



Biodegradation of pharmaceuticals by microorganisms

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Abstract

The presence of pharmaceutical residues in water poses a serious threat to human health. The fate of many of these pharmaceuticals in the environment has not been thoroughly investigated. Sulfamethoxazole, sulfamethizole, trimethoprim and carbamazepine are among these pharmaceuticals with significant bioactivity that are considered to be persistent pollutants. The biodegradation of these compounds has been studied in this project in order to assess the fate of these pharmaceuticals in the environment. An easily degradable carbon source was added in these biodegradation experiments to optimize co-metabolism as a removal mechanism. Five microorganisms were used to determine if the selected drugs were biodegradable and, also, to identify the metabolites arising from their biodegradation.

It was demonstrated that biodegradation occurred for sulfamethoxazole, sulfamethizole and carbamazepine. Trimethoprim showed a high resistance to biodegradation. It appeared that the microorganism *Rhodococcus rhodochrous* showed a particular ability to degrade the pharmaceuticals. The presence of metabolites was confirmed by HPLC and mass spectra analyses.

Sommaire

La présence de résidus de produits pharmaceutiques dans l'eau représente une sérieuse menace pour l'environnement et la santé humaine. Le devenir de ces produits pharmaceutiques dans l'environnement n'a pas été adéquatement étudié. Le sulfaméthoxazole, le sulfaméthizole, le triméthoprimé ainsi que le carbamazépine sont parmi ces composés pharmaceutiques qui ont une importante bioactivité et sont considérés comme polluants persistants. Dans ce projet, la biodégradation de ces produits a été étudiée afin d'évaluer le devenir de ceux-ci dans l'environnement. Une source de carbone facilement biodégradable a été utilisée lors des expériences afin de stimuler le mécanisme d'élimination par co-métabolisme. Cinq microorganismes ont été utilisés afin d'évaluer la biodégradabilité des produits pharmaceutiques sélectionnés et aussi identifier les métabolites résultant de leur biodégradation.

Il a été démontré que la biodégradation est survenue pour le sulfaméthoxazole, le sulfaméthizole ainsi que le carbamazépine. Le triméthoprimé a quant à lui démontré une forte résistance à la biodégradation. Le microorganisme *Rhodococcus rhodochrous* a démontré une habileté particulière à dégrader les produits pharmaceutiques. La présence de métabolites a également été confirmée par analyse HPLC et spectrométrie de masse.

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Table of Contents

| | |
|--|------|
| Table of Contents | iv |
| List of Figures | vi |
| List of Tables | viii |
| List of Equations | ix |
| List of Equations | ix |
| I. Introduction | 1 |
| II. Review of Literature..... | 3 |
| 2.1 Rationale | 3 |
| 2.1.1 Problems related to pharmaceuticals..... | 3 |
| 2.1.2 Pharmaceutical regulations..... | 5 |
| 2.1.3 Consequences of pharmaceutical accumulation | 7 |
| 2.2 Pharmaceuticals studied..... | 8 |
| 2.2.1 Carbamazepine (CBZ)..... | 8 |
| 2.2.2 Sulfamethoxazole (SMX) and Trimethoprim (TMP) | 10 |
| 2.2.3 Sulfamethizole (SMZ)..... | 12 |
| 2.3 Metabolites..... | 12 |
| 2.4 Removal mechanisms of selected pharmaceuticals..... | 13 |
| 2.4.1 Photochemical degradation..... | 13 |
| 2.4.2 Biodegradation in activated sludge and pure culture..... | 14 |
| 2.5 Role of microorganisms acclimation & co-metabolism | 16 |
| 2.6 Microorganisms selected for this study..... | 17 |
| 2.6.1 <i>Rhodococcus rhodochrous</i> | 17 |
| 2.6.2 <i>Pseudomonas putida</i> and <i>Pseudomonas fluorescens</i> | 18 |
| 2.6.3 <i>Bacillus subtilis</i> | 18 |
| 2.6.4 <i>Aspergillus niger</i> | 18 |
| 2.6.5 <i>Sphingomonas herbicidovorans</i> | 19 |
| III. Materials & Methods..... | 20 |

| | | |
|-------|---|----|
| 3.1 | Biodegradation experiment..... | 20 |
| 3.1.1 | Media preparation | 20 |
| 3.1.2 | Enrichment and acclimation | 20 |
| 3.1.3 | Pharmaceutical solutions..... | 21 |
| 3.1.4 | Flask preparation..... | 22 |
| 3.1.5 | Controls in biodegradation experiments | 22 |
| 3.1.6 | Monitoring growth..... | 23 |
| 3.2 | Sampling procedure & normalization of the concentrations..... | 23 |
| 3.3 | Effect of heat on pharmaceuticals | 24 |
| 3.4 | Addition of supplementary carbon source | 24 |
| 3.5 | HPLC methods & isolation of metabolites | 25 |
| 3.6 | Identification of metabolites..... | 26 |
| IV. | Results..... | 27 |
| 4.1 | Effect of pharmaceuticals on microorganisms' growth | 27 |
| 4.2 | HPLC analysis: Calibration curve method limits..... | 28 |
| 4.3 | Effect of autoclaving on pharmaceuticals..... | 29 |
| 4.4 | Effect of evaporation on sample measurements..... | 30 |
| 4.5 | Biodegradation of trimethoprim | 32 |
| 4.6 | Biodegradation of carbamazepine..... | 33 |
| 4.7 | Biodegradation of sulfamethoxazole | 34 |
| 4.8 | Biodegradation of sulfamethizole | 37 |
| V. | Discussion..... | 39 |
| 5.1 | Method development | 39 |
| 5.2 | Biodegradation studies | 41 |
| VI. | Conclusion..... | 49 |
| VII. | References | 51 |
| VIII. | Appendices..... | 56 |

List of Figures

| | |
|---|----|
| Figure 1 : Chemical structure of carbamazepine | 9 |
| Figure 2 : Chemical structure of sulfamethoxazole | 10 |
| Figure 3 : Chemical structure of trimethoprim | 11 |
| Figure 4 : Chemical structure of sulfamethizole..... | 12 |
| Figure 5: Growth Comparison | 27 |
| Figure 6: Calibration Curves..... | 29 |
| Figure 7: Example of measured concentrations..... | 31 |
| Figure 8: Example of normalized concentrations | 31 |
| Figure 9: Biodegradation experiments of trimethoprim with <i>Rhodococcus rhodochrous</i> and <i>Pseudomonas putida</i> | 32 |
| Figure 10: Biodegradation experiment of trimethoprim with <i>Aspergillus niger</i> | 33 |
| Figure 11: Biodegradation experiment of carbamazepine with <i>Rhodococcus rhodochrous</i> and <i>Aspergillus niger</i> | 34 |
| Figure 12: Biodegradation experiment of sulfamethoxazole..... | 36 |
| Figure 13: Mass spectrum of the first metabolite of sulfamethoxazole..... | 36 |
| Figure 14: Mass spectrum of the second metabolite of sulfamethoxazole | 37 |
| Figure 15: Biodegradation experiment of sulfamethizole | 38 |
| Figure 16: Structure of the first metabolite of sulfamethoxazole | 45 |
| Figure 17 : Potential structure of the second metabolite of sulfamethoxazole | 48 |
| Figure 18 : HPLC chromatogram of a sample of carbamazepine degraded by <i>Aspergillus</i> <i>niger</i> | 59 |
| Figure 19: HPLC chromatogram of a sample of carbamazepine degraded by <i>Rhodococcus</i> <i>rhodochrous</i> | 60 |
| Figure 20: HPLC chromatogram of a sample of sulfamethoxazole degraded by <i>Rhodococcus rhodochrous</i> | 60 |
| Figure 21: HPLC chromatogram of an abiotic control of sulfamethoxazole..... | 61 |
| Figure 22: Mass spectrum of the first metabolite of sulfamethoxazole (QTOF 2) | 61 |
| Figure 23: MSMS of the first metabolite of sulfamethoxazole | 62 |

| | |
|--|----|
| Figure 24: MSMS of pure sulfamethoxazole..... | 62 |
| Figure 25: MSMS of the second metabolite of sulfamethoxazole..... | 63 |
| Figure 26: HPLC chromatogram of an abiotic control of sulfamethizole | 63 |
| Figure 27: HPLC chromatogram of a sample of sulfamethizole | 64 |

List of Tables

| | |
|---|----|
| Table 1: Microorganism information..... | 21 |
| Table 2: Concentrations (ppm) of pharmaceuticals used..... | 22 |
| Table 3: Effect of pharmaceuticals on microbial growth..... | 28 |
| Table 4: LOD and LOQ for the HPLC methods developed | 29 |
| Table 5: Effect of autoclave process on SMX and SMZ samples | 30 |
| Table 6 : Isotopic distribution (%) of the peak with an m/z 255.1 and for the theoretical molecule with a formula of $C_{10}H_{11}O_4N_2S$ | 44 |
| Table 7: Fragments observed from msms analysis of metabolite 2 (m/z 255) | 46 |
| Table 8 : Measured and theoretical isotopic distribution for the second metabolite (M+H) isolated from SMX biodegradation experiment..... | 47 |
| Table 9 : Metabolites of CBZ from human and rat urine | 56 |
| Table 10: Metabolites of CBZ from abiotic degradation..... | 57 |
| Table 11: Reported metabolites of TMP..... | 57 |
| Table 12: Reported metabolite of SMX..... | 58 |
| Table 13: Composition of the MMSM | 59 |
| Table 14 : Summary of result obtained from biodegradation experiments | 64 |

List of Equations

| | |
|--|----|
| Equation 1 : Expected Introductory Concentration | 6 |
| Equation 2 : Normalized Concentration | 23 |
| Equation 3: Volume | 24 |
| Equation 4 : Normalized Volume | 24 |
| Equation 5: Evaporated Volume | 24 |
| Equation 6 : Isotopic Mass [M+1] | 44 |
| Equation 7 : Isotopic Mass [M+2] | 44 |
| Equation 8 : Ring plus Double Bonds rule | 46 |

I. Introduction

The importance of the pharmaceutical industry has constantly increased over the last 50 years reflecting the increasing demand for pharmaceuticals. Ultimately, these drugs are either excreted by human, or simply disposed of, and end up in the environment. Hence, the accumulation into the environment of pharmaceuticals is now recognized as a serious problem. Many countries, in North America and Europe, have reported the presence of low concentration of pharmaceuticals in their sewage, surface and ground water.

Although the concentrations reported are very low (in ng/L to µg/L) many pharmaceuticals have a significant bioactivity and can have an effect even at low concentrations. The eventual repercussions to flora, fauna, and on human health may be considerable. As well the ultimate fate of many pharmaceuticals is unknown and this uncertainty adds to the risks associated with the presence of pharmaceuticals in the environment. One reason for this lack of data has been that, in the past, it was difficult to conduct analyses at low concentration levels such as those observed for these compounds. This is no longer true as numerous analytical methods have been developed that overcome this problem. To understand the consequences of pharmaceuticals polluting the environment, it is imperative to know the fate of these drugs and this includes knowing whether they are biodegradable, to what extent, and whether there are any metabolites that are created during the biodegradation.

The goal of this research was to study the biodegradation of selected pharmaceuticals by common microorganisms in order to shed some light on the fate of the pharmaceuticals in the environment. The pharmaceuticals selected for this study have been repeatedly found in the environment and are considered to be micro pollutants. These include three antibiotics, sulfamethoxazole, sulfamethizole and trimethoprim as well as one antiepileptic drug, carbamazepine. The specific objectives of this project are:

- 1) To determine the biodegradability of the selected pharmaceuticals by common microorganisms growing at the expense of a more easily metabolized carbon source
- 2) To identify any metabolites arising from the biodegradation

II. Review of Literature

This section first defines the problems associated with the presence of pharmaceuticals in the environment followed by an overview of the occurrence of various pharmaceuticals in the environment. The next section is a brief presentation of the relevant regulations and the potential consequences of pollution by pharmaceuticals. A description of each pharmaceutical studied, including their use, occurrence and natural removal mechanisms is then presented. Next, microorganisms selected for this study are described. The last section is a description of the microbial degradation of xenobiotics by co-metabolism.

2.1 Rationale

2.1.1 Problems related to pharmaceuticals

Pharmaceuticals include any substance or mixture of substances for use in the diagnosis, treatment, mitigation or prevention of a disease, disorder or abnormal physical state, or its symptoms in human beings or animals (Enick and Moore, 2007). Thus, these products include a wide range of structures, functions, behaviours and activity. As an example more than 3000 active ingredients are approved in Europe just for human medical care (Zwiener, 2007). Most of the pharmaceuticals produced ultimately make their way into the environment polluting the flora and fauna to different extents. The presence of pharmaceutical residues in the environment was reported for the first time in the late 1970s (O. A. H. Jones, *et al.*, 2005).

It has been shown that pharmaceuticals are introduced into the environment by a diverse range of pathways. The main one is human excretion after consumption. In this case pharmacokinetic studies have shown that a significant proportion, up to 50%, of the original compound may be excreted unchanged (Ternes, 1998). The rest is usually excreted in the form of a conjugate, which can revert back to the initial compound in

sewers or sewage works (Jones, *et al.*, 2004). The other major source of pharmaceuticals in the environment is their improper disposal. For example, when a medication has expired, people dispose of it in a toilet or in garbage (Ternes, *et al.*, 2002). Leaching from landfill sites to groundwater has been demonstrated in some studies (Jones, *et al.*, 2004). Often the pharmaceuticals pass into sewages and end up in water bodies without any treatment. For instance, Canadian cities were reported to have an average discharge of 3.25 billions litres per day of essentially untreated sewage into surface water or ocean (Daughton and Ternes, 1999).

Passage through a wastewater treatment plant does not guarantee the removal of pharmaceuticals. Many studies have shown the inefficiency of wastewater treatment plants at the removal of pharmaceuticals (Ternes, 1998). Ternes reported that the removal efficiency after passage through a wastewater treatment unit for 32 pharmaceutical residues ranged from 7 to 96 %. The removal process is inadequate for many pharmaceuticals simply because wastewater treatment units have not been designed to treat such compounds (Kolpin, *et al.*, 2002). Modifications, such as increasing the solid retention time for a typical wastewater plant that uses biological treatment has been proposed (Oppenheimer, *et al.*, 2007), as it promotes the growth of a more diverse biological community able to degrade xenobiotic compounds (Grady, *et al.*, 1999). However studies have shown that this solution did not significantly increase the removal for all drugs tested (Clara, *et al.*, 2005). In recent years there have been many proposals to remediate the situation and to include major changes in the typical wastewater treatment process. Most of these employ an oxidation process or a combination of these called advanced oxidation processes (Benítez, *et al.*, 2008). These techniques are based on the production of reactive and oxidizing radicals, which degrade pharmaceuticals. Ozonation is one technique that can successfully remove many pharmaceuticals, especially compounds with an activated ring or carbon-carbon double bond (Nakada, *et al.*, 2007). Unfortunately the cost related to these kind of advanced technologies is significant and they pose maintenance problems (Jones, *et al.*, 2007), which make them economically unfeasible for many municipalities.

The failure to remove pharmaceuticals from wastewater raises concerns for researchers due to their potential accumulation in the environment. Although pharmaceuticals represent a small fraction of all the chemicals that are rejected into the environment, special care must be taken regarding their accumulation for four reasons (Enick and Moore, 2007)

- 1) Pharmaceuticals are ubiquitous and disseminate well
- 2) Pharmaceuticals are specifically designed to act on biological systems
- 3) Pharmaceuticals are known to cause a wide range of side effects in non-target organisms
- 4) Pharmaceuticals can cause chronic toxicity at low concentrations ($\mu\text{g/L}$ to ng/L)

Since concerns regarding the accumulation of drugs have been raised, many studies have reported the presence of pharmaceuticals in different countries and water plants throughout the world (O. A. H. Jones, *et al.*, 2005). The usual concentrations stated are in the magnitude of ng/L , and rarely exceed the drinking-water-guidelines (Kolpin, *et al.*, 2002). However, many compounds have not been regulated yet.

2.1.2 Pharmaceutical regulations

The procedure used to determine the guidelines for water quality gives rise to a vigorous debate among regulators, environmentalist and industries, each one defending their own point of view. The stakes are crucial on each side; protection of the environment including human and animal health on one side and billions of dollars in market on the other. One of the major systems of regulation in the world is held in the United States under the jurisdiction of Food and Drug Administration (FDA). Any company wishing to commercialize a new pharmaceutical must submit a request to this organization. The Canadian agency Health Canada, which is in charge of the regulation in Canada, works and collaborates with the FDA. The environmental assessment

procedure for a new drug is divided in two steps. Firstly, the manufacturer is asked to make an estimation of the expected introductory concentration (EIC) entering the environment based on five years of production, as shown by equation 1 (Sungpyo and Aga, 2007):

$$\text{Equation 1 : EIC (ppb)} = A \times B \times C \times D$$

where A (kg/year) is the amount of active ingredient produced, B (day/L) is one divided by the amount of entering public treatment works, C is the number of year divided 365 days, and D is a conversion factor ($10^9 \mu\text{g/kg}$). If the EIC is less than 1 $\mu\text{g/L}$, the drug is categorized as acceptable and excluded from further environmental tests. If the EIC is estimated to be over 1 $\mu\text{g/L}$, data on the environmental fate and ecotoxicological tests are asked. The usual tests assess the microbial respiration and acute toxicity to at least one algal, one invertebrate and one fish species (Jones, *et al.*, 2004). Finally, if the pharmaceuticals have the potential to bioaccumulate, chronic tests are required. The regulations have faced many criticisms and in the opinion of many they need to be modified (Daughton and Ternes, 1999). For instance, criticism has been directed at the lack of clear standards regarding the limit that requires an assessment and the lack of inclusion of the risk to terrestrial biota (Sungpyo and Aga, 2007).

Due to the large quantity of pharmaceuticals present in an effluent, it is complicated to design tests that really assess its toxicity. Moreover, pharmaceuticals are usually designed to act specifically on a target metabolic system, but the effects on other species are not necessarily the same and often unpredictable. For instance, the analgesic ibuprofen has shown antibacterial and antimycotic properties, and certain antidepressants have been shown to affect the spawning of shellfish (Jones, *et al.*, 2004). Therefore, given the vast array of mechanisms of action of drugs and the possible side effects, it is evident that the usual toxicity tests merely indicate the level of immediate harmfulness for the flora and fauna (Daughton and Ternes, 1999).

2.1.3 Consequences of pharmaceutical accumulation

Scientists are particularly apprehensive about the contamination of drinking water by pharmaceuticals. In the US, among the 170 drinking standards or health advisories relating to organic compounds in potable water supply, none are related to pharmaceuticals (Webb, *et al.*, 2003). The contamination of drinking water by pharmaceutical waste is a risk that can lead to problems with public health.

The concentration of pharmaceuticals in tap water is on the order of ng per litre, which is much lower than the doses prescribed in therapeutic treatment usually in the order of mg per pill. Hence, no pharmacological effects are expected at these concentrations (Åke and Bo, 2005) and it explains the negative results obtained by researchers regarding the acute toxic effect tests at these concentrations (Jones, *et al.*, 2004). However, a few studies have reported the likelihood of profound effects for pharmaceuticals with highly specific mechanisms, even at extremely low concentrations (Daughton and Ternes, 1999).

Some researchers have established methods to measure the indirect exposure to pharmaceuticals via drinking water (Jones, *et al.*, 2004). Their conclusion is that for more than 90% of the drugs tested, there was a very large margin between daily intake and the therapeutic dose. Their conclusions indicated, for many drugs, that the ingestion of 2 litres of tainted water every day over a lifetime does not correspond to a single prescribed dose (Oliver A. Jones, *et al.*, 2005). Chronic effects, which occur over a long period of time, are more likely, but little is known about the effect of long term exposure. Studies of this type may take several years to be conclusive.

Possible effects of long term exposure to pharmaceuticals include abnormal physiological processes and reproductive impairment (Jobling, *et al.*, 1998), increased incidences of cancer (Davis and Bradlow, 1995) and the potential for increased toxicity in chemical mixtures (Kolpin, *et al.*, 2002). Synergic effects of chemical mixtures represent an important risk which is difficult to evaluate. A combination of pharmaceuticals can

enhance the side effects of one of them. For instance, a mixture of ibuprofen, prozac and ciprofloxacin has been shown to be harmful to plankton, aquatic plants and fish at concentration 10 to 200 times lower than the typically prescribed dose (Jones, *et al.*, 2004). Still very little is known about the synergic effects because of the wide range of possible chemical mixtures (Ternes, 1998).

Many negative effects are related to the intrinsic nature of a pharmaceutical. Thus, the presence of antibiotics is likely to be of concern because of their influence on any biological system. More importantly, development of pathogen resistances particularly worries scientists. It is estimated that more than 70% of bacteria are insensitive to at least one antibiotic (Hirsch, *et al.*, 1999). Antibiotics also have the potential to affect the microbial community in a way that compromises the degradation of organic material due to nitrification and denitrification (Jones, *et al.*, 2004). The increase in allergic responses to antibiotics has also been of concern (Daughton and Ternes, 1999).

2.2 Pharmaceuticals studied

Since the fate of most of pharmaceuticals in the environment is not known, the number of drugs that could have been included in our research was fairly high. The research focused on four pharmaceuticals, each of which has been found in the environment. The compounds are: carbamazepine, sulfamethoxazole, sulfamethizole and trimethoprim.

2.2.1 Carbamazepine (CBZ)

Carbamazepine, also known as 5H-dibenz[b,f]azepine-5-carboxamide, is an important pharmaceutical that was introduced in the early 1960's by Scindler and Geigy. Its structure is shown in figure 1. CBZ has a widespread use in the medical field and is mainly used for the treatment of epilepsy, which is the second most common central neuron system disease. In addition, CBZ is a mood stabilizing drug used to treat other brain disorders such as bipolar behavior, a serious disease affecting 1.2% of adults in the

US (Miao and Metcalfe, 2003). The annual prescription of CBZ in Germany is approximately 80 tons per year (Ternes, 1998). The efficiency of the drug is dramatic as it reduces the frequency of complex partial seizures in epilepsy by 83%.

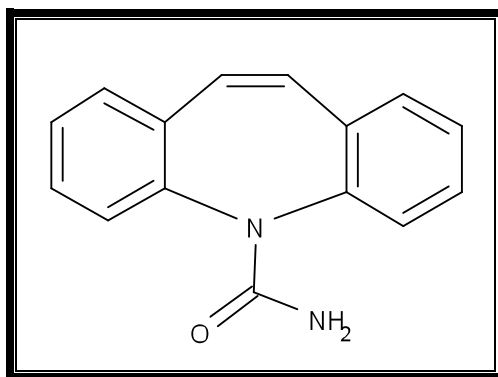


Figure 1 : Structure of carbamazepine

Investigations done on carbamazepine proved that it inhibits the voltage dependence of Na⁺ channels of neurons. The carbamazepine molecules bind themselves to the Na⁺ channels, giving a more negative membrane potential that slows recovery from the inactive state (Porter, 1990). Hence, only a few sodium channels are available to be opened which makes the brain cells less excitable.

Carbamazepine is removed mainly by the hepatic metabolism and is degraded by the cytochrome P450 system (Miao and Metcalfe, 2003). So far, thirty-three metabolites of carbamazepine have been reported from human and rat urine. Some of these are shown in table 10 in the appendix. Pharmacokinetic studies reveal that only 1-2% of the carbamazepine is excreted unmetabolized (Ternes, 1998). Studies have shown that antiepileptic drugs are associated with an increased risk of major congenital abnormalities (E. Bettina Samrén, 1999) in the offspring of women using the drug during pregnancy, which raises concerns about the contamination of the drinking water by this compound. The intake of CBZ by drinking water in Germany is evaluated at 30 ng per litre (Webb, *et al.*, 2003). The maximum concentration of CBZ reported in Canada in drinking water is 35 ng per litre and about 258 ng per litre in the United States (Oliver A. Jones, *et al.*, 2005).

2.2.2 Sulfamethoxazole (SMX) and Trimethoprim (TMP)

Sulfamethoxazole (figure 2), or 4-Amino-N-(5-methyl-3-isoxazolyl)-benzenesulfonamide, is a sulfonamide antibiotic. Sulfonamides have been, and continue to be, extensively used because of their low cost of production, relatively low toxicity and their efficiency (Connor, 1998). Sulfamethoxazole used to be prescribed as a single antibiotic, but the development of antibiotic resistance has changed the way it is prescribed today. Since 1973 in the United States, sulfamethoxazole is combined with trimethoprim, to enhance the antibacterial efficiency (Karpman and Kurzrock, 2004). Trimethoprim, (2,4-Diamino-5-(3,4,5-trimethoxybenzyl)pyrimidine) shown in figure 3, is a derivative of pyrimidine. The drugs separately are bacteriostatic, meaning that they hamper the growth of the bacteria, but together they have a synergic effect and they become bactericidal (Connor, 1998). Sulfamethoxazole, which is less active than trimethoprim, enhances the activity of the latter. The combination of the drugs (SMX-TMP), called co-trimoxazole, is fixed in a ratio of 5 sulfamethoxazole to 1 trimethoprim (Zhou and Moore, 1994).

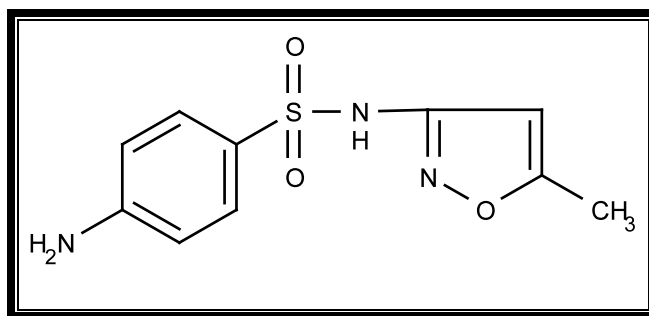


Figure 2 : Structure of sulfamethoxazole

Co-trimoxazole is principally used to cure urinary and respiratory tract infections (Connor, 1998). The combination is popular in medicine and, as an example, the amount prescribed in Germany per year is approximately of 20 tons of co-trimoxazole (Hirsch, *et al.*, 1999). It is also in the top 200 list of prescribed drug in Canada (Cavallucci, 2007). Co-trimoxazole is particularly active against gram negative bacilli and certain

Staphylococcus species (Karpman and Kurzrock, 2004). It inhibits two sequential two steps of the bacterial folate synthesis. The folate synthesis is essential to the formation of purines, which are precursors of deoxyribonucleic acid (Connor, 1998). The first step of the sequential mechanism is the inhibition by SMX of the synthesis of dihydrofolic acid from para-aminobenzoic acid. TMP inhibits the subsequent conversion to tetrahydrofolic acid, the active form of folic acid (Connor, 1998).

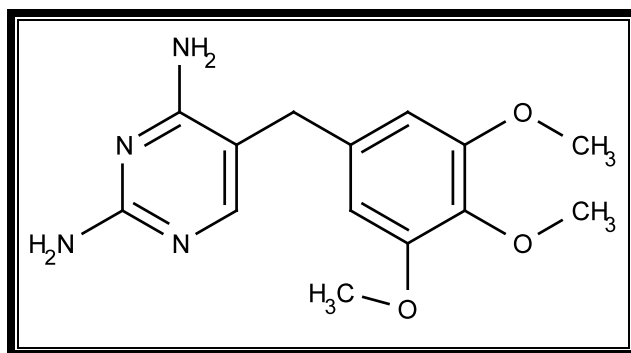


Figure 3 : Structure of trimethoprim

Approximately half of the ingested TMP is excreted unchanged in urine. SMX is mainly transformed in the liver by acetylation and about 20% is excreted unchanged in urine (Karpman and Kurzrock, 2004). A list of the known human metabolites is presented in the appendices. Co-trimoxazole has been related to adverse effects such as phototoxic and photoallergic skin reactions (Zhou and Moore, 1994). Although TMP is the key ingredient in the formulation of co-trimoxazole, most of the side effects are attributed to SMX (Karpman and Kurzrock, 2004).

The poor removal efficiency observed in the sewage treatment plant results in concentrations of SMX up to 110 ng per litre and 470 ng per litre for TMP in Sweden (Åke and Bo, 2005). In ground waters, traces of SMX (0.47 µg per litre) have been detected (Hirsch, *et al.*, 1999). Traces of SMX and TMP have been found in US streams with respectively 1.9, and 0.71 µg per litre (Kolpin, *et al.*, 2002).

2.2.3 Sulfamethizole (SMZ)

Sulfamethizole, 4-Amino-N-(5-methyl-1,3,4-thiadiazol-2-yl)benzenesulfonamide, shown in figure 4, has a very similar structure to SMX. It is also a sulfonamide and it is used in the treatment of urinary tract infections. It can be also used in combination with TMP. The metabolism is also comparable and a mixture with TMP increases the efficiency of the medication. Traces of SMZ have been found in US streams of 0.13 μg per litre (Kolpin, *et al.*, 2002).

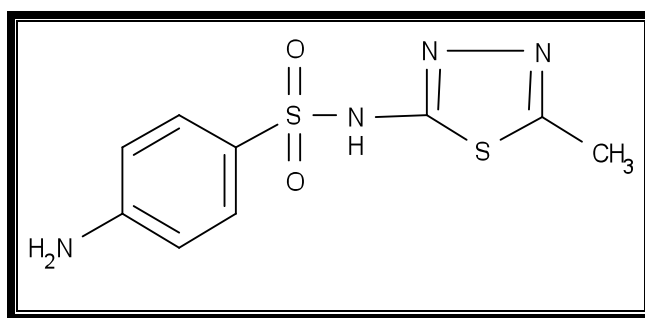


Figure 4 : Structure of sulfamethizole

2.3 Metabolites

There are concerns about the metabolites that arise from the degradation of pharmaceuticals. These can come from a partial transformation by the human body, by the microorganisms in the environment or from an uncompleted mineralization during an oxidation or advanced oxidation processes. Research indicated that the by-products of biodegradation are, in many cases, similar to their parent drug. For instance the metabolites of TMP formed in activated sludge treatment have a structure very similar to TMP (Sungpyo and Aga, 2007). The antibacterial activity of these metabolites still has to be tested. In the case of CBZ, one of its principal human metabolites, 10,11-dihydro-10,11-epoxycarbamazepine, is also an anticonvulsant having similar activity to CBZ (Miao and Metcalfe, 2003). The accumulation of the by-products in the environment may also lead to health problems. One degradation product of CBZ created by an

oxidation experiment was acridine, which is in a category of compounds having mutagenic and carcinogenic activities (Vogna, *et al.*, 2004). Hence in many cases the absence of particular pharmaceuticals in an effluent is not enough to guaranty the complete mineralization of the compound and therefore, it is necessary to look for its metabolites. The lack of data regarding these transformation products is of concern and, for this reason, testing for metabolites is one objectives of this study.

2.4 Removal mechanisms of selected pharmaceuticals

Certain attempts have been made to evaluate the degradation or removal mechanisms of the compounds being studied. The aim was to understand the fate of these in engineered systems and in the environment and to elucidate the by-products created. In a very few cases, the biodegradation of pharmaceuticals was studied but only in activated sludge systems as described in the following sections.

2.4.1 Photochemical degradation

The abiotic degradation that most pollutants undergo is by photodegradation and hydrolysis. As pharmaceuticals are usually resistant to hydrolysis, because they are designed for oral intake, it is not surprising that most abiotic degradation occurs by photodegradation (Andreozzi, *et al.*, 2003).

Carbamazepine transformation by sunlight has been investigated by Vogna (Vogna, *et al.*, 2004). Acridine, a toxic compound, has been detected as a by-product in every experiment. They also considered the effect of nitrates and humic acid, two photosensitizers, which are commonly present in wastewater. Their presence enhanced the conversion of CBZ by generating reactive oxygen species. In the same sort of experiment, it was found that the half life of CBZ is more than 100 days in winter, which demonstrates its persistence (Andreozzi, *et al.*, 2003). The half life estimated for SMX was 2.4 days. In photolysis experiments, SMX decomposed quickly in aqueous solution to give rise to five metabolites (Zhou and Moore, 1994). One of the reported metabolites

was aniline, a known toxic compound. The action of sunlight on TMP has been explored and it appears that five metabolites arise from the transformation (Bergh JJ, 1989). The toxicity still has to be tested. However, other studies have shown that TMP is stable in sea water, which absorbs more UV radiation than fresh water, under sunlight exposure (Tore Lunestad, *et al.*, 1995). Experiments conducted with SMZ showed no significant degradation when exposed to sunlight (Numan, *et al.*, 2002).

2.4.2 Biodegradation in activated sludge and pure culture

Activated sludge treatment is the most common process for the treatment of wastewater. Two elimination processes act simultaneously in the activated sludge. Either there is sorption to solids by the pharmaceuticals, or biodegradation is caused by the microorganisms. The elimination process depends on the characteristics of the xenobiotic compound, such as its partitioning coefficient. Hence, for some compounds like ibuprofen and mefenamic acid, studies have shown that the sorption is not significant and most of the removal is due to biodegradation (Jones, *et al.*, 2007). Although, for other compounds, such as ciproflaxin, an antibiotic, sorption has been found as the main removal mechanism (Sungpyo and Aga, 2007). Generally the more simple a molecule is, the more chance there is for biodegradation to take place (Jones, *et al.*, 2004). During biodegradation, the xenobiotic compounds can be transformed in three different ways: mineralization, hydrophobic or hydrophilic transformation. A complete mineralization is accomplished if the compound is transformed into carbon dioxide. The partition into the sludge will increase if the drug is transformed into a more hydrophobic compound, and it will likely remain in water if it becomes more hydrophilic. While biodegradation of the parent compounds have been studied for many pharmaceuticals, the formation of the metabolites has not been elucidated (Sungpyo and Aga, 2007).

Carbamazepine is one of the pharmaceuticals with the lowest removal efficiency, around 7 percent, when treated with activated sludge treatment (Ternes, 1998). Therefore CBZ is not affected either by sorption or by the microorganisms. One study has shown positive results regarding the biodegradation of CBZ by a pure culture. The

experiment was done to assess the persistence and toxicity of CBZ in the environment. It was found that the concentration of CBZ decreased during the growth of the algae *Ankistrodesmus braunii* (Andreozzi, *et al.*, 2002). The sorption mechanism was excluded as no significant amount of CBZ was found in the algae cells. The study did not look at the metabolites created or if complete mineralization was achieved.

Since antibiotics have numerous adverse effects on the environment, many studies have been done regarding their biodegradability. Activated sludge has shown to degrade many sulfonamides (Halling-Sorensen, 2000). The results vary according the source of the activated sludge.

The removal efficiency obtained in wastewater treatment plants for SMX differs greatly depending on the conditions and the sludge. Some researchers reported removal efficiencies close to 100 %, but others showed a negative removal (Göbel, *et al.*, 2007). The negative value can be explained by the presence in the influent of metabolites of SMX, like N-4-acetylsulfamethoxazole, which can revert back to the parent structure. Sorption to the activated sludge was found to be of minor importance. Closed bottle tests, conducted with microorganisms from activated sludge, using only SMX as a carbon source, revealed the drug to have poor biodegradability (A. Al-Ahmad, 1999). However the experimental conditions were not representative of what is found in the environment. No results of the biodegradation of SMX by pure cultures have been reported.

Although TMP is also an antibiotic, the removal efficiency obtained in wastewater treatment plants was less than that of the sulfonamides. In fact TMP showed a high resistance to biodegradation. The results are not uniform and the maximum removal efficiency obtained in activated sludge was around 20 percent (Göbel, *et al.*, 2007). Again the sorption (less than 6 percent, which is with in the range of the uncertainty) was considered to be negligible. The same trend was observed in laboratory experiment. However, nitrifying bacteria have shown the ability to degrade TMP with a removal of 75 percent (Eichhorn, *et al.*, 2005).

2.5 Role of microorganism acclimation & co-metabolism

The adaptation of the biomass consists of an acclimatized period in which the microorganisms are exposed to the target chemical to degrade. The microorganisms then have time to develop enzymes to degrade more effectively this compound, which is usually not a readily used carbon or nitrogen source (Jones, *et al.*, 2007). In many experiments reported, the biomass, which usually was the activated sludge, was acclimatized to the pharmaceuticals studied. An acclimation period was reported in the case of SMX and TMP. In the case of SMX the degradation was almost completed (Drillia, *et al.*, 2005), but the removal efficiency for TMP was not enhanced, even after a prolonged adaptation of several weeks (Eichhorn, *et al.*, 2005).

In activated sludge, microorganisms compete for the primary carbon source, which is usually the compound that is present at the highest concentration among the more easily degradable carbon sources. The dominant compounds in the influent are likely to be lipids or proteins while the pharmaceuticals are present in low concentrations. The biodegradation is therefore likely achieved by microorganisms with oligotrophic metabolism (Jones, *et al.*, 2007). These microorganisms are slow growing which explains why an increase in the solid retention time in wastewater treatment plants enhances the removal efficiency. These microorganisms are also expected to be found in environments characterized by low concentrations of carbon sources (Daughton and Ternes, 1999).

Another mechanism for the biodegradation of the xenobiotic compound is co-metabolism. The microorganisms in this case partially convert the drug but do not use it as a carbon source or at least as a primary carbon source (Jones, *et al.*, 2007). In some studies, the removal of some antibiotics, like TMP and SMX, has been attributed to co-metabolism during the nitrification process (Sungpyo and Aga, 2007). The biodegradation of SMX in activated sludge with acetate as a readily carbon source has been studied (Drillia, *et al.*, 2005). It has been found that SMX can serve both as carbon

and nitrogen source. During biodegradation experiments in the presence of another readily available carbon source, acetate, SMX served as the nitrogen source. When ammonium was added, SMX remained intact. There was also evidence of diauxic growth. After the consumption of the acetate, the SMX was then found to degrade. A delay was observed between the consumption of the two carbon sources, presumably due to the time required by the microorganisms to develop suitable enzymes.

Co-metabolism mechanisms have been studied successfully with others xenobiotic compounds. For example, studies were done with specifically plasticizers (Gartshore *et al.*, 2003). These chemicals, like the pharmaceuticals, are a subject of concern because of their accumulation in the environment. Researches have demonstrated that many microorganisms were able to degrade plasticizers such as di-2-ethylhexyl phthalate (DEHP) and di-2-ethylhexyl adipate (DEHA) (Nalli, *et al.*, 2005) even though these plasticizers are not readily biodegradable. Instead, biomass grew on another, more easily utilized, carbon source and the enzymes present in this biomass caused some biotransformation of the plasticizers. The pure cultures used in this way were shown to degrade the plasticizers and metabolites were identified.

2.6 Microorganisms selected for this study

Six microorganisms were selected for our study. Each one was known to be able to degrade similar pharmaceuticals. The following section gives a brief overview of each bacterium.

2.6.1 *Rhodococcus rhodochrous*

Rhodococcus rhodochrous is a bacterium commonly found in soils. The *Rhodococcus* species have been reported to be able to grow on a wide range of compounds. (Bergey and Holt, 1994). For instance it has been shown to have the ability to assimilate pyridine and some steroids. Moreover, the microorganism has been isolated from soil polluted with petroleum, consistent with an ability to grow on different

substrates. These microorganisms have been reported to be sensitive to some antibiotics, but to a lesser extent to sulfonamides.

2.6.2 *Pseudomonas putida* and *Pseudomonas fluorescens*

The *Pseudomonas* species are found in soils and water. They have the ability to proliferate even when the amount of nutrient is very low. Both microorganisms have been recognized to be able to degrade xenobiotic compounds. *Pseudomonas putida* has been able to transform several pesticides such as desmedipham and promecarb (Knowles and Benezet, 1981), and also acridine, a derivative of carbamazepine (Pia Arentsen, *et al.*, 2005). In other experiments regarding the biodegradation of N-heterocycles, a microorganism that is closely related to *Pseudomonas fluorescens* has been able to break down carbazole, which has a similar structure to carbamazepine (Gieg, *et al.*, 1996).

2.6.3 *Bacillus subtilis*

The bacillus species are known to be very resistant to many adverse conditions. They are psychrophilic to thermophilic, and they can proliferate at a wide range of pH. Another noticeable characteristic is their ability to create endospores under unfavourable conditions. Most species are commonly found in nature. *Bacillus* have been shown to transform many xenobiotic compounds such as picolinic acid and isonicotinic acid (Kaiser, *et al.*, 1996). It has also been reported that the degradative capacity of *Bacillus subtilis* regarding some pesticides like desmedipham, phenmedipham and promecarb, that have similar functional group to carbamazepine, is relatively high (Knowles and Benezet, 1981).

2.6.4 *Aspergillus niger*

Among the microorganisms selected for our study, *Aspergillus niger* is the only fungus. The *Aspergillus* species are ubiquitous in the environment. They are isolated from soil, plant debris and indoor air. Many species are thermotolerant and grow rapidly

on various substrates (Concordia University, 2005). Although the ability of *Aspergillus* to degrade xenobiotic compounds has not been thoroughly investigated, it has been found that some species are able to break down the pesticide carbaryl, which has an amide group like carbamazepine (Dorough, 1976). Moreover, naphthalene, an aromatic hydrocarbon, has been found to be metabolized by *Aspergillus niger*.

2.6.5 *Sphingomonas herbicidovorans*

Most of the studies done on *Sphingomonas herbicidovorans* have shown that it is an excellent herbicide degrader (Dorough, 1976). Moreover other studies have reported the ability of *Sphingomonas* species to break down acridine, a derivative of carbamazepine (Pia Arentsen, *et al.*, 2005).

III. Materials & Methods

3.1 Biodegradation experiment

3.1.1 Media preparation

Prior to experimentation, all microorganisms were grown in an optimal nutrient medium to ensure that a healthy population was obtained. The medium used was Brain Heart Infusion (BHI), (Becton, Dickinson and Company) which was prepared according to the recipe of the manufacturer at 37 grams/L in deionised water. For the biodegradation experiments, a minimum mineral salt media (MMSM) was used. The composition of MMSM is presented in the appendices in table 14. As MMSM media contains only the minimum requirements for the microorganisms, a carbon source, glucose (A&C, CAS # 50-99-7), as well as a protein supplement, yeast extract (Fisher, CAS # 8013-01-2), were added to the shake flasks. The concentrations of glucose and yeast extract were 3 and 0.5 grams per litre respectively. All media were autoclaved (AMSCO, model 3021-S) at 121 degrees Celsius at a pressure of 10 PSI for 15 minutes before inoculation. The glucose solution was autoclaved separately to prevent side reactions with other nutrients.

3.1.2 Enrichment and acclimation

Microorganisms were purchased from the American Type Culture Collection (ATCC) and frozen for future experiments. The initial inocula used for the biodegradation experiments were either taken from the freezer or from previously grown biomass on agar plates or flasks. Microorganisms were grown twice in flasks with the BHI media and then transferred twice into flasks containing the MMSM. The microorganisms used in the experiments, as well as the time to achieve stationary phase in MMSM are shown in table 1. After an acclimation period was done. MMSM was also used as the media in the acclimation period. The same additives were used, but in this

case, the pharmaceutical was added at the concentration used in the biodegradation studies. The concentrations of the pharmaceutical were not monitored during the acclimation period. The microorganisms were allowed to grow twice in the MMSM in the presence of the pharmaceuticals. Finally they were transferred in flasks for the beginning of the biodegradation experiment. Triplicate experimental flasks were prepared. Usually, the pharmaceuticals were added at the onset of the experiment, however, in some cases, the pharmaceutical was added during the stationary phase.

Table 1: Microorganism information

| Microorganism | ATCC number | Time to achieve stationary phase [days] |
|-------------------------------------|-------------|---|
| <i>Rhodococcus rhodochrous</i> | 13808 | 3 |
| <i>Pseudomonas putida</i> | 12633 | 3 |
| <i>Pseudomonas fluorescens</i> | 13525 | 1 |
| <i>Aspergillus niger</i> | 16888 | n.d. |
| <i>Sphingomonas herbicidovorans</i> | 700291 | 4 |
| <i>Bacillus subtilis</i> | 6051 | 2 |

3.1.3 Pharmaceutical solutions

Each pharmaceutical was studied separately. The stock solutions of carbamazepine (Sigma, CAS # 298-46-4), sulfamethoxazole (Sigma, CAS # 723-46-6) and sulfamethizole (Sigma, CAS # 144-82-1) were prepared in distilled water and autoclaved prior to inoculation. The trimethoprim (Sigma, CAS # 738-70-5) stock solution was prepared at about 12500 ppm in dimethylsulfoxide (DMSO, Sigma, CAS # 67-68-5) and kept under nitrogen. The stock solutions were used to obtain the calibration curves and for biodegradation experiments. The concentrations used for the

biodegradation experiments and for the calibrations are shown in table 2. All dilutions were done in distilled water.

Table 2: Concentrations (ppm) of pharmaceuticals used

| Pharmaceuticals | Stock solution | Biodegradation flask | Range of the calibration curve |
|------------------------|-----------------------|-----------------------------|---------------------------------------|
| Carbamazepine | 100 | 10 | 3-20 |
| Sulfamethoxazole | 400 | 40 | 9-45 |
| Sulfamethizole | 400 | 40 | 25-60 |
| Trimethoprim | 12500 | 80 | 25-87.5 |

3.1.4 Flask preparation

Experiments were conducted in 500 ml Erlenmeyer flasks equipped with foam caps to prevent contamination. During the experiments, the flasks were kept in an incubator shaker (New Brunswick Scientific Company, model Innova 44) at 26° Celsius and at 150 rpm. The total volume of medium used was 100 ml. For experiments conducted with sulfamethizole, sulfamethoxazole and trimethoprim, the shake flasks were covered with aluminium foil to prevent photochemical degradation from occurring during the experimentation. All bacterial manipulations were done in a laminar flow hood (The Baker Company, model VBM600) using sterilized equipment.

3.1.5 Controls in biodegradation experiments

As previously mentioned, pharmaceuticals can undergo abiotic degradation. In addition, microorganisms release metabolites during their growth, which are unrelated to the pharmaceutical. Three different types of controls were prepared for the biodegradation experiments; the biotic, the abiotic and the control with dead biomass. The biotic control monitored the growth of the biomass without the pharmaceutical being

present. It was prepared to detect metabolites that were not associated with the pharmaceuticals. The second type of controls, abiotic, were made with the drug present in the media but without any microbe. The abiotic control was used to determine the degradation of the pharmaceuticals in the conditions used during the experiments. Finally the dead biomass control contained autoclaved biomass and pharmaceuticals were added to test for sorption.

3.1.6 Monitoring growth

The growth curves for all microorganisms, except *Aspergillus niger*, were obtained by measuring optical density. To do this, 1-ml samples of broth were collected over the period of the experiment and diluted with 2-ml of distilled water. The optical density was measured using a UV spectrometer (Varian, model Cary 50) at a wavelength of 600 nm. No quantitative growth was observed for *Aspergillus niger*.

3.2 Sampling procedure & normalization of the concentrations

To account for the evaporation of water from the experimental flasks, the flasks were weighed prior to the onset of the experiment and then before and after each sample was collected. Unless otherwise mentioned, concentrations were normalized using these weights and the procedure is described below. Samples (2 to 4 ml) were taken from the flasks and transferred to plastic centrifuge tubes. Samples were then centrifuged (IEC, model micromax) for 10 minutes at 10,000 RPM. The supernatant was then filtered with 0.45 micron PVDF filters (Fisher, cat # 097203) at which point the samples were ready for HPLC analysis.

The normalized concentration ($C_N(t)$) in ppm was defined by the following equation:

$$\text{Equation 2 : } C_N(t) = \frac{C_M(t) * V(t)}{V_N(t)}$$

Where $C_M(t)$ is the measured concentration by HPLC, $V(t)$ is the volume in the flask at time (t) and $V_N(t)$ is the normalized volume, as defined by equations 2, 3 and 4. The density was assumed equal to 1g/ml to convert the measured masses into volume.

$$\text{Equation 3: } V(t) = \frac{\text{mass of flask}(t)_{\text{before sampling}} - \text{mass of empty flask } (t = 0)}{\rho}$$

$$\text{Equation 4 : } V_N(t) = V(t) + \sum_0^t V_{\text{Evap}}(t)$$

Where $V_{\text{Evap}}(t)$ is the total volume evaporated at time t , which is given by :

$$\text{Equation 5: } V_{\text{Evap}}(t) = \frac{\text{mass of flask}(t-1)_{\text{after sampling}} - \text{mass of flask } (t)_{\text{before sampling}}}{\rho}$$

3.3 Effect of heat on pharmaceuticals

In order to study the thermal effects due to autoclaving on CBZ, SMX and SMZ, different solutions of the standards were prepared and then analyzed and compared using HPLC. For example, for the stock solution prepared in 3.1.3, two flasks, labelled A and B, were prepared in distilled water at concentration used for the biodegradation experiment. Flask A was autoclaved. Another solution of same concentration (flask C) was prepared in methanol waster 1:1 but not autoclaved. Two millilitres of each solution was filtered with a PVDF filter and put in an HPLC vial. The peak area and retention time of the solution were compared using the HPLC.

3.4 Addition of supplementary carbon source

The easily degradable carbon source is essential for growth of the bacterial population and is subject to rapid consumption. Hence lack of the primary carbon source towards the end of an experiment could prevent further biodegradation of the pharmaceutical. Therefore, in some experiments, in order to avoid this, glucose was added after stationary phase was achieved to see whether further biodegradation was

possible. The addition of supplementary carbon source will be discussed in sections of concern.

3.5 HPLC methods & isolation of metabolites

Due to the availability of the equipment, HPLC analyses were performed using two different HPLCs. The first one was an Agilent 1100 equipped with a variable wavelength detector. The second instrument was an Agilent 1200 semi-preparative HPLC set up with a diode array detector. The HPLC method developed for each pharmaceutical is described separately.

For carbamazepine an Eclipse XDB-CN 5 μ m (4,6x150 mm) column was used. Elution conditions were 70 % of a 25 mM ammonium acetate buffer adjusted to pH 3.0 with formic acid (solvent A) and 30% acetonitrile (solvent B). The flow rate was 1.0 ml/min and the detection wavelength was set to 220 nm. The injection volume was 25 μ l and the temperature was kept at 20 °C. The total analysis time was 10 minutes.

For sulfamethoxazole and sulfamethizole an Eclipse XDB-C18 5 μ m (4,6x250 mm) column was used. The elution conditions were 80 % of 20 mM NH₄COOH buffer at pH 3.5 (solvent A) and 20% acetonitrile (solvent B) for 10 minutes, then 30% of B for 5 minutes followed by 45% of B for 5 minutes. The flow rate was 0.7 ml/min and the detection wavelength was set at 273 nm. The injection volume was 5 μ l and the temperature kept at 40 °C. The total analysis time was 20 minutes.

Finally for trimethoprim an Eclipse XDB-C18 5 μ m (4.6 x 250 mm) column was used. The elution conditions were 85 % of methanol/water (60:40) at pH 3.0 (solvent A) and water (solvent B) 15%. The flow rate was 1.2 ml/min and the wavelength was set at 230 nm. The injection volume was 5 μ l and the temperature was kept at 35 °C. The total analysis time was 8 minutes.

Metabolites were collected using the 1200 Agilent semi-preparative HPLC. The metabolites were collected on a time basis. The injection volume was set to 25 μ l in order to maximize collection of the by-product. Samples were then concentrated under nitrogen.

3.6 Identification of metabolites

Mass spectra analyses were performed. Two different mass spectra analysers were used. The first one was a Micromass QTOF 2 obtained from Waters Ltd. The ionization was achieved by nanospray at a flow of 1 μ l per minute. The recording of the spectra was done in positive mode. The energy varied between 10 to 20 eV. The second apparatus was an IonSpec 7.0 Tesla FTMS equipped with a Z-spray source from Waters. Again the positive mode was used to do the analyses.

IV. Results

4.1 Effect of pharmaceuticals on microorganisms' growth

The toxicity of each drug on microorganisms was studied by comparing growth curves of bacteria exposed to the drug to a control growth curve of bacteria not exposed. Figure 5 presents the results obtained for *Sphingomonas herbicidovorans* exposed to carbamazepine. Similar trends were observed for other microorganisms exposed to the pharmaceuticals studied (data not shown). A longer lag phase was observed for some combination of microorganisms/pharmaceuticals as summarized in table 3.

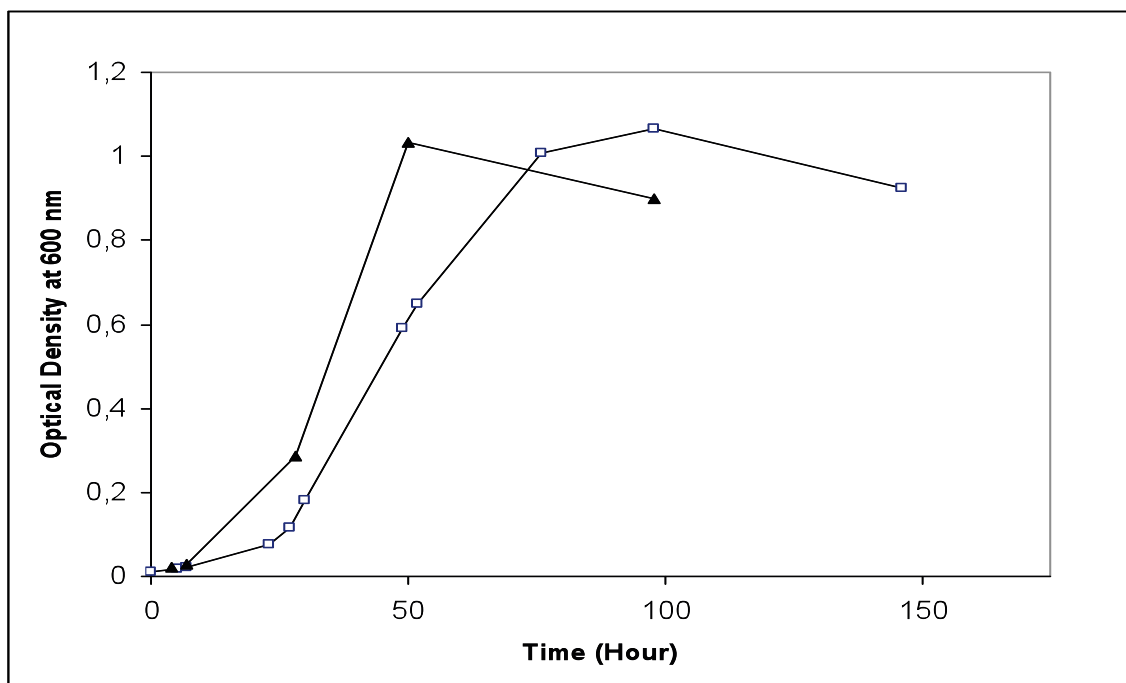


Figure 5: Comparison of the growth curve of *Sphingomonas herbicidovorans* when exposed (□) and not exposed (▲) to carbamazepine in MMSM media with glucose as easily degradable carbon source

Table 3: Effect of pharmaceuticals on microbial growth

| Microorganism | Pharmaceutical | Effect |
|-------------------------------------|-----------------------|----------------|
| <i>Bacillus subtilis</i> | CBZ | None |
| <i>Pseudomonas putida</i> | CBZ | None |
| <i>Pseudomonas putida</i> | CBZ | Not determined |
| <i>Pseudomonas fluorescens</i> | CBZ | Not determined |
| <i>Rhodococcus rhodochrous</i> | CBZ | None |
| <i>Rhodococcus rhodochrous</i> | SMX | Delay 2-3 days |
| <i>Rhodococcus rhodochrous</i> | SMZ | Delay 2-3 days |
| <i>Rhodococcus rhodochrous</i> | TMP | Not determined |
| <i>Aspergillus niger</i> | TMP | None |
| <i>Aspergillus niger</i> | CBZ | None |
| <i>Sphingomoans herbicidovorans</i> | CBZ | None |

4.2 HPLC analysis: Calibration curve method limits

The calibration curves obtained from the analysis of pharmaceuticals concentration on the HPLC are presented in the figure 6 (where the y axis UVA is the UV absorbance unit). The limit of detection (LOD) and limit of quantification (LOQ), shown in table 4, were calculated as follow: The LOD was defined as $3 \cdot S_{y/x} / m$, where m is the slope of the calibration curve and $S_{y/x}$ is the standard deviation of the regression. The LOQ was defined as $10 \cdot S_{y/x} / m$.

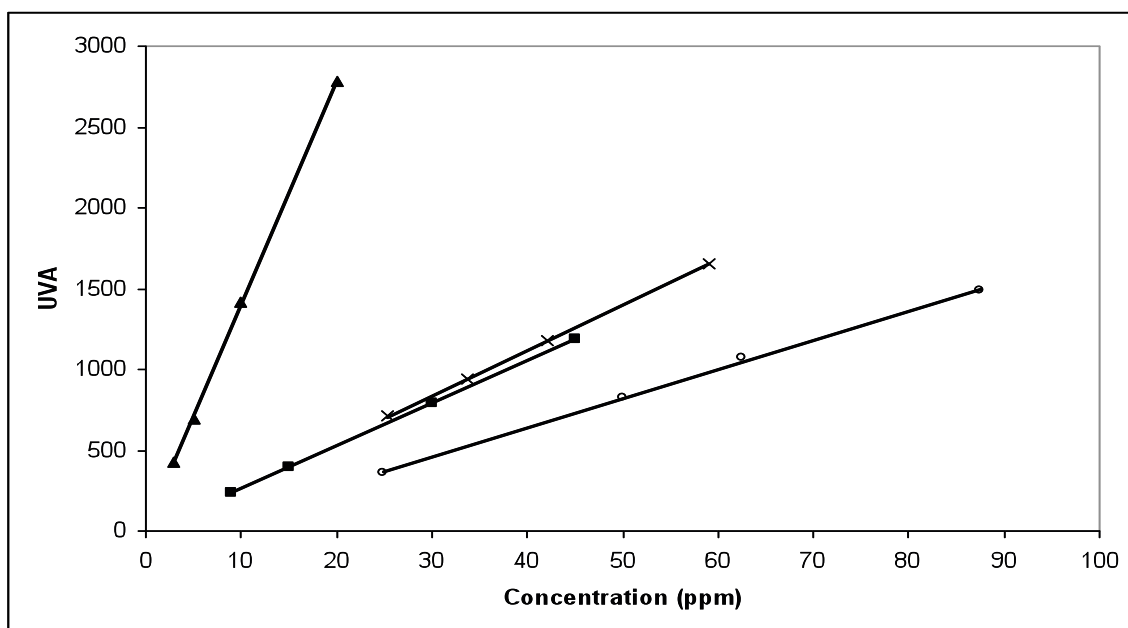


Figure 6: Calibrations curves (CBZ (▲, $R^2=0.9999$), SMZ (X, $R^2=1$), SMX (■, $R^2=1$), TMP (○, $R^2=0.9989$))

Table 4: LOD and LOQ for the HPLC methods developed

| Pharmaceutical | LOD (ppm) | LOQ (ppm) |
|----------------|-----------|-----------|
| CBZ | 0.32 | 1.08 |
| SMZ | 0.25 | 0.81 |
| SMX | 0.02 | 0.08 |
| TMP | 3.14 | 10.49 |

4.3 Effect of autoclaving on pharmaceuticals

The retention times and the peak areas obtained for SMX and SMZ standards, autoclaved and not autoclaved, are presented in table 5. Standard A and B came from the same stock solution whereas the standard C was prepared separately in methanol/water (1:1) solution. The same type of result was obtained for CBZ (not shown).

Table 5: Effect of autoclave process on SMX and SMZ samples

| | Sulfamethoxazole (SMX) | | Sulfamethizole (SMZ) | |
|---------------------|------------------------|--------|----------------------|---------------|
| | Retention time | Area | Retention time | Area |
| A Autoclaved | 6.279 | 1137.5 | 5.743 | 858.8 |
| B Not autoclaved | 6.269 | 1043 | 5.756 | 828.8 |
| C Not autoclaved | 6.268 | 1114.4 | 5.743 | Not available |

4.4 Effect of evaporation on sample measurements

Figure 7 shows the concentration of one duplicate abiotic control obtained for carbamazepine over 25 days. This increasing trend was observed in all biodegradation experiments and reflects water evaporation occurring in long experiments.

Figure 8 presents the normalized concentration along with the measured concentrations for the abiotic controls shown in figure 7. The normalized concentrations were obtained by applying the procedure described in section 3.2 to correct the values for water lost. Unless otherwise specified, results presented in the following sections were normalized.

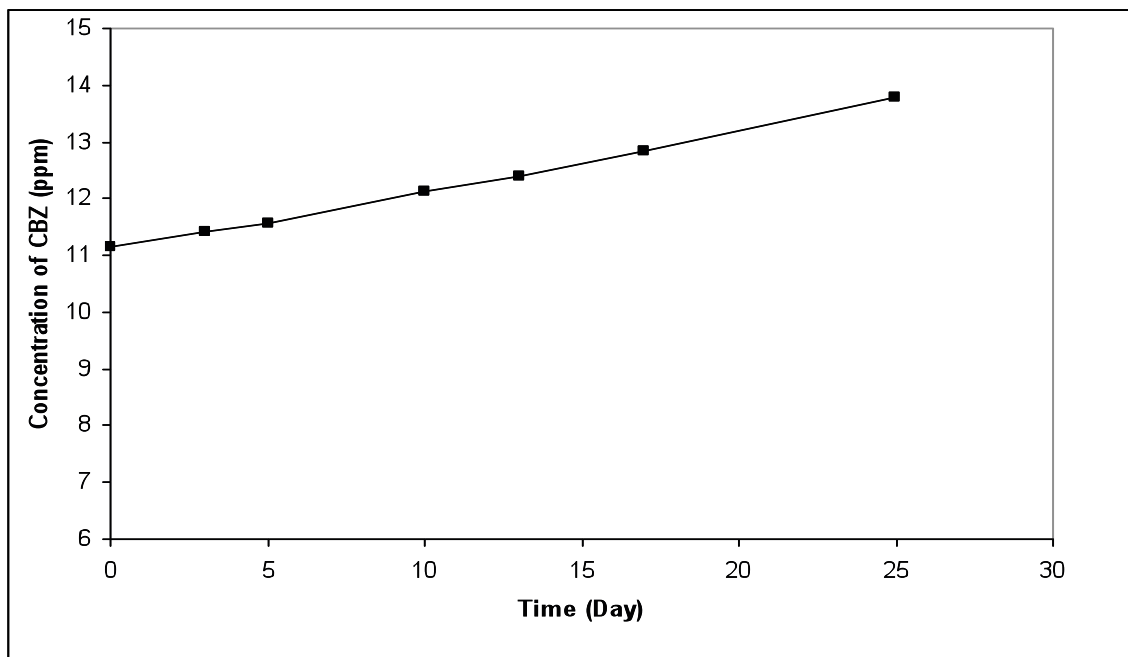


Figure 7: Increasing concentration of one abiotic control of CBZ due to evaporation during long experimentation

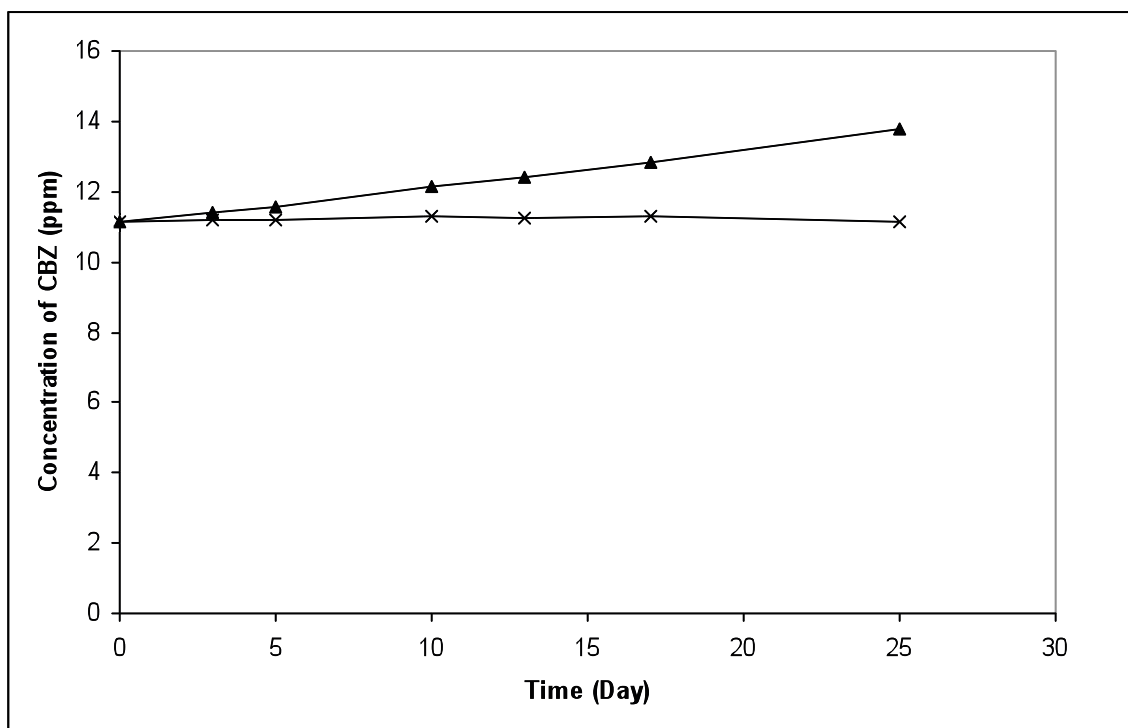


Figure 8: Normalized (X) and measured (▲) concentrations for abiotic control of CBZ

4.5 Biodegradation of trimethoprim

Three bacteria growing on glucose were exposed to TMP: *Rhodococcus rhodochrous*, *Pseudomonas putida* and *Aspergillus niger*. Figure 9 shows the evolution of the concentration when bacteria *Rhodococcus rhodochrous* and *Pseudomonas putida* were tested. The data presented here were not corrected for the lost of water. The results of the experiment with *Aspergillus niger* are presented separately in figure 10 since the experiment was conducted for a longer period. The measurements are corrected for the evaporation of water and are presented along with an abiotic control.

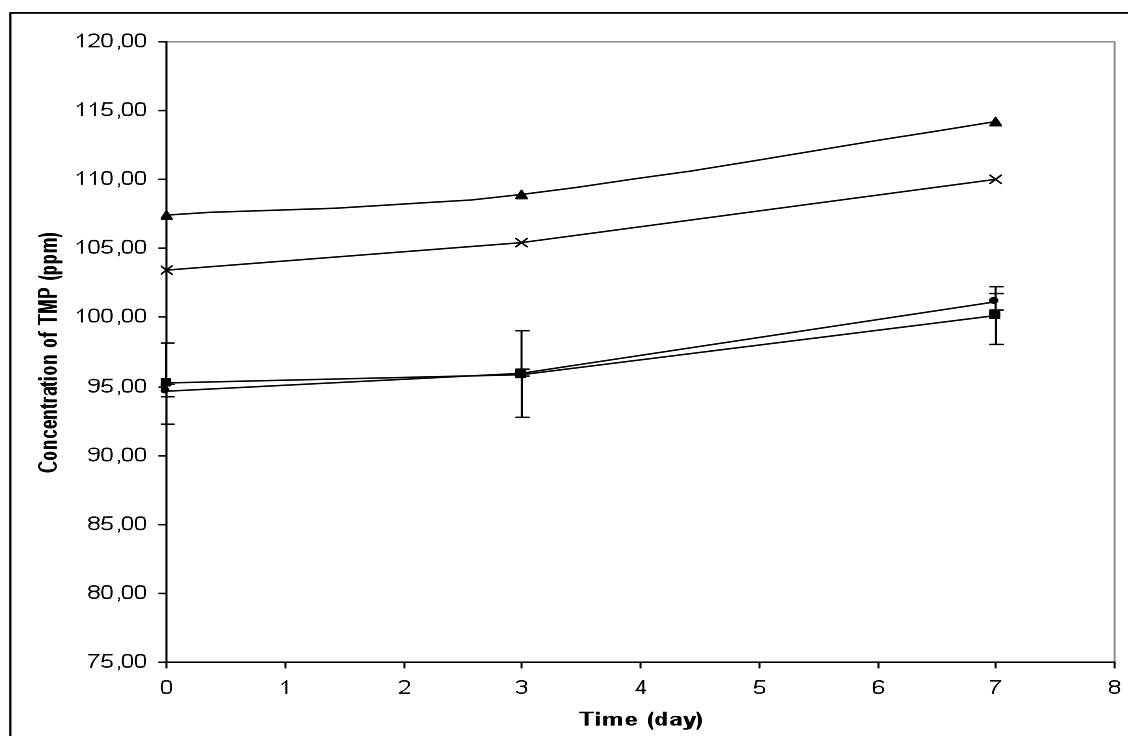


Figure 9: Biodegradation experiment for TMP by *Rhodococcus rhodochrous* [(sample (■), control(X)] and *Pseudomonas putida* [sample (●), control (▲)] in MMSM

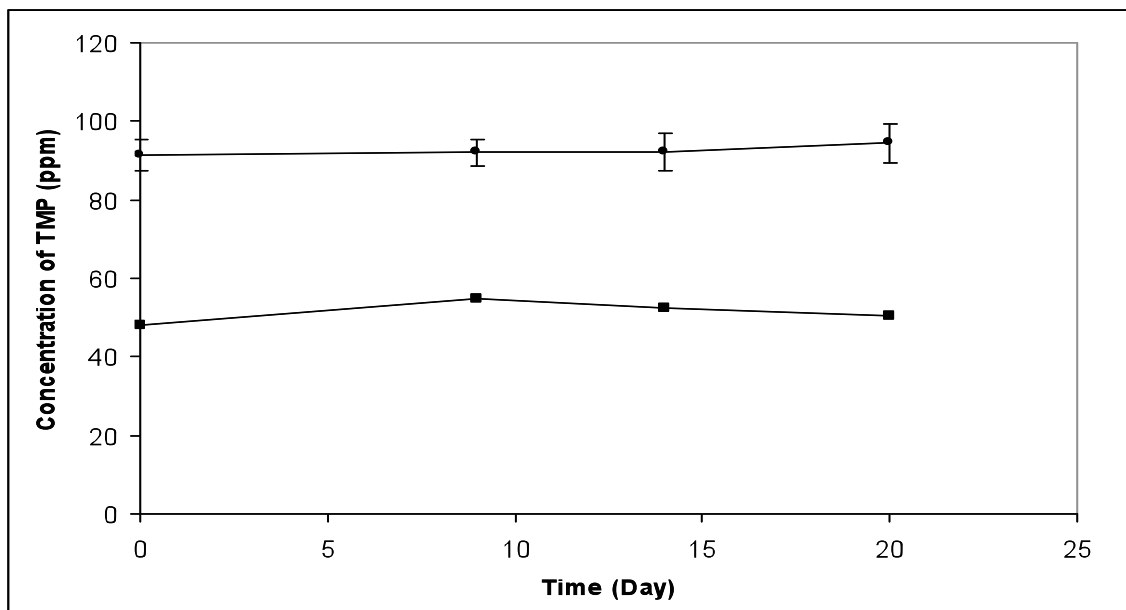


Figure 10: Biodegradation experiment of TMP with *Aspergillus niger* [sample (●), abiotic control (■)] in MMSM

4.6 Biodegradation of carbamazepine

Six different microorganisms were exposed to carbamazepine. The results obtained from the microorganisms *Pseudomonas putida*, *Pseudomonas fluorescens*, *Sphingomonas herbicidovorans* and *Bacillus subtilis* grown in presence of CBZ, were similar. The graphs are not shown since biodegradation was not achieved. The decrease in concentration of controls and samples were of the same order, namely of 5% or less.

The results were different for the two other microorganisms *Aspergillus niger* and *Rhodococcus rhodochrous*. As shown in figure 11, a significant decrease in concentration was observed when *Aspergillus niger* grew in presence of CBZ. The pharmaceutical was added during the stationary phase and the experiment was carried out for 10 days. The overall decrease in concentration was about 20 %. However, the concentration in the control using dead biomass control dropped by as much as 10%. An example of a chromatogram obtained at the end of the experimentation is presented in the appendices (figure 18). Two additional small peaks were observed at retention time of 3.79 and 3.99 minutes. These peaks were not observed in any control.

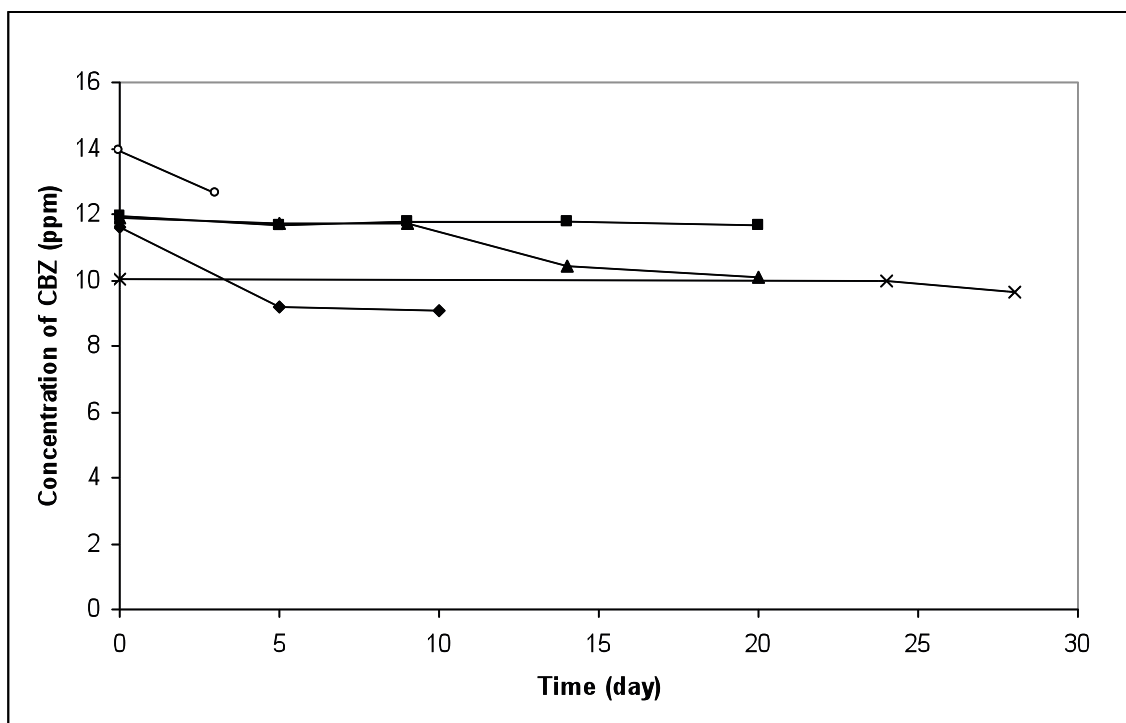


Figure 11: Biodegradation experiment of carbamazepine with *Rhodococcus rhodochrous* (duplicate samples (■ and ▲)) and *Aspergillus niger* (abiotic control (x), sample (◆) and dead biomass control (○)) in MMSM

The microorganism *Rhodococcus rhodochrous* showed also the ability to degrade CBZ. The decrease in concentration observed was of 19 %. The abiotic degradation obtained in control flasks was about 4% and the sorption from biomass was negligible. Degradation was not observed in every sample as shown in figure 11. The same peaks (retention time) as those from the biodegradation with *Aspergillus niger* appeared at the end of the experimentation as shown in figure 19 in the appendices.

4.7 Biodegradation of sulfamethoxazole

The first microorganism tested against SMX, *Rhodococcus rhodochrous* gave positive results. The biodegradation experiment was done for a period of 36 days. The degradation observed in the abiotic control was of 33%, which was almost equal to the

decreased observed in control with dead biomass (31%). During the same period of time, the concentration in samples dropped by 55%. The results are presented in figure 12. It should be noted that in this experiment, the carbon source was replenished after day 14 in order to evaluate the possibility of an enhanced biodegradation. Hence glucose was added to 2 samples and 2 abiotic controls, while water was added to the remaining sample and control (nothing was added to the control with dead biomass). The HPLC chromatograms of the sample and of the abiotic control are shown in the appendices (figure 20 & figure 21). Metabolites were observed for the sample at retention times around 15 and 19 minutes. These two metabolites were collected for further analysis.

Figure 13 presents the mass spectrum for the metabolite collected at a retention time of 15 minutes. The mass spectrum was recorded with the IonSpec 7.0 Tesla FTMS. The peaks at an m/z of 415 and 702 were impurities or noise from the apparatus. Another mass spectrum of the same sample was recorded one month later with the Micromass QTOF 2 and is presented in the appendices (figure 22). From the latter, msms analysis was performed on peak with an m/z of 255 (figure 23). The msms of the standard is also presented in the appendices (figure 24). The spectra presented for the standard is the sum of its own ms and of its msms spectra recorded on the Micromass QTOF 2, which means that the relative intensities are arbitrary and do not correspond to the ones obtained during the analysis. Finally the mass spectrum of the second metabolite, with a retention time of 19 minutes on the HPLC, is presented in figure 14. Its msms spectrum is presented in appendix (figure 25).

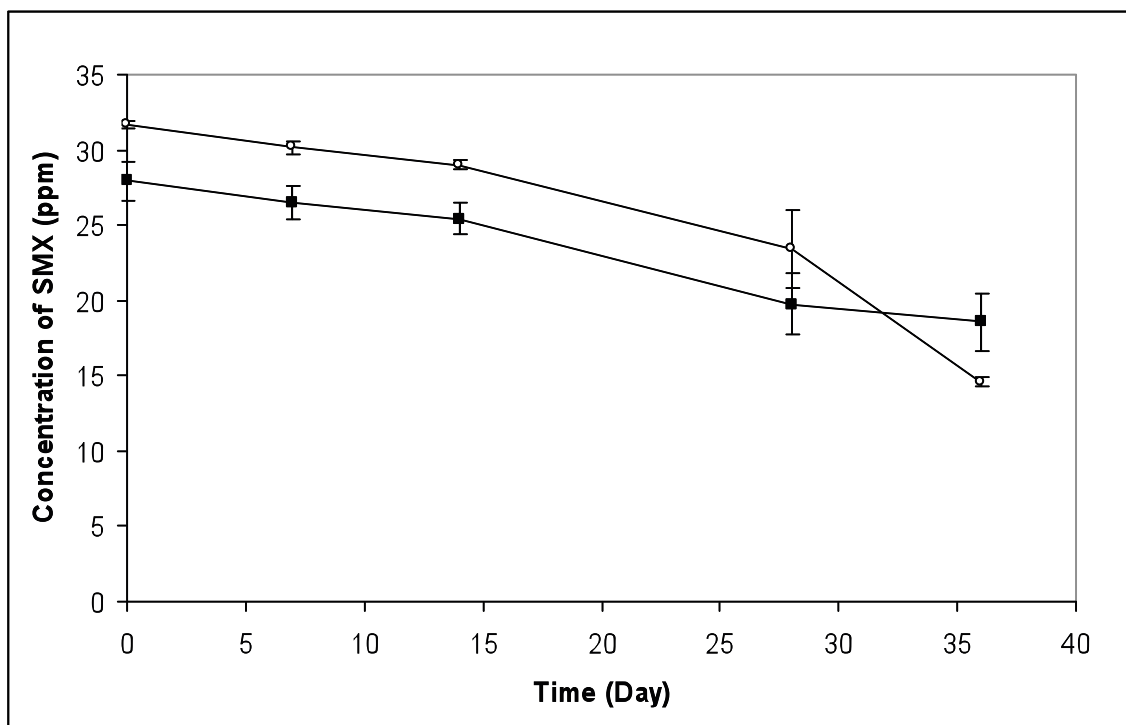


Figure 12: Biodegradation of SMX by *Rhodococcus rhodochrous* (sample (○), abiotic control (■)) in MMSM

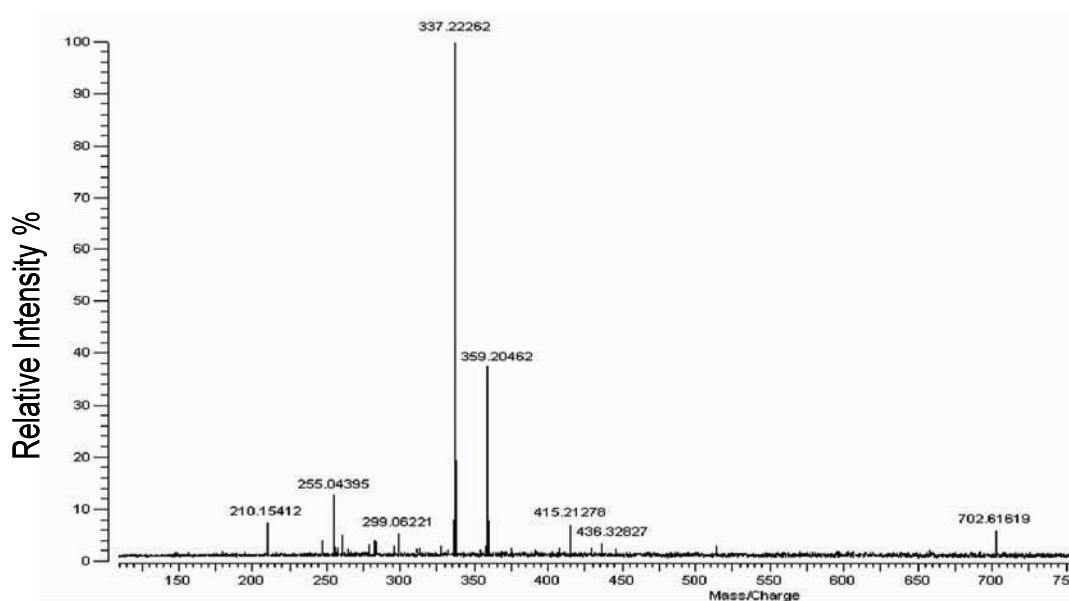


Figure 13: Mass spectrum of SMX metabolite at retention time of 15 minutes

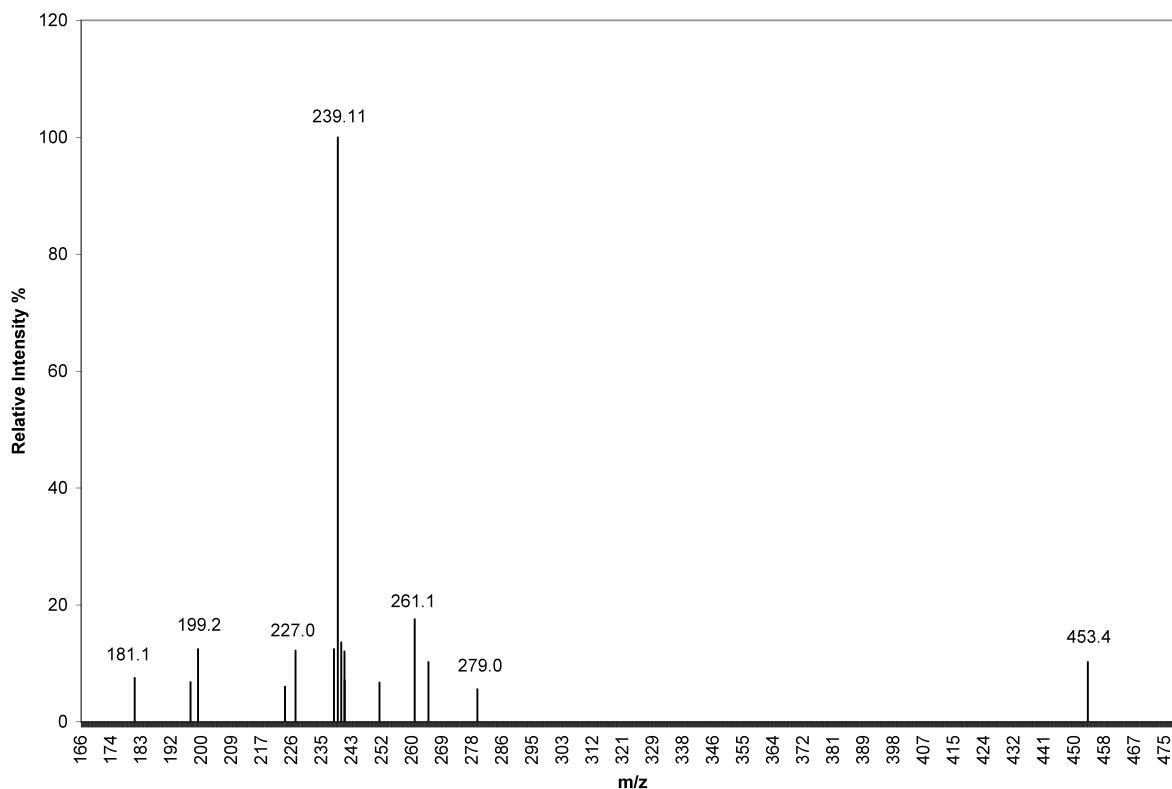


Figure 14: Mass spectrum of SMX metabolite at retention time of 19 minutes

4.8 Biodegradation of sulfamethizole

The experimentation was conducted only with *Rhodococcus rhodochrous*. A biodegradation of 14% was measured during an experiment carried out over 12 days. Figure 15 shows the concentration measured over time. The degradation in the abiotic control was around 6%. HPLC chromatograms of the abiotic control and of the sample are presented in the appendices and the metabolites observed are pointed out (figure 26 and 27). However it was not possible to perform analysis on metabolites.

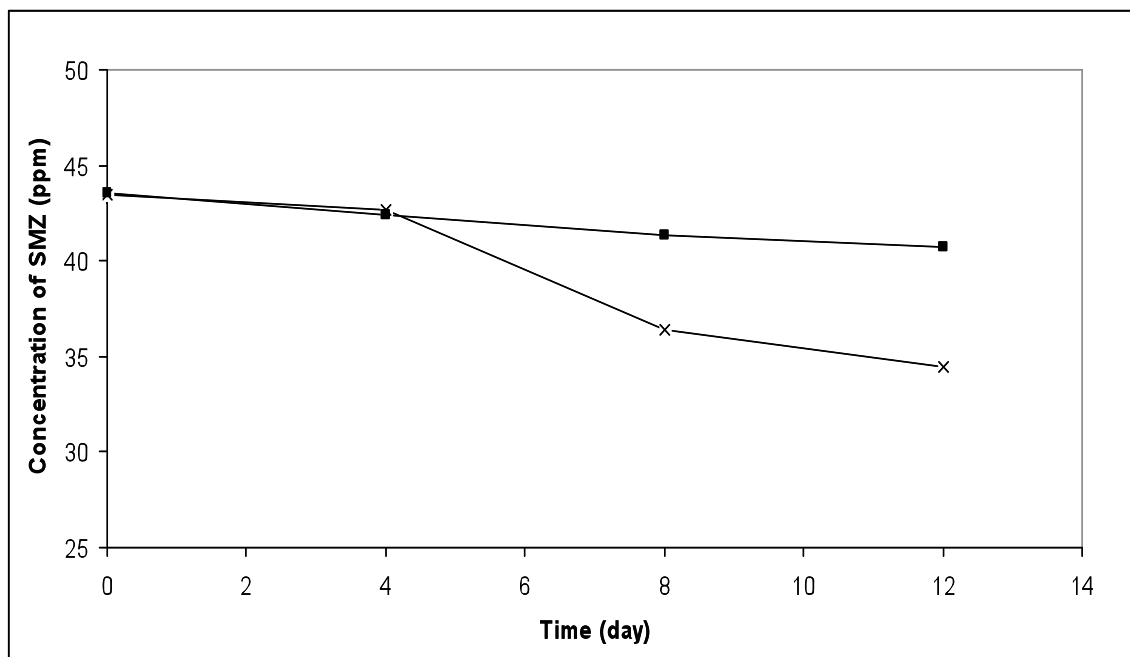


Figure 15: Biodegradation of SMZ by *Rhodococcus rhodochrous* (sample (x), abiotic control (■)) in MMSM

V. Discussion

5.1 Method development

Determining the exact effect of a pharmaceutical on biomass is a very complex problem. For this work, it was necessary to consider whether the compounds being tested for biodegradability were causing toxic effects. Such toxicity may reflect the bioactivity of the drug. The assessment of the toxicity was done by comparing the growth of biomass exposed to not exposed to the drug. Figure 5 represents an example of a growth curve of biomass exposed to one of the drugs in question. Since the growth curve was similar to the control, it indicates that there was not significant inhibition of growth at the concentration used. In particular the final optical density reached the same value whether the microorganism was exposed to the drug or not. Some of the pharmaceuticals studied did exhibit a greater tendency to impede the growth rate, which in some cases created a delay of two or three days for the microorganisms to reach the stationary phase. This trend was observed even when an acclimation period was allowed for the biomass. The results of the toxicity assessment are summarized in table 3. Although the delays observed might have slightly decreased the amount of biodegradation observed, the toxicity was considered negligible for the biodegradation experiments.

During the biodegradation experiments small changes in concentration needed to be measured precisely. This leads to the importance of having a stable and linear calibration curves for the HPLC. The correlation coefficients obtained for these calibration curves are close to one in all cases and reflect the precision of the methods.

The sterilization of the solution containing the drugs was important to prevent the flasks from being contaminated but the process may also destroy the drugs. Thus, it was necessary to prove that the heat involved during autoclaving could not have induced a transformation of the drug. As an example, carbamazepine is known to be a thermolabile

molecule. Although the temperature usually reported for its rearrangement is higher than the temperature reached during the autoclaving process it was necessary to ensure that the drug was not affected and remained unchanged before doing the biodegradation experiments. The same analyses were done with SMX and SMZ even though they were not reported to be heat sensitive. The results for SMX and SMZ are presented in table 5. It can be seen that the autoclaved samples showed higher peak areas than the non-autoclaved samples. These differences were due to the evaporation during the autoclaving process. The decrease in volume observed was usually about 10%, which corresponds to the increase in concentration. The sample C prepared in a mixture of methanol and water gave approximately the same peak area as expected. The retention times are very similar for the data presented and the HPLC chromatograms did not reveal any new peaks. This confirms that the autoclaving process did not alter the pharmaceuticals.

One major problem to solve was the loss of water due to evaporation during the long periods of time required for the biodegradation experiments. Even though the incubator shaker was design to minimize the evaporation, a typical pattern of an increase in concentration, was seen and an example of this is shown in figure 7. A few different solutions to this problem were considered. One option was to add an internal standard and compute a ratio of the sample to standard signal but there were many drawbacks to this option. First the standard had to be of similar structure to give the same type of signal. In addition the standard had to be inexpensive, which was not the case for many internal standards proposed in the literature. The stability of the standard had to be determined and, this leads to the main problem; the standard could have been degraded during the biodegradation process and left metabolites in the medium. A second solution was to add a rubber lid to seal the flasks which would have prevented evaporation. However, this would have restricted the respiration of the biomass, which would have hindered the biodegradation.

Hence, it was decide to normalize the concentration over time and report it with respect to the initial volume, or the initial concentration, by adding the quantity of water

evaporated to the sample. This volume correction was done by weighing each sample. The precision of the method was mainly determined by the precision of the balance used, which was 3 significant digits. For the purpose of the calculation, an assumption was made of 1 g/ml for the density of the solution, which should be a good approximation for a dilute aqueous system. This approach worked very well as can be seen for the abiotic experiments in figure 8. The normalized data are very close to horizontal lines, as expected.

5.2 Biodegradation studies

Trimethoprim showed a strong resistance to biodegradation as none of the bacteria tested were able to break it down. TMP is light sensitive but the abiotic control showed that even photo-degradation did not occur in the time of the experiments. Most of the samples were not weighed. Nevertheless, the evaporation did not have as much of an effect as in other experimentations because the concentration of TMP used was much higher and was thus less affected by small modifications in volume.

Two duplicate samples were prepared in experiments conducted with *Pseudomonas putida*. The only change was a small increase in concentration due to the fact that the data were not normalized for evaporation (figure 9). However, the exact same trend was observed for the control with dead biomass. This means that neither sorption of TMP nor biodegradation occurred. The results obtained with *Rhodococcus rhodochrous* were very similar since a small increase in concentration was observed due to the evaporation, which was also present in the control.

The experiment conducted with *Aspergillus niger* and TMP is an example of normalizing the data. Although the abiotic control had a concentration lower than the one used in sample, the values were in the range of the calibration curve and still acceptable. Again, there was no evidence of biodegradation. The negative results for biodegradation of TMP by *Rhodococcus rhodochrous*, *Pseudomonas putida* and

Aspergillus niger were consistent with the HPLC chromatograms (data not shown) as these showed no new peaks and, thus no evidence of metabolite formation.

It was expected that carbamazepine would be difficult to degrade as it is a persistent compound in the environment. Hence, the microorganisms *Pseudomonas putida*, *Pseudomonas fluorescens*, *Sphingomonas herbicidovorans* and *Bacillus subtilis* were not able to breakdown CBZ. The small decreases in concentration that were observed were also seen in control flasks and attributed to the abiotic degradation of CBZ. The sorption of the drug was not found significant. Finally, there was no evidence of metabolite production over time on HPLC chromatograms.

However, two microorganisms were able to co-metabolise CBZ when they were growing on glucose as a carbon source. A rapid decrease in concentration (20%) was observed with *Aspergillus niger*. Half of this was attributed to sorption by comparing to the control with dead biomass. Hence, 10% of the decrease in concentration was related to the biodegradation. About the same amount of biodegradation was observed with *Rhodococcus rhodochrous* (15%). This biodegradation was consistent with the appearance of new peaks in the HPLC that could be attributed to metabolites. However, the concentrations were too small to allow identification.

Carbamazepine was not easily degradable as biodegradation was not observed in every flask. Although the experiment was reproduced more than once, there were always discrepancies in the results, as biodegradation occurred in some flasks whereas nothing happened in others. However, a possible explanation is a mutation of the microorganisms that could have happened in some flasks, which may have allowed them to achieve biodegradation.

The degradation obtained in the case of SMX is more difficult to interpret because after the extended experiment, 33% degradation was observed for the abiotic control. Since almost the same value was obtained for the control with dead biomass the sorption was again considered negligible. In the actual experiments, the decrease in concentration

was about 55% and this leaves 22% due to biodegradation. The small error bars indicate good reproducibility among the samples. This biodegradation corresponds to values obtained in previous experimentation (data not shown).

An experiment was done with SMX in which the carbon source, glucose in solution, was replenished after day 14. There was a small drop in the concentration when glucose was added to flasks. The addition of the extra glucose did not increase the biodegradation (figure 12).

The metabolites arising from the abiotic degradation and biodegradation of SMX are not the same (figure 20 and 21). For the abiotic control, one peak appeared around 12 minutes whereas two were seen around 15 and 19 minutes for the sample. According to the retention times obtained for these metabolites, it is reasonable to think that the structures are not very similar to one another. Further analysis on the abiotic metabolites has not been performed.

The mass spectra of the SMX metabolite (retention time of 15 minutes) indicated that the compound collected was not pure. As shown in figure 13, one compound had an m/z of 337.226 uma and the others were present in trace amounts. The main component (337.226) was not stable as it was not detected in a second analysis performed on the same sample (figure 22). The two mass spectra shared one peak with an m/z of 255 uma. Hence only the peak with an m/z of 255 uma was investigated. The weight of this metabolite indicated that the structure was possibly similar to SMX. The exact weight of the metabolite was 254.03612 uma and it is 253.05211 uma for SMX. The comparison of the spectra of this metabolite and of SMX confirmed the similarity of the two molecules. A useful tool, which helps to solve for the structure is the nitrogen rule. This rule stipulates that when the molecular weight is odd, the molecule must contain an odd number of nitrogen atoms. Inversely if the molecular weight is even, then the molecule has no or an even number of nitrogen atoms (Dass, 2007). The conclusion from this rule is that the metabolite had an even number of nitrogen atoms. The primary amine of the SMX is the most likely nitrogen atom to be lost as the other two are more firmly bound in

the compound (figure 2). Replacing this amine with a hydroxyl functional group, which has a mass of 17.00 uma would give the correct molecular weight. The mass difference is less than 1 ppm.

The isotopic distribution was consistent with the proposed structure for this metabolite. The isotopic distribution evaluates the augmented mass due to the presence of isotopes such C¹³ or deuterium. The isotopic distribution is presented in table 6 and has been calculated by the following equations for the formula C_XH_YO_NN_ZS_M (Ham, 2008):

$$\text{Equation 6 : \% [M+1]} = 1.11x + 0.015y + 0.37z + 0.037n + 0.8m$$

And

$$\text{Equation 7 : \% [M+2]} = ((1.08x)^2)200 + 4.4m + 0.2n$$

Table 6 : Isotopic distribution (%) of the peak with an m/z 255.1 and for the theoretical molecule with a formula of C₁₀H₁₁O₄N₂S

| Mass | Formula of C ₁₀ H ₁₁ O ₄ N ₂ S | Measured |
|---------------|--|----------|
| M+1 (256 uma) | 12.95 | 13.45 |
| M+2 (257 uma) | 5.78 | 6.44 |

The values in table 6 match very well. Figure 16 presents the structure of the metabolite, which is hydroxy-N-(5-methyl-1,2-oxazol-3-yl)benzene-1-sulfonamide. It should be noted that the position of the hydroxyl group in the aromatic ring has not been confirmed but the most likely position is shown.

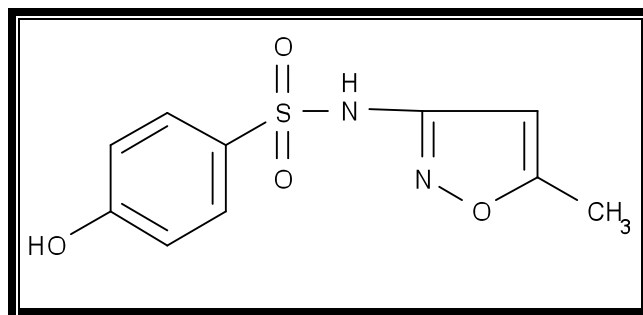
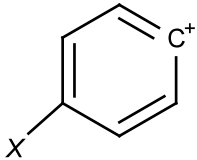
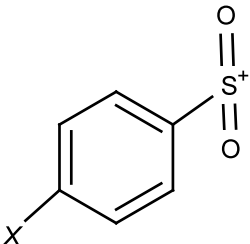
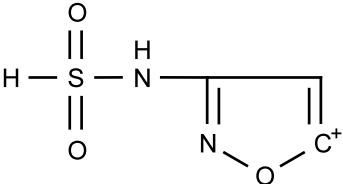


Figure 16: SMX proposed metabolite

Finally to confirm the structure the fragmentation of the metabolite and of the standard were compared. Table 7 proposes different fragments identified from the breakdown of the SMX standard and of the collected metabolite (m/z 255.04395 uma, figure 23). Some structures of the fragments were not found. For most of the fragments observed the mass difference is either one or zero. The unit variation is explained by the substitution of the amine functional group by the hydroxyl on the SMX.

Mass spectrometry was also performed on the second metabolite collected from SMX biodegradation (retention time of 19 minutes). The compound had an m/z of 239.11 uma. This second metabolite was stable and resistant to fragmentation. The fragmentation pattern that was observed did not correspond to the one of SMX and it has not been possible to determine the structure. Although there is strong evidence of the presence of an alcohol because of the break down pattern, which corresponds to the loss of water (18 uma). The molecular peak ($M+H$) obtained from the Micromass QTOF was of 239.107 uma. From the nitrogen rule, the number of nitrogen atoms had to be zero or even. The most reasonable formula that meets the above conditions is $C_8H_{19}O_4N_2S$. The exact mass of this chemical structure is 239.10655 uma.

Table 7: Fragments observed from msms analysis of metabolite 2 (m/z 255)

| Fragment | Mass with X=NH ₂ (uma) | Mass with X=OH (uma) |
|--|--------------------------------------|-------------------------|
|  | 92.1 | 93.1 |
|  | 156.0 | 157.0 |
|  | 147.0 | 147.0 |
| unknown | 99.1 | 99.1 |
| unknown | 108.1 | 109.1 |

The chemical formula proposed for metabolite 2 is validated by 3 arguments; the first one is that it follows the nitrogen rule, the second is that the exact mass agreed the mass measured, and finally the third one is that it is composed of the same elements that SMX is made of. However, one striking difference between the chemical formula and SMX is the degree of unsaturation, or the number of hydrogen. The ring plus double bonds (r+db) (Ham, 2008) rule illustrates this discrepancy. The rule for an electrospray ionization process is:

$$\text{Equation 8 : } r + db = x - 1/2 y + 1/2 z + 1/2$$

Where x, y and z are the coefficients for a general formula C_xH_yO_NN_z.

Applying equation 6, a value of 6 is obtained for SMX but a value of 0 is calculated for the chemical formula proposed for the second metabolite. This means that the SMX structure underwent a major transformation. For instance, the aromatic ring of SMX could no longer be present with such formula. The degree of unsaturation calculated made the determination of the structure even more difficult. Even with the right formula, the possible number of isomer makes the determination of the structure unfeasible without hints from the msms spectrum.

The isotopic distribution can also be used to validate the formula proposed. Table 10 compares the obtained and calculated isotopic distributions with the same equation used previously (equation 4 and 5). The errors between the theoretical and the measured isotopic peaks are larger than the difference observed for the first metabolite (table 8). However the isotopic distribution is only an approximation and should not be used as an absolute tool of identification.

Table 8 : Measured and theoretical isotopic distribution for the second metabolite (M+H) isolated from SMX biodegradation experiment

| Mass | Formula of C ₈ H ₁₉ O ₄ N ₂ S | Measured |
|----------------|---|----------|
| | % | % |
| M+1 (240.1 g.) | 10.85 | 13.57 |
| M+2 (241.1 g.) | 5.57 | 7.16 |

Although a chemical formula seems to fit for the second metabolite, the exact structure could not be established because of the numerous possible isomers. Figure 17 shows one of the potential structures of the chemical formula C₈H₁₉O₄N₂S.

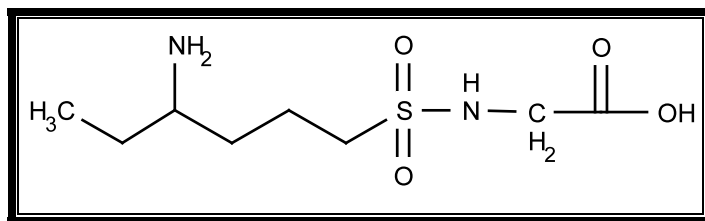


Figure 17 : One potential structure for the chemical formula $C_8H_{19}O_4N_2S$

A biodegradation of 14% was obtained when *Rhodococcus rhodochrous* was exposed to SMZ. However SMZ was not easily biodegraded. In fact biodegradation was not observed in some flask. Figure 15 shows an example of biodegradation of SMZ. It appears that the biodegradation began right after the stationary phase. It suggests that the drug might be consumed after the consumption of the glucose. A glucose assay test could have indicated if it has happened or not. Metabolites were unstable and had degraded by the time isolation was achieved.

VI. Conclusion

Analytical techniques were developed to ensure precise measurements of the concentration of the pharmaceuticals during biodegradation experiments. It was shown that the monitoring of the concentrations was greatly affected by the evaporation of water. Hence a method to overcome to water loss was developed that gave excellent results. The approach was to weigh samples and use these data to normalize concentrations. This technique was used for the subsequent biodegradation experiments.

Biodegradation was observed for most of the drugs tested. The data obtained reflected the low biodegradability of the drugs studied. Only trimethoprim showed total resistance to biodegradation. Carbamazepine was resistant to biodegradation by many microorganisms but slightly degraded by two microorganisms, *Rhodococcus rhodochrous* and *Aspergillus niger*. This resulted in the observation of two metabolites in the HPLC chromatograms. Both were present in very low concentrations and neither was identified.

Sulfamethoxazole was degraded by *Rhodococcus rhodochrous* (22%). Two metabolites were seen in HPLC chromatograms and these were isolated using a HPLC fraction collector. One of these was identified as (hydroxy-N-(5-methyl-1,2-oxazol-3-yl)benzene-1-sulfonamide). The exact position of the hydroxyl group is not known. The complete identification of the second metabolite was not achieved. However, a chemical formula was proposed ($C_8H_{19}O_4N_2S$).

Sulfamethizole was not easily biodegrade. *Rhodococcus rhodochrous* was able to co-metabolize this drug in some experiments. The highest level of biodegradation observed was of 14%. Metabolites were observed but these were unstable and not identified.

Co-metabolism was not found to significantly increase the amount of biodegradation of the pharmaceuticals tested. In a few cases, small amounts of metabolites were observed and a few of these were identified and found to be different from those observed in earlier work for direct biodegradation using the pharmaceutical as the sole carbon source.

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VIII. Appendices

Table 9 : Metabolites of CBZ from human and rat urine

| | |
|---|--|
| Hydroxy-CBZ, I | Hydroxy-10,11-dihydroxy-10,11-dihydroiminostilbene |
| Hydroxy-CBZ, II | Dihydroxyiminostilbene |
| 3-Hydroxy-CBZ | 3-Hydroxy-10,11-dihydroxy-10,11-dihydroiminostilbene |
| 2-Hydroxy-CBZ | Methylsulfinyliminostilbene |
| 2-Hydroxy-1-methoxy-CBZ | Methylsulfonyliminostilbene |
| 2-Hydroxy-3-methoxy-CBZ | N-Hydroxy-(methylthio)-iminostilbene |
| 1,2-Dihydroxy-CBZ | Methylthiohydroxyiminostilbene |
| 2,3-Dihydroxy-CBZ | Methylsulfinylhydroxyiminostilbene, I |
| Dihydroxy-CBZ, I | Methylsulfinylhydroxyiminostilbene, II |
| Dihydroxy-CBZ, II | Methylsulfinylhydroxyiminostilbene, III |
| Cis-10,11-Dihydroxy-10,11-dihydro-CBZ | Methylsulfinylhydroxyiminostilbene, IV |
| Trans-10,11-Dihydroxy-10,11-dihydro-CBZ | 9-Hydroxymethylacridan |
| Trans-2,3-Dihydroxy-2,3-dihydro-CBZ | Hydroxy-9-hydroxymethylacridan |
| 1,4-Dihydroxy-1,4-dihydro-CBZ | 10-Hydroxy-10,11-dihydroiminostilbene |
| Hydroxy-10,11-dihydroxy-10,11-dihydro-CBZ, I | 2-Hydroxyiminostilbene |
| Hydroxy-10,11-dihydroxy-10,11-dihydro-CBZ, II | 10-Hydroxyiminostilbene |

(Lertratanangkoon and Horning, 1982)

Table 10: Metabolites of CBZ from abiotic degradation

| Metabolites | Degradation process |
|---------------------------|--|
| Anthranilic acid | Ozonation ^a |
| Acridine | Sunlight ^b /Advanced (H ₂ O ₂ +UV) ^b |
| Acridine-9-carboxaldehyde | Advanced (H ₂ O ₂ +UV) ^b |
| Iminostilbene | Advanced (H ₂ O ₂ +UV) ^b |
| Acridone | Advanced (H ₂ O ₂ +UV) ^b |
| 2-hydroxybenzoic acid | Advanced (H ₂ O ₂ +UV) ^b |
| 2-hydroxyphenol | Advanced (H ₂ O ₂ +UV) ^b |
| 2-aminobenzoic acid | Advanced (H ₂ O ₂ +UV) ^b |
| 1-hydroxyacridine | Advanced (H ₂ O ₂ +UV) ^b |
| 2-hydroxyacridine | Advanced (H ₂ O ₂ +UV) ^b |

a (Andreozzi, et al., 2002), *b* (Vogna,et al., 2004)

Table 11: Reported metabolites of TMP

| Metabolites | Degradation process |
|--|---|
| α-hydroxytrimethoprim | Nitrifying bacteria ^a /Biotic ^c |
| 2,6-diamino-5-hydroxy-5-(3,4,5-trimethoxy-benzyl)-5,6-dihydro-1H-pyrimidin-4-one | Nitrifying bacteria ^a |
| 2,4-diolTMP | Abiotic ^b |
| 4-amino-TMP | Abiotic ^b |
| 2-amino-TMP | Abiotic ^b |
| 5-[(3,4,5-trimethoxyphenyl)carbonyl]pyrimidin-2,4-diamine | Abiotic ^b |
| 2-amino-5-[(4-hydroxy-3,5-dimethoxyphenyl)methyl] pyrimidin-4-ol | Abiotic ^b |
| 1-NO-TMP | Biotic ^c |
| 2-NO-TMP | Biotic ^c |
| 3-OH-TMP | Biotic ^c |
| 4-OH-TMP | Biotic ^c |

a (Eichhorn, et al., 2005), *b* (Bergh JJ, 1989), *c*(van 't Klooster, et al., 1992)

Table 12: Reported metabolite of SMX

| Metabolites | Degradation process |
|--|--------------------------------|
| SMX-N ₁ -glucuronide | Human metabolite ^a |
| N ₄ -hydroxy-SMX | Human metabolite ^{ab} |
| N ₄ -acetyl-SMX | Human metabolite ^a |
| 5-methylhydroxy-SMX | Human metabolite ^a |
| N ₄ -acetyl-5- methylhydroxy-SMX | Human metabolite ^a |
| 4-NO-SMX | Human metabolite ^b |
| 4-NO ₂ -SMX | Human metabolite ^b |
| SMX-AZ | Human metabolite ^b |
| SMX-AD | Human metabolite ^b |
| 4-amino-N-(5-methyl-oxazol-2-yl) benzenesulfonamide | Photochemical ^c |
| 1-[(4-aminobenzene)sulfonyl]-3- (2- oxopropyl)urea | Photochemical ^c |
| Aniline | Photochemical ^c |
| 3,5-dimethyl-1,2-oxazole | Photochemical ^c |
| 4-aminobenzene-1-sulfonic acid | Photochemical ^c |

a (Vree, et al., 1994), b (Monika Lovrek, 1998), c (Zhou and Moore, 1994)

Table 13: Composition of the MMSM

| Salt | Supplier | CAS # | Concentration in distilled water (g/L) |
|--|----------|------------|--|
| NH ₄ NO ₃ | Fisher | 6484-52-2 | 4 |
| KH ₂ PO ₄ | Fisher | 7778-77-0 | 4 |
| Na ₂ HPO ₄ | Fisher | 7558-79-4 | 6 |
| MgSO ₄ • 7 H ₂ O | A&C | 10034-99-8 | 0.2 |
| CaCl ₂ • 2 H ₂ O | Acros | 10035-04-8 | 0.01 |
| FeSO ₄ • 7 H ₂ O | Acros | 7782-63-0 | 0.01 |
| Na ₂ EDTA | Fisher | 6381-92-6 | 0.014 |

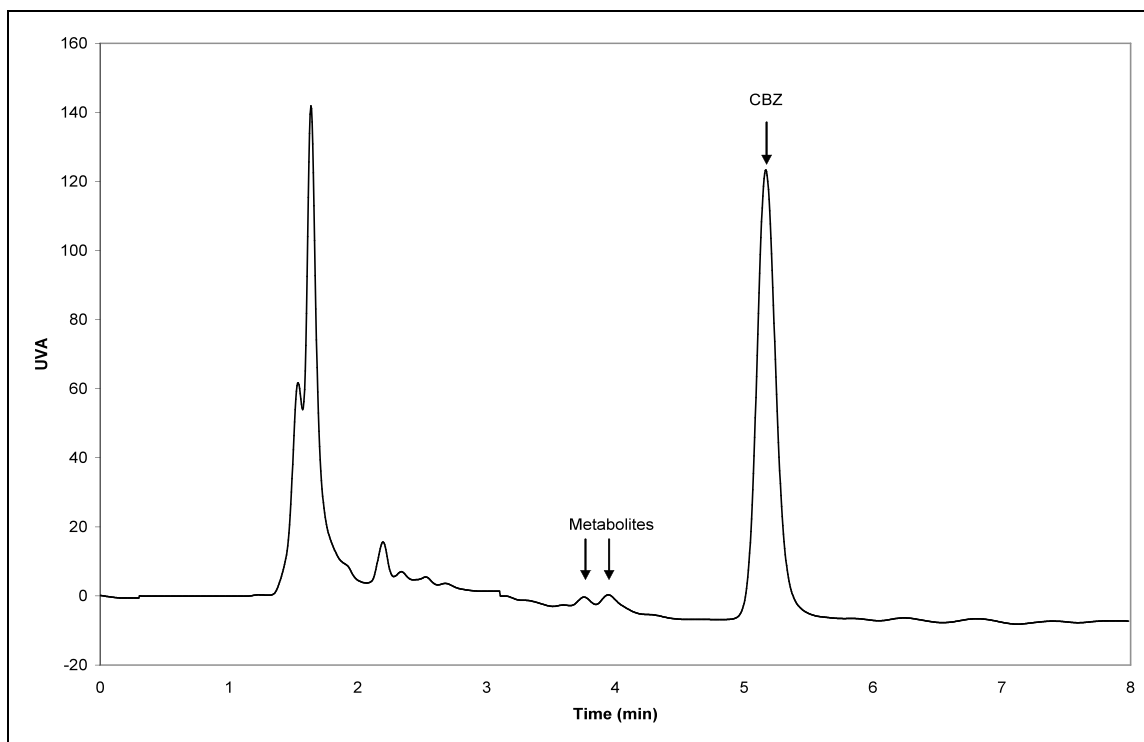


Figure 18 : Chromatogram of one CBZ sample obtained at the end of the biodegradation experiment conducted with *Aspergillus niger*

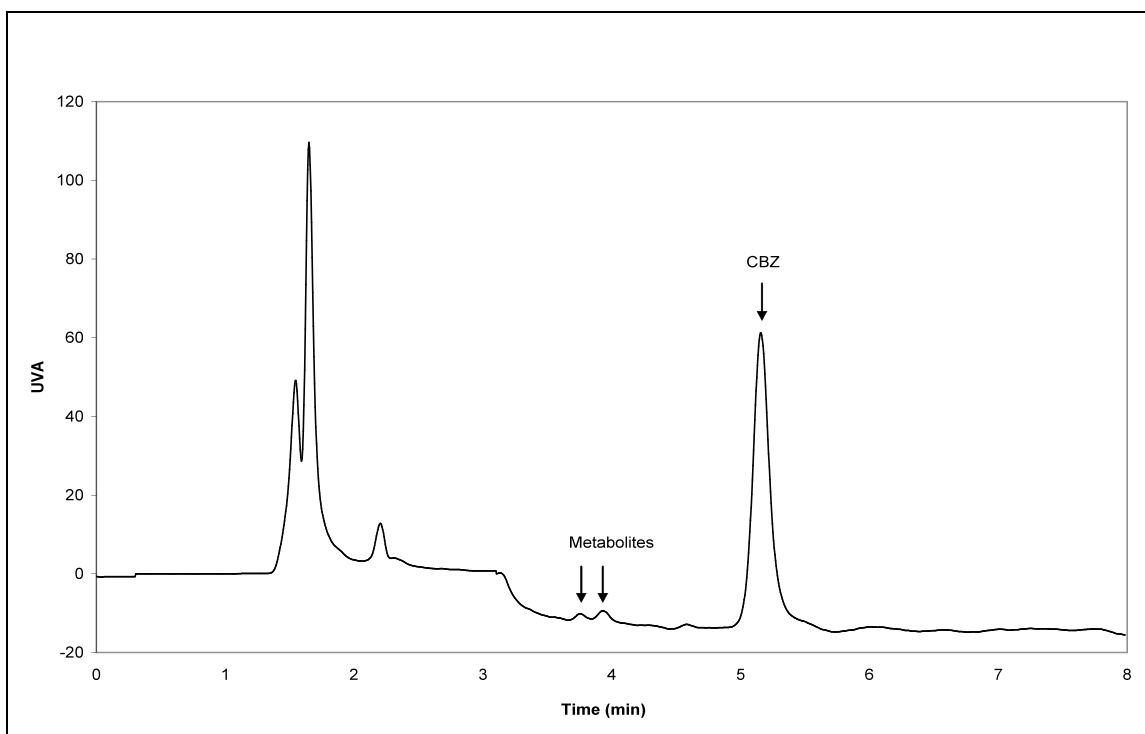


Figure 19: Chromatogram of one CBZ sample obtained at the end of the biodegradation experiment conducted with *Rhodococcus rhodochrous*

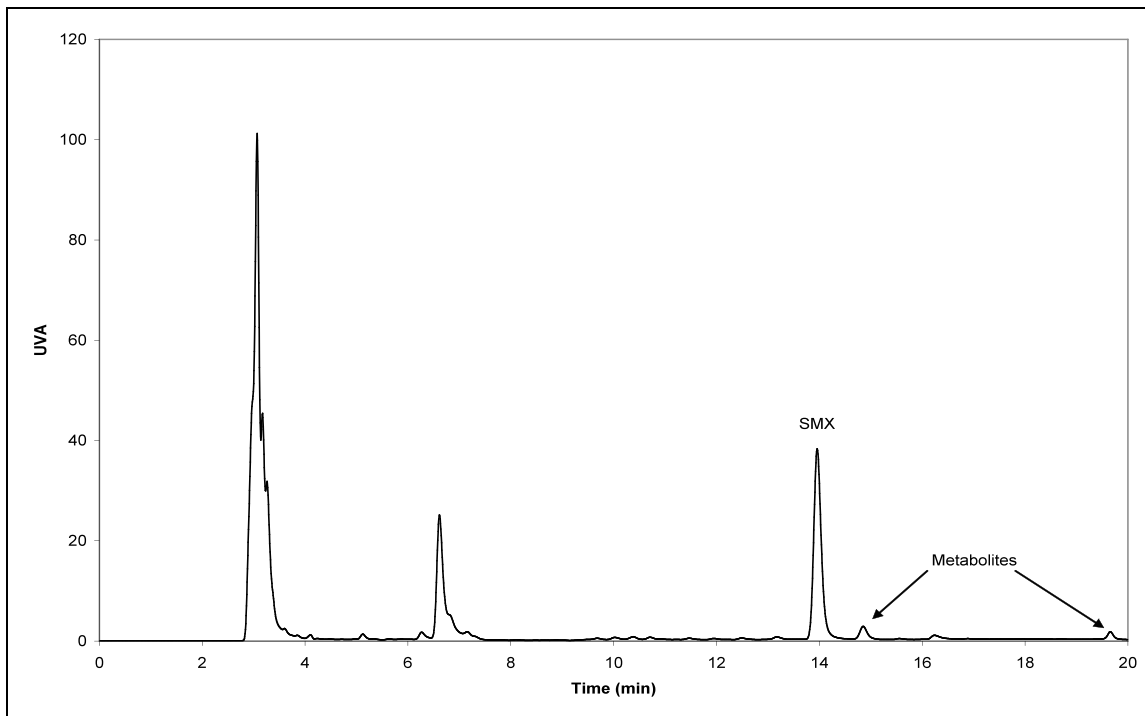


Figure 20: Chromatogram of SMX sample degraded by *Rhodococcus rhodochrous*

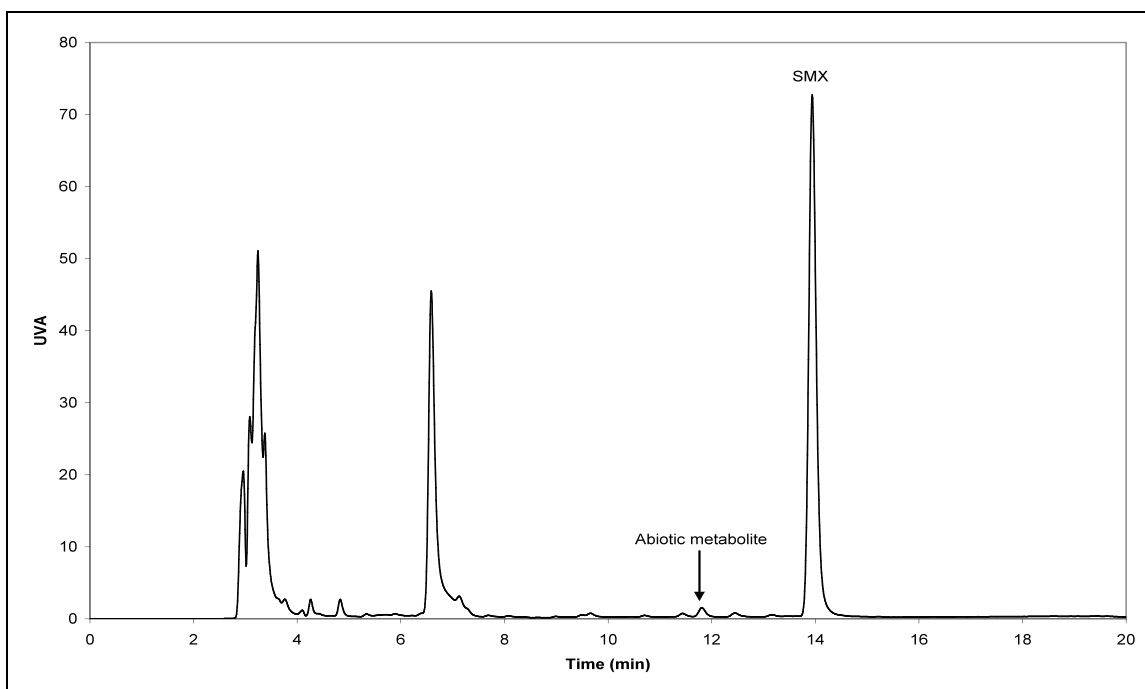


Figure 21: Chromatogram of SMX abiotic control

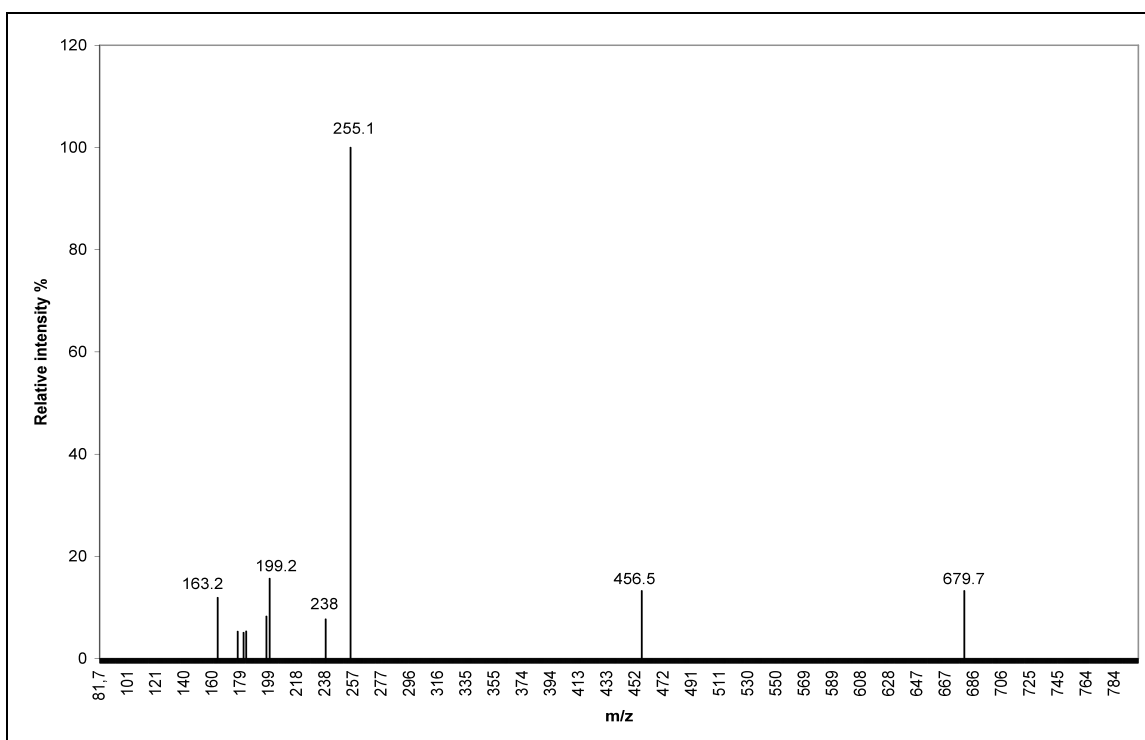


Figure 22: Mass spectra of SMX metabolite at retention time of 15 minutes recorded with Micromass QTOF 2

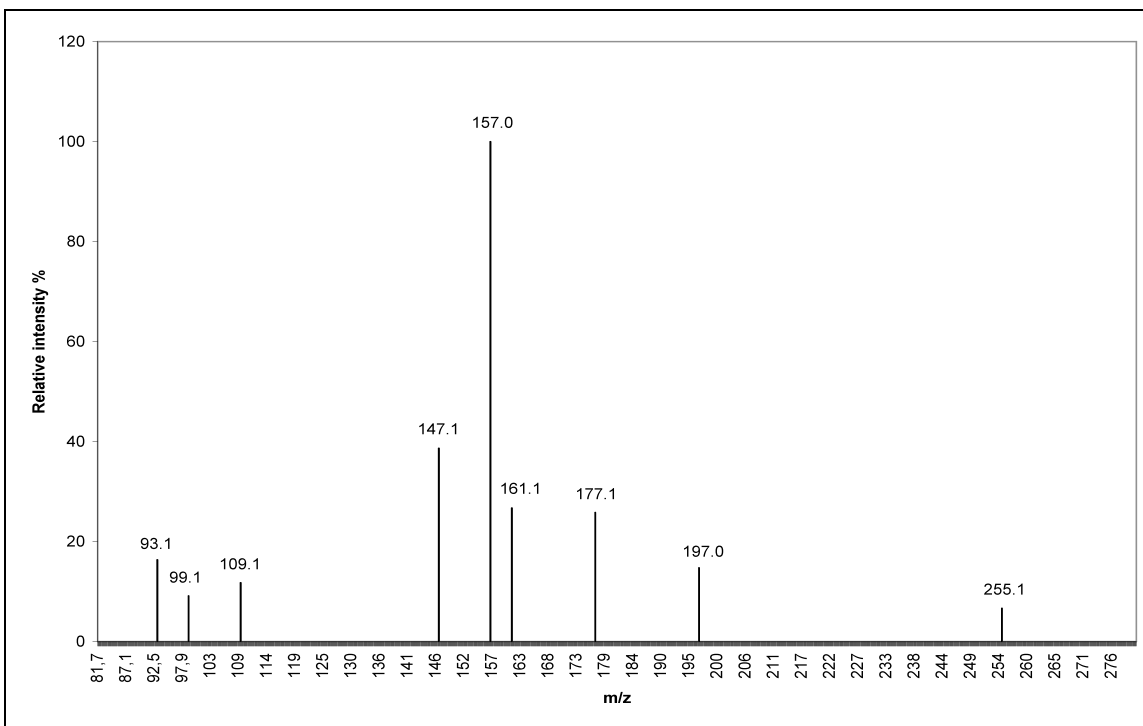


Figure 23: Mass spectra (MSMS) of peak with m/z of 255 recorded with Micromass QTOF 2

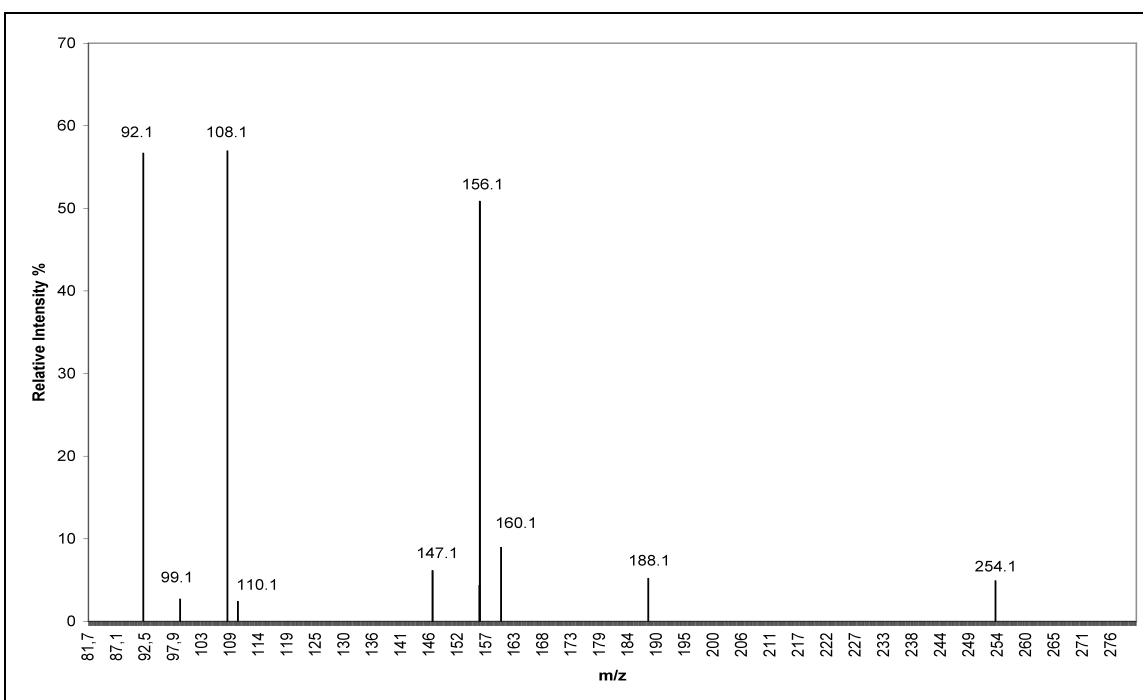


Figure 24: Mass spectra (MSMS) of SMX standard recorded with Micromass QTOF 2

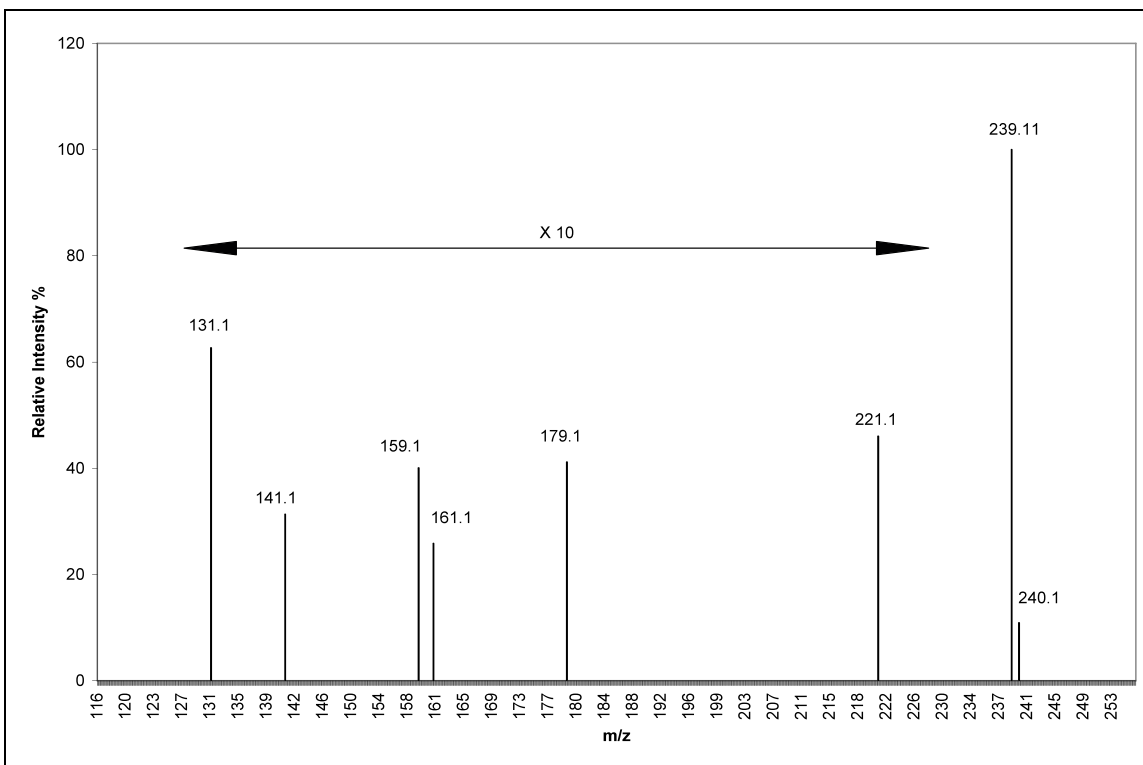


Figure 25: MSMS of the second SMX metabolite (Retention time of 19 minutes)

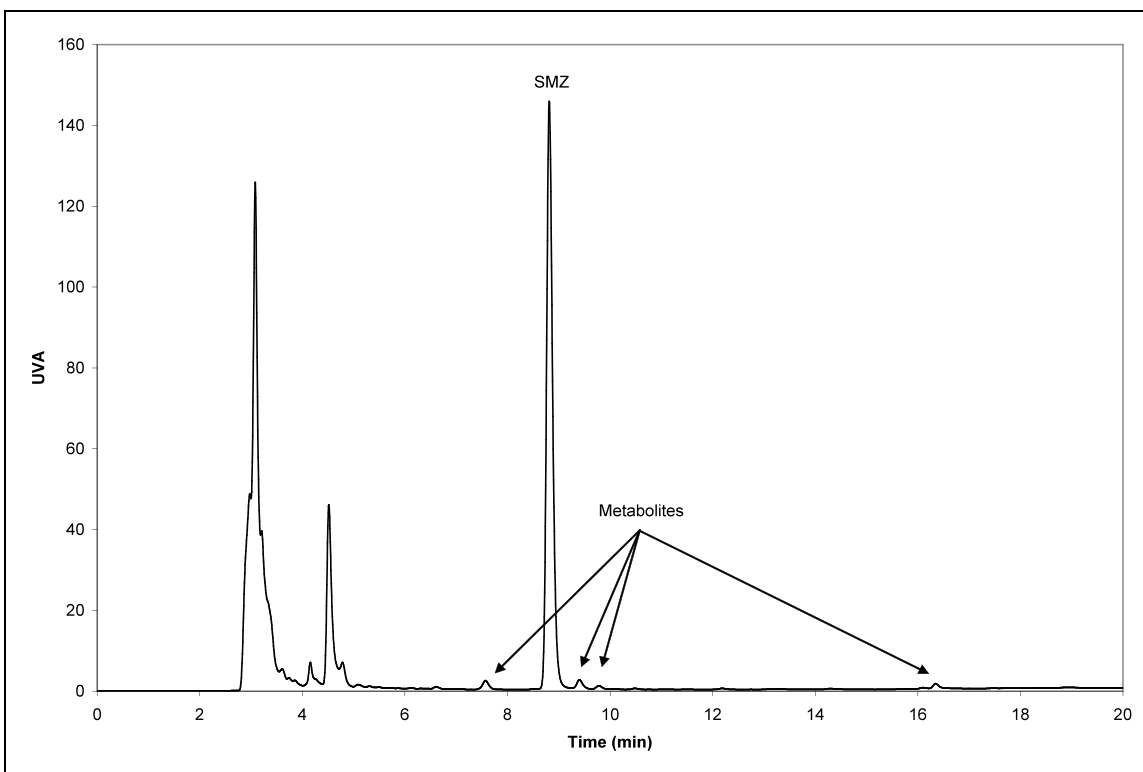


Figure 26: HPLC chromatogram of SMZ abiotic control

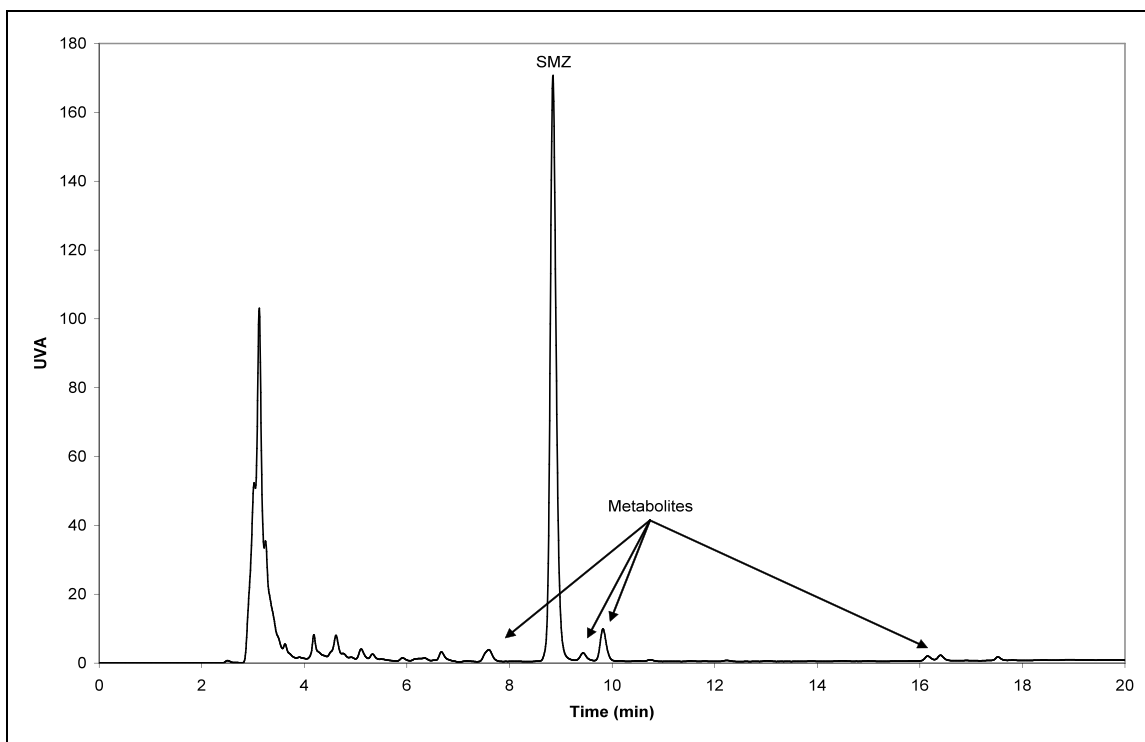


Figure 27: HPLC chromatogram of SMZ sample

Table 14 : Summary of result obtained from biodegradation experiments

| Microorganisms | CBZ | SMZ | SMX | TMP |
|-------------------------------------|------------|------------|------------|------------|
| <i>Rhodococcus rhodochrous</i> | 15% | 14 % | 22 % | 0 % |
| <i>Aspergillus niger</i> | 10% | --- | --- | 0 % |
| <i>Sphingomonas herbicidovorans</i> | 0 | --- | --- | --- |
| <i>Bacillus subtilis</i> | 0 | --- | --- | --- |
| <i>Pseudomonas fluorescens</i> | 0 | --- | --- | 0 % |
| <i>Pseudomonas putida</i> | 0 | --- | --- | --- |