Indeed, it is possible to observe both abrupt and longer-term changes (Floch *et al.*, 2011).

1.3.5 Selected pesticides and their microbial degradation

1.3.5.1 Pesticides selection

In this study, five pesticides were chosen among a list of 9 pesticides for which the parent compound or metabolites are frequently found in Walloon groundwater. In the whole list, two where directly avoided because they are currently banned. Two others were not selected because they are both contained in the same commercial product, which makes their investigation more complicated. The selected pesticides are then : metamitron, metazachlor, bentazon, s-metolachlor and chlortoluron. These parent compounds or their metabolites are characterized by high leaching behavior and pollution potential. Persistence (DT_{50}) and mobility (k_{oc}) are important parameters that characterize their leaching and pollution behavior (Table 1.1).

Table 1.1: Fate characteristics of metamitron, metazachlor, bentazon, s-metolachlor/metolachlor, chlortoluron and their principal metabolites in soils.

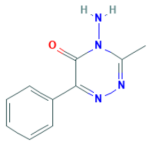
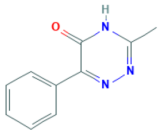
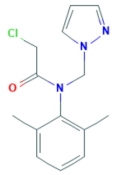
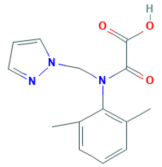
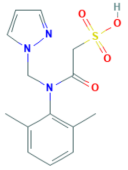
Active substance	Important soil metabolites	DT_50 (field dissipation studies)	Mobility : k_{oc}	Persistence	Human toxicity	Eco-toxicity	Reference
Metamitron	Metamitron 	2.2 - 45.5 days	Moderate to very high : 22.4 - 392 mL/g	Low to moderate	Yes	Yes	EFSA (2008)
	Deamino-metamitron 	22.8 - 45.2 days	Medium to high : 66 - 392 mL/g	moderate	No	No	EFSA (2008)
	M3	/	/	/	/	/	EFSA (2008)
Metazachlor	Metazachlor 	2 - 14.4 days	Medium to very high : 53.8–220 mL/g	Low to moderate	Yes	/	Brancato *et al.* (2017)
	Metazachlor oxanilic acid (479M04) 	49.9 - 66 days	high to very high : 1–94 mL/g	Moderate to very high	No (si ADI <0.33 mg/kg.j) Acceptable Daily intake	/	Brancato *et al.* (2017)
	Metazachlore ethan sulfonic acid (479M08) 	43.4 - 116.4 days	Very high : 4–78.5 mL/g	Moderate to very high	No (If ADI <0.2 mg/kg.j)	/	Brancato *et al.* (2017)
	479M09	/	Very high : 4.9–6.8 mL/g	moderate	Yes	/	Brancato *et al.* (2017)
	479M11	/	Very high : 18.1–23.5 mL/g	moderate	Yes	/	Brancato *et al.* (2017)

Table 1.1 continued from previous page

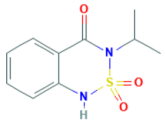
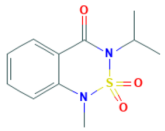
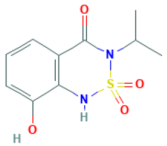
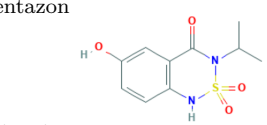
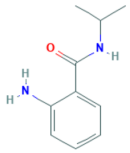
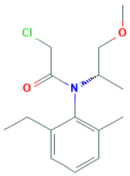
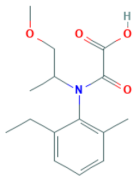
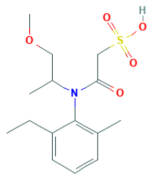
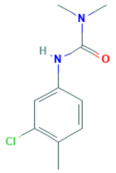
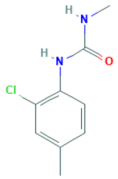
Active substance	Important soil metabolites	DT_50 (field dissipation studies)	Mobility : k_{oc}	Persistence	Human toxicity	Eco-toxicity	Reference
	479M12	/	Very high : 5.1–12 mL/g	Moderate to very high	No (si ADI <0,.8 mg/kg.j)	/	Brancato *et al.* (2017)
Bentazon	Bentazon 	3.9 - 26.4 days	Medium to high : 3 - 176 mL/g	Low to moderate	Yes	Yes	EFSA (2015)
	N-methyl-bentazon 	33-153 days	Medium : 205 - 312 mL/g	/	/	Yes	EFSA (2015)
	8-OH bentazon 	/	/	/	/	/	
	6-OH bentazon 	/	/	/	/	/	
	AIBA (2-amino-N-isopropyl benzamid) 	/	/	/	/	/	
	S-metholachlor 	11 - 31 days	Medium : 110 - 369 mL/g	Low to moderate	/	/	EFSA (2019)

Table 1.1 continued from previous page

Active substance	Important soil metabolites	DT_50 (field dissipation studies)	Mobility : k_{oc}	Persistence	Human toxicity	Eco-toxicity	Reference
S-metholachlor	Metolachlor oxanilic acid (CGA 51202/ CGA351916) 	19.8 - 165 days (en laboratoire)	High to very high : 2.82 - 62 mL/g	Moderate to very high	/	/	EFSA (2019)
	Metolachlor Ethane sulfonic acid (CGA 354743/ CGA380168) 	94 - 169 days (Laboratory)	Very high: 3 - 22 mL/g	Moderate to very high	/	/	EFSA (2019)
Chlortoluron	Chlortoluron 	26 - 42 days	Medium : 108 - 384 mL/g	moderate	/	/	European Commission (2005)
	3-(3-chloro-p-tolyl)-1-methylurea 	/	/	/	/	/	European Commission (2005)

1.3.5.2 Metamitron

Metamitron is one of the most common herbicides for the control of annual broadleaf weeds and grasses in beet crops (Janaki *et al.*, 2013). This acts as an inhibitor of photosynthesis by disrupting the transport of electrons in the photosystem I (EFSA, 2008). It belongs to the chemical class of triazinones (Pohanish, 2014).

Metamitron is considered moderately to highly mobile in soils. It is weakly adsorbed to soil and organic matter. It therefore presents a risk of leaching to water resources (Rosenbom *et al.*, 2015). When not leached, metamitron is rapidly mineralised by soil micro-organisms and by photochemical degradation (Wang *et al.*, 2017a). In laboratory degradation studies, the half-life of metamitron ranges from 2.2 to 45.5 days in soil (20°C and pF2). Deamino-metamitron is the main pesticide metabolite of metamitron. It is slightly less mobile than its parent compound and its half-life in soil in laboratory degradation studies ranges from 22.8 to 45.2 days (20°C and pF2) (EFSA, 2008). Laboratory soil degradation studies with radiolabelled metamitron have shown that microbial mineralization is the main transformation process for metamitron (Figure 1.6)(Wang *et al.*, 2017a).

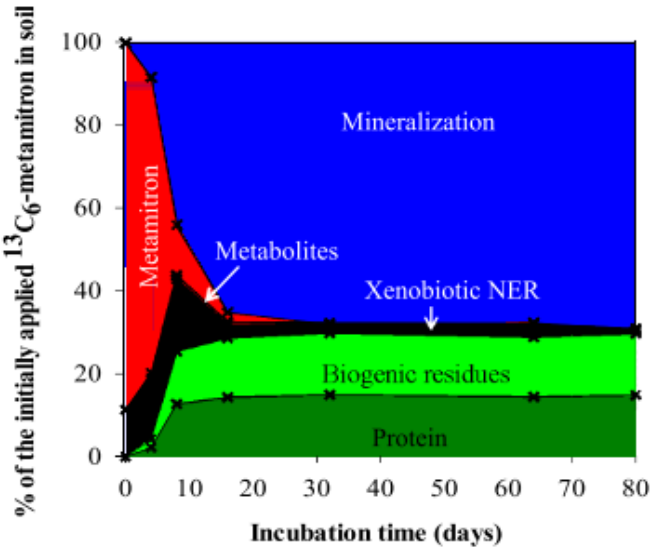


Figure 1.6: Transformation of C^{13}metamitron in soil in the presence of microorganisms: figure of Wang *et al.* (2017a).

In addition, two biotic degradation routes for metamitron were identified, each associated with a strain of bacteria isolated from the soil (Wang *et al.*, 2017a). These routes are the "Desamino-metamitron route" performed by *Arthrobacter sp.* DSM 20389 and the "Rhodococcus route" realised by *Rhodococcus sp.* 0246b. (Figure 1.7) (Parekh *et al.* 1994; Engelhardt *et al.* 1982; Wang *et al.* 2017a). These two strains catalyse each metamitron reaction step with one specific enzyme such as metamitron 2,3-dioxygenase, 2,3-dihydro-2,3-dihydroxy-metamitron dehydrogenase, 2,3-dihydroxymetamitron 1,2-dioxygenase, metamitron amidohydrolase and benzoylformate (Daniel Norat and Michael Turnbull, 2008). Both degrading strains are aerobic gram-positive soil bacteria belonging to the phylum of Actinobacteria (Parekh *et al.* 1994; Engelhardt *et al.* 1982). Moreover, a strain "Rhodococcus sp. MET" capable of degrading metamitron through two new distinct degradation pathways has been reported (Fang *et al.*, 2016).

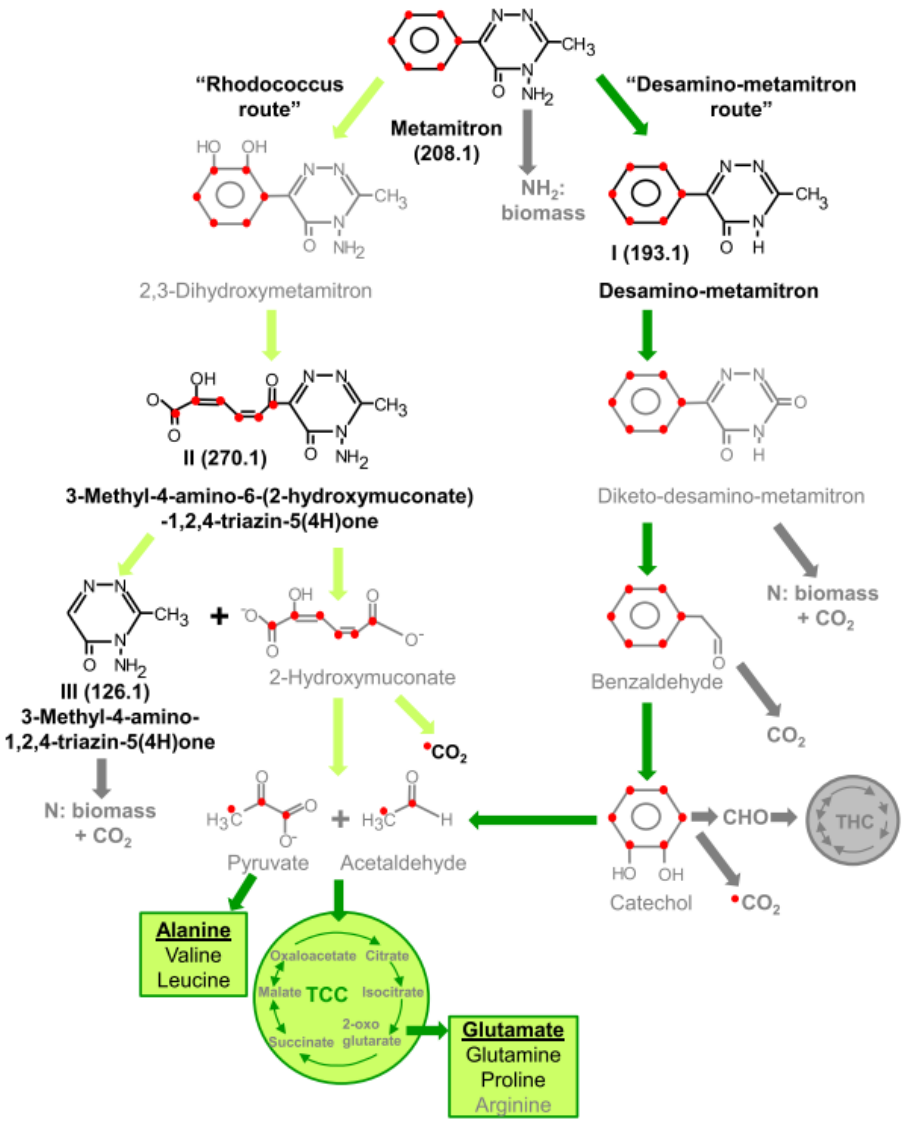


Figure 1.7: Suspected biological degradation pathways of metamitron in soils : Figure from Wang *et al.* (2017a).

Very recently, Wang *et al.* (2021) showed that these isolated strains are certainly not the only ones to degrade metamitron. Other gram-negative bacteria are expected to be even more important in the degradation of metamitron. Indeed, in a soil microcosm experiment, the living biomass of gram-negative bacteria prevails directly (4 days) after the addition of metamitron over the biomass of Actinobacteria. Meanwhile, Actinobacteria are highly concentrated in ^{13}C from the radiolabelled metamitron after only 8 and 16 days. This suggests that gram-negative bacteria use metamitron quickly after addition and are the primary degraders in soils while Actinobacteria use metamitron more slowly and are likewise consumers of primary metamitron degraders. This highlights the fact that metamitron degradation does not take place unilaterally but could be the result of complex interactions and syntrophic relationships between primary degraders and consumers (Wang *et al.*, 2021).

1.3.5.3 Metazachlor

Metazachlor is an herbicide commonly used in rapeseed (*Brassica napus* L. subsp. napus) crops to control the emergence of annual broadleaf weeds and grasses. It acts as an inhibitor of the synthesis of long-chain fatty acids causing a loss of membrane rigidity and leading to cell leakage and poor division (Karier *et al.*, 2017). Metazachlor belongs to the chloroacetamide class of herbicides. The literature on the fate of this herbicide in soils is limited. (Szpyrka *et al.*, 2020).

Metazachlor is considered moderately to highly mobile in soils with low to moderate persistence. The half-life varies from 10.92 to 12.68 days in a field clay soil. The half-life drops to 5.7 days under microcosm laboratory conditions. Under laboratory conditions, higher and more stable temperatures and a more constant water content led to higher biodegradation of metazachlor (Szpyrka *et al.*, 2020).

In soils, the two main metabolites are metazachlor oxanilic acid (479M04) and metazachlor ethane sulfonic acid (479M08). Others exist to a lesser extent such as 479M09 [2], 479M11[3] and 479M12[4]. In addition, much of the metazachlor is found in soils as NER (43,2% of the applied radioactivity (AR) after 100 days). Finally, only 6.9 % of the AR is found in mineralized form after 100 days. The two main metabolites (479M04 and 479M08) are not readily degradable (DT_{50} of 49.9 - 66 days and 43.4 - 116.4 days respectively) and tend to leach into deeper horizons (Brancato *et al.*, 2017).

Metazachlor degrades biotically and abiotically in the environment. Abiotic degradation occurs mainly by indirect photolysis (Mantzos *et al.*, 2017). To our knowledge, no strain of micro-organism capable of degrading metazachlor has been identified to date. Only one strain of bacteria (strain HL1) capable of desulphonating metazachlor ethane sulphonic acid (479M08) and recovering the sulfur into proteins has been isolated (Laue *et al.*, 1996).

The overall effect of the application of metazachlor, at high dose level, is to decrease the biomass and enzymatic activity of micro-organisms. Only oligotrophic micro-organisms respond positively over 60 days to the application of metazachlor. Dehydrogenase, catalase, urease and phosaphatase enzymatic activities were negatively correlated with metazachlor application after 30 days. This overall loss of biomass and enzyme activity after metazachlor application may be due to the toxicity of the metabolite metazachlor ethane sulfonic acid (479M08) (Baćmaga *et al.*, 2014).

1.3.5.4 Bentazon

Bentazon is an herbicide used to control annual broadleaf weeds and sedges, notably in cereals, rice, soybeans and sugarcane crops (Pohanish, 2014). Bentazon acts as a photosynthesis inhibitor by blocking electron transfer in photosystem II (Mine and Matsunaka, 1975). It belongs to the benzothiazinone class of herbicides (Pohanish, 2014).

Bentazon is moderately to highly mobile in soils with a low to moderate persistence. Its field half-life in Europe varies from 3.9 to 26.4 days (EFSA, 2015). N-methyl-bentazon, 8-OH bentazon, 6-OH bentazon and AIBA are the most frequently found metabolites of bentazon in soils.

In laboratory degradation studies, bentazon is degraded to N-methyl-bentazon (max 5,7% of AR), which is the main metabolite and is commonly found in surface and ground water. This second compound is more persistent than the parent compound (Dt_{50} 33 - 153 days in laboratory degradation studies) and has a moderate mobility. Moreover, after 120 days of incubation in laboratory degradation studies, bentazon is completely mineralized for only 11% of the AR. Eventually, a large part is found as non-extractable residues (73% of the AR) after 120 days (EFSA, 2015). These non-extractable residues are bound to the organic matter of the soil which may forms numerous adsorption cites (Boivin *et al.*, 2004). Three bentazon metabolites (8-OH bentazon, 6-OH bentazon and AIBA) are permanently incorporated into the soil organic matter via biotic and abiotic humification (Figure 1.8). This was verified by the addition of the two OH-metabolites to soil, which became non-extractable within a few hours (Huber and Otto, 1994).

[2] N-(2,6-dimethylphenyl)-N-(1H-pyrazol-1-ylmethyl)aminocarbonylmethylsulfinyl acetic acid
[3] methyl N-(2,6-dimethylphenyl)-N-(1H-pyrazol-1-ylmethyl) aminocarbonylmethyl sulfoxide
[4] N-[(2-hydroxycarbonyl-6-methyl)phenyl]-N-(1Hpyrazol-1-ylmethyl)oxalamide

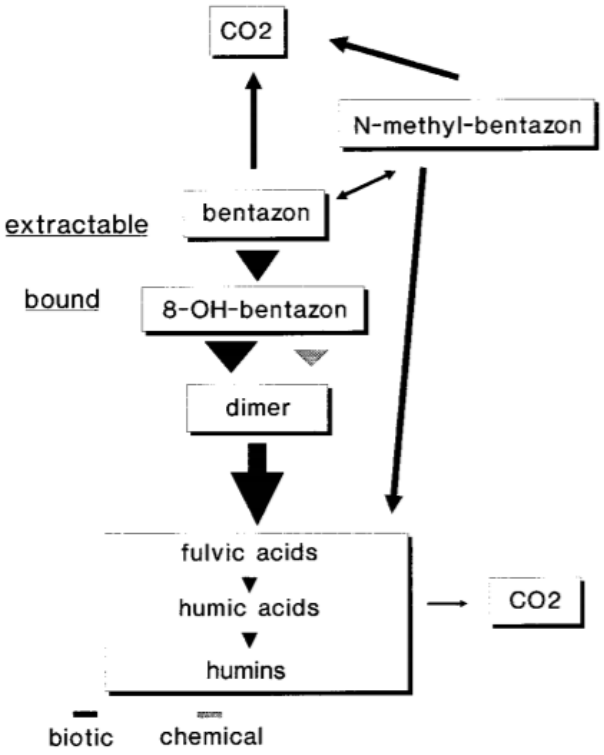


Figure 1.8: Proposed model of bentazon transformation in soils. The width of the arrows indicates the relative quantitative importance of the pathway: Figure from Knauber *et al.* (2000)

Degradation of bentazon occurs both biotically and abioticall. Bentazon is degraded by hydrolysis and photolysis to N-methyl-bentazon, 6-OH bentazon and 8-OH bentazon (Song *et al.*, 2019). Two fungal strains identified as *Phanerochaete chrysosporium* and *Ganoderma lucidum* were able to degrade bentazon (Table 1.2) (Castillo *et al.*, 2000; Da Silva Coelho *et al.*, 2010). Both strains belong to the heterogeneous group of white rot fungi which are lignin-degrading basidiomycetes. They are able to produce the enzymes Lignin peroxidase, Mn peroxidase or Laccase which are capable of attacking a wide range of recalcitrant lignin-related compounds, including herbicides (Da Silva Coelho *et al.*, 2010).

1.3.5.5 S-Metolachlor

S-metolachlor is an herbicide of the chloroacetamide class. It is commonly used against annual grasses and certain broadleaf weeds in maize, beet, potato, beans, soybeans, sugarcane, cotton and other crops (Pohanish, 2014). S-metolachlor is actually a chiral molecule and therefore forms a racemic mixture of 80-100% of the S-enantiomer and 0-20% of the R-enantiomer. This differs from metolachlor, currently banned in Europe, by the composition of the racemic mixture which is 50 % S-enantiomer and 50 % R-enantiomer (MA *et al.*, 2006; Long *et al.*, 2014). S-metolachlor reduces the application rate by 35% compared to metolachlor because it is more concentrated in the S-enantiomer, which is the only one with phyto-active properties (Rose *et al.*, 2018). This acts on weeds by inhibiting protein synthesis (Pohanish, 2014).

S-metolachlor is moderately mobile in soils with a low to moderate persistence (European Commission, 2021). The two main residues of s-metolachlor in soils are obviously the same as for metolachlor and are metolachlor ethane sulfonic acid (CGA380168/CGA354743) and metolachlor oxanilic acid (CGA351916/CGA51202) (Figure 1.9). They are also known to potentially contaminate groundwater due to their high mobility (Schuhmann *et al.*, 2019).

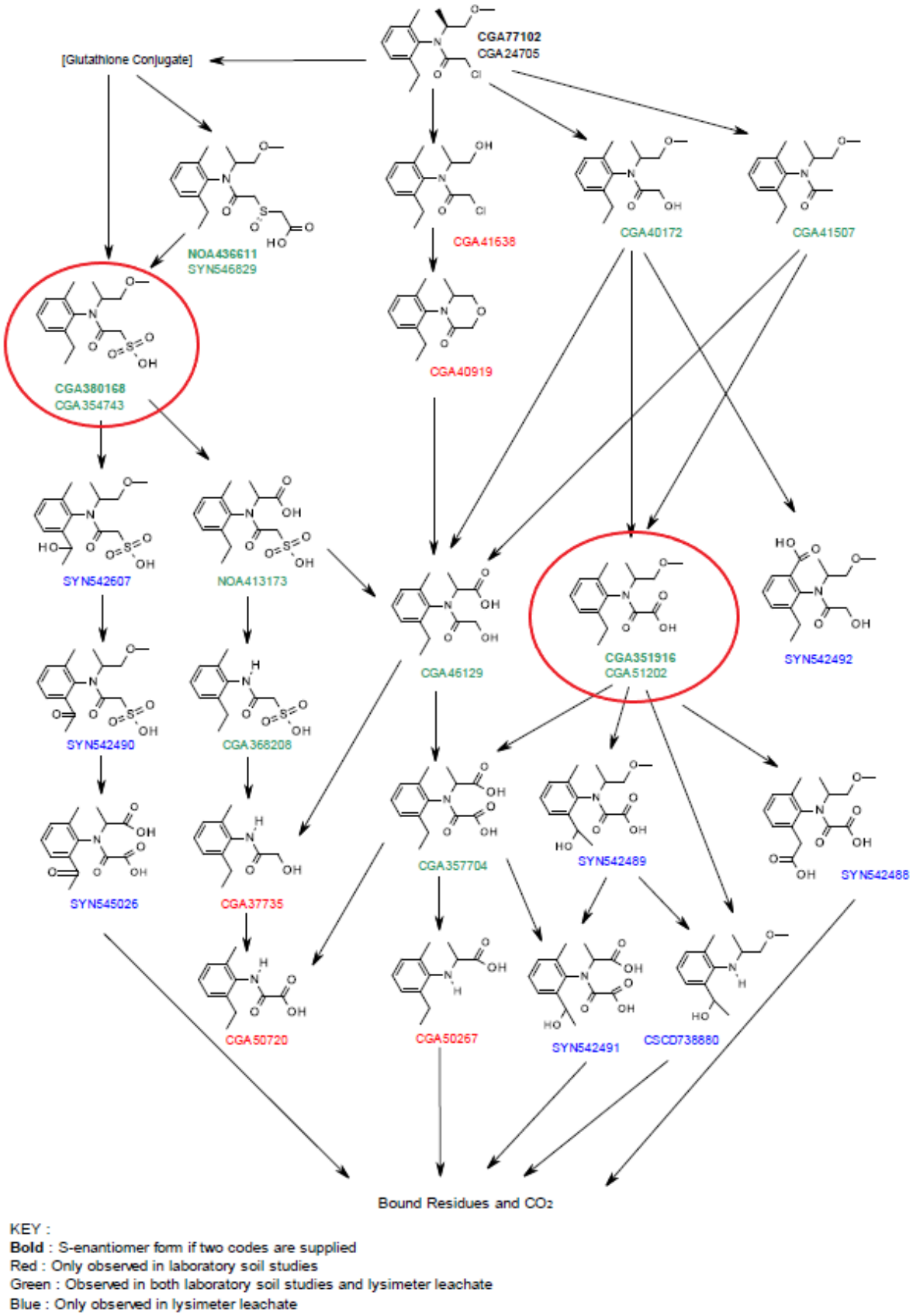


Figure 1.9: Proposed degradation pathways of s-metolachlor in soils : Figure of EFSA (2019).

Fungal and bacterial strains have been reported to degrade s-metolachlor mainly by co-metabolic reactions (Zemolin *et al.*, 2014; Stamper and Tuovinen, 1998). McGahen and Tiedje (1978) first described the

metabolism of metolachlor by the fungus *Chaetomium globosum*. In the following years, numerous fungal and bacterial strains able to degrade metolachlor have been isolated such as *Actinomyces sp.*, *Fusarium sp.*, *Mucor racemosus*, *Bacillus circulans*, *Bacillus megaterium*, *Rhizopus*, *Streptomyces sp.*, *Phanerochaete chrysosporium*, *Rhizoctonia praticola*, *Syncephalastrum racemosum*, *Aspergillus flavus*, *Aspergillus terricola*, *Candida xestobii*, *Bacillus simplex* and *Penicillium oxalicum* (Table 1.2) (Saxena *et al.*, 1987; Liu *et al.*, 1988; Liu *et al.*, 1991; Sanyal and Kulshrestha, 2002; Xu *et al.*, 2008; Munoz *et al.*, 2011; Chang *et al.*, 2020). In most cases, the initial stage of microbial degradation of metolachlor involves hydrolytic or reductive dechlorination (Liu *et al.*, 1991; Zemolin *et al.*, 2014; Chang *et al.*, 2020).

1.3.5.6 Chlorotoluron

Chlortoluron is an herbicide of the phenylurea class. It is used against annual grasses and most broadleaf weeds in winter cereals (winter barley, winter wheat, spelt and triticale) and in plant nurseries (fruit bushes, apple and pear trees). It acts by inhibiting electrons transport in PSII (Ryan, 1981).

Chlorotoluron is moderately mobile and has a moderate persistence in soils. The main metabolite of chlortoluron in soils is 3-(3-chloro-p-tolyl)-1-methylurea. In degradation laboratory studies, 16 days after application of chlortoluron to soil, 23% of the AR is found in 3-(3-chloro-p-tolyl)-1-methylurea. After 100 days, only 6.4 to 13.3% of chlortoluron is in fully mineralized form. Chlortoluron is also largely found as NER in soils (28.2-62.6% of AR after 100 days) (European Commission, 2005).

Several bacterial and fungal strains capable of degrading chlorotoluron have been isolated in previous years (Table 1.2). Most of them have the ability to degrade other phenylurea herbicides such as diuron, isoproturon, linuron, monolinuron, monuron, fluometuron, fenuron, metobromuron or chlorbromuron (Khadrani *et al.*, 1999; Sørensen *et al.*, 2001; Turnbull *et al.*, 2001; Tixier *et al.*, 2002; Sun *et al.*, 2009; Badawi *et al.*, 2009; Sharma *et al.*, 2010; Wang *et al.*, 2017b).

Table 1.2: Reported microbial strains degrading metamitron, metazachlor, bentazon, s-metolachlor/metolachlor and chlortoluron.

Active substance	Degrading microorganisms	Type	Isolated from or source	Degradation rate in liquid medium	Purpose or reaction	Source
Metamitron	*Arthrobacter sp.* DSM 20389	Bacterium	Soil	Almost 100% in 14 days (C0=80 ppm)	/	Engelhardt *et al.* (1982)
	Rhodococcus sp. 0246b	Bacterium	Soil	100% removed in 5 days (C0=20 ppm)	Carbon source	Parekh *et al.* (1994)
	Rhodococcus sp. MET	Bacterium	Activated sludge	100% removed in 12 h (C0=9 ppm), 11% removed in 12 h (C0=97 ppm)	Carbon and energy source	Fang *et al.* (2016)
Metazachlor	/	/	/	/	/	/
Bentazon	*Phanerochaete chrysosporium*	Fungus	Culture Collection	/	/ (Lignin peroxydase Mn peroxydase enzymes)	Castillo *et al.* (2000)
	Ganoderma lucidum	Fungus	Culture Collection	55% removed in 10 days (C0=600 ppm)	/ (Laccase and Mn peroxydase enzymes)	Da Silva Coelho *et al.* (2010)
	Methanotrophic microorganisms culture	Bacteria	Groundwater	53% removed in 21 days (C0= 1,7-1,8 ppm)	Cometabolic	Hedegaard *et al.* (2018)
S-metolachlor /metolachlor	*Chaetomium globosum*	Fungus	Soil	45% removed in 6 days (C0=100 ppm)	/	McGahen and Tiedje (1978)
	Actinomyces sp.	Bacterium	Soil	59% removed in 9 days (C0=50 ppm)	Cometabolic	Saxena *et al.* (1987)
	Fusarium sp.	Fungus	Soil	69% removed in 9 days (C0=50 ppm)	Cometabolic	Saxena *et al.* (1987)
	Mucor racemosus	Fungus	Soil	79% removed in 9 days (C0=50 ppm)	Cometabolic	Saxena *et al.* (1987)
	Bacillus circulans	Bacterium	Soil	>30% removed in 9 days (C0=50 ppm)	Cometabolic	Saxena *et al.* (1987)
	Bacillus megaterium	Bacterium	Soil	>30% removed in 9 days (C0=50 ppm)	Cometabolic	Saxena *et al.* (1987)
	Rhizopus	Fungus	Soil	/	Cometabolic	Liu *et al.* (1988)
	Streptomyces sp.	Bacterium	Soil	41% removed in 16 days (C0=100 ppm)	Cometabolic, dechlorination	Liu *et al.* (1991)
	Phanerochaete chrysosporium	Fungus	Culture collection	28,1% removed in 16 days (C0=100 ppm)	Dechlorination	Liu *et al.* (1991)
	Rhizoctonia praticola	Fungus	Culture collection	26,8% removed in 16 days (C0=100 ppm)	Dechlorination	Liu *et al.* (1991)
	Syncephalastrum racemosum	Fungus	Soil	13,5% removed in 16 days (C0=100 ppm)	Dechlorination	Liu *et al.* (1991)

	Aspergillus flavus	Fungus	Soil	68,76% removed in 7 days (C0=19,14 ppm)	Dechlorination, N-dealkylation and amide bond cleavage	Sanyal and Kulshrestha (2002)
	Aspergillus terricola	Fungus	Soil	44,12% removed in 7 days (C0=19,44 ppm)	Dechlorination, N-dealkylation and amide bond cleavage	Sanyal and Kulshrestha (2002)
	Mixed culture of *Bacillus luciferensis*, *Stenotrophomonas acidaminiphila*, *Kocuria erythromyxa* and *Pseudomonas sp.*	Bacteria	Soil and sludge	39% removed in 21 days (6,7 ppm)	/	Xu *et al.* (2008)
	Candida xestobii	Yeast	Soil	60% removed in 4 days (C0=50 ppm)	Catabolism for carbon source	Munoz *et al.* (2011)
	Bacillus simplex	Bacterium	Soil	30% removed in 9 days (C0= 50 ppm)	Catabolism for carbon source	Munoz *et al.* (2011)
	Penicillium oxalicum	Fungus	Activated sludge	88,6% removed in 16 days (C0= 50ppm)	Cometabolic	Chang *et al.* (2020)
Chlortoluron	*Oxysporus sp.*	Fungus	Soil or decayed wood	76% removed in 5 days (C0 =100 ppm)	/	Khadrani *et al.* (1999)
	Bjerkandera adusta	Fungus	Soil or decayed wood	71% removed in 5 days (C0 = 100 ppm)	/	Khadrani *et al.* (1999)
	Athelia decipiens	Fungus	Soil or decayed wood	49% removed in 5 days (C0 =100 ppm)	/	Khadrani *et al.* (1999)
	Coniophora arida	Fungus	Soil or decayed wood	41% removed in 5 days (C0 =100 ppm)	/	Khadrani *et al.* (1999)
	Sphingomonas sp. SRS2	Bacterium	Soil	/	/	Sørensen *et al.* (2001)
	Arthrobacter globiformis D47	Bacterium	Soil	/	/	Turnbull *et al.* (2001)
	Arthrobacter sp. N2	Bacterium	Soil	100 % removed in 30 h (C0= 40 ppm)	/	Tixier *et al.* (2002)
	Sphingobium sp. YBL2	Bacterium	Soil	>80 % removed in 24 h (C0 = 10 ppm)	/	Sun *et al.* (2009)
	Mortierella sp.	Fungus	Soil	60% removed in 26 days (C0 = 9 ppm)	/	Badawi *et al.* (2009)
	Micrococcus sp. PS-1	Bacterium	Soil	>60 % removed in 48 h (C0=250 ppm)	/	Sharma *et al.* (2010)
	Neurospora intermedia DP8-1	Fungus	Sugarcane root	/	Carbon source	Wang *et al.* (2017b)

1.4 Sterilization techniques in laboratory soil degradation studies

To describe the fate and the transformation behavior of a pesticide in soils, laboratory degradation studies can be performed. Methods can be normalized into guidelines. One of the most commonly used is the "Test No. 307: Aerobic and Anaerobic Transformation in Soil" performed under static conditions (OECD, 2002). However, more and more studies are trying to approach field conditions by using micro-lysimeters or soil columns to simulate pesticide transport in unsaturated zones, such as that proposed by Heistermann *et al.* (2003). Guildelines also exist for this kind of experimentation (OECD, 2004; USEPA, 2008). In this case, the DT50 and sorption coefficient (k_{oc}) can only be obtained by inverse modelling (Dubus *et al.*, 2004; Roulier and Jarvis, 2003).

To estimate abiotic degradation rates of pesticides, tests may be performed under sterile conditions. However, many guidelines, such as those of the OECD, do not provide a standard method for sterilizing soils (Lees *et al.*, 2018). The purpose of the sterilization is to remove all microbial activity from the soil during the time of the experiment (Lees *et al.*, 2018). Autoclaving (Zhang *et al.*, 2013), gamma irradiation (Pinheiro *et al.*, 2015) and addition of sodium azide (Zhang *et al.*, 2013) were successfully used to sterilize soils.

For sterile experiments to be comparable with non-sterile experiments, it is important that the sterilization method does not change physico-chemical properties of the soil that might control the fate of the pesticide (Lees *et al.*, 2018). These are the pH, the dissolved organic carbon (DOC), the cation exchange capacity (CEC), the soil structure, the ionic strength and the particle size (Lees *et al.*, 2016).

Among the sterilization techniques, autoclaving (moist heat) is maybe the easiest and cheapest to perform. Each microbiology laboratory has its own autoclave (Trevors, 1996). Sterilizing soil by autoclaving consists in several successive autoclaving cycles (2 or 3) at 120°C at 1.1 atm for 20 to 60 minutes (depending on the soil amount) with 24-hour rest periods between each cycle (Lees *et al.*, 2018; Berns *et al.*, 2008 Trevors, 1996). The 24-hour delay between autoclave cycles ensures that heat-resistant spores can germinate and be destroyed in the next cycle (Miyaki *et al.*, 1996).

Despite the efficiency and ease of the method, autoclaving can affect the physicochemical properties of the soil. Autoclaving can increase extractable N, NH_4, NO_3, P, S, Mn and organic matter. Moreover, free radicals involved in the abiotic degradation of persistent organic compounds can be destroyed by autoclaving (Wolf and Skipper, 1994). Autoclaving can destroy soil aggregates, thereby increasing the clay fraction and lowering the silt fraction. However, in Luvisols, this phenomenon is not dramatically pronounced because the aggregates are well bound. Finally, autoclaving can lead to a significant increase in dissolved organic carbon (DOC) due to the lysis of microorganisms, the degradation of soil organic matter and the release of entrapped organic matter. The pH may be slightly lowered due to the release of dissolved organic acids (Berns *et al.*, 2008).

1.5 Research objectives

Based on the fact that pesticides can end up in water bodies and deteriorate the structure and functions of aquatic systems, despite the current precautions taken in their use, it is necessary to acquire more knowledge about their fate in soils. Among the processes that control the fate of pesticides, biodegradation by micro-organisms may be one of the most important. However, pesticides biodegradation is reported to be mostly linked to a few key species that exhibit degradation capabilities. The identification of these key species, associated degradation pathways, degrading enzymes/genes and controlling factors *in vitro* is essential for understanding the processes involved in microbial biodegradation of pesticides.

However, it is necessary to translate this laboratory knowledge to real-life conditions to assess the role played by these key species in the environment. To bridge this gap between *in vitro* and field conditions, leaching studies in undisturbed soil columns are methods that may provide a close approach to field conditions. Working with undisturbed soil structure is more relevant for pesticides leaching studies as it take into account various properties (such as porosity, surface area, water retention, hydraulic conductivity and biologic activity) that govern the mobility and the degradation of pesticides. However, carrying out these studies under abiotic conditions without disturbing the physico-chemical properties of the soil is an obstacle to overcome in order to understand the real contribution of degrading micro-organisms in undisturbed soil columns.

Regarding these declarations, the first objective of this work was to isolate the cultivable microorganisms present in the soils of the AIL EcoFoodSystem experimental plots of Gembloux Agro-Bio Tech, potentially playing a role in the degradation of metamitron, metazachlor, bentazon, s-metolachlor/metolachlor and chlortoluron. The second objective was to select the most promising ones on the basis of their ability to produce potential degrading enzymes and to grow in the presence of pesticides as sole carbon source. The third objective was to identify and evaluate the ability of selected microorganisms to degrade pesticides in liquid medium. The last objective was to develop a method to evaluate the microbial degradation of pesticides in intact cores soil column. The feasibility of autoclaving (moist heat) as a sterilization method was examined by evaluating the effect of autoclaving on soil structure, sorption and hydraulic properties.

2 Materials and Methods

2.1 Soil sampling, handling and storage

Plots along the cemetery from the AIL EcoFoodSystem experiment in Gembloux, Belgium (50°33'57"N - 4°42'29"E) were sampled on March 2, 2021. The plots sampled are plot nb. 3 : Reference T2.1 (NO-PHYTO) and plot nb. 2: Phyto T2.1 (PHYTO) and were cultivated with *Brassica napus* L. sown on 04/09/2020 (Figure 2.1). Both plots have already received herbicides in recent years, but only the plot PHYTO will receive them again. The plot PHYTO also received 2,5 L/ha of Butisan S (metazachlor) which was not the case for the plot NO-PHYTO. The historic of herbicides application on both plots is presented in appendices A-1. The first 25 centimeters of soil were sampled using a 32 mm diameter auger (van der Bom *et al.*, 2018). A composite sample was taken from each plot using 20 drilling points along a W-shaped path (Figure 2.1). The auger and sampling equipment were disinfected with alcohol before each sampling. The two composites samples were stored in closed plastic bags and transported to the laboratory to be sieved at 2 mm. The equipment used (sieves, trays and rammers) was also disinfected with alcohol before each sieving. Each composite sample was separated into three sub-samples of about 300g each in order to make 3 replicates. Each replica was stored in closed plastic bags at 4°C. Samples could be stored for about 8 weeks in these conditions (Černohlávková *et al.*, 2009).

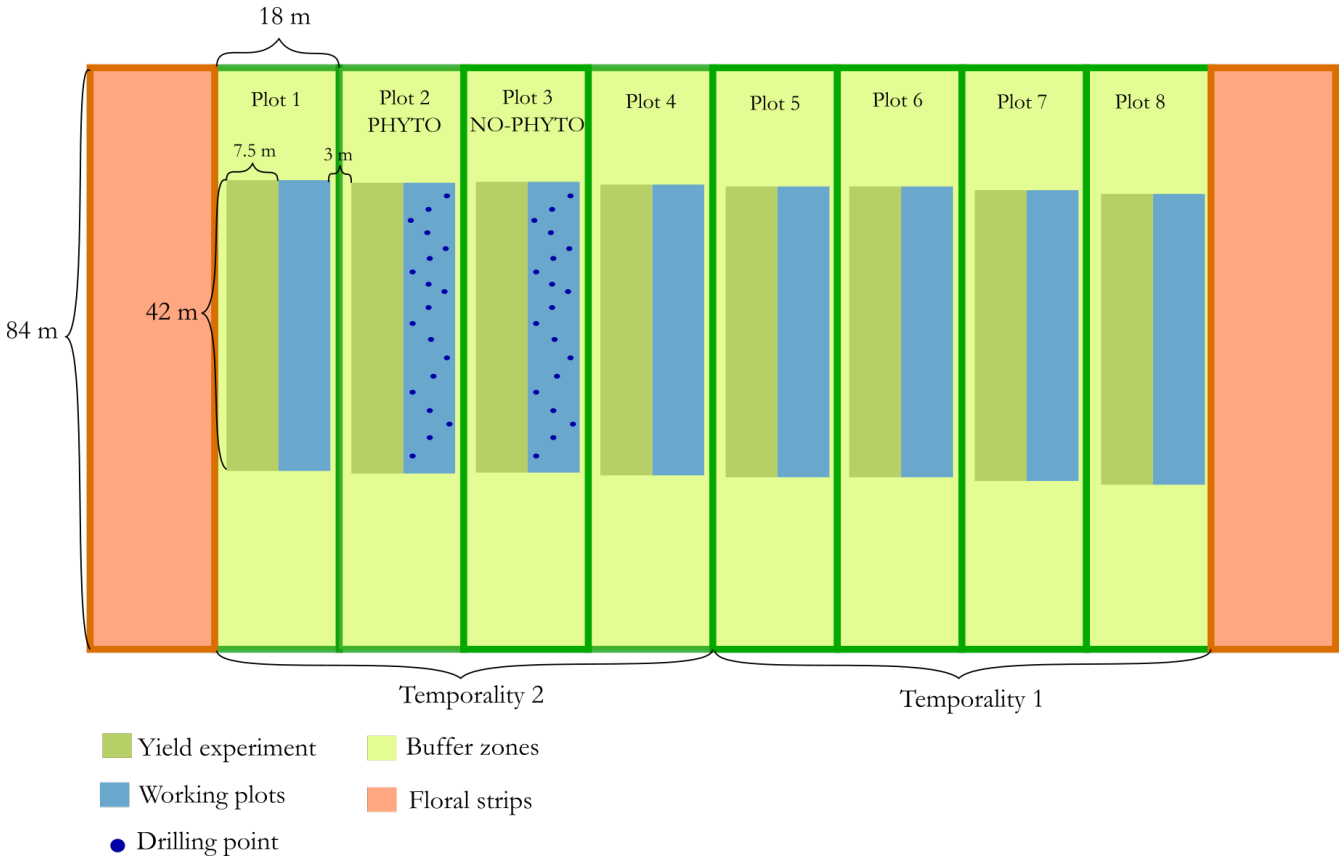


Figure 2.1: Soil sampling design : blue plots were sampled.

2.2 Herbicides

Each herbicide consists in a formulated commercial product which contains the active substance but also other additives such as adjuvants and synergists (Table 2.1). Herbicides were Chloortoluron 500 SC (active substance (AS): chlortoluron), Goltron 700 SC (AS: metamitron), Butisan S (AS: metazachlor), Dual Gold (AS: s-metolachlor) and Basagran SG (AS: bentazon). Herbicides were diluted 25 times and 250 times in distilled water. Herbicides were not filter sterilized because at this level of dilution, water and the herbicides formed an immiscible solution containing larger colloids than the filter pore size (0.22 µm).

Commercial product	Active substance	Concentration active substance	Hazardous additives
Chloortoluron 500 SC	Chlortoluron	700 g/L (42-46% W/W)	- Ethylene glycol (3-6 % W/W) - 1,2-Benzisothiazole-3(2H)-one (<0,005 % W/W)
Goltron 700 SC	Metamitron	700 g/L (58,3% W/W)	- Glycerol (4-6% W/W) - Propane-1,2-diol (2-4% W/W) - Reaction mass of : 5-chloro-2-methyl-4isothiazolin-3-one and 2-methyl-4-ospthiazolin-3-one (<0,01% W/W)
Butisan S	Metazachlor	500 g/L (43,5% W/W)	- Propane-1,2-diol (<20% W/W) 2-méthylisothiazol-3(2H)-one (<0,01% W/W) - Benzenesulfonic acid, hydroxy-, formaldehyde-based polymer, phenol and urea, sodium salt (5% W/W) - 2-méthylisothiazol-3(2H)-one (<0,01% W/W)
Dual Gold	S-Metolachlor	960 g/L (86,5% W/W)	- Solvent naphtha (petroleum), highly arom. (1-5% W/W) - Calcium dodecylbenzene sulphonate (1-5% W/W) - Poly(oxy-1,2-eth anediyl),-[2,4,6-tris(1-phenylethyl) phenyl]-hydroxy (1-5% W/W) - 2-methylpropan-1-ol (1-2% W/W)
Bazagran SG	Bentazon	Solid (87% W/W)	- Sodium salt (9,6% W/W)

Table 2.1: Commercial herbicides with related active substances and hazardous additives.

2.3 Media

Mineral salt medium (MSM), firstly developed by (Rousseaux *et al.*, 2001) to isolate atrazine-degrading bacteria, was prepared by adding (for 1 l of autoclaved medium) :

- 1,6 g K_2HPO_4 ;
- 0,4 g KH_2PO_4 ;
- 0,2 g $MgSO_4.7H_2O$;
- 0,1 g $NaCl$;
- 0,0265 g $CaCl_2.H_2O$;
- 1 ml of autoclaved salt stock solution composed by : 2 g/l H_3BO_3 ; 1,8 g/l $MnSO_4.H2O$; 0,356 g/l $ZnSO_4.7H_2O$; 0.165 g/l $CuSO_4.5H_2O$; 0,25 g/l $Na_2MoO4.2H_2O$;
- 1 ml vitamin stock solution composed by : 100 mg/l of thiamine-HCl (Vitamin B1), 40 mg/l de biotin (vitamin B8) ;
- 1 ml of $FeSO_4.6H_2O$ stock solution 5g/l;
- 1L distilled water

Vitamin and $FeSO_4$ stock solution were filter (0.22 µm pore size) sterilized and added in the medium after autoclaving at 120°C during 20 min.

The herbicide for which we want to isolate degrading strains was added to liquid MSM after autoclaving to reach a final concentration of 0.106 mM of the active substance to serve as carbon and nitrogen sources. The pH of the MSM was checked and reaches 7,5.

Solid MSMA (MSM agar) was also prepared using the same protocol and adding 15 g/L of agar (Difco™) prior autoclaving at 120°C. Preliminary tests using solid MSMA added with increasing concentration of herbicide was carried out to determine the threshold concentration where herbicides have not any effect on the growth of selected microorganisms on plates. The 1.06 mM concentration of one herbicide (metamitron, metazachlor, bentazon, s-metolachlor or chlortoluron) was found to be this threshold concentration, was added to the autoclaved MSMA prior plating on petri dishes.

Solid Luria Bertani (LB) was used for cultivation and morphological characterization of isolated strains and was prepared with tryptone (10 g/L), yeast extract (5 g/L), NaCl (5 g/L) agar Difco™ (15 g/L), distilled water prior autoclaving for 20 min at 120°C.

Solid Potato Dextrose Agar (PDA) was also used for cultivation and morphological characterization of isolated fungi and was prepared with 39 g/L Potato Dextrose Agar (Scharlab) and distilled water prior autoclaving 20 min at 120°C.

Liquid TY was also used for cultivation of isolated strains and was prepared with (5g/L), yeast extract (3 g/L), $CaCl_2$ (66 mg/L) and distilled water prior autoclaving 20 min at 120°C.

2.4 Enrichment and isolation of metamitron, metazachlor, bentazon, s-metolachlor and chlortoluron degrading strains

Enrichment of herbicides-degrading strains was performed as described by (Romdhane *et al.*, 2016) with slight modifications. 12.5 g of wet soil (10 g dry weight) was added to sterilized flasks with 90 ml of sterilized MSM. The herbicide was added to reach a final concentration of 0.106 mM. The herbicide for which we want to isolate (metamitron, metazachlor, bentazon, s-metolachlor and chlortoluron) served as carbon and nitrogen sources. Flasks were incubated at 28°C in the dark on an orbital shaker (75 to 130 rpm in function of flask size) to avoid any sedimentation. The experiment was conducted in three replicates. Three controls were also carried out. The first one was a control without soil (CWS) and consist in 90 ml of MSM + 0,106 mM of the herbicide. It enables to ensure that isolated degraded strains originate from soil samples and not from any contamination in the lab or from the herbicide itself (because it has not been sterilized). The other two controls, were performed with 12,5 g of each soil (NO-PHYTO & PHYTO) in 90 ml of MSM without any herbicide (CP & CN-P) to ensure that selection was driven trough the herbicide. Four successive enrichment cultures of 7 days each were performed (in total 28 days). Enrichment cycles were performed by inoculating 90 mL of fresh MSM + 0,106mM of herbicide with 10 mL of the preceding enrichment cycle (Figure 2.2).

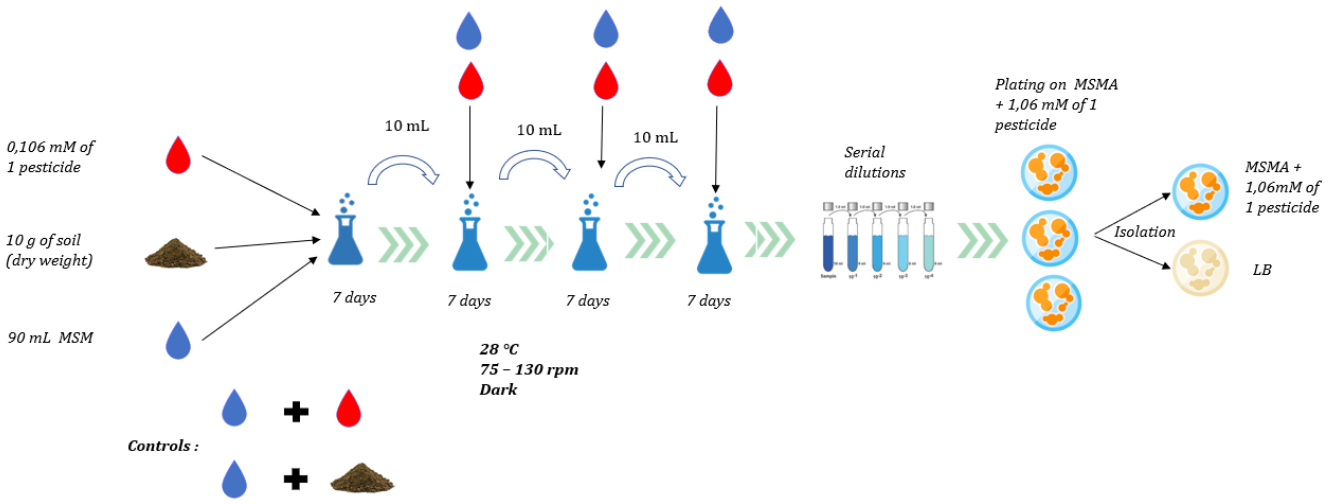


Figure 2.2: Schematic representation of the enrichment procedure.

At the end of the second, the third and the fourth enrichment cultures 100 µl aliquot of serial dilutions (10^{-3} and 10^{-4}) of the solution were plated on solid MSMA. Petri dishes were incubated for 4 days at 28°C in the dark. 1 ml of the enrichment solutions were also conserved in 30% glycerol at -80°C. Variations in abundance, colony type and growth were visually observed on the Petri dishes between successive enrichment cultures. Colonies from the fourth enrichment culture showing high growth and different morphology to the naked eye were isolated on both solid MSMA and solid LB and let to grow for 48 hours at 28°C in the dark. It was visually ensured that the isolated colonies were different from those from the control without soil.

2.5 Screening of potential degrading strains

From the isolated pure cultures, a selection was made before carrying out degradation studies in liquid media. This selection was based on :

1. The ability to convert metamitron to red by pure cultures isolated with metamitron.
2. The enzymatic Phenol Oxidase (PO) activity of each pure culture assayed on ABTS substrate.
3. The ability of each pure culture to use the pesticide (for which it has been isolated) for its growth.

2.5.1 Metamitron colour change test with isolated strains

At the end of the fourth enrichment culture with metamitron, a reddish/pink colour appeared in all flasks inoculated with PHYTO and NO-PHYTO soil. The three controls (CWS, CP & CN-P) remained colourless. The objective of this test was to see if strains isolated from the 4th metamitron enrichment solution were able to cause this colour change.

A total of 19 strains isolated from the 4th metamitron enrichment solution were inoculated from LB plates to 150 ml sterile flasks containing 90 ml MSM + 0.106mM metamitron. A negative control was performed with 90 ml MSM + 0.106mM metamitron without any microbial inoculation. A positive control was performed by inoculating 90 ml of MSM + 0.106mM metamitron with 0.5 ml of 4th metamitron enrichment culture solution (PHYTO). All flasks were incubated for 21 days at 28°C in the dark on an orbital shaker 75 rpm. The color of every flask was compared visually with the negative and the positive control. The experiment was carried out in a single repetition.

2.5.2 Phenol oxidase activity with ABTS test

The phenol oxidase enzyme activity of each isolate was assessed on the substrate ABTS (2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt) using the method described by (Huang *et al.*, 2013). Each of the isolated strains was transferred from LB plates into 15 ml Falcon™ conical centrifuge tubes containing 3 ml autoclaved TY. *Escherichia coli* was also grown to serve as a negative control (Huang *et al.*, 2013). The inoculation rate was not controlled. Tubes were incubated for 48 h at 37°C in an orbital shaker at 200 rpm in the dark. After the incubation all tubes were centrifuged at 8000 rpm for 10 minutes. 2 mL of supernatants were collected.

The ABTS 3mM solution was prepared at pH 3 using 0.1M McIlvaine buffer. McIlvaine 0.1M buffer was prepared by mixing 4.11 volumes of 0.2M Na_2HPO_4 stock solution with 15.89 volumes of 0.1M citric acid stock solution (McIlvaine, 1921). The pH was verified using a pH meter and corrected with Na_2HPO_4 or citric acid.

Phenol oxidase activity was assessed in 96-well plates by adding rapidly 150 µL of the ABTS 3mM solution with 50 µL of supernatants. Absorption at 420 nm was measured after 10 minutes of incubation at 30°C using a microplate spectrophotometer (Thermo Scientific Multiskan GO). The measurement was carried out in triplicate. The blank was performed with the raw TY. The negative control was performed with *Escherichia coli*. A 3 mg/L solution of Laccase from *Trametes versicolor* ≥0.5 U/mg (Sigma, 38429) was used as positive control. Blank, positive and negative controls were carried out in 8 replicates.

A calibration curve using 0; 0.25; 0.5; 1; 1.5; 2 and 3 mg/L solutions of laccase from *Trametes versicolor* ≥0.5 U/mg (Sigma, 38429) was performed to transform the absorption at 420 nm to a laccase concentration in mg/L. 150 µL of the ABTS 3mM solution was added with 50 µL of laccase solution. Absorption at 420 nm was measured after 10 minutes of incubation at 30°C. For each laccase concentration the absorption was measured in 8 replicates. The calibration curve was fitted using a linear regression model. The phenol oxidase enzyme activity of each supernatant was expressed in mg laccase per L of supernatant.

2.5.3 Microbial growth with pesticide as sole carbon and nitrogen source

2.5.3.1 Bacterial growth

The bacterial growth of each isolated pure culture was determined in MSM containing 50 mg/L of the pesticide for which the culture was isolated. For this purpose, each bacterium was suspended by scratching the pure culture on a LB plate in MSN medium. The bacterial suspension was transferred to 50mL sterile tubes. Serial dilutions of each suspension were performed in MSM and OD_{630} was measured determine the dilution required to achieve an OD_{630} of 0.1 ± 0.02. Next, 10 mL of MSM containing 50 mg/L of pesticides was inoculated with the appropriate amount of bacterial suspension to achieve an OD_{630} of 0.1 ± 0.02. Bacterial growth was measured in a 96-well plate after the addition of 200 µL of MSM solution containing the pesticide and the bacterial culture. The 96-well plate was closed and maintained in the dark at 28°C. The OD at 630 nm was measured after 0, 1, 2, 4, 6, 24, 48, 72, 96, 120 and 144 h (6 days) using a microplate spectrometer (Labsystem Multiskan RC), each time before 10 seconds of agitation at 1000 rpm. The growth was measured in 4 replicates. A negative control was carried out with *Escherichia coli* in 4 replicates. A blank was performed with fresh MSM containing 50 mg/L of the pesticide in 8 replicates.

2.5.3.2 Fungal growth

Fungal growth was assessed on solid MSMA containing 1.06 mM of the herbicide for which the culture was isolated. For this purpose, a 0.5 cm diameter agar plus covered by fungi on solid MSMA was

transferred to fresh MSMA containing 1.06 mM of the herbicide. Growth was determined by measuring the diameter of the colony every 24 h for 7 days. The experiment was repeated in triplicates.

2.6 Degradation of metamitron, metazachlor, bentazon, s-metolachlor and chlortoluron by selected cultures

Selected pure cultures (3 for each pesticide) (table 2.2) were assessed for their ability to degrade pesticides in liquid media. 100 ml sterile Erlenmeyer flasks containing 20 ml sterile MSM were supplemented with a pesticide (metamitron, metazachlor, bentazon, s-metolachlor or chlortoluron) at a concentration of 50 mg/L as the sole source of carbon and nitrogen. Each flask was inoculated with the suspension of the chosen strain, that was expected to degrade the pesticide contained in it, to obtain a biomass level of $OD_{630} = 0.1 \pm 0.02$ (using the same method as in 2.5.3.1). For each pesticide, two non-inoculated treatments were carried out. The first was kept at 4°C in the dark during the experiment and was considered as the initial concentration of pesticides. The second was used as an abiotic control and was maintained under the same conditions as the inoculated flasks. The experiment was performed in a single replica. In total, 20 flasks were incubated in a rotating shaker at 28 ± 2 °C and 150 rpm for 120 hours. They were kept in the dark by wrapping them with aluminium paper. 120 hours after inoculation, pesticide residues in each flask were measured.

Because the method of pesticide residue analysis allows the measurement of concentrations of several pesticides within the same sample, the 25 samples were combined into only 5 composed samples for analysis. One composed sample was made by adding 5 ml of solution from each sample into Falcon 50mL Conical Centrifuge Tubes (table 2.2). Each composed sample was centrifuged at 8000 rpm for 10 min and 15 ml of supernatants were analysed for pesticide residues by the CRA-W laboratory.

Pesticide	Metamitron	Metazachlor	Bentazon	S-metolachlor	Chlortoluron
Sample 1	MET 8	MZ 3	BTZ 23	SM 14	CHL 1
Sample 2	MET 12	MZ 11	BTZ 14	SM 12	CHL 9
Sample 3	MET 17	MZ 1	BTZ 12	SM 2	CHL 4
Sample 4	MET initial	MZ initial	BTZ initial	SM initial	CHL initial
Sample 5	MET abiotic control	MZ abiotic control	BTZ abiotic control	SM abiotic control	CHL abiotic control

Table 2.2: Composed samples for pesticides residues analysis : sample 1, 2 and 3 were flasks containing the selected cultures for pesticides degradation, sample 4 was the initial pesticides concentration and sample 5 was the un-inoculated abiotic control.

Briefly, pesticide residue analysis was carried out by Acquity (Milford, USA) ultra performance liquid chromatography-diode array detector (UPLC-DAD). The chromatographic separation was performed in a Acquity BEH C18 (2.1 × 50 mm, 1.7 μm) column maintained at 50°C. The injection volume was 1μl. The mobile phase A was composed by 0.1% formic acid in H_2O and mobile phase B was composed by acetonitrile. The flow rate of the mobile phase was 0,8 ml/min. The gradient flow of mobile phases was 90:10% (phase A:B) at 0 min until 0.5 min. It then shifted to 10:90% (phase A:B) until 8 min, remained constant until 9 min, followed by a return to 90:10% (phase A:B) in 10 seconds and maintained until 10 min. The expected retention time was 1.06 min for metamitron, 2.76 min for bentazon, 2.89 min for chlortoluron, 3.39 min for metazachlor and 4.44 min for metolachlor. Pesticides detection was carried out by the Acquity UPLC Photodiode Array (PDA) Detector at 230 nm wavelength. The analytical error is estimated to be 5% maximum. The preparation of samples is probably the cause of more inaccuracies (from CRA-W internal source).

The bacterial growth was monitored during the experiment using the same method presented in the section 2.5.3.1. The OD at 630 nm was measured after 0, 1, 2, 4, 6, 24, 48, 72, 96 and 120h using a the same spectrometer, each time before 10 seconds of agitation at 1000 rpm. A negative control was carried out with *Escherichia coli*. A blank was performed with fresh MSM containing 50 mg/L of the pesticide. The growth was measured in 3 replicates.

2.7 Identification of isolated cultures

2.7.1 Morphological identification

Selected bacteria were gram-colored using the Gram-Color | 111885 - Merck Millipore Stain set. The method used was the same as that provided by the manufacturer. The gram-coloration was observed under an immersive optical microscope (ZEISS Primostar).

Fungi were routinely grown on PDA and their development was first observed eye naked. They were then observed using an immersive optical microscope. Identification down to the genus was proposed by comparing their shape and pores with the "Illustrated Genera of Imperfect Fungi" 4th edition (Barnett and Hunter, 1998).

2.7.2 16S rDNA identification

The selected bacterial strains were identified by sequencing their 16S rDNA. The PCR was performed in a 47 µL reaction volume which contained :

- 0,5 µL of 25 µM 16S DNA A1 forward primer (5'-AGAGTTTGATCMTGGCTCAG-3') (Takema and Naruo, 1998).
- 0,5 µL of 25 µM 16S DNA B1 reverse primer (5'-TACGGYTACCTTGTTACGACTT-3') (Takema and Naruo, 1998).
- 1,0 µL of 1000 U/µL Mango Taq DNA polymerase (Bioline).
- 1,0 µL of 10mM DNA templates (Eurogentec).
- 1,5 µL of 50 mM MgCl−2 solution (Eurogentec).
- 10 µL of colorless PCR buffer (Bioline)
- 32,5 µL of sterile Milli-Q water

Bacterial DNA was physically extracted by directly adding the bacterial cells from LB plates to the reaction volume and vigorously mixing/crushing. Two positive controls were carried out by adding 2 µl of bacterial DNA solution into the 47 µL reaction volume. One negative control was performed by adding 2 µL of sterile Milli-Q water into the 47 µL reaction volume.

PCR reactions were performed in a thermocycler (Eppendorf Mastercycler Nexus Gradient Thermal Cycler):

- For 2 min at 94°C.
- Followed by 30 successive cycles of : 1 min at 94°C, 1 min at 50°C and 2 min at 70°C.
- Followed by 10 min (extension step) at 72°C

DNA was purified using the QIAquick PCR Purification Kit (QIAGEN) and following the protocol prescribed by the manufacturer. The quality of the DNA was verified by electrophoresis on an agarose gel. Briefly, agarose gel was prepared with 1 g of agarose in 100 mL of TAE buffer (242 g tris base 0,04M, 57.1 ml glacial acetic acid and 37,2 g EDTA in 1 L of distilled water) and 10 µL of GelRed® Nucleic Acid Gel Stain (Biotium). Then 7 µL of each purified DNA sample was added with 2 µL of TriTrack DNA Loading Dye 6X (Thermo Scientific™) were placed in wells in the agarose gel. 5 µL of GeneRuler 100 bp Plus DNA Ladder (Thermo Scientific™) was placed on each side wells of the agarose gel. Electrophoresis was carried out for 45 min with the PowerPac Basic (BIO-RAD) at 140 mA. DNA migration was visualized using the UV gel documentation system "Vilber Lourmat E-BOX CX5 TS". The concentration of purified 16S rDNA was quantified with a spectrophotometer (NanoDrop ND-1000 Spectrophotometer).

The 16SA1 rDNA was sequenced using Sanger technology by the firm Macrogen Europe. The sequences of the 16SA1 rDNA were compared to closest sequences provided by the Nucleotide collection database using "Basic Local Alignment Search Tool" (BLAST: https://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.8 Development of a method to evaluate the degradation of pesticides by degrading-strains on intact core soil columns

The experiment consist in 4 types of columns (AL, AH, NAL & NAH) :

- AL : Low flow rate - Autoclaved columns.
- AH : High flow rate - Autoclaved columns.
- NAL : Low flow rate - Non-Autoclaved columns.
- NAH : High flow rate - Non-Autoclaved columns.

Each type of column was performed in 3 repetitions.

2.8.1 The soil mini-columns experimental setup

The soil mini-columns setup was mainly inspired by the setup presented by Cheyns *et al.* (2010). The experiment was carried out on mini soil columns of 3 cm diameter and 10 cm height. One column consists in a Falcon 50mL cut at the bottom. Intact soil cores of 7 cm height were taken directly from the soil to the column by drilling it into the soil to preserve the soil structure. A random sample of 12 columns was taken from plot 6 : Reference T1.2. where sugar beet (*Beta vulgaris*) was currently grown. The soil characteristics for the first 30 cm depth are presented in the table 2.3.

pH H2O	7,8
Total carbon (g/100g)	1,4
Clay % (<0,002 mm)	12
Fine silt % (0,002 - 0,02 mm)	29,5
Coarse silt % (0,02 - 0,05 mm)	50,9
Fine sand % (0,05 - 0,2 mm)	4,8
Coarse sand % (0,2 - 2 mm)	2,7
Textural class	Silt Loam

Table 2.3: Soil characteristics : 0 - 30 cm.

The columns were left straight at all times to avoid disturbing the structure. A cheesecloth and a Falcon tube cap drilled with a 1 cm diameter hole were placed at the bottom of the each column to retain the soil. A second 50 mL falcon tube was screwed directly to the cap under the column to collect the percolates. The columns were closed at the top with a cap (not completely) to prevent evaporation and vacuum formation. The pressure under the column corresponded to the atmospheric pressure.

Figure 2.3: Mini-column design.

Figure 2.4: Mini-column design.

2.8.2 $CaCl_2$ leaching on autoclaved and non-autoclaved soil mini-columns

Of the 12 columns, 6 columns were autoclaved once for 35 min at 120°C (1.1 atm). The columns were left to rest for 24 hours and were autoclaved again using the same procedure. The 2 autoclave cycles with 24 hours rest in between were necessary for the heat-resistant spores to germinate and be destroyed during the second autoclave cycle(Miyaki *et al.*, 1996).

Firstly, the effect of autoclaving on the columns was assessed visually (deformation of the column, soil structure, preferential pathways). Then, 25 mL of demineralized water was slowly added to each column and left to drain for 24 hours. The next day, the saturated hydraulic conductivity K_{Sat} is measured on each soil column. To achieve it, demineralized water was added to maintained at a height of 1 cm above the soil top layer to maintain a constant pressure head of 1cm. Once steady flow at the bottom of the column was achieved, the time required to infiltrate 20 ml of water in saturated flow was measured to determine the flow rate. The K_{Sat} was then determined for each column using Darcy's law (2.1):

$$K_{Sat} = \frac{-J}{\frac{dH}{dz}} = \frac{Q}{A}\frac{L}{L+p} \tag{2.1}$$

- J : Water flux within the column [$m.s^{-1}$].
- $\frac{dH}{dz}$: Total water load gradient [].
- Q : The water saturated flow rate within the column [$m^3.s^{-1}$].
- A : The section area of the column : 7,069 $\times$ 10^{-4} [m^2].

- L : The length of the column, 0.07 [m].
- p : Water height at the top of the column, 0.01 [m].

24 h later, a 10 mL pulse of 5 g/L $CaCl_2$ is applied to each column. The next day and for 14 days, water was added with a rate of 5 ml/day (7,07 mm/day) for low flow rate columns (AL & NAL) and 10 ml/day (14,15 mm/day) for high flow rate columns (AH & NAH).

Every day, the volume of water percolated, the pH and the electrical conductivity of the solution were measured with a Hach Lange HQ40d multimeter (figure : 2.5). The electrical conductivity was plotted against the $CaCl_2$ concentration using a calibration curve. The calibration curve was made by plotting the electrical conductivity of 10, 5, 2.5, 1, 0.5, 0.25, 0.1 and 0 g.L^{-1} $CaCl_2$ solutions. The calibration curve was fitted using a linear regression model by minimizing the sum of the squared errors.

Figure 2.5: Maintenance of the columns with the multimeter.

The $CaCl_2$ breakthrough curve (BTC) of each column was modelled using the classical convection-dispersion equation (CDE) (2.2) assuming steady state flow, a homogeneous soil and neglecting the chemical or physical non-equilibrium. The transport parameters v, R, D and pulse duration were inverse estimated by fitting the CDE equation (2.2) to the experimental BTC using the CXTFIT program with the STANMOD software (Simunek *et al.*, 1999). Non-degradation was assumed.

$$R\frac{\partial c_r}{\partial t} = D\frac{\partial^2 c_r}{\partial x^2} - v\frac{\partial c_r}{\partial x} \tag{2.2}$$

Where c_r is the concentration of the liquid phase [g.L^{-1}], D is the dispersion coefficient [cm².day^{-1}], x [cm] is distance ant t is time [day]. v is the average pore-water velocity [cm.day^{-1}] :

$$v = \frac{q}{\theta} \tag{2.3}$$

Where q is the average water flux [cm.day^{-1}] and θ is the average volumetric water content [cm³.cm^{-3}]. q is estimated by the total water volume leached divided by the duration of the experiment.

R is the retardation factor [-]:

$$R = 1 + \frac{\rho_b K_d}{\theta} \tag{2.4}$$

Where ρ_b is the bulk density of the soil [g.cm^{-3}] and K_d is the adsorption coefficient [cm^3.g^{-1}] of the linear isotherm described as :

$$s = K_d c_r \tag{2.5}$$

3 Results

3.1 Enrichment and isolation of metamitron, metazachlor, bentazon, s-metolachlor and chlortoluron degrading strains

Colonies of the fourth CWS enrichment solution growing on MSMA were different from those of the PHYTO and NO-PHYTO enrichment solutions in term of morphology. It was not the case for the CP and CN-P.

In total, 85 pure cultures were isolated from the fourth enrichment cultures (table 3.1). A total of 19 pure cultures resistant to metamitron (MET 1 → 19), 14 to metazachlor (MZ 1 → 14), 22 to bentazon (BTZ 1 → 23), 15 to s-metolachlor (SM 1 → 15) and 15 to chlortoluron (CHL 1 → 15) were isolated. 46 cultures were isolted from the PHYTO plot while 39 were isolated from the NO-PHYTO plot. All isolated cultures were able to grow on both solid MSMA and solid LB within 48h of incubation, with the exception of the culture SM 12, which was unable to grow on solid LB.

Plot	Metamitron	Metazachlor	Bentazon	S-metolachlor	Chlorotoluron	Total
PHYTO	9	7	10	9	11	46
NO-PHYTO	10	7	12	6	4	39
Total	19	14	22	15	15	85

Table 3.1: Total number of cultures isolated from the fourth enrichment cycle.

At the end of the fourth enrichment culture with metamitron, a light reddish colour was observed in the PHYTO and NO-PHYTO flasks, while the controls remained colourless (figure 3.1). This observation was not reported with the enrichment solutions containing the other pesticides.

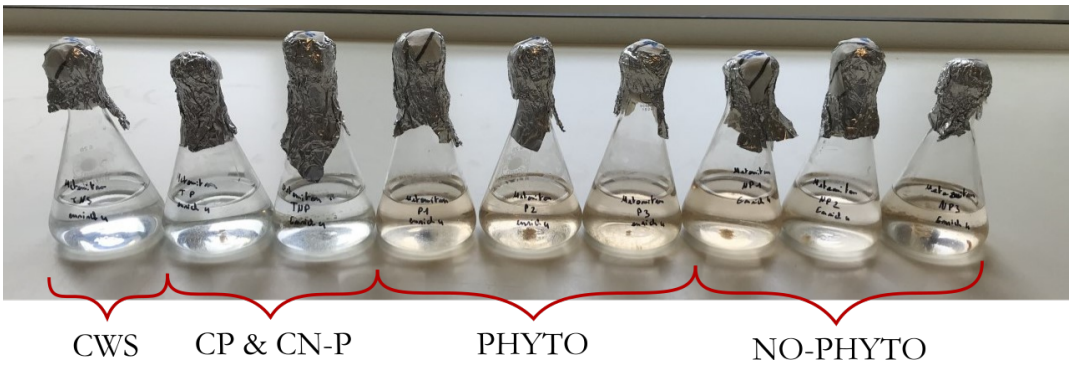


Figure 3.1: Fourth enrichement cultures with metamitron : CWS = Control without soil, CP & CN-P = Controls without metamitron, PHYTO = Fourth enrichement cultures with metamitron and soil from PHYTO plot, NO-PHYTO = Fourth enrichement cultures with metamitron and soil from NO-PHYTO plot.

3.2 Screening of potential degrading strains

From the 85 isolated pure cultures, a selection of 15 pure cultures was made before carrying out degradation studies in liquid media and identification. This selection was based on :

1. The ability to convert metamitron to red by pure cultures isolated with metamitron.
2. The enzymatic Phenol Oxidase activity of each pure culture assayed on ABTS substrate.
3. The ability of each pure culture to use the pesticide (for which it has been isolated) for its growth.

3.2.1 Metamitron colour change test with isolated strains

It was hypothesized that the colour change of the fourth enrichment culture with metamitron may be due to the appearance of a metabolite of metamitron or a metabolite of an additive contained in Goltron 700 SC. The objective of the next test was to determine whether the strains isolated from the 4th metamitron enrichment solution were able to cause this colour change separately.

After 7 days of incubation, no colour change was observed in the solution of all flasks except in the positive control (figure 3.2). The same observation is made after 14 and 21 days. The slight coloration observed in the figure 3.2 on days 14 and 21 is due to the reflection of light and the colour of the micro-organisms growing in the flasks.

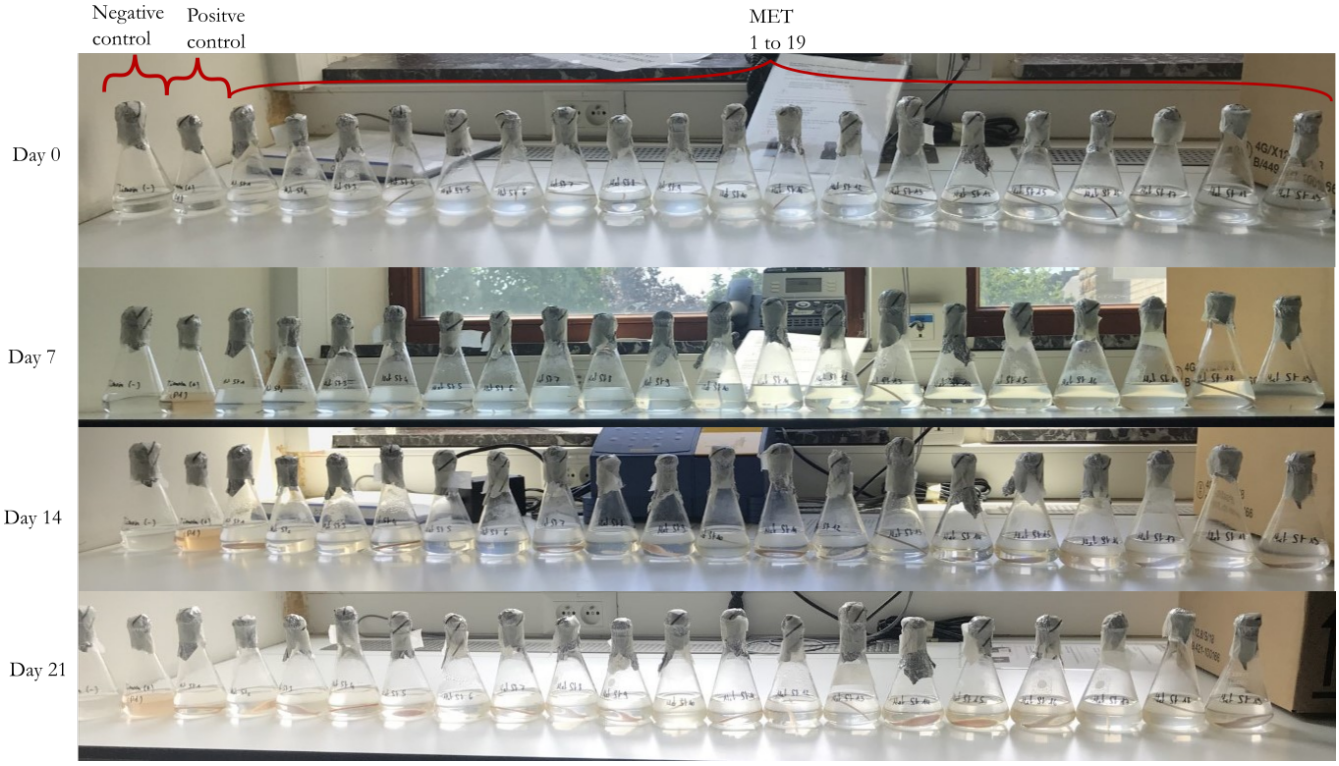


Figure 3.2: Metamitron colour change test with isolated strains.

3.2.2 Phenol oxidase activity with ABTS test

The calibration curve performed showed a linear relationship between the absorbance (420 nm) and the laccase concentration (figure 3.3). This relationship is modeled by the equation : Abs = 0.07977 * [Laccase] + 0.1121. The goodness of the fitting of the linear regression is evaluated by the R^2 (0,9952)

and the Root Mean Square Error (or Sy.x) (0,005377). The 3 mg/l laccase solution became visibly green 10 min after exposure to ABTS and was used as positive control (figure 3.4).

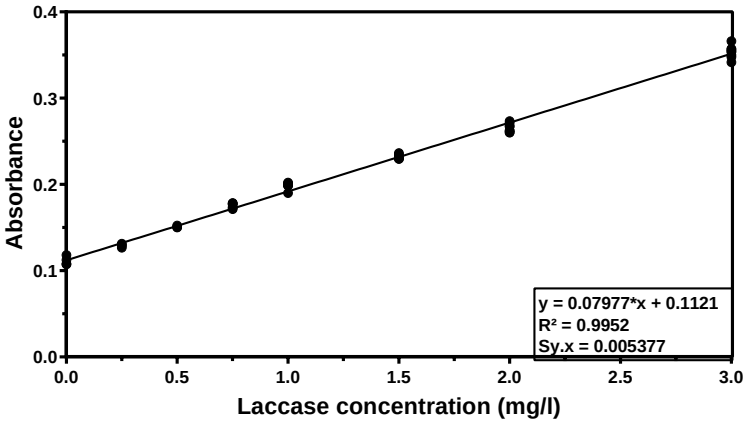


Figure 3.3: Laccase-Abs calibration curve.

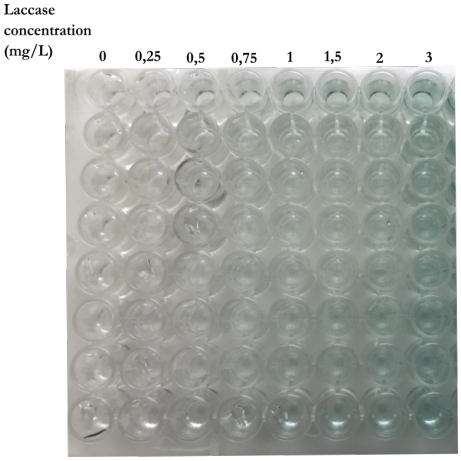


Figure 3.4: 96 wells plate used for the Abs-Laccase concentration calibration curve.

None of the supernantants of the 85 isolated strains became became visibly green 10 min after exposure to ABTS. However, among the 85 isolated strains, 27 showed a significantly higher PO activity than *Escherichia coli* used as negative control. The relative concentration of laccase and the significance of the strains concerned are shown in Figure 3.5. The strain showing the highest PO activity was BTZ 2, with a laccase equivalent 388,5 % higher than *Escherichia coli*.

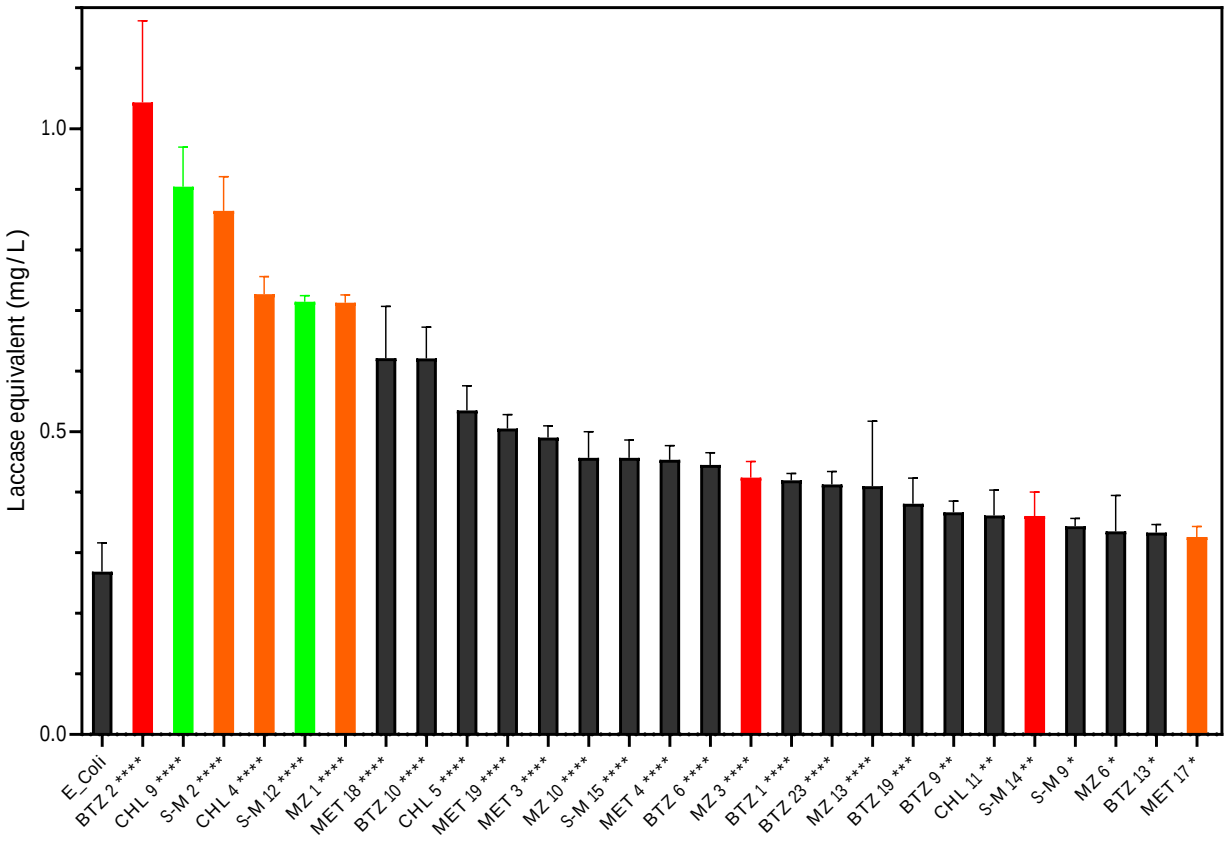


Figure 3.5: PO activity of microbial strains with significant higher PO activity compared to *E. Coli* - Unpaired t-test ; p-Value < 0.05 = *, p-Value < 0.005 = **, p-Value < 0.0005 = ***, p-Value < 0.0001 = ****. Error bars show the standard deviation. Colored bars show the selected strains (See table 3.2).

3.2.3 Microbial growth with pesticide as sole carbon and nitrogen source

3.2.3.1 Bacterial growth curves with metamitron as carbon and nitrogen source

The growth curves of isolated metamitron-resistant bacteria in sterile MSM supplemented with 50 mg/L metamitron (Goltron 700 SC) at 28°C are shown in figure 3.6. The red, green and orange curves represent the growth of selected strains; MET 8, MET 12 and MET 17, respectively.

All cultures were inoculated at an OD_{630} between 0.068 and 0.0945. Between 0 and 1 hour after inoculation, the OD_{630} of each solution decreased by an average of 0.01. This should be due to an error in the OD_{630} measurement rather than bacterial decay, as this is also observed in the blank control. Between 1h and 24h after inoculation, several strains showed exponential growth such as MET 8, MET 12, MET 14, MET 17, MET 11, MET 18 and MET 19. After 24 hours of growth, most of the inoculated strains reached a plateau or a decay phase. Only the MET 8 strain was able to grow steadily until 96 h and reach a peak with an OD_{630} of 0.1985 ± 0.0222.

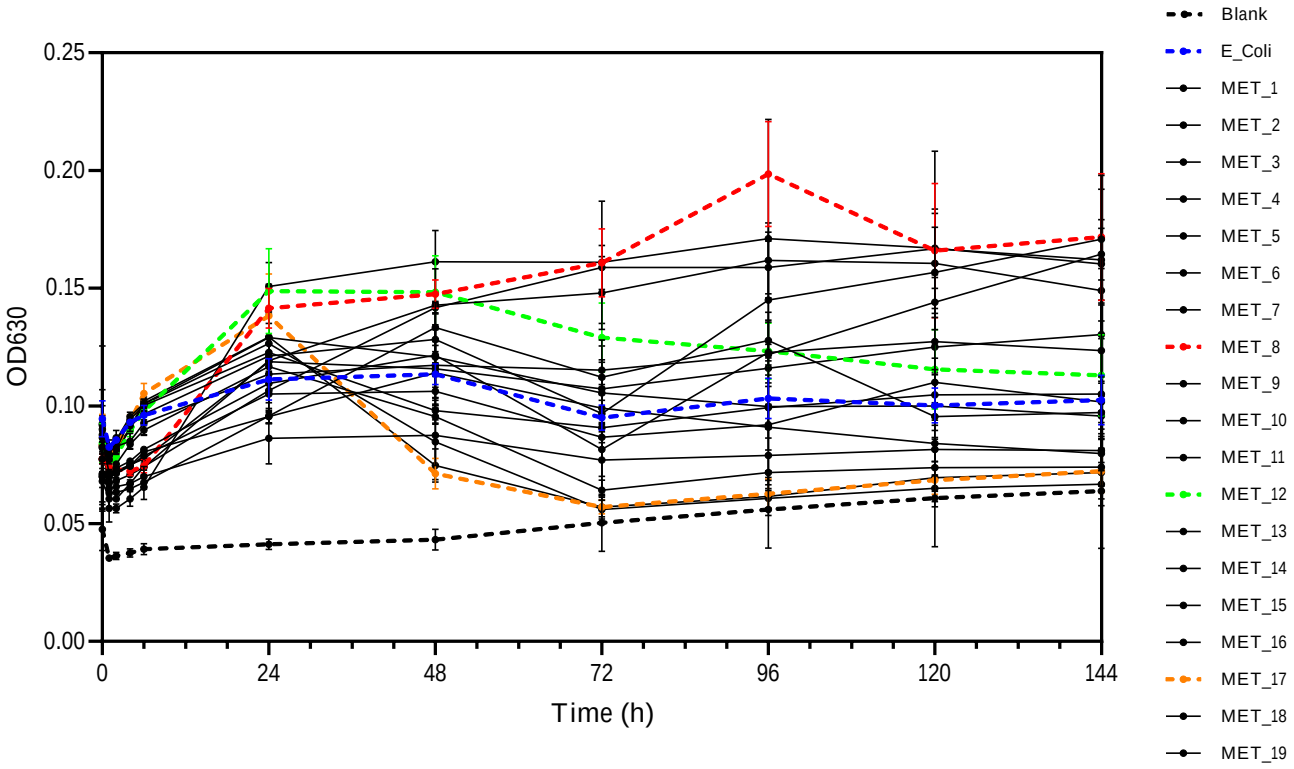


Figure 3.6: Growth curves of isolated metamitron-resistant bacteria. Error bars show the standard deviation.

3.2.3.2 Bacterial growth curves with metazachlor as carbon and nitrogen source

The growth curves of isolated metazachlor-resistant bacteria in sterile MSM supplemented with 50 mg/L metazachlor (Butisan S) at 28°C are shown in Figure 3.7. The red, green and orange curves represent the growth of selected strains; MZ 3, MZ 11 and MET 1, respectively.

All cultures were inoculated at an OD_{630} between 0.0475 and 0.0795. In general, the strains isolated with metazachlor grew quiet poorly with it as carbon and nitrogen source. However, some strains were able to grow within the first 48h after the inoculation such as MZ 1, MZ 11, MZ 4 and MZ 14. After 48h,

the OD_{630} decreased until 96h before increasing again up to 120h. Over the next few hours, the OD_{630} of most strains oscillated with a maximum amplitude of 0.03 (OD_{630}) and a period of 48 h.

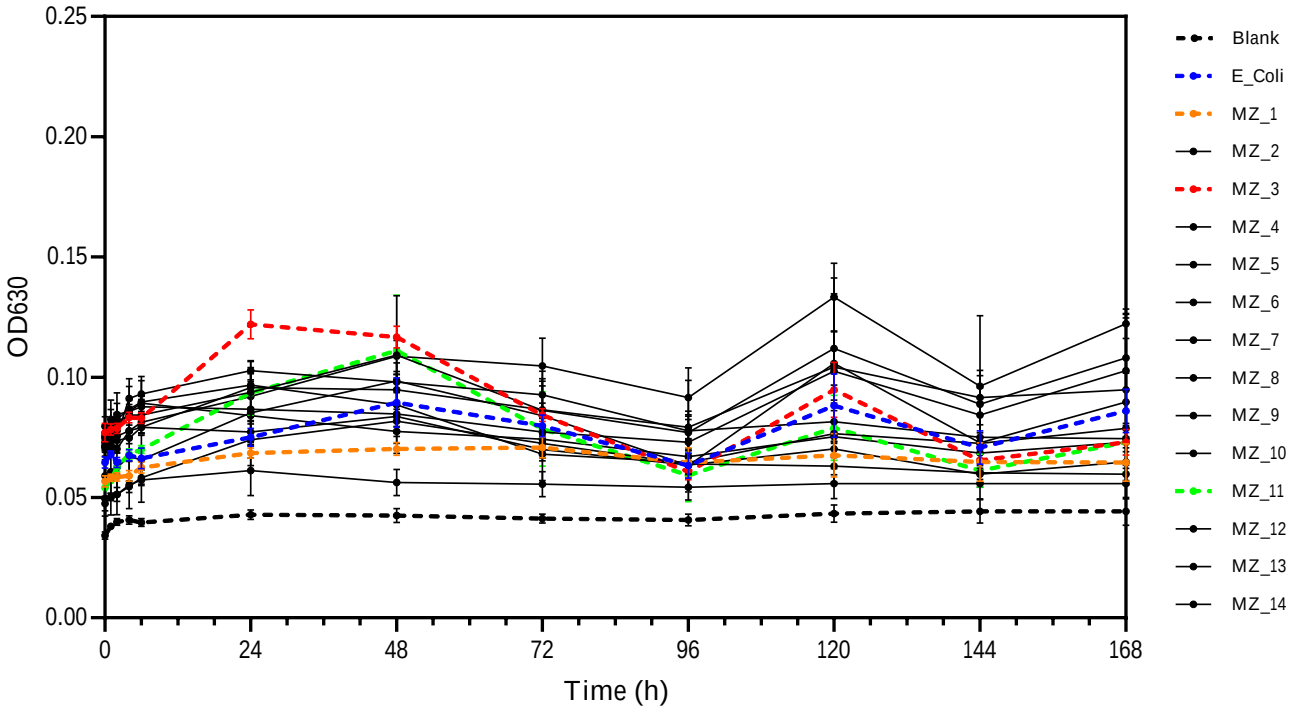


Figure 3.7: Growth curves of isolated metazachlor-resistant bacteria. Error bars show the standard deviation.

3.2.3.3 Bacterial growth curves with bentazon as carbon and nitrogen source

The growth curves of isolated bentazon-resistant bacteria in sterile MSM supplemented with 50 mg/L bentazon (Bazagran SG) at 28°C are shown in Figure 3.8. The red, green and orange curves represent the growth of selected strains; BTZ 3, BTZ 14 and BTZ 12, respectively.

All cultures were inoculated at an OD_{630} between 0.05625 and 0.08475. Several strains showed high growth potential in sterile MSM supplemented with 50 mg/L bentazon compared to *Escherichia coli*. The two strains showing the highest growth were BTZ 14 and BTZ 12.

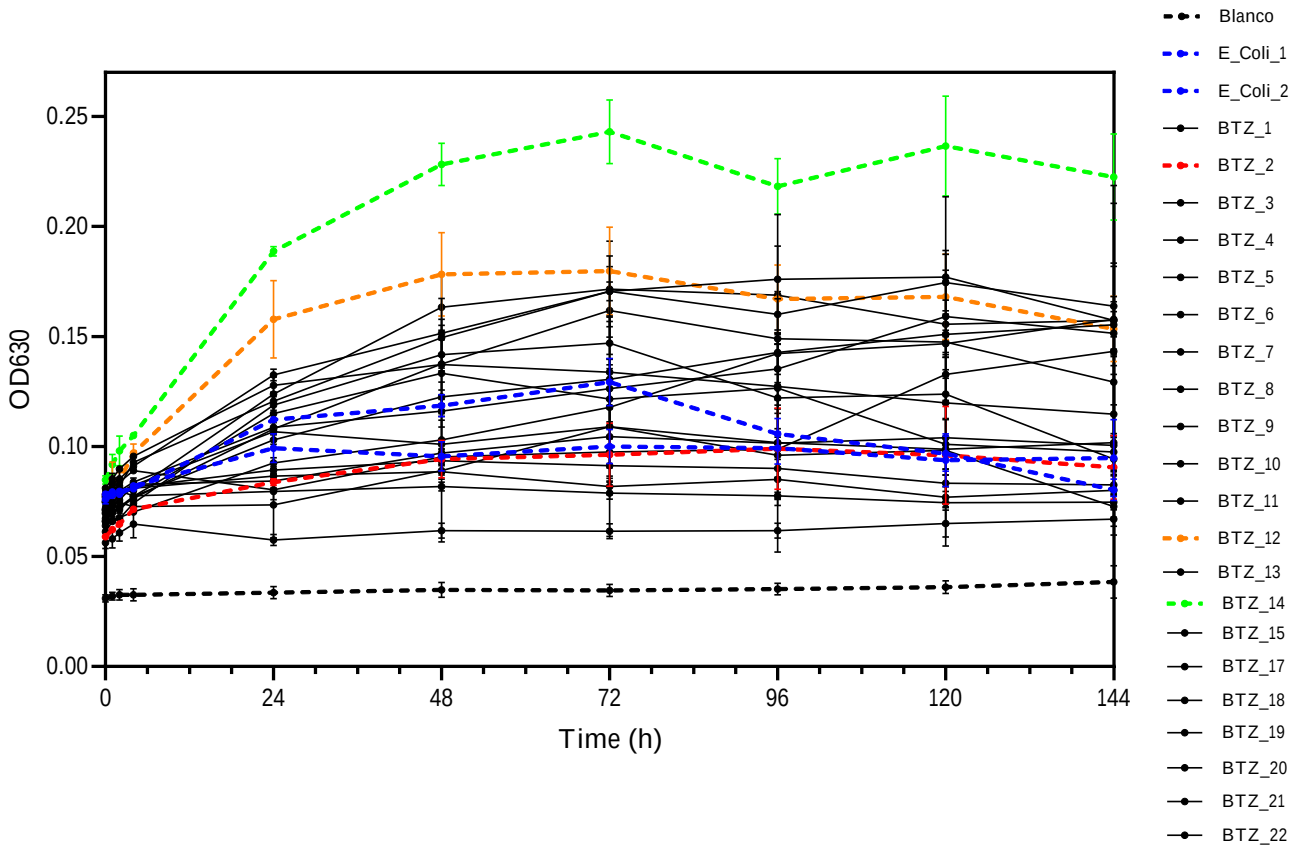


Figure 3.8: Growth curves of isolated bentazon-resistant bacteria. Error bars show the standard deviation.

3.2.3.4 Bacterial growth curves with s-metolachlor as carbon and nitrogen source

The growth curves of isolated s-metolachlor-resistant bacteria in sterile MSM supplemented with 50 mg/L s-metolachlor (Dual Gold) at 28°C are shown in Figure 3.9. All cultures were inoculated at an OD_{630} between 0.06 and 0.1, with the exception of the SM 12 strain which was inoculated with only a few cells. Indeed, the SM 12 strain was not readily cultivable on solid LB and did not provide enough biomass to be inoculated at an OD_{630} of 0.1 after 48h of growth on LB.

Among all bacteria, only the strains SM 12 and SM 14 were able to grow readily compared to *Escherichia coli*. SM 14 had an exponential growth phase during the first 24 hours and a stationary/decreasing phase during the following five days. During the exponential phase, SM 14 was able to grow from an OD_{630} of 0.095 ± 0.05 to an OD_{630} of 0.165 ± 0.07. The SM 12 strain also had a high growth potential and could grow from an OD_{630} of 0.035 ± 0.0005 to an OD_{630} of 0.051 ± 0.016 in 24 hours. The strain SM 2 showed a similar growth to *Escherichia coli*. Other strains also grew similarly to *Escherichia coli* for the first 6 hours but then showed a decay, such as SM 15. Some could not develop at all and perished.

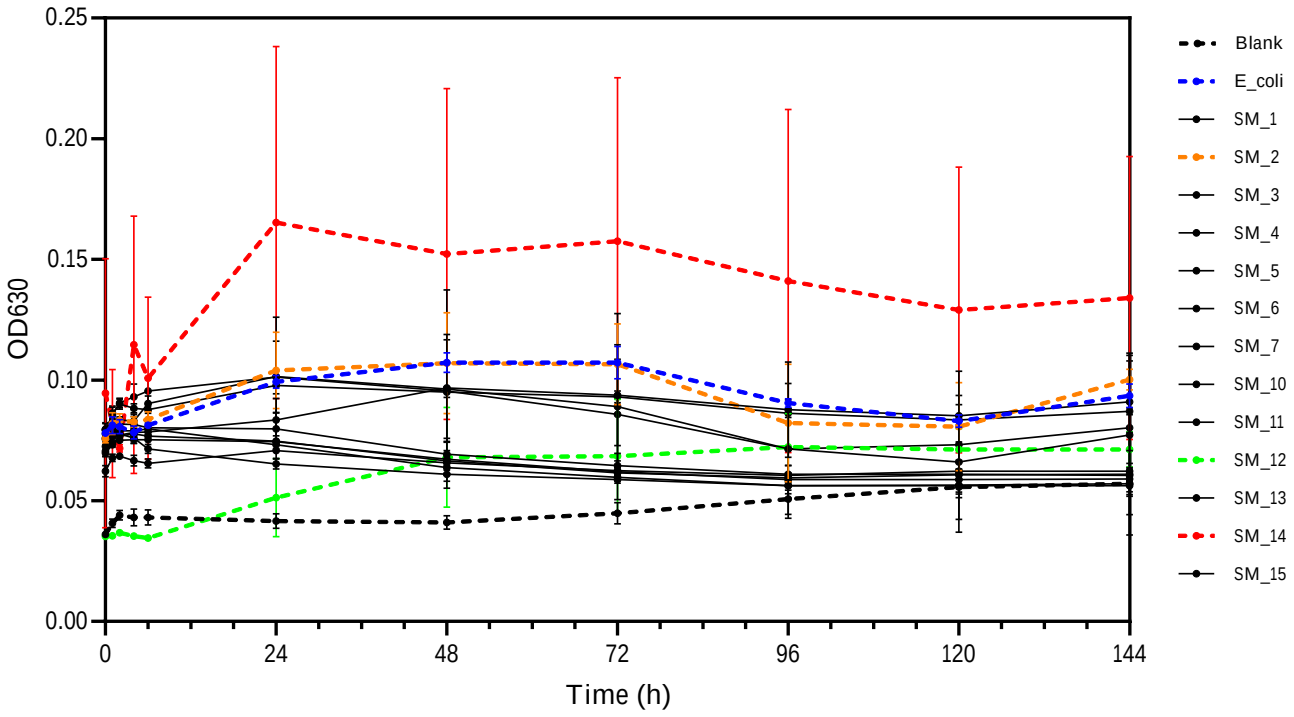


Figure 3.9: Growth curves of isolated s-metolachlor-resistant bacteria. Error bars show the standard deviation.

3.2.3.5 Bacterial growth curves with chlortoluron as carbon and nitrogen source

The growth curves of isolated chlortoluron-resistant bacteria in sterile MSM supplemented with 50 mg/L chlortoluron (Chloortoluron 500 SC) at 28°C are shown in figure 3.10. All cultures were inoculated at an OD_{630} between 0.056 and 0.119. Compared to the bacteria isolated with the other four pesticides, the strains isolated with chlortoluron grew very poorly in the presence of the pesticide as the only carbon and nitrogen source. The red, green and orange curves represent the growth of selected strains; CHL 1, CHL 9 and CHL 4, respectively.

Of all the bacteria, only the strain CHL 1 could grow faster than *Escherichia coli* in the presence of Chlortoluron. CHL 1 showed exponential growth for the first 6 hours, then steady growth up to 72 hours and finally decay. Other strains had similar or lower growth potential than *Escherichia coli*.

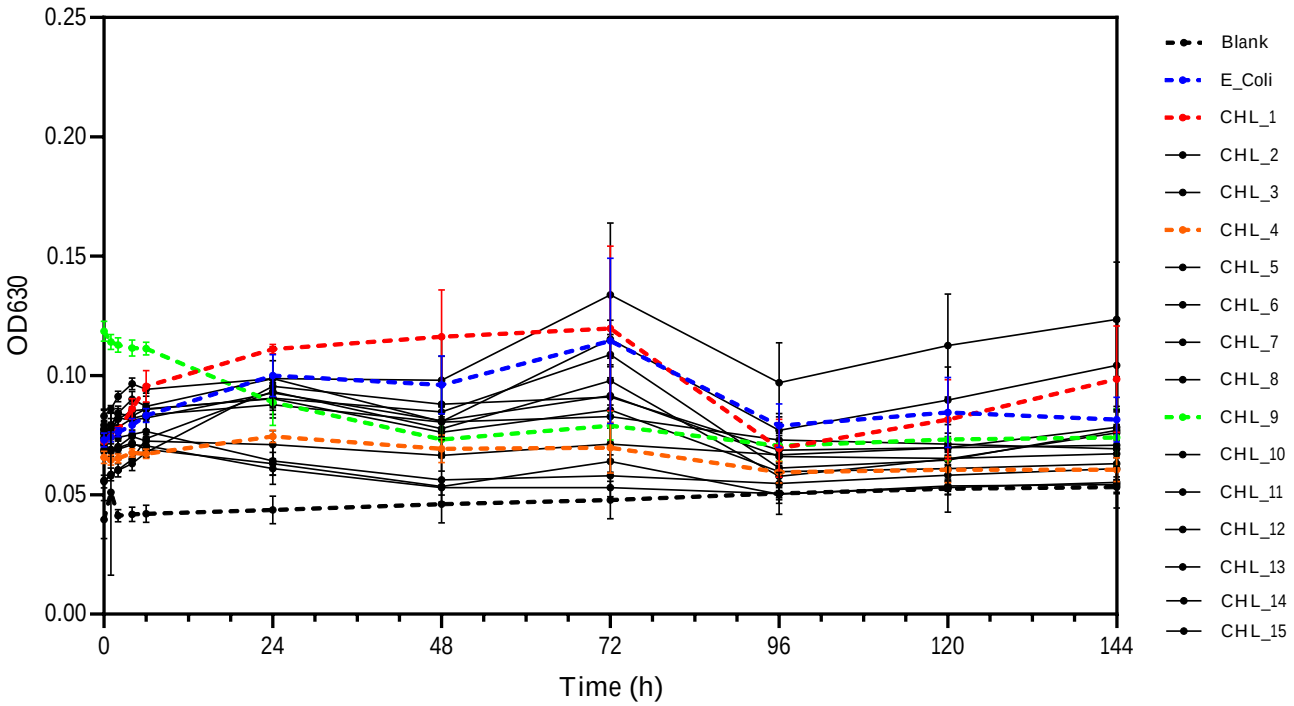


Figure 3.10: Growth curves of isolated chlortoluron-resistant bacteria. Error bars show the standard deviation.

3.2.3.6 Fungal growth

The fungal growth of the three isolated fungi was assessed by measuring the diameter on solid MSMA containing 1.06 mM s-metolachlor (for SM 8 and SM 9) and 1.06 mM bentazon (for BTZ 23). All 3 fungi were able to grow on MSMA. For the strains isolated with s-metolachlor, the SM 9 strain grew faster than SM 8 (Figure 3.11).

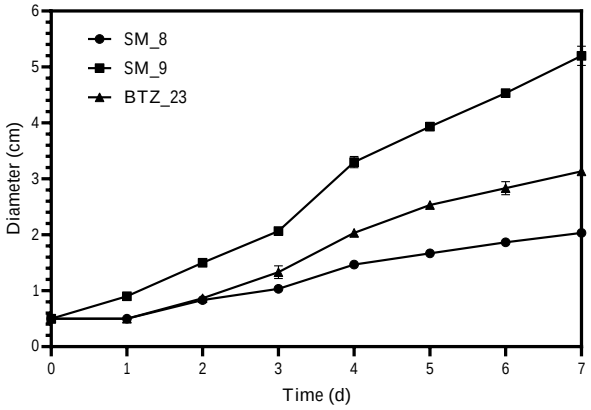


Figure 3.11: Fungal growth of the isolated strains SM 8, SM 9 and BTZ 23 on solid MSMA. Error bars show the standard deviation.

3.2.4 Selected strains

In total, 15 strains (3 for each pesticide) were selected to perform pesticide degradation studies and identification. For the selection, the ability to convert metamitron to red by the pure cultures isolated with metamitron was not considered. Indeed, none of these pure cultures were able to transform metamitron into a reddish color. Thus, the selection of candidates is made on a case-by-case basis on the criteria of growth with the pesticide as source of carbon/nitrogen and PO activity (Figure 3.2).

Fungal strains were not retained for the pesticides degradation studies despite some potential for growth on MSMA (Figure 3.11) and the PO potential activity for BTZ 23 and SM 9 (Figure 3.5). However, identification of fungi down to the genus was attempted on the basis of morphology.

Table 3.2: List of strains showing growth potential and significant PO activity. Colored cells show selected strains. Colors are kept constant in following figures.

Ranking	Metamitron		Metazachlor		Bentazon		S-Metoclachlor		Chlortoluron	
	Growth	PO activity	Growth	PO activity	Growth	PO activity	Growth	PO activity	Growth	PO activity
1	MET 8	MET 18	MZ 3	MZ 1	BTZ 14	BTZ 2	S-M 14	S-M 2	CHL 1	CHL 9
2	MET 12	MET 19	MZ 11	MZ 10	BTZ 12	BTZ 10	S-M 12	S-M 12		CHL 4
3	MET 14	MET 3	MZ 4	MZ 3	BTZ 17	BTZ 6	S-M 15	S-M 15		CHL 5
4	MET 17	MET 4	MZ 14	MZ 13	BTZ 7	BTZ 1	S-M 2	S-M 14		CHL 11
5	MET 11	MET 17		MZ 6	BTZ 11	BTZ 23		S-M 9		
6						BTZ 19				
7						BTZ 9				
8						BTZ 13				

Metamitron

For the selection of the 3 metamitron strains, MET 17 was directly chosen because it showed both significant PO activity and growth potential with metamitron as the only carbon/nitrogen source (Figures 3.5 & 3.6). Strain MET 8 was chosen because of its high growth potential, even though it did not show significant PO activity. MET 12 was preferred to MET 14, although the latter also had a high growth potential. This is because MET 14 had significantly lower PO activity than MET 12 (result not shown).

Metazachlor

MZ 3 was directly chosen because it showed both significant PO activity and growth potential with metazachlor as the only carbon/nitrogen source (Figures 3.5 & 3.7). MZ 1 was chosen for its PO activity. MZ 11 was chosen for its ability to grow with metazachlor as the only carbon/nitrogen source and without having an oscillating dynamic (See discussion 4.1.2).

Bentazon

The BTZ 2 strain (isolated with bentazon) showed the highest PO activity and was selected as a highly potential bentazon-degrader (Figure 3.5). BTZ 14 was chosen because it showed the highest growth among the 82 isolated bacteria (Figure 3.8). BTZ 12 was also chosen for its high growth potential.

S-metolachlor

SM 14, SM 12 and SM 2 were chosen because they all presented significant PO activity and the potential to grow with s-metolachlor as the only carbon/nitrogen source (Figures 3.5 & 3.9). Comparatively, SM 14 showed the highest growth potential while SM 2 showed the highest activity on the ABTS assay. The growth of SM 12 was also significant as it was inoculated at a much smaller OD_{630} rate than the other strains and was able to grow steadily for 6 days.

Chlortoluron

For chlortoluron, the strains CHL 1 was chosen because it was the only one to show growth potential with chlortoluron as source of carbon/nitrogen (Figure 3.10). As no other strain could grow with chlortoluron as source of carbon/nitrogen, the strains CHL 4 and CHL 9 were chosen for their activity PO (Figure 3.5).

3.3 Identification of selected cultures

3.3.1 Identification of selected bacteria

Electrophoresis of the purified 16S rDNA PCR product showed the presence of DNA sequences of approximately 1500 base pairs for each bacterial strain. Positive controls showed the same pattern.

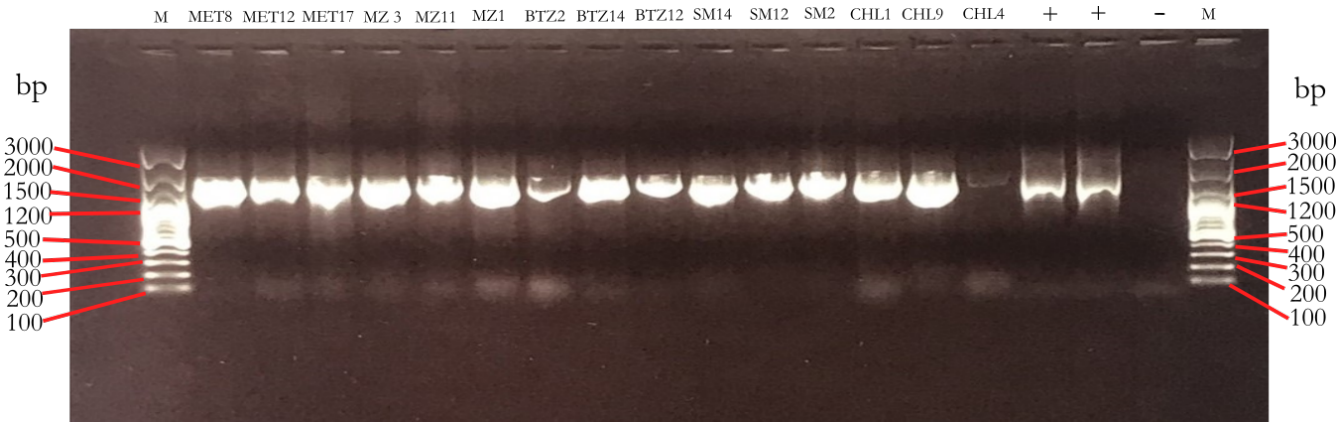


Figure 3.12: Agarose gel electrophoresis of purified PCR product of 16S rDNA from selected bacterial strains; M = DNA ladder; + = positive control; - = negative control.

Among the 15 selected bacteria, 13 were identified by their 16S rDNA. The MET 17 and CHL 4 strains could not be identified by 16S rDNA sequencing. Indeed, for these DNA samples, the sequencing traces showed two or more overlapping peaks at the same location, which is typical of mixed template sequencing (Figure 3.13 vs 3.14). This may be due to the presence of two or more DNA templates in the sequencing reaction, which may come from non-pure colonies.

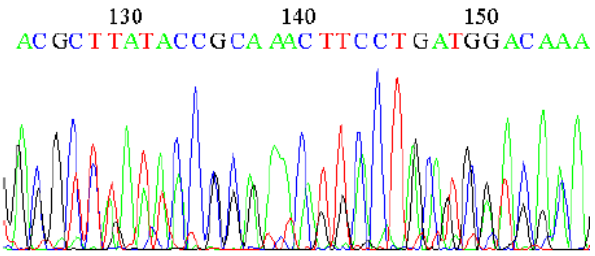


Figure 3.13: Sequencing trace of MET 17 : Mixed template.

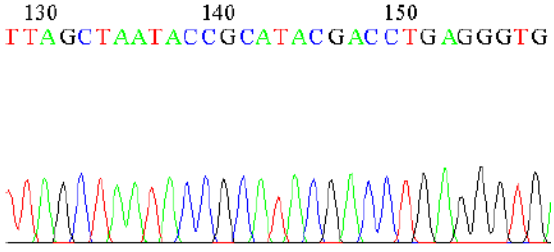


Figure 3.14: Sequencing trace of MET 8 : Single template.

Metamitron

The MET 8 16S rDNA sequence showed high similarity with several *Cupriavidus metallidurans* strains (Table 3.3) and was identified as a strain from the same species. Probable identification of the MET 12 strain is a strain from the *Pseudomonas* genus. The exact species of MET 12 is still uncertain as the 16S DNA matched with both *Pseudomonas* sp. and *Pseudomonas vancouverensis* strains (Table 3.3). The gram coloration confirmed that both bacteria were gram-negative (Figure 3.15).

Metazachlor

The MZ 3 and MZ 1 strains were both identified as *Pseudomonas* genus strains. The MZ 3 strain showed the highest similarity with several Pseudomonas sp. strains and should be a Pseudomonas sp. (Table 3.3). The exact species of MZ 1 is still unclear as it showed the same similarity with several Pseudomonas sp. and Pseudomonas poae strains. The MZ 11 strain was best described as a *Variovorax*

genus bacterium. The species identification remains uncertain as the 16S rDNA sequence showed 96% of similarities with *Variovorax paradoxus* and *Variovorax* sp. yged136. All MZ strains were confirmed as gram-negative bacteria (Figure 3.15).

Bentazon

The strain BTZ 2 was identified as a strain from the *Pseudomonas* genus. Indeed, its 16S rDNA sequence was similar with one strain of *Pseudomonas helmanticensis* and several strains of *Pseudomonas* sp. (Table 3.3). BTZ 14 and BTZ 12 were both identified as strains from the *Cupiravidus* genus. The the probable species of BTZ 14 is *Cupriavidus* sp. as it showed high similarity with four *Cupriavidus* sp. strains. The species of BTZ 12 is more uncertain as it showed similarities with *Cupriavidus* sp. and *Cupriavidus necator*. All MZ strains were confirmed as gram-negative bacteria (Figure 3.15).

S-metolachlor

SM 14 was identified as a strain of the *Ancylobacter* genus (Table 3.3). The best match was for one strain of *Ancylobacter defluvii*. The SM 12 was best related with an uncultured beta proteobacterium, affiliated with the *Duganella* and *Pseudoduganella* genus. This is consistent with the fact that SM 12 is not readily culturable on solid LB. The SM2 strain was identified as a strain from the *Pseudomonas* genus. The exact species remains uncertain as the 16S rDNA sequence showed the same similarity with several *Pseudomonas* species. All SM strains were confirmed as gram-negative bacteria (Figure 3.15).

Chlortoluron

The strain CHL 1 was closest to a strain of *Arthrobacter* sp. (Table 3.3) and was the only gram-positive isolated bacteria (Figure 3.15). The strain CHL 9 had the closest phylogenetic affiliation with *Sphingobium* sp. Z007.

Table 3.3: Phylogenetic affiliation of selected strains based on 16S rDNA sequencing. All E-values were equivalent to 0.

Strain	Closest phylogenetic affiliation	Query coverage	Identities	Max Score	Accession	Gram
MET 8	Cupriavidus metallidurans strain AU4057	98%	934/964(97%)	1611	AY860234.1	negative
MET 12	Bacterium strain BS0333	89%	1278/1305(98%)	2246	MK823521.1	negative
	Pseudomonas sp. XBGSY2	89%	1278/1305(98%)	2244	KJ184897.1	negative
	Pseudomonas vancouverensis strain OB155	89%	1279/1307(98%)	2241	KF424309.1	negative
	Pseudomonas vancouverensis strain A-18	89%	1279/1307(98%)	2241	HQ202824.1	negative
MET 17	?	?	?	?	?	negative
MZ 3	Pseudomonas sp. SRWI 108	99%	1181/1213(97%)	2047	CP077086.1	negative
	Uncultured Pseudomonas sp. clone 96	99%	1181/1213(97%)	2047	JF500973.1	negative
	Pseudomonas sp. strain MTR-48-522	99%	1181/1213(97%)	2047	FN666552.1	negative
	Pseudomonas sp. strain MTR-48-521	99%	1181/1213(97%)	2047	FN666551.1	negative
MZ 11	Variovorax paradoxus strain DJG-7	96%	1263/1322(96%)	2084	MF101074.1	negative
	Variovorax sp. yged136	96%	1263/1322(96%)	2084	EF419341.1	negative
MZ 1	Pseudomonas sp. strain CP19	100%	1076/1078 (99%)	1978	MH813400.1	negative
	Pseudomonas poae strain HTM601-1	100%	1076/1078 (99%)	1978	MG835948.1	negative
	Pseudomonas sp. BR3-39	100%	1076/1078 (99%)	1978	EU853186.1	negative
	Pseudomonas sp. BR2-22	100%	1076/1078 (99%)	1978	EU853183.1	negative
	Pseudomonas sp. BR2-02	100%	1076/1078 (99%)	1978	EU853182.1	negative
BTZ 2	Pseudomonas helmanticensis strain 2-1R	99%	1237/1258 (98%)	2191	MK070159.1	negative
	Pseudomonas sp. strain PCH128	99%	1237/1258 (98%)	2191	MF774114.1	negative
	Pseudomonas sp. Tibet-YD5003-3	99%	1237/1258 (98%)	2191	KF805078.1	negative
	Pseudomonas sp. Dma28	99%	1237/1258 (98%)	2191	JQ977565.1	negative
	Pseudomonas sp. Dra16	99%	1237/1258 (98%)	2191	JQ977132.1	negative
BTZ 14	Cupriavidus sp. strain GJT	100%	1220/1242 (98%)	2156	MH900172.1	negative
	Cupriavidus sp. strain B4	100%	1220/1242 (98%)	2156	MF093186.1	negative
	Cupriavidus sp. strain S2-10	100%	1220/1242 (98%)	2156	KY357340.1	negative
	Cupriavidus sp. GT 4-03	100%	1220/1242 (98%)	2156	KM253091.1	negative
	Cupriavidus sp. strain NyZ417	100%	1220/1242 (98%)	2154	MW040526.1	negative
BTZ 12	Cupriavidus sp. strain MA118	99%	1233/1253(98%)	2187	MH699183.1	negative
	Uncultured Cupriavidus sp. clone DDGJ16	99%	1233/1253(98%)	2187	KR095620.1	negative
	Cupriavidus necator strain DZBT04	99%	1233/1253(98%)	2187	KM191297.1	negative
	Cupriavidus necator strain DZBT08	99%	1233/1253(98%)	2187	KM087341.1	negative
	Cupriavidus sp. SaCRH15	99%	1233/1253(98%)	2187	JX233515.1	negative
SM 14	Ancylobacter defluvii strain SK 15	99%	1115/1119 (99%)	2041	NR_133695.1	negative
	Ancylobacter sp. HS 7	99%	1112/1118 (99/%)	2036	GQ365188.1	negative
	Ancylobacter sp. Y25-102	98%	10971099 (99%)	2017	KT452770.1	negative
SM 12	Uncultured beta proteobacterium clone vj1_6a	97%	1206/1229(98%)	2130	JQ867274.1	negative
SM 2	Pseudomonas sp. Gwa5-10 \\\end{tabular}	100%	1229/1248(98%)	2185	KF578427.1	negative
	Pseudomonas fluorescens strain G20-18	100%	1229/1248(98%)	2185	CP075566.1	negative
	Pseudomonas sp. S10724	100%	1229/1248(98%)	2185	JX173286.1	negative
	Pseudomonas migulae strain T4	100%	1229/1248(98%)	2185	EU111685.2	negative
CHL 1	Arthrobacter sp. strain C20	99%	1236/1266(98%)	2152	MW867036.1	positive
CHL 9	Sphingobium sp. Z007	99%	1217/1245(98%)	2132	FN293045.1	negative
CHL 4	?	?	?	?	?	negative

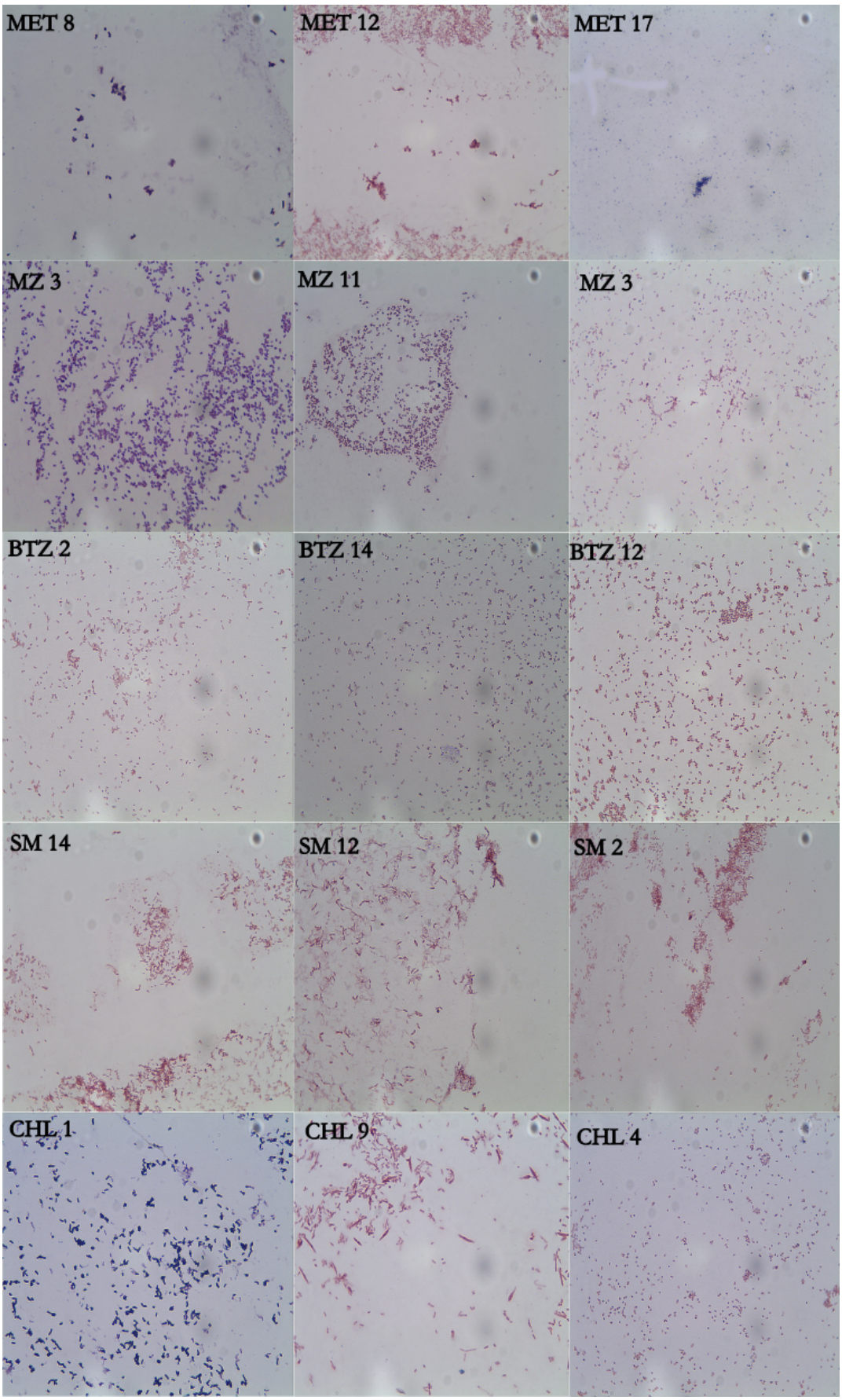


Figure 3.15: Pictures of selected strains after gram coloration. Red/pink colored strains are gram - and purple/blue are gram +.

3.3.2 Identification of fungal strains

An attempt to identify the genus of the 3 isolated fungi was made by means of morphological visualisation. This identification is not 100% reliable and confirmation should be carried out using molecular tools.

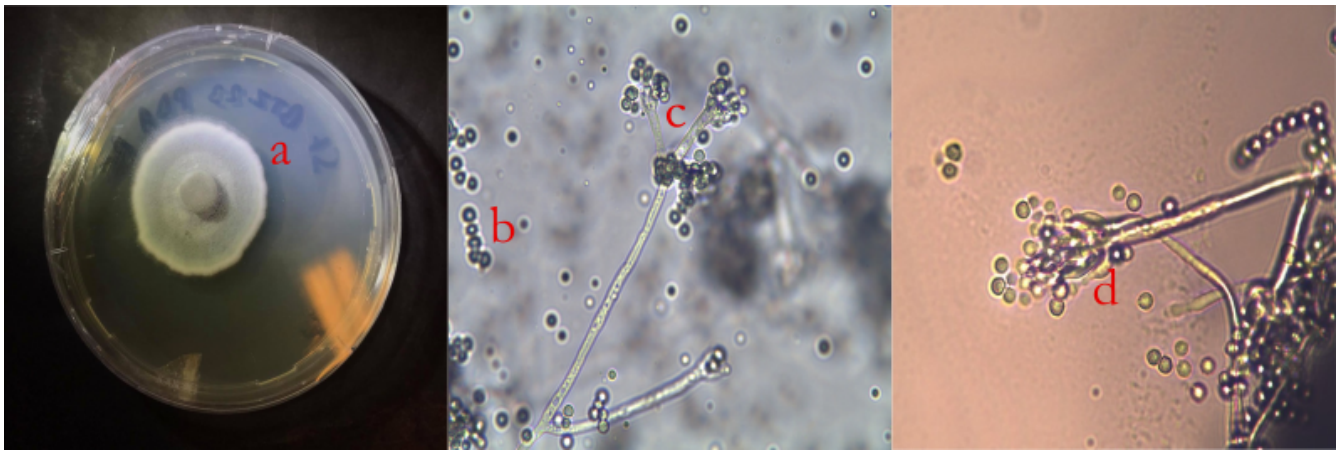


Figure 3.16: Pictures of the fungal strain BTZ 23 identified as *Penicillium* : (a) colony shape on PDA, (b) spore, (c) conidiophores and (d) bottle-shaped phialides.

The BTZ 23 strain produced 1-celled round spores. Colonies, spores, and other tissues were green to colourless. Spores were produced in unbranched chains. Conidiophores were simple or branched but not swollen at the apex. The spores are carried by bottle-shaped phialides clusters (Figure 3.16). It is then suggested that BTZ 23 is a strain of the genus *Penicillium*.

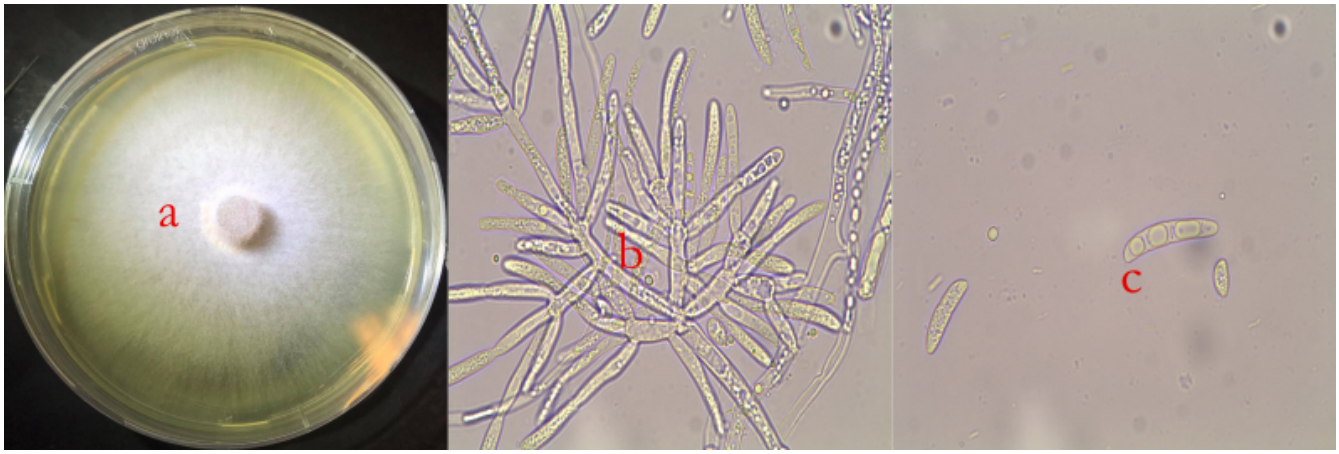


Figure 3.17: Pictures of the fungal strain SM 9 identified as *Fusarium* : (a) colony shape on PDA, (b) sporodochia and (c) macroconidia (spore).

The SM 9 strain produced spores with more than one cell with visible septa. Spores (macroconidia) were colorless to green, canoe shaped produced in clustered conidiophores forming sporodochia (Figure 3.17). SM 9 is therefore strongly suspected to be a strain of the genus *Fusarium*.

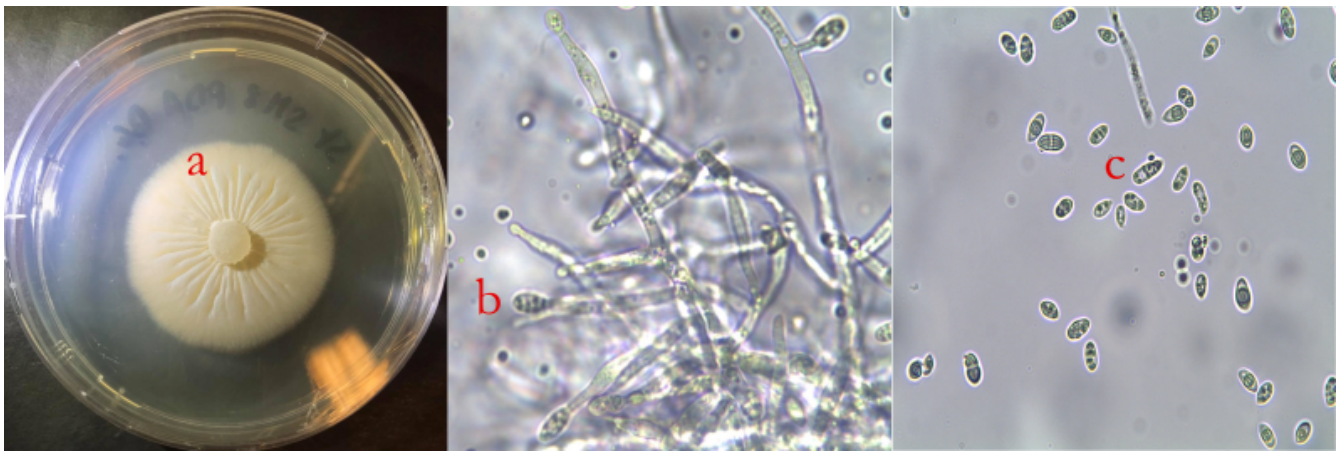


Figure 3.18: Pictures of the fungal strain SM 8 identified as *Fusarium* : (a) colony shape on PDA, (b) chlamydospores (spore) and (c) microconidia (spore).

The SM 8 strain produced minimum two different spores forms. The first spores were one or two-celled, spindle-shaped to ovoid and straight. The latter were multi-celled, rather round and terminaly or intercalary attached to the mycelium (Figure 3.18). These two spores may be microconiconidia and chlamydospores of a strain of the genus *Fusarium*.

3.4 Degradation of pesticides by selected cultures

3.4.1 Degradation of metamitron by MET 8, MET 12 and MET 17 strains

The metamitron concentration in the abiotic control was 42.55 mg/L. All three strains were defined a possible metamitron-degraders as they were able to decrease the pesticide concentration by more than 5% compared to "initial concentration" and abiotic flasks (Figure 3.19). The ranking of degradation performance was as follows: MET8 > MET17 > MET 12. The remaining metamitron concentration in the flasks inoculated with MET 8 > MET 17 > MET 12 was 10.89% > 8.25% > 7.56% and 8.58% > 5.81% > 5.17% lower than in the "initial concentration" and abiotic flasks, respectively.

The OD_{630} of the abiotic control increased steadily from 0.04 to 0.57 in 5 days (Figure 3.20), which was not the case for the degradation studies with the other pesticides. The growth rates of MET 8, MET 12 and MET 17 were higher than for *Escherichia coli* (Figure 3.20). MET 12 grew for up to 24 hours and then slowly decayed, whereas MET 8 and MET 17 grew for up to 48 hours and then declined more rapidly.

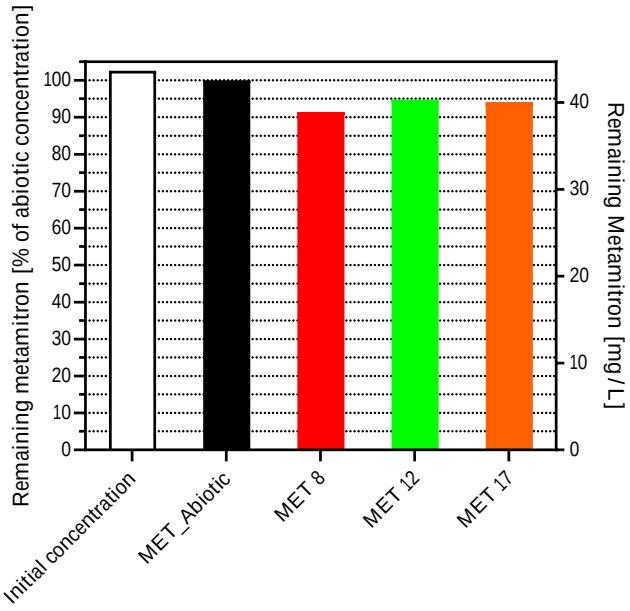


Figure 3.19: Degradation of metamitron by the stain MET 8, MET 12 and MET 17 in liquid MSM.

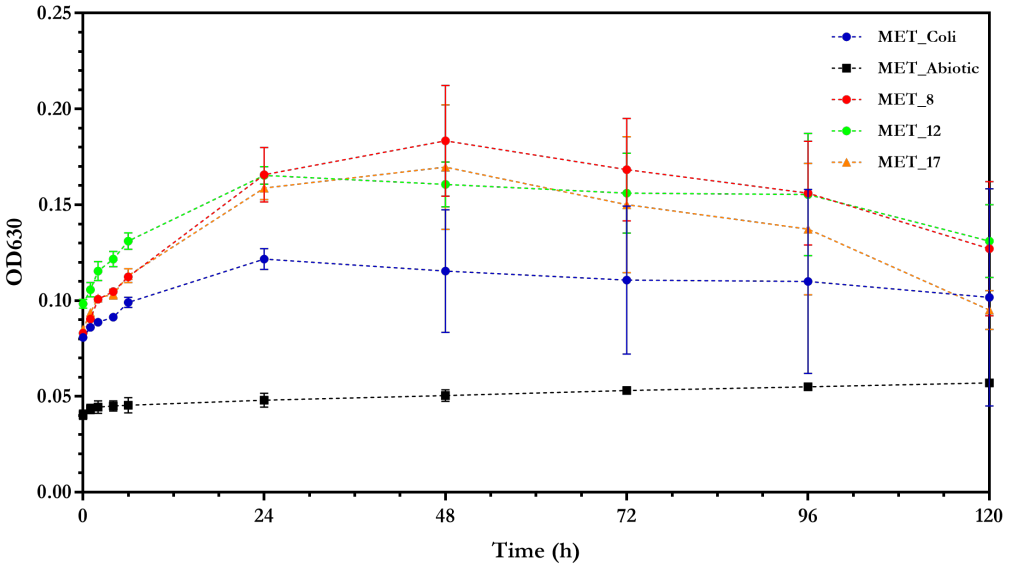


Figure 3.20: Growth curves of the stains MET 8, MET 12 and MET 17 during the degradation study of metamitron in liquid MSM. Error bars show the standard deviation.

3.4.2 Degradation of metazachlor by MZ 3, MZ 11 and MZ 1 strains

The metazachlor concentration in the abiotic control was 43.93 mg/L and was higher than in the "initial concentration" flask. The concentration of metazachlor was 5.57%, 1.36% and 3.41% lower than the abiotic control in flasks inoculated with MZ 3, MZ 11 and MZ 1, respectively (Figure 3.21).

Among the strains tested, only the MZ 3 strain was able to grow more significantly than *Escherichia coli* in liquid MSM + metazachlor (Figure 3.22). The MZ 11 strain grew more poorly than in the growth test for the screening of potential degrading strains (Figure 3.7).

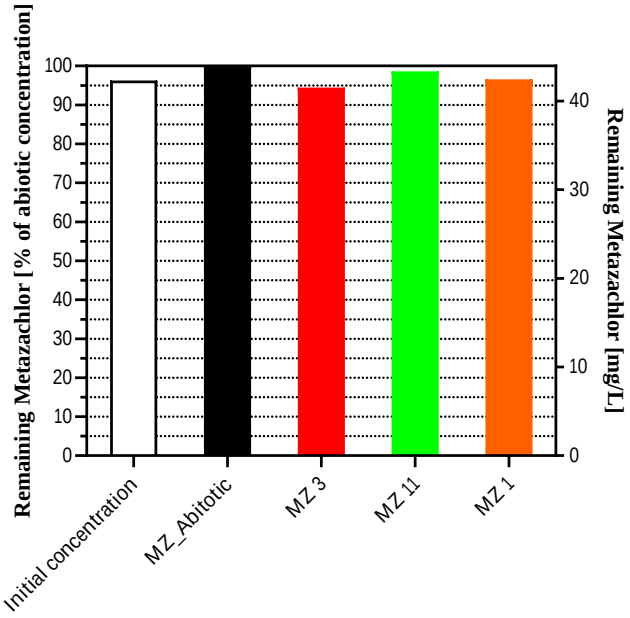


Figure 3.21: Degradation of metazachlor by the MZ 3, MZ 11 and MZ 1 strains in liquid MSM.

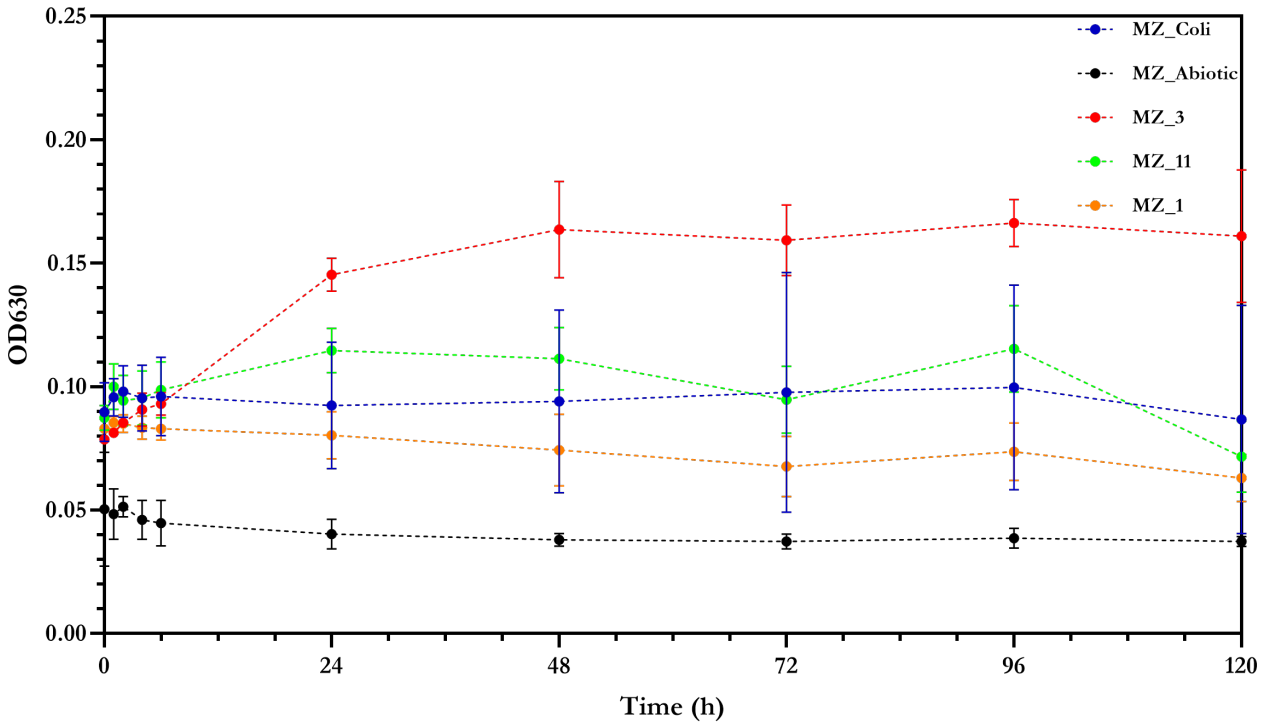


Figure 3.22: Growth curves of the stains MZ 3, MZ 11 and MZ 1 during the degradation study of metazachlor in liquid MSM. Error bars show the standard deviation.

3.4.3 Degradation of bentazon by BTZ 2, BTZ 14 and BTZ 12 strains

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The bentazon concentration in the abiotic control was 52.85 mg/L. The concentration of bentazon was 0.85% and 2.55% lower than the abiotic control in flasks inoculated with BTZ 2 and BTZ 12, respectively (Figure 3.23). The concentration of bentazon was 1.51% higher in flask inoculated with BTZ 14 than in the abiotic control. The bentazon concentration in all inoculated flasks was higher than in the "initial concentration" flask.

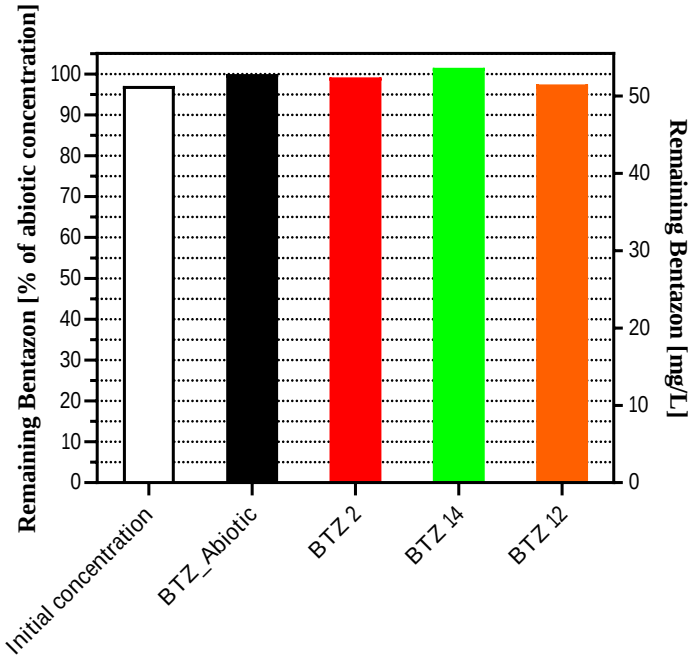

Figure 3.23: Degradation of bentazon by the BTZ 2, BTZ 14 and BTZ 12 strains in liquid MSM.

The growth of BTZ 2, BTZ 14 and BTZ 12 strains showed a similar trend to the growth test for the screening of potential degrading strains (Figure 3.8). BTZ 12 and BTZ 14 showed high growth up to 24 hours after inoculation, a plateau up to 96 hours and then a decline (Figure 3.24). BTZ 2 showed a much lower growth potential.

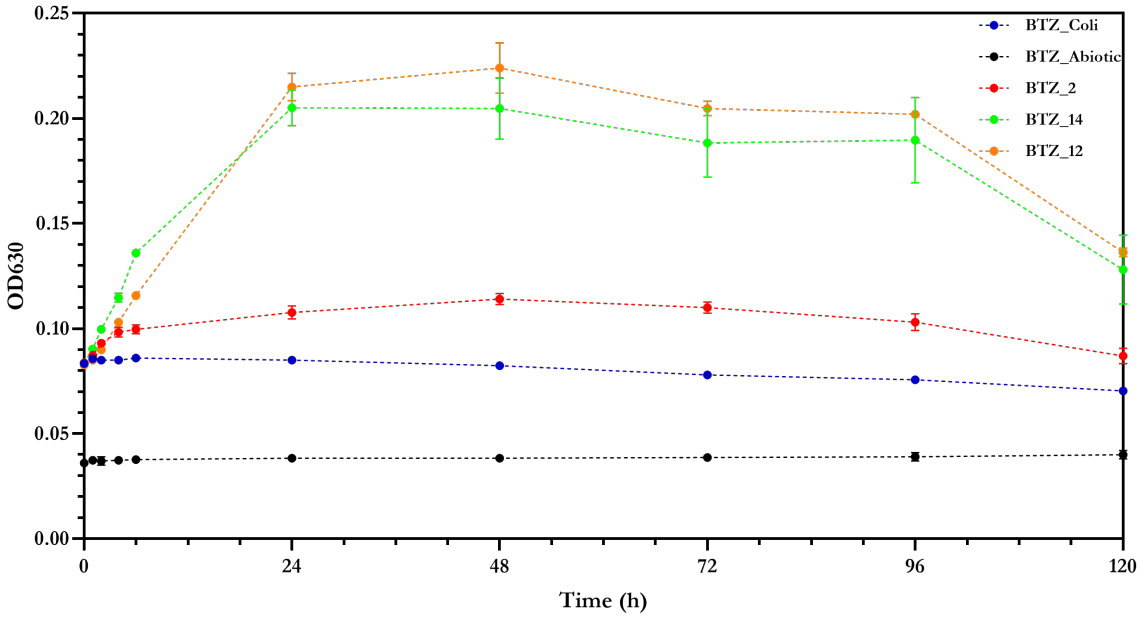

Figure 3.24: Growth curves of the stains BTZ 2, BTZ 14 and BTZ 12 during the degradation study of bentazon in liquid MSM. Error bars show the standard deviation.

3.4.4 Degradation of s-metolachlor by SM 14, SM 12 and SM 2 strains

All three strains were defined a possible metolachlor-degraders as they were able to decrease the metolachlor concentration by more than 5% compared to "initial concentration" and abiotic flasks (Figure 3.25). The ranking of degradation performance was as follows: SM 12 > SM 2 > SM 14. The remaining metolachlor concentration in the flasks inoculated with SM 12 > SM 2 > SM 14 was 17.52% > 13.82% > 6.80% and 22.55% > 19.07% > 12.47% lower than in the "initial concentration" and abiotic flasks, respectively. The SM 12 strain was the most efficient in degrading pesticides as it decreased the concentration of metolachlor the most with the lowest inoculation rate (OD_{630}).

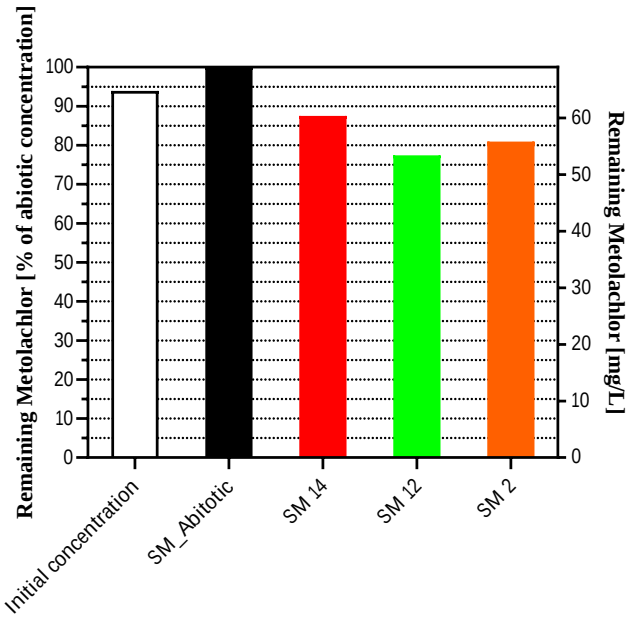

Figure 3.25: Degradation of s-metolachlor by the SM 14, SM 12 and SM 2 strains in liquid MSM.

The SM 12 strain was inoculated at an OD_{630} of 0.05 while the two other strains were inoculated at an OD_{630} of 0.08. Indeed, the bacterial biomass of SM 12 was not sufficiently important in the LB plates to inoculate the 20 mL of MSM at the same OD_{630}. SM 12 showed significant growth up to 6 h and decreased slowly over the next four days. As observed in the growth curves for the screening of potential degrading strains (3.9), the SM 14 strain showed a high intrinsic variability in growth. The SM 14 growth was, on average, lower in the degradation study than in the screening of potential degrading strains. The SM 2 strains showed no particular growth compared to *Escherichia coli*.

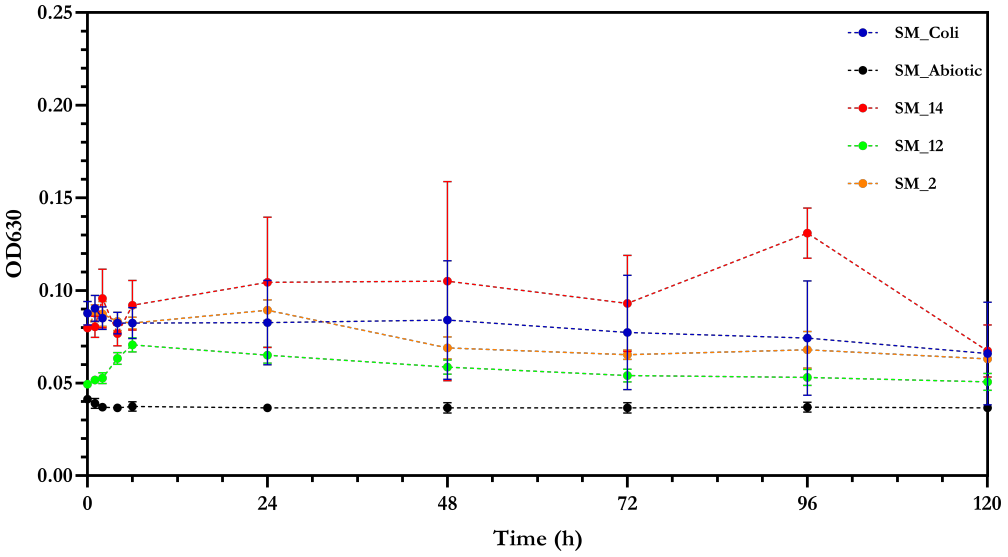

Figure 3.26: Growth curves of the stains SM 14, SM 12 and SM 12 during the degradation study of s-metolachlor in liquid MSM. Error bars show the standard deviation.

3.4.5 Degradation of chlortoluron by CHL 1, CHL 9 and CHL 4 strains

The chlortoluron concentration in the abiotic control was 33.55 mg/L. The concentration of chlortoluron was 4.62%, 0.15% and 3.87% lower than the abiotic control in flasks inoculated with CHL 1, CHL 9 and CHL 4, respectively (Figure 3.27).

The growth curves of CHL 1, CHL 9 and CHL 4 were consistent with the previous observations (Figure 3.10). CHL 9 and CHL 4 showed no particular growth compared to *Escherichia coli* (Figure 3.28). A slight growth was observed for CHL 1 during the first 24 hours after inoculation.

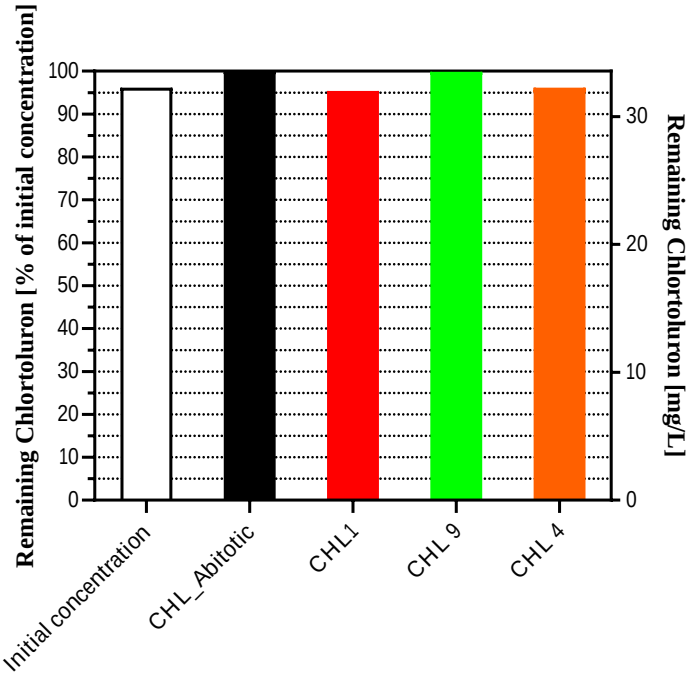

Figure 3.27: Degradation of chlortoluron by the CHL 1, CHL 9 and CHL 4 strains in liquid MSM.

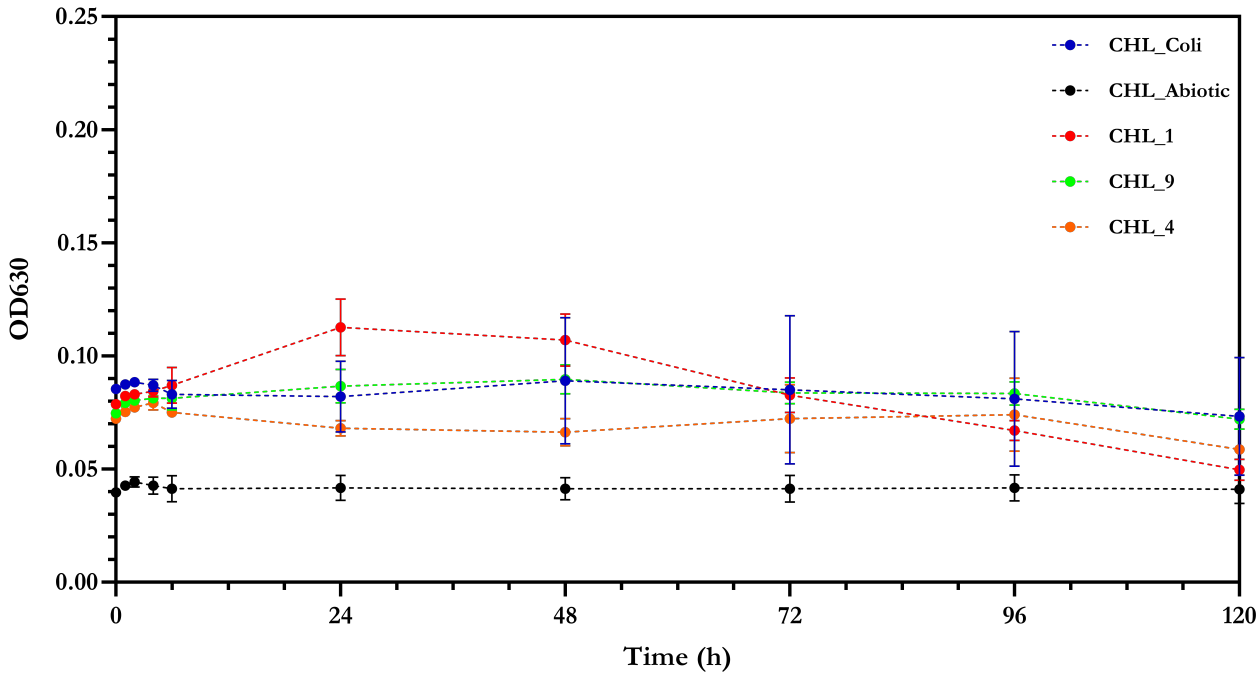

Figure 3.28: Growth curves of the stains SCHL 1, CHL 9 and CHL 4 during the degradation study of chlortoluron in liquid MSM. Error bars show the standard deviation.

3.5 Soil mini-column experiment

3.5.1 Saturated hydraulic conductivity

Among the 12 soil columns, two non-autoclaved soil columns showed high saturated hydraulic conductivity (K_{sat}) due to preferential water flow paths (Figure 3.29). These two K_{sat} values were defined as outliers because they were 1.5 times the interquartile range above the third quartile.

Figure 3.29: Soil column presenting preferential flow paths.

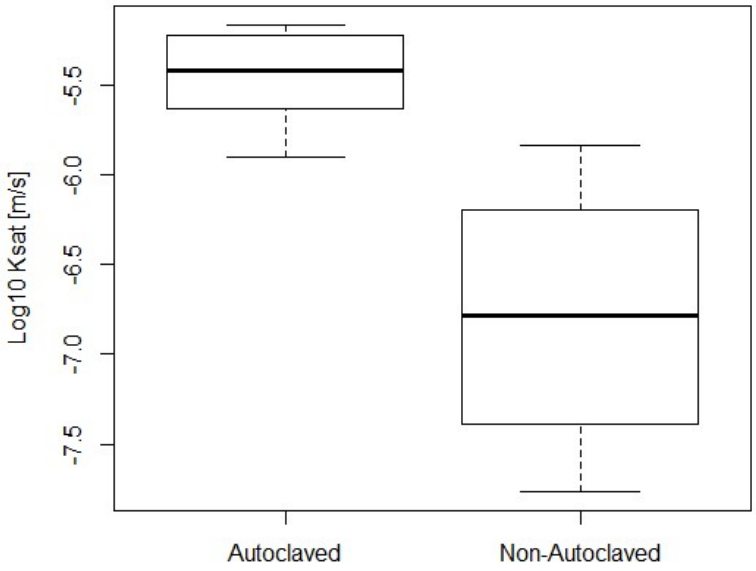

Figure 3.30: Boxplot of the saturated hydraulic conductivity of autoclaved and non-autolaved soil columns.

After eliminating outliers, normality and equality of the two variances were confirmed. The t-student test revealed that the K_{sat} of autoclaved columns was significantly higher than non-autoclaved columns (p-value = 0.01307) (Figure 3.30). The K_{sat} of autoclaved columns ranged from 1.27×10^{-6} m.s^{-1} (min) to 6.88×10^{-6} m.s^{-1} (max) which correspond to values found for a loam. For the non-autoclaved columns the K_{sat} ranged from 1.72×10^{-8} m.s^{-1} (min) to 1.47×10^{-6} m.s^{-1} (max).

3.5.2 Calcium chloride leaching

3.5.2.1 Calcium chloride concentration vs electrical conductivity calibration curve

The calibration curve performed showed a linear relationship between the calcium chloride concentration and the electrical conductivity (Figure 3.31). The intercept of the linear regression was set at 0.55 μS/cm, which is the electrical conductivity value measured for 0 mg/L $CaCl_2$ in demineralized water. The slope was found to be 1368 μS.cm^{-1}.g^{-1} of $CaCl_2$.

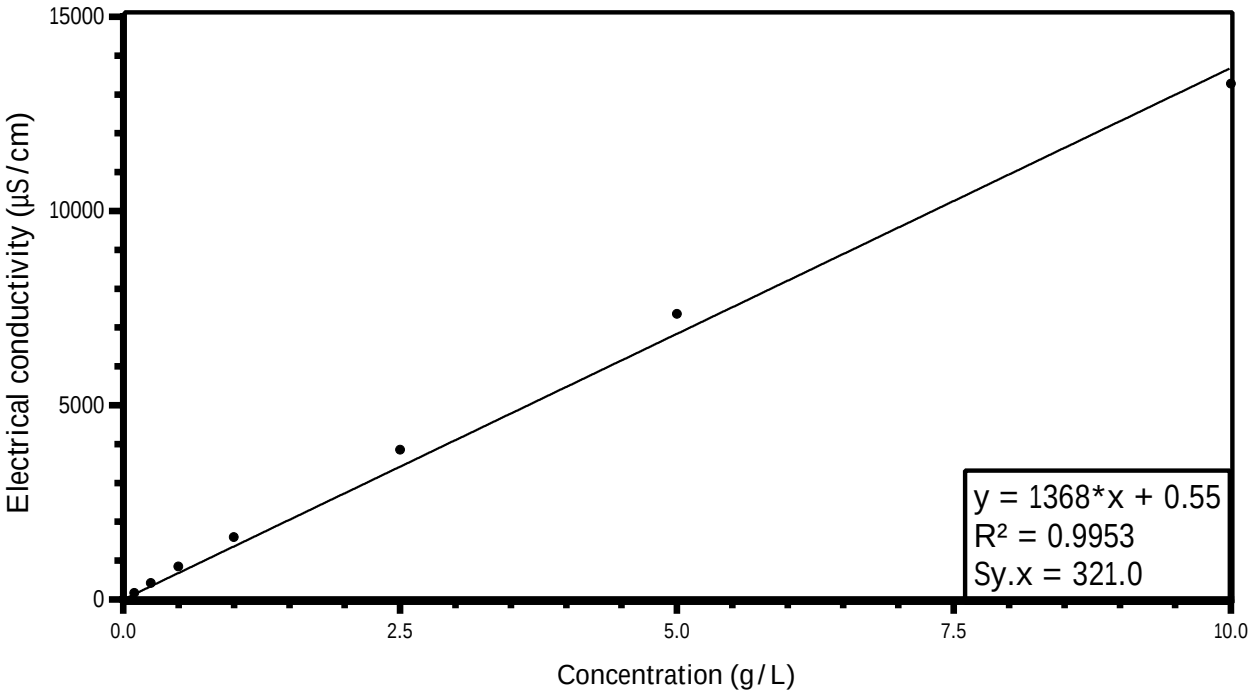

Figure 3.31: Calibration curve of the $CaCl_2$ concentration vs the electrical conductivity. Goodness of the fitting is evaluated by the R^2 and the Sy.x (or RMSE).

3.5.2.2 Calcium chloride BTCs of non-autoclaved and autoclaved mini-columns

Only the calcium chloride BTCs of high flow rate non-autoclaved and autoclaved columns (NAH & AH) were fitted to the CDE (2.2) model using the CXTFIT program. Indeed, two columns of the low flow rate modality showed high preferential flow paths and were directly excluded from the study. Moreover, the low flow rate modality was far from the steady state flow assumed in the CDE equation. The high flow modality comes closer to these conditions with two columns (NAH-1 and NAH-2) that showed steady state saturated flow due to low infiltration rates. The other 4 columns (NAH-3, AH-1, AH-2 & AH-3) did not show steady state flow with higher infiltration rates but were assumed to be.

The modelled BTCs could describe the experimental data with determination coefficient between 0.86 to 0.99 (Figure 3.32). The fit to NAH-1 BTCs is considerably poorer due to a lack of data on day 2.

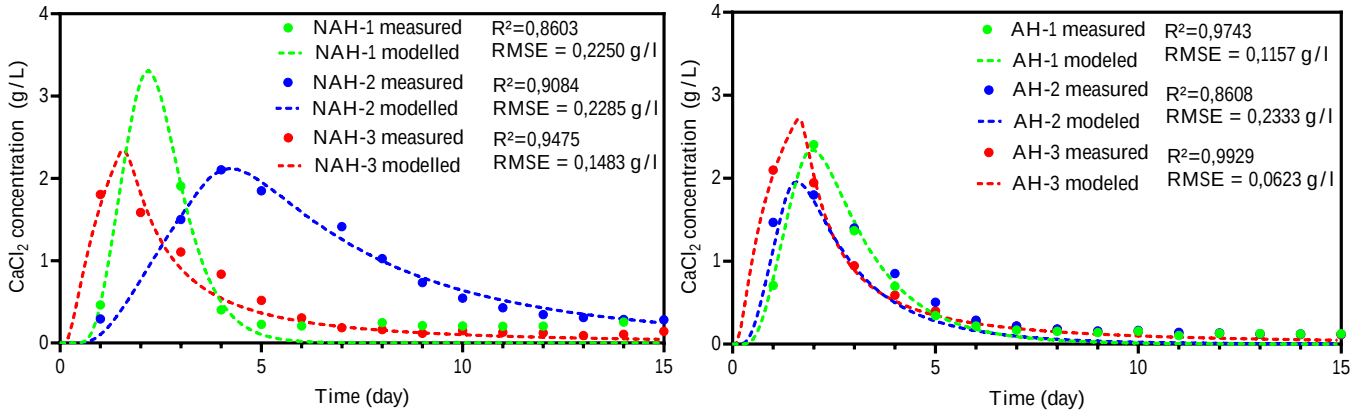

Figure 3.32: Observed and modelled BTCs of $CaCl_2$ for non-autoclaved (NAH) and autoclaved (AH) columns supplied by a high water flow rate (1.415 cm/j). Concentrations of the leachates is plotted against the time.

The estimated transport parameters v, R, D and pulse duration are presented in the table 3.4.

Table 3.4: Estimated transport parameters for $CaCl_2$ in the non-autoclaved and autoclaved mini-columns using the CDE model.

Column	k_sat (m/s)	q (cm/day)	v (cm/day)	D (cm^2/day)	R (-)	Pulse duration (day)	λ = D/v (cm)	θ = q/v ($cm^3.cm^3$)
NAH-1	2,80E-07	0,8009	2,9870	1,8170	0,7863	1,23	0,6083	0,2681
NAH-2	9,52E-08	0,6877	2,9900	9,4700	2,7900	2,978	3,1672	0,2300
NAH-3	1,47E-06	1,2750	9,7600	103,0000	5,6500	1,37	10,5533	0,1306
AH-1	1,27E-06	1,4500	30,8000	48,1000	9,9300	1,19	1,5617	0,0471
AH-2	6,07E-06	1,4520	3,6400	8,7700	1,1900	1,51	2,4093	0,3989
AH-3	3,86E-06	1,4380	4,6460	76,0000	2,8800	1,52	16,3582	0,3095

The total amount of $CaCl_2$ eluted seems to be greater than the amount applied in all columns except for NAH-1. This excess may be due to the desorption of naturally occurring electrolytes in the soil. The most probable reason that can explain why NAH-1 showed a different pattern is the lack of data on day 2 which may be due to a leakage in the column. As soon as NAH-1 is excluded from the observations, no clear effect of autoclaving on the total amount of eluted $CaCl_2$ can be found.

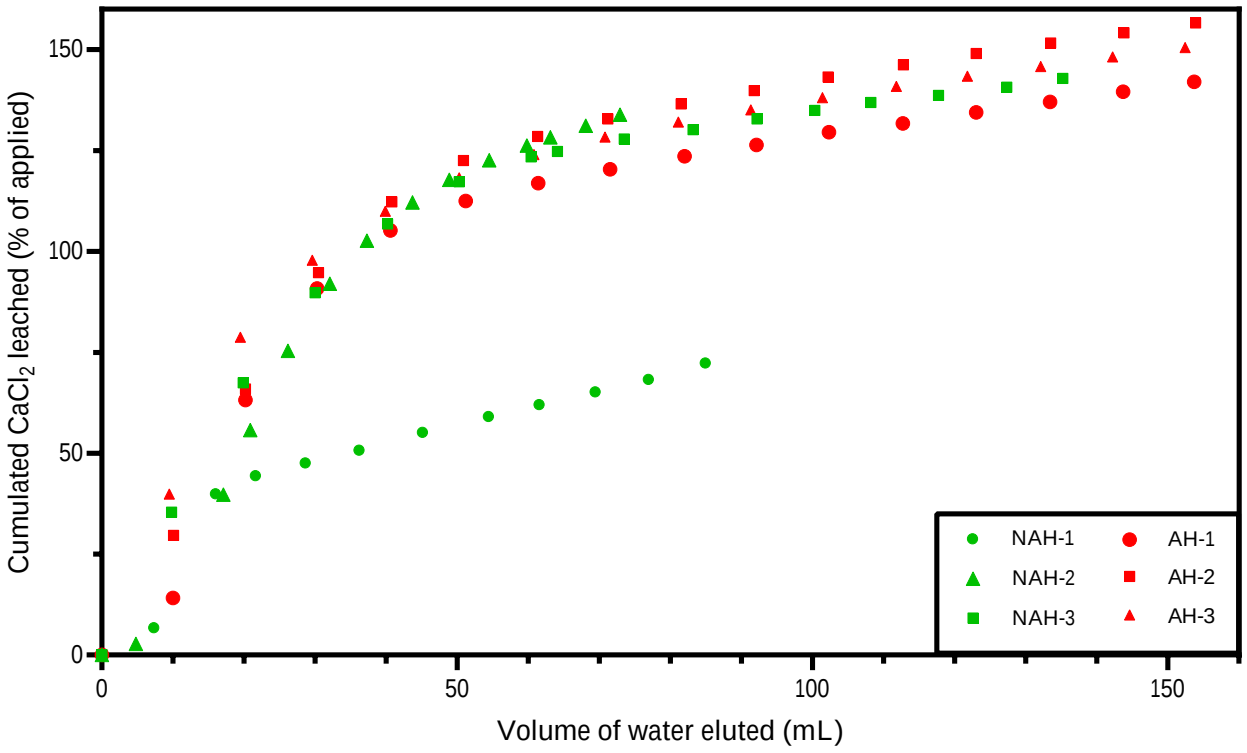

Figure 3.33: Cumulated $CaCl_2$ leached during the 15 days of experiment against the volume of water eluted.

3.5.2.3 Effect of autoclaving on leachate pH

It was observed that the leachate from autoclaved columns became yellowish in colour, compared to non-autoclaved columns (Figure 3.34). This may be due to the release of dissolved organic carbon after autoclaving, which has been reported to slightly decrease the soil pH due to an augmentation of dissolved organic acids (Berns *et al.*, 2008).

To verify this hypothesis, the pH of the first 10 ml of leachate from non-autoclaved and autoclaved columns was compared. Because variances in non-autoclaved column was much higher than in autoclaved columns, the non-parametric equivalent of the independent two-sample t-test was used (Mann-Whitney U test). Despite the mean pH was lower for the autoclaved columns, no significant pH difference (0.05 level of significance) was found since the U-value (=12) was above the critical value of the Mann-Whitney U-table (=5) (Figure 3.35).

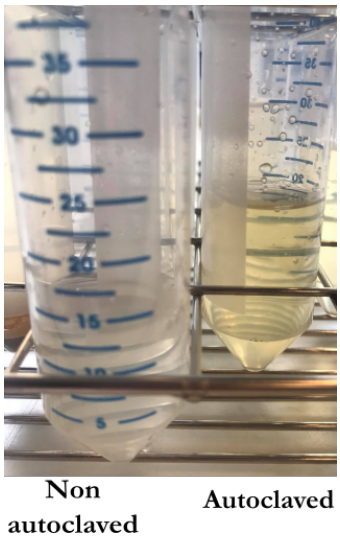

Figure 3.34: Picture of the leachates from non-autoclaved and autoclaved columns.

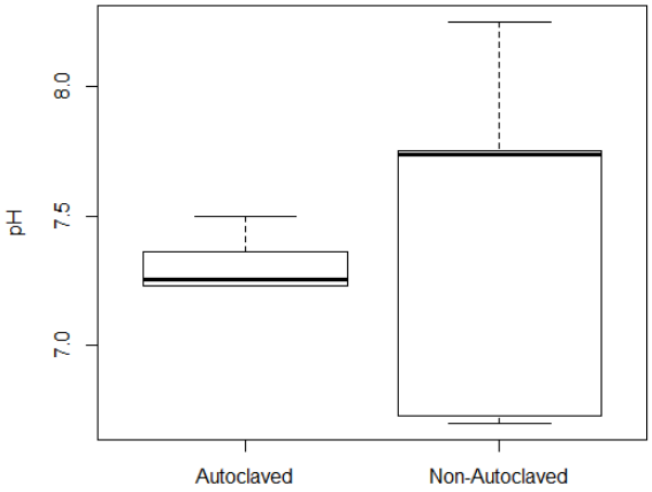

Figure 3.35: Boxplot of pH of leachates from non-autoclaved and autoclaved columns.

3.5.2.4 Saturated hydraulic conductivity and Calcium chloride leaching

Pearson correlation between the K_{sat} and the amount of $CaCl_2$ leached was studied using the method described in http://www.biostat.ulg.ac.be/pages/Site_r/corr_pearson.html. The mass of $CaCl_2$ leached was selected after passing 20 ± 2 ml of water through the column after the $CaCl_2$ pulse. After a logarithmic transformation of the K_{sat}, a significant correlation was found between the $\log_{10}K_{sat}$ and the amount of $CaCl_2$ leached with 20 ± 2 ml of water (p-value = 0.001119) (Figure 3.36). The Pearson correlation coefficient R was found to be 0.8190.

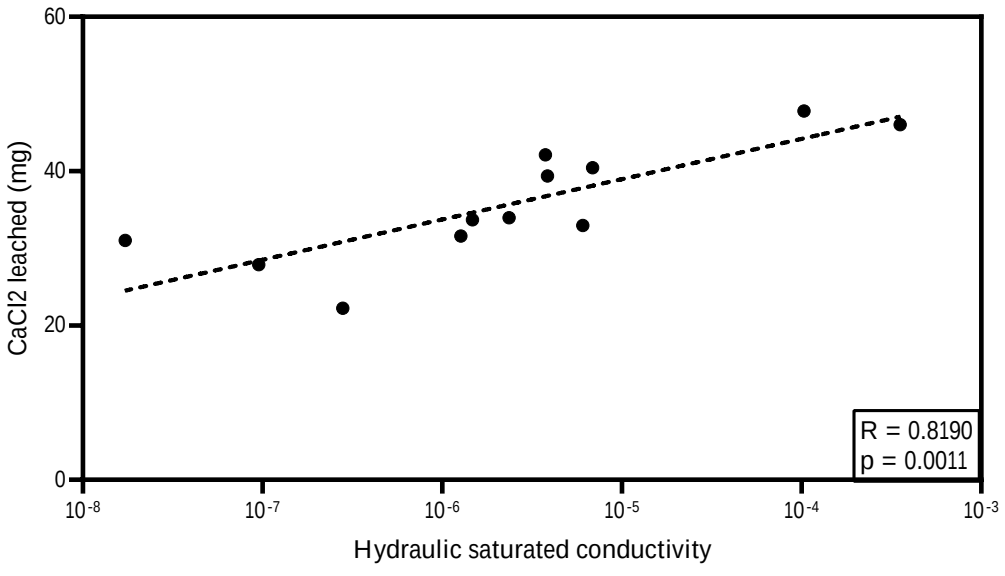

Figure 3.36: Correlation between the $\log_{10}K_{sat}$ and the mass of $CaCl_2$ leached with 20 ± 2 ml of water.

4 Discussion

4.1 Isolation an characterization of pesticides degrading strains

Of the 15 strains selected from the 85 isolated, 7 were retained as possible pesticide degraders, until statistically confirmed by repeated studies. This means that 8 of the 15 selected strains studied and 78 of the 85 strains isolated are probably not degraders, making the selectivity of the enrichment cultures rather low. Turnbull *et al.* (2001) also found that only 16% of isolated diuron-resistant strains were able to degrade diuron. Indeed, in selective enrichment cultures some non-degraders microbes may thrive using dead cells or predating microbes as an alternative source of carbon (Turnbull *et al.*, 2001). Other microbes, called "cheaters", that do not produce exo-enzymes can also use the degradation products provided by the "cooperators" as a source of nutrients (Schuster *et al.*, 2010). In addition, the presence of impurities in the agar may provide an additional source of nutrients for resistant non-degrading microbes that have survived during the enrichment cultures (Turnbull *et al.*, 2001). Moreover, additional carbon source in the pesticides commercial product used for the enrichment cultures (such as ethylene glycol, glycerol, propane-1,2-diol, ...Table 2.1) may be used by microbes. It is also possible that degrading microbes lose their ability to degrade pesticides after repeated subcultures (Parekh *et al.*, 1996). The opposite phenomenon is also possible with horizontal transfers of pesticides-degradation genes during the enrichment cycles and subculturing (Mandelbaum *et al.*, 1995; Bazhanov *et al.*, 2016).

During the pesticide degradation studies in liquid MSM, it was attempted to use initial solutions with a pesticide concentration of 50 mg/L. However, it seems that this exact concentration was not reached, certainly due to inaccuracies in pipetting and in the concentration in the pesticide stock solutions provided. This may also be due to the non-purity of the pesticide stock solutions, due to their degradation in the container. For the metazachlor, bentazon, s-metolachlor and chlortoluron studies, the initial concentration of pesticides was lower than the abiotic control, which was not expected. This is certainly due to both the inaccuracies of the pesticide analysis method (which is a maximum of 5%) and impressions in the preparation of the pesticides solutions. Moreover, the sample called "initial concentration" does not correspond to the real initial concentration because it was analysed at the same time that the other samples. The only difference between the "initial concentration" and the abiotic control is the incubation condition. The "initial concentration" was maintained at 4°C without agitation while the abiotic control was under the same conditions as the test samples (at 28°C and 150 rpm agitation). As the abiotic control and the test samples were kept under the same conditions, the test samples may be more comparable to the abiotic controls than to the "initial concentration" sample. Nevertheless, possible pesticide degraders were defined as the ones able to decrease the pesticide concentration by more than 5% of the initial concentration and of the concentration in the abiotic control. However, no statistical interpretation can be made as the degradation studies were only carried out on a single replica. This provides an exploratory approach before statistically valid tests can be carried out in further work.

4.1.1 Isolation, selection and identification of potential metamitron-degrading strains

In the fourth enrichment culture with metamitron, the reddish coloration observed in the PHYTO and NO-PHYTO flasks was suspected to be due to the appearance of a metabolite of metamitron or a metabolite of an additive to Goltron 700SC (Figure 3.1). To our knowledge, this has never been observed. As none of the isolated colonies with metamitron has been able to cause the colour change separately (Figure 3.2), the origin of this phenomenon remains unexplained. Without attempting to draw any conclusions, as no single isolated organism was able to perform this transformation this could be due to a microbial degrading consortia. Indeed, consortia have been reported to be more effective in pesticides degradation (Bollag and Liu, 1991), influenced by metabolic cooperative interactions between species (Smith *et al.*, 2005). A second possibility is that a high degrading strain was present in the fourth enrichment culture with metamitron but could not be isolated because of its inability to grow on MSMA.

This colour change may also be the reason for the increase in OD_{630} of the abiotic control during the growth test for screening of potential degrading strains, as well as during the degradation study (Figure 3.6 & 3.20). Indeed, it is unlikely that this increase is due to bacterial contamination due to the repetition of these observations. Furthermore, in the degradation study, the abiotic concentration was lower than the "initial concentration". A slow abiotic degradation of metamitron was found under similar conditions (Parekh *et al.*, 1994; Fang *et al.*, 2016). This increase may be due to an increase in the 630 nm ray reflection of the solution due to the abiotic transformation of the metamitron. This abiotic transformation reflected by a steady increase in OD_{630} was also observed in some inoculated samples. This is observed after the degradation phase, when there are probably no more living cells, as shown in the MET 17 growth curve (Figure 3.6).

Even tough MET 8 (*Cupriavidus metallidurans*) > MET 17 (consortium) > MET 12 (*Pseudomonas*) were found to be probably able to degrade metamitron, their degradation rates were lower than those performed by the metamitron-degrading strains previously described (Engelhardt *et al.*, 1982; Parekh *et al.*, 1994; Fang *et al.*, 2016). The *Cupriavidus metallidurans* CH34 strain, which is geneticaly close to MET 8, is known as a trace elements resistant strain and has been reported to be able use aromatic compounds as sole carbon and energy source (Millacura *et al.*, 2017). Many strains of *Pseudomonas* capable of metabolising herbicides have been isolated (Singh and Singh, 2014). Indeed, Pseudomonas are the most widely studied species in the degradation of phenolic compounds, such as pesticides (Wasi *et al.*, 2013). All these potential metamitron-degrading bacteria are gram-negative (Figure 3.15). Until now, the metamitron degraders isolated from soil were all gram-positive *Actinobacteria* (Engelhardt *et al.*, 1982; Parekh *et al.*, 1994 ; Fang *et al.*, 2016). However, it has recently been found that soil metamitron-degraders can be dominated by gram-negative bacteria that act as primary degraders, with gram-positive bacteria being their consumers (Wang *et al.*, 2021).

The PO activity produced by the metamitron-degrading strains seems to have no effect on the degradation rates of metamitron. Indeed, the MET 8 strain, which has no PO activity, show the highest degradation rate. Thus degradation rates may be more related to the growth behaviour with metamitron as carbon and/or nitrogen source. Indeed, previously isolated metamitron-degrading bacteria were able to use it as sole carbon source (Engelhardt *et al.*, 1982; Parekh *et al.*, 1994; Fang *et al.*, 2016). However, the growth curves of MET 8, MET 12 and MET 17 compared to the analysis of metamitron residues may suggest that metamitron was not the limiting factor for their growth (Figure 3.20). Indeed, the growth curves flatten out after 48 hours while there is still metamitron in the flasks after 5 days. This may indicate that the exponential growth of MET 8, MET 12 and MET 17 during the first hours would have been enabled by an alternative carbon source present in the Goltron 700 SC. Indeed, the *Escherichia coli* growth curve also showed an increase of OD_{630} during the first 24 h suggesting the presence of an alternative source of carbon. This may indicate that the ethylene glycol in the Goltron 700 SC formulation could be a more readily available carbon source (Table 2.1). *Escherichia coli* was found to use poly-ethylene

glycol to sustain its growth (R *et al.*, 1995) while ethylene glycol showed no impact on several *Cupriavidus metallidurans* strains growth (Houdt *et al.*, 2018). Several *Pseudomonas putida* were also found to be able to metabolize ethylene glycol (B *et al.*, 2012).

Another limiting factor may be the absence of nitrogen to supply growth. Indeed, Parekh *et al.* (1994) found that the metamitron metabolizing strain *Rhodococcus* sp.0246b could not grow in presence of metamitron as sole carbon source without an additional source of nitrogen. Growth curves and degradation kinetics, with and without an additional carbon/nitrogen source, could be interesting to perform. This may allow to determine whether MET 8, MET 12 and MET 17 use metamitron as sole carbon/nitrogen source or whether they degrade it cometabolically.

4.1.2 Isolation, selection and identification of potential metazachlor-degrading strains

Seven metazachlor-resistant strains were isolated from the PHYTO plot while the other seven came from the NO-PHYTO plot. Indeed, no clear difference was observed in the abundance of colonies on MSMA Petri dishes inoculated with the fourth enrichment culture solution from PHYTO and NO-PHYTO plot. As a reminder, the PHYTO plot received 2.5 L/ha of Butisan S (metazachlor) a few weeks before the sampling, which was not the case for the NO-PHYTO plot. This may indicate that the recent application of Butisan S had no visible effect on the presence of metazachlor resistant strains in the PHYTO plot. A previous study indicated that application of the recommended dose of Fuego 500 SC (metazachlor) after 30 days did not affect the counts of oligotrophic soil bacteria, organotrophic bacteria, actinomycetes, Azotobacter spp. and fungi (Baćmaga *et al.*, 2014).

When selecting potential metazachlor-degrading strains, several bacterial growth curves with metazachlor as carbon and nitrogen source showed an oscillating dynamic (Figure 3.7). This oscillating dynamic is common and sustained when the population is small, nutrient supply is limited and mortality is high (Khatri *et al.*, 2012): this was the case here. Indeed, oscillating pattern arises from nutrient excesses and deficiencies, relative to the bacterial population. The bacterial population grows rapidly whenever there is an excess of nutrients. The population then exceeds the amount of nutrients available, creating a sudden nutrient deficit. Thus, the population rapidly decreases and undershoots the nutrient supply, and creating a nutrient surplus with dead cells (Allen and Waclaw, 2018). Metazachlor resistant strains presenting this oscillating growth pattern, which was the case here, may indicate that they were able to survive during enrichment cultures using dead cells as an alternative carbon/nitrogen source. However, *Escherichia coli* showed this oscillating dynamic with metazachlor but not with the other pesticides, which is still unexplained.

It seems that no significant abiotic degradation was occurring in abiotic flask which is consistent with the fact that metazachlor is not susceptible to direct photolysis and hydrolysis in water (Mantzos *et al.*, 2017; Ulrich *et al.*, 2021). MZ 11 (*Variovorax*) was not able to degrade metazachlor (Figure 3.21) despite the fact that two Variovorax sp. strains named as WDL1 and SRS16 were previously recognised as liuron (phenylurea herbicide) degraders (SR *et al.*, 2005; Horemans *et al.*, 2013). MZ 1 (*Pseudomonas*) strain, which had the highest PO activity also could not degrade metazachlor. The ability of the strain MZ 3 (*Pseudomonas sp.*) is more unclear. Indeed, the metazachlor concentration in flasks with the MZ 3 strain was lower (1,89% and 5,57%) than in both the "initial concentration" and the abiotic flasks. As the analytical error may be estimated at 5%, further statistically valid degradation studies are needed to determine whether or not MZ 3 can degrade metazachlor. The MZ 3 strain was also the only one to be able to grow during the degradation experiment (Figure 3.22). This could indicate that metazachlor is degraded to favour the growth of certain oligotrophic bacteria of the genus *Pseudomonas*. Indeed, it has previously been found that the application of high doses of metazachlor is positively correlated with the abundance of oligotrophic soil bacteria (Baćmaga *et al.*, 2014). In particular, it has been shown that

under natural oligotrophic conditions, bacteria of the genus *Pseudomonas* are predominantly present and that some can degrade pesticides (Lopez *et al.*, 2005). This is consistent with the fact that MSM is a medium that aims to mimic oligotrophic soil conditions and that 5 of the 13 bacteria identified in this study are members of the genus *Pseudomonas*.

To date, no metazachlor-degrading micro-organisms have been isolated. The discovery of a metazachlor-degrading strain may allow the determination, for the first time, of an individual metazachlor biodegradation pathway in soils (Mantzos *et al.*, 2016).

4.1.3 Isolation, selection and identification of potential bentazon-degrading strains

To date, the isolated bentazon-degrading micro-organisms have mostly been fungi. One bentazon resistant fungal strain (BTZ 23) was visually identified as a member of the *Penicilium* genus (Figure 3.16). Previously, the strain *Penicilium oxalicum* MET-F-1 have been found to be able to degrade the herbicide metolachlor (Chang *et al.*, 2020). Moreover, two basidiomycetes fungi identified as *Phanerochaete chrysosporium* and *Ganoderma lucidum* are able to degrade bentazon by laccase, lignin- and Mn-peroxidase activity (Castillo *et al.*, 2000; Da Silva Coelho *et al.*, 2010). As BTZ 23 showed similar ABTS oxidative enzymes to these two bentazon-degrading fungi, it would be interesting to investigate this isolated strain further.

The bentazon degradation studies with BTZ 2 (*Pseudomonas*), BTZ 14 (*Cupriavidus* sp.) and BTZ 12 (*Cupriavidus*) suggest that none of these bacteria were able to degrade bentazon (Figure 3.23). The BTZ 2 high enzymatic ability is not a surprise as an other *Pseudomonas* was found to produce the enzyme CumA which is a laccase like enzyme able to oxidise ABTS (Sheng *et al.*, 2017). However, the laccase-like enzymes produced by BTZ 2 that oxidized ABTS were not able to catalyze the bentazon degradation reaction, as opposed to the laccase of *Ganoderma lucidum* (Da Silva Coelho *et al.*, 2010). Indeed, many different laccase-like enzymes exist and have not the same properties (Arregui *et al.*, 2019). Furthermore, it was found that the laccase produced by *Polyporus pinsitus* could improve the binding of bentazon with humic substances, making it inoffensive (Kim *et al.*, 1997). The same reaction could be catalyzed by the oxidative enzymes produced by BTZ 2 and should be investigated by carrying out studies of bentazon transformation by BTZ 2 in presence of humic monomers such as catechol.

The two *Cupriavidus* strains BTZ 14 and BTZ 12 were able to grow on MSM with bentazon as carbon source but without degrading it. This may be due to the presence of an alternative source of carbon to support growth. As described for the metamitron degrading strains, this source of carbon may come from an organic additive of Bazagran SG. However, no organic additives are reported by the manufacturer of Bazagran SG. Another reason may be the ability of BTZ 14 and BTZ 12 to use CO_2 as sole carbon source. Indeed several *Cupriavidus* sp. and *Cupriavidus necator* strains have been found to be able to fix CO_2 and use it as carbon source (Agarwal *et al.*, 2019; Garrigues *et al.*, 2020; Panich *et al.*, 2021). This autotrophic ability could therefore have allowed them to survive and thrive during enrichment cultures.

4.1.4 Isolation, selection and identification of potential metolachlor-degrading strains

Two fungal strains (SM 8 and SM 9) resistant to metolachlor were identified as strains of the genus *Fusarium* by visualization (Figure 3.18 & 3.17). It is consistent with the fact that a strain of *Fusarium* sp. isolated from metolachlor-contaminated soil was previously found to degrade metolachlor cometabolically (Saxena *et al.*, 1987). Indeed, the isolated metolachlor-degrading microbes were mostly fungi and have been shown to play an important function in the dissipation of metolachlor in soils (White *et al.*, 2010;

Vryzas *et al.*, 2012; Sun *et al.*, 2020; Chang *et al.*, 2020). Confirmation is provided by a recent soil column study that found that *Fusarium* fungi were stimulated by metolachlor application at low concentration (Sun *et al.*, 2020).

The concentration of metolachlor in the abiotic flask was 6.5% higher than the "initial concentration". This may indicate the total error range as no significant abiotic degradation of metolachlor was observed previously by Saxena *et al.* (1987), Liu *et al.* (1991), Sanyal and Kulshrestha (2002) and Munoz *et al.* (2011). In soils, metolachlor was found to degrade only biologically (Accinelli *et al.*, 2001). SM 12 (which is a *β-Proteobacteria* close to *Duganella* and *Pseudoduganella* genus) could degrade metolachlor with the same order of magnitude as the metolachlor-degrading bacteria previously described (Saxena *et al.*, 1987; Munoz *et al.*, 2011). It was found in a soil column study that metolachlor application increased the amount of *Proteobacteria* in the soil (Li *et al.*, 2020).

The growth of the strain SM 14 (*Ancylobacter*) with metolachlor as only carbon source was found to be similar as the metolachlor-metabolizing strain *Bacillus simplex* found by Munoz *et al.* (2011). However, its degradation rate was lower. *Ancylobacter defluvii* strain SK 15 which is closely related with SM 14 was quite recently identified by Poroshina *et al.* (2013). The latter strain was cytochrome-oxidase positive and could use methanol and CO_2 as a source of carbon and energy. The use of CO_2 or 2-methylpropan-1-ol (contained in Dual Gold) as extra carbon source may indicate the SM 14 could grow as well as high metolachlor users with lower degradation rates.

SM 2 (*Pseudomonas*) did not grown with metolachlor as sole carbon and nitrogen source (Figure 3.26) which may indicate that it degrade it cometabolically. This is consistent with the fact that bacterial strains of *Actinomyces sp.*, *Bacillus circulans*, *Bacillus megaterium* were previously found to degrade metolachlor cometabolically (Saxena *et al.*, 1987). Moreover, Xu *et al.* (2008) found that mixed cultures containing different strains of *Pseudomonas* sp. and *Pseudomonas putida* were able to degrade metolachlor.

The fact that all three strains could degrade metolachlor may indicate that enzymatic PO activity assayed on ABTS is related to metolachlor degradation. Indeed, SM 14, which had the highest growth potential and the lowest PO activity, showed the lowest degradation rates. However, SM 12 did not show the highest PO activity but was able to degrade metolachlor the most. This can be explained by the fact that the PO activity of SM 12 may be underestimated as it was measured after growth on TY medium, which may not be an appropriate culture medium for SM 12 as it is not very cultivable on conventional media. Previous studies found that s-metolachlor degradation rate is correlated with soil dehydrogenase and phosphomonoesterase activities (Wołejko *et al.*, 2017). We may hypothesize that the oxidative enzymes produced by these three degrading strains tested on ABTS may be involved in the oxidation of the glutathione-metolachlor conjugate to metolachlor ESA (White *et al.*, 2010). This can be verified by analysing the metabolites of metolachlor during the degradation study. The presence of several metabolites may also attest to the ability of these degrading strains to perform successive degradation reactions of metolachlor or not. Indeed, most isolated metolachlor-degrading strains failed to mineralize it and actually contribute to one or only few degradation steps. To date, only the yeast *Candida xestobii* has been found to completely degrade metolachlor (Munoz *et al.*, 2011).

4.1.5 Isolation, selection and identification of potential chlortoluron-degrading strains

Abiotic concentration may indicate that no significant abiotic degradation of chlortoluron occurred during the degradation experiment (Khadrani *et al.*, 1999; Badawi *et al.*, 2009). Despite CHL 1 (*Arthrobacter* sp.) and CHL 9 (*Shpingobium* sp.) are both related to species that have previously been identified as degrading chlortoluron (Tixier *et al.*, 2002; Sun *et al.*, 2009), neither was able to degrade chlortoluron in this study (Figure 3.27). The same observation was made for CHL 4. It thus seems that the enzymatic

activity of CHL 9 and CHL 4 measured with ABTS assay is not related with chlortoluron degradation. *Arthrobacter globiformis* D47 was shown to degrade a wide range of phenylurea herbicides using a single degradation gene with broad substrate specificity (Turnbull *et al.*, 2001). This gene encodes a hydrolase that only catalyses the hydrolysis of the carbonyl group of phenylurea, which may not be related to the enzymes revealed by the ABTS test.

It was also found that chlortoluron degradation by *Sphingomonas* sp. strain SRS2 may give a rise to a reddish color in growth medium due to an unidentified metabolite. This reddish color was not observed during the chlortoluron enrichment cultures. This may indicate that no degradation strains, performing the same degradation pathway that *Sphingomonas* sp. SRS2 was present in our enrichment cultures. This ineffectiveness of the enrichment culture may be due to the presence of 1,2-Benzisothiazole-3(2H)-one which is a strong biocide contained in Chloortoluron 500 SC. Microtox assay revealed that chlortoluron is not that toxic against *Vibrio fischeri* (Tixier *et al.*, 2002). The higher bacterial toxicity of 1,2-Benzisothiazole-3(2H)-one compared to chlortoluron may have led to the selection of strains resistant to 1,2-Benzisothiazole-3(2H)-one rather than to chlortoluron. Moreover, 1,2-Benzisothiazole-3(2H)-one may be the reason of the overall bacterial inhibition observed during the growth of CHL isolated bacteria in MSM + Chloortoluron 500 SC (Figure 3.10).

Finally, all these observations are consistent with the general fact that repeated exposure to the same pesticide leads to microbial adaptation to the degradation of that same pesticide (Rouchaud *et al.*, 2000; Arbeli and Fuentes, 2007). Indeed, chlortoluron was the only one of the 5 herbicides that during the last 20 years has not been applied on the plot from which the strains were isolated.

4.2 Soil mini-column experiment

Hydraulic conductivity

The effect of autoclaving on several soil physico-chemical properties was studied in a soil mini-columns experiment. Our results suggest that the saturated hydraulic conductivity K_{sat} was significantly affected by autoclaving (Figure 3.30). Berns *et al.* (2008) reported the destruction of aggregates after autoclaving due to the breakdown of the polysaccharides holding the micro-aggregates. Contrary, it has been suggested previously that autoclaving may enhance the aggregation of clay into larger particles leading to reduction of surface area (Salonius *et al.*, 1967; Lotrario *et al.*, 1995). These differences may be due to different methods for aggregate determination (Berns *et al.*, 2008) as well as the relative importance of aggregates formation/destruction which may be influenced by soil types. Moreover, (Lotrario *et al.*, 1995) suggested that small soil pores may collapse after autoclaving due to pressurization and depressurization. We may speculate that the increase in hydraulic conductivity is due to the destruction of polysaccharides, weakening the soil aggregates against the pressurization and depressurization, leading to the collapse of small pores and its cracking. This is especially true since the destruction of polysaccharides may also reduce pore clogging, resulting in a higher conductivity (Sujatha *et al.*, 2021).

In a pesticide leaching experiment, the hydraulic conductivity of the soil can be important as it can control the sorption behaviour of pesticides. Indeed, pesticide sorption is a time-dependent process where the contact time between the soil and the pesticide is directly related to the pore water velocity (Kah and Brown, 2007). Thus, an increase in hydraulic conductivity caused by soil autoclaving can directly increase the pore water velocity, which leads smaller contact times and consequently a reduction in the adsorption of pesticides. This is illustrated here by the positive correlation found between the leaching behaviour of $CaCl_2$ and the hydraulic conductivity of the soil mini-columns (Figure 3.36).

Moreover, autoclaving seems to reduce the variability of soil hydraulic conductivity which is high in non-autoclaved columns. High variability is inherent in undisturbed soil columns and is exacerbate at small-scale by the uneven distribution of hydraulically active biopores and fissures. As the development

of heterogeneous pore spaces is induced by different levels of aggregation (Skvortsova and Rozhkov, 2011), the destruction of aggregates by autoclaving may result in a more homogeneous matrix leading to much uniform infiltration properties. Thus, repeatability between studies under autoclaved soil columns seems to be improved.

pH of the leachate

In this study, the pH of the soil leachate was not significantly affected by autoclaving despite a lower average pH for the autoclaved columns (Figure 3.35). It has already been reported that autoclaving lowers soil pH due to the release of humic acids from the soil organic matter (Shaw *et al.*, 1999; Berns *et al.*, 2008). Previous studies found that pH changes after autoclaving may depend on the soil buffering capacity and is thus soil dependent (Trevors, 1996). Dissolved organic matter was not measured but our visual observations may suggest a higher presence of dissolved organic matter in the leachate from autoclaved columns (Figure 3.34). The same visible change in the aqueous phase was observed by Berns *et al.* (2008). The uneven presence of dissolved organic matter and dissolved humic acid between autoclaved and non-autoclaved soils can lead to changes in the behaviour of pesticides in the soil solution. Indeed, pesticides may bind with dissolved organic matter leading to uneven sorption behaviour between autoclaved and non-autoclaved soils (Carmosini and Lee, 2009; Kim *et al.*, 1997).

It also seems that the variability of soil pH was reduced by autoclaving. Lowest pH were observed in soils with preferential flow paths while highest pH were observed in columns with the lowest hydraulic conductivity. Thus, pH variability seems to be linked with soil hydraulic conductivity. This decrease in pH variability can be explained by the fact that pH was measured after the $CaCl_2$ pulse. Indeed, autoclaved soil seems to have a more homogeneous structure, leading to more homogeneous infiltration rates and probably more homogeneous Ca^{2+} leaching behaviour. As Ca^{2+} is known for its buffering capacity of soils (BACHE, 1984), a more homogeneous Ca^{2+} contact time in autoclaved columns has probably led to more similar pH values. This buffering capacity of Ca^{2+} may also explain that no dramatic pH decrease was observed in the autoclaved columns.

Calcium chloride BTCs

The NAH1 modelled BTC curve converged to an estimated $R < 1$ which leads to a negative adsorption coefficient according to the equation 2.4 (See table 3.4). This means that the lower the concentration of $CaCl_2$ in the soil solution, the more $CaCl_2$ is adsorbed on the soil surface, which is contrary to thermodynamic laws. The BTC curve modelled by AH1 has converged to a high value of v which leads to an unreasonably low value of θ. Moreover, estimated longitudinal dispersivity λ was pretty high for NAH-3 and AH-3 columns. Wrong parameter estimation may also be due to unsteady flow within soil columns.

Furthermore, the mass balance of $CaCl_2$ was not met in almost all columns. Indeed, the cumulative mass of $CaCl_2$ leached was higher than the mass of $CaCl_2$ added at the top of most columns (Figure 3.33). This may be due to uneven adsorption/desorption and leaching of electrolytes from the soil column. Indeed, the electrical conductivity used to measure the concentration of the marker ($CaCl_2$) is actually sensitive to the presence of all electrolytes in the solution. Measuring other electrolytes than $CaCl_2$ in the leaching solution has probably led to biased $CaCl_2$ BTCs.

Given the presence of all these aberrations, it would be inappropriate to draw conclusions from these modelled parameters. Previous work reported the increase in the sorption coefficient of C-2,4-dichloro-UL-phenol through autoclaved soil compared to untreated soil (Shaw *et al.*, 1999). This increase in asdorption properties was supposed to be due to an indistinguishable combination of changes in pH, organic carbon and dissolved organic carbon.

5 Conclusion and outcomes

A total of 85 pesticide-resistant (19 for metamitron, 14 for metazachlor, 22 for bentazon, 15 for s-metolachlor and 15 for chlortoluron) strains were isolated from the plots of the AIL EcoFoodSystem experiment in Gembloux. These were obtained after four successive enrichment cultures with pesticides as sole carbon and nitrogen source. 82 strains were identified as bacteria while 3 were Ascomycota fungi. Among these 85 isolated strains, 27 presented a significant phenol oxidase activity compared to *Escherichia coli* on ABTS substrate. Moreover, the growth curves of the 82 isolated bacterial strain was performed on minimal medium with pesticides as sole carbon and nitrogen source. Growth curves of fungi were obtained on solid MSMA with pesticide as sole carbon and nitrogen source. On the basis of their ability to oxidise ABTS and to grow with pesticide as sole carbon and nitrogen source, 15 potential pesticide-degrading strains were selected to assess their pesticide degradation ability.

One uncultured beta proteobacterium SM 12 affiliated with the *Duganella* and *Pseudoduganella* genus, one strain *pseudomonas* SM 2 and one *Anclylobacter* SM 14 were highly suspected to be able to degrade metolachlor. They were respectively able to decrease the concentration of metolachlor remaining in liquid MSM with metolachlor as the only carbon and nitrogen source by $22.55\% > 19.07\% > 12.47\%$ after 5 days of incubation compared to an abiotic control. One *Cupriavidus metallidurans* MET 8 strain, one unidentified consortium MET 17 and one *Pseudomonas* MET 12 were also suspected to be able to degrade metamitron. They respectively could decrease the metamitron remaining concentration in liquid MSM with metamitron as the only carbon and nitrogen source by $8.58\% > 5.81\% > 5.17\%$ after 5 days of incubation compared to an abiotic control. There is some evidence suggesting that the MZ 3 strain identified as belonging to the genus *Pseudomonas* may be capable of degrading metazachlor. This should be confirmed with repeated degradation assessments. Pending verification, this would be the first isolated strain capable of degrading metazachlor.

These promising results only encourage further studies on these strains. Indeed, the first priority should be to carry out repeated degradation studies in liquid medium. The pesticides used for these studies should be the pure active substances instead of the formulated commercial products. Degradation studies should also be carried out in the presence of additional carbon and nitrogen sources to determine whether these strains degrade pesticides for growth or cometabolically. Moreover some key parameters such as pH, temperature and pesticides concentration may be adjusted to find optimal degradation conditions. Degradation kinetics can also be performed. Linear kinetics often reveal co-metabolism, while exponentially decreasing kinetics may reveal metabolism. These kinetic may be modeled by linear, single first order or Monod equations to find key degradation parameters of the isolates. Such studies, however, require a large number of laborious chromatographic pesticide analyses. In the case of metolachlor the colorimetric immunoassay (ELISA) procedure can be used to determine its concentration in water samples. This may allow repeated degradation studies with the 3 potential metolachlor-degrading strains isolated with lower precision but higher sample numbers. The capability of metolachlor degradation by the two isolated fungal strains (SM 8 and SM 9) may also be tested. Moreover, degradation pathways performed by isolated strains may be deduced from the pesticides metabolites found in the reaction solutions. The complete genome sequencing and genome functional annotation of a degrading strain may allow to find potential degradation genes encoding for degradation enzymes. Each gene coding for a specific enzyme may be linked to a specific reaction occurring in the degradation pathway of a pesticide.

Finally, it is necessary to transfer this laboratory knowledge to field conditions in order to assess the role played by these key species in the leaching behaviour of pesticides. To make this possible, an attempt was made to develop a pesticide leaching study in undisturbed soil columns which may be conducted under sterile conditions. In this study a focus was made on the effect of autoclaving on soil properties and the feasibility of using this method of sterilization for a pesticide leaching study in undisturbed soil columns.

Here was presented the effect of autoclaving on a soil column study. Although no clear interpretation can be made on the basis of the calcium chloride BTCs, this study has revealed that the autoclaving process has influenced the physicochemical properties of the soil. Soil hydraulic conductivity was found to be changed by autoclaving. No clear effect of leachate pH was found, but previous studies suggest that it could be influenced, as well as particle aggregation and soil organic matter. All these changes can lead to incomparable sorption profiles between biotic and abiotic experiments. This may lead to inaccurate assessments of the fate of pesticides under soil column experiments. Given the impact of autoclaving on the soil structure, it should be not recommended to use this method of sterilisation for intact soil cores. Gamma irradiation was found to be a less destructive sterilization method for soil structure (Lees *et al.*, 2018). The latter should be investigated for our purpose. Indeed, all sterilisation methods seem to have a different impact depending on the characteristics of the soil. However, sterilization may not be complete every time. For these reasons, the impact and effectiveness of the sterilization technique should be extensively tested before and during any soil abiotic experiments.

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Appendices

Table A-1: Historic exposure of the cemetery plot to Metamitron, Metazchlor, Bentazon and S-metolachlor.

Active substance	**Date**	**Dose (g/ha)**
Metamitron	13-04-11	/
Metamitron	11-05-11	/
Metamitron	06-05-16	352,16
Metamitron	25-05-16	451,49
Metazachlor	09-09-04	594,29
Metazachlor	12-09-06	551,72
Metazachlor	17-10-06	662,07
Metazachlor	14-09-07	1200
Metazachlor	07-10-13	691,88
Metazachlor	24-10-13	313,65
Bentazon	01-07-05	700
Bentazon	11-06-08	400
S-metolachlor	02-06-10	192
S-metolachlor	29-05-18	183,99
S-metolachlor	06-06-18	297,21