Biodegradation of 17α-ethinylestradiol in Wastewater

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Abstract

Pharmaceutical substances such as 17α-ethinylestradiol (EE2), the active component of contraceptive pill, are often not eliminated during wastewater treatment and are not biodegraded in the environment. In this Master's project, analytical techniques for analyzing the low concentration of EE2 during biodegradation were developed and the biodegradability of 17α-ethinylestradiol was studied using five bacteria. A preliminary identification of the produced metabolites was also completed.

A low cost solid phase extraction (SPE) procedure was developed and optimized to obtain reproducible analyte recoveries in order to pre-concentrate the EE2 samples prior to HPLC analysis. The limit of analysis (LOA) was significantly lowered from 1.73 ppm to 0.173 ppm upon implementation of the combined SPE/HPLC method. *R. erythropolis* degraded EE2 up to 47% of its initial concentration in 13 h, while after 65 h, *R. equi* degraded EE2 up to 39% of its initial concentration. No significant EE2 degradation was observed using *Rhodococcus rhodochrous* and *Rhodococcus zopfii*. Preliminary analysis of EE2 degradation products confirmed the presence of phenol among other possible metabolites of high molecular weight.

Sommaire

Plusieurs composés pharmaceutiques tel que le 17α-ethinylestradiol (EE2), l'ingrédient actif des contraceptifs oraux, ne sont pas efficacement éliminés lors du traitement des eaux usées et se biodégradent peu dans l'environnement. Au cours de ce projet de maîtrise, des techniques analytiques ont été développées afin de mesurer de faibles concentrations d'EE2 et la biodégradabilité d'EE2 a été étudiée en utilisant cinq types de bactéries. Une identification préliminaire des produits de dégradation a également été effectuée.

Une méthode d'extraction en phase solide a été développée afin de préconcentrer les échantillons avant l'analyse par HPLC. L'optimisation de la méthode a permis d'obtenir une méthode reproductible offrant un haut pourcentage de récupération de l'analyte et ce, à faible coût. La préconcentration de l'échantillon a aussi permis de réduire la limite d'analyse par HPLC de 1.73 ppm à 0.173 ppm. *R. erythropolis* a dégradé jusqu'à 47% de la concentration initiale d'EE2 en 13 h, tandis qu'après 65 h, *R. equi* a engendré une dégradation atteignant 39% de la concentration initiale d'EE2. Aucune dégradation significative a été observée en utilisant *R. rhodochrous* et *R. zopfii*. Finalement, l'analyse préliminaire des produits de dégradation confirme la présence de phénol parmi d'autres produits de dégradation de poids moléculaires élevés.

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I. Introduction

There has been increasing concern over the past ten years regarding the occurrence and fate of low-level concentrations of pharmaceuticals, hormones, and other organic contaminants in the aquatic environment. Over 80 pharmaceutical compounds have been detected at μg/L concentration levels in surface, sewage and ground waters in countries such as Canada, Brazil, Germany and the United States, among others (Heberer, 2002; Boyd et al., 2003). Several studies have demonstrated some evidence that pharmaceutical substances are often not eliminated during waste water treatment and additionally are not biodegraded in the environment. Pharmaceutical contaminants are also released into the environment through untreated human and animal excreta, manure run-off as well as unused pharmaceuticals that have not been properly disposed. Within the various pharmaceutical categories, particular attention is being focused on hormones and endocrine-modulating substances. Hormonal activity has become a widely recognized mechanism of toxicity, and studies have shown that exposure to endocrine-modulating substances can impair reproductive function in adults of either sex, lead to irreversible abnormalities when administered during development, or in the most extreme cases cause cancer (Jobling et al., 1998). Exemplifying these traits is the synthetic estrogen, 17α-ethinylestradiol (EE2), the main ingredient in the contraceptive pill, which has been found at detectable levels in sewage, ground and surface waters.

Despite improved research on the assessment and management of risks associated with pharmaceuticals in the environment, few studies have been directed at analyzing pharmaceutical biodegradation in addition to measuring the toxicity and estrogenic activity of metabolites formed during waste water treatment. Thus the main objectives of the project were to:

1. Develop analytical techniques for analyzing the low concentration of EE2 during biodegradation;
2. Identify microorganisms capable of degrading 17α-ethinylestradiol;
3. Identify the metabolites formed in order to evaluate their toxicity.

The literature review, presented in section II, begins by examining the impact of pharmaceuticals and more specifically estrogens in the environment, along with an overview of the biodegradation of pharmaceuticals using activated sludge treatment. General information about EE2 is presented followed by a discussion of previous studies completed on the biodegradation of EE2. Lastly, the selected microorganisms for biodegradation experiments are described. Analytical techniques that have been developed in order to quantify EE2 in addition to identifying metabolites are outlined in section III. This section also includes the overall approach and methods pertaining to the degradation experiments. A discussion of the results obtained is presented in section IV. Recommendations and conclusions are provided in the final sections of the thesis.

II. Literature Review

2.1 Rationale

Pharmaceuticals and hormones are a class of emerging environmental contaminants that are extensively and increasingly being used in human and veterinary medicine (Fent et al., 2006). In Germany, some pharmaceuticals are used in quantities of more than 100 tons/year (Ternes et al., 2002). Table 1 provides the daily quantities of estrogens excreted by humans. Industrial contamination is another source of contamination and occurs as a result of accidental discharges during production, however it is usually minimized due to the high value of the chemicals (Winkler et al., 2001). This would indicate that the domestic source represents the main and most consistent pathway of contamination. Surplus drugs in households, for example, are disposed to waste water.

Table 1: Daily excretion (µg) of estrogenic steroids in humans (Ying et al., 2002)

Category	Estrone (E1)	17β-Estradiol (E2)	Estriol (E3)	*17α-ethinylestradiol (EE2)*
Males	1.6	3.9	1.5	-
Menstruating females	3.5	8	4.8	-
Menopausal females	2.3	4	1	-
Pregnant women	259	600	6000	-
Women	-	-	-	*35*

Of environmental concern is not necessarily a high production volume of a certain pharmaceutical per se, but the environmental persistence and critical biological activity of that pharmaceutical (Fent et al., 2006). Most antibiotics are designed so

that they retain their chemical structure long enough to complete their therapeutic work and this combined with their continuous input could enable them to persist in the environment for lengthy periods of time (Roberts and Thomas, 2006). Pharmaceuticals may not be completely metabolized during wastewater treatment producing metabolites or conjugates as well as residual concentrations of the pharmaceutical. Estrogens are excreted mainly as conjugates of sulfuric and glucuronic acids. Conjugates can however be cleaved in sewage treatment plants due to the presence of various bacteria resulting in the release of active parent compound as shown for estradiol and EE2 (Ternes et al., 1999b).

17α-ethinylestradiol (EE2), a synthetic estrogen used in the oral contraceptive pill, as well as natural estrogens such as 17-β-estradiol (E2), are detected in ecologically relevant amounts in sewage effluents, surface water, river water, bed sediments, and in activated and digested sludge (Weber et al., 2005). In fish, as in all other vertebrates, estrogens play an important role in many reproductive and developmental processes, including sexual differentiation. For example, less than 1 ng/L EE2 and even as little as 0.1 ng/L can stimulate male rainbow trout to produce an egg yolk protein normally only associated with sexually mature female called vitellogenin (Jurgens et al., 2002); and 4 ng/L caused male fathead minnows to fail to develop normal secondary sexual characteristics such as gonad development, sex determination, and reproductive maturity (Lange et al., 2001). In a study performed in the United Kingdom, wild male roach found in rivers and estuaries that receive municipal wastewater effluent showed increased vitellogenin and oocytes within testicular tissue, a condition termed “intersex” (Parrott and Blunt, 2005). In the case of male zebrafish, full life cycle exposure to just 3 ng/L of EE2 caused gonadal feminization resulting in limited reproduction abilities (Fent et al., 2006). While no effects related to the presence of EE2 or other estrogens in wastewater effluents have been documented on humans it has been hypothesized that the statistically derived decrease in sperm counts over the last decades, increasing incidents of testicular cancer and other disorders regarding male infertility may be caused by the intake of

estrogens via food or drinking water (Ternes et al., 1999a). It is due to these reasons that EE2 needs to be more efficiently eliminated during waste water treatment.

In today's society, the majority of sewage treatment plants (STPs) were constructed in the 1960s and were designed to meet simple water quality criteria such as biological oxygen demand (BOD) and total organic carbon (TOC) (Hallas and Heitkamp, 1995); they were not designed to remove or eliminate pharmaceuticals and hormones from effluents. As a result, there exists a wide variety of transport pathways for pharmaceuticals and hormones to enter and persist in sewage, ground and surface waters (Kolpin et al., 2002). Elimination rates of pharmaceuticals can vary depending on several factors such as hydraulic retention time, season and performance of the STP. Estrogens, in particular EE2, have been continually detected at the ng per liter level in sewage treatment effluents. In ten Canadian municipal wastewater effluents (MWWEs) EE2 was discharged into the environment at concentrations up to 42 ng/L (Ternes et al., 1999a). The median concentration for EE2 and E2 in Canadian STP effluents were 9 and 6 ng/L, respectively (Ternes et al., 1999a). The maximum concentration of EE2 from four MWWEs in Michigan was measured to be much lower at 0.8 ng/L (Parrott and Blunt, 2005). In another study, Baronti et al. (2000) measured E2 and EE2 concentrations up to 25 and 13 ng/L, respectively, in untreated sewages in the Tiber River located in Rome, Italy. In seven STPs in the UK, EE2 and E2 were detected in sewage treatment effluents in concentrations ranging from 0 – 7 ng/L and 1 – 50 ng/L, respectively, while effluents from three Dutch wastewater treatment plants contained EE2 concentrations of 0.2 – 7.5 ng/L (Svenson et al., 2003). Ternes et al. (1999a) also measured estrogen levels in sixteen German municipal sewage treatment effluents and found concentrations of EE2 up to 15 ng/L. Lastly, and certainly most alarming, is that EE2 was detected in groundwater and in raw and purified drinking water in Germany up to concentrations of 2.4 ng/L (Heberer, 2002).

The continued exponential growth of the human population has created a greater demand for implementing advanced wastewater technologies or in the very least

improving existing processes so as to protect our water resources from residual pharmaceuticals and hormones, such as EE2. Additionally, due to analytical difficulties associated with the determination of such low estrogen concentrations in the aquatic environment studies regarding the occurrence and abundance of estrogens have been limited. While techniques have been developed to measure the low concentrations of EE2 in water samples most methods are too expensive for large amounts of samples that are typically involved in research projects. This raises the concern for the development of more cost effective and overall more efficient analytical techniques.

2.2 EE2

As previously stated, EE2, a synthetic estrogen, is the main component in the oral contraceptive pill. The contraceptive pill contains between 30 and 50 µg of EE2 per pill. EE2, its molecular structure seen in Figure 1, is also used in the stockbreeding industry as a hormonal agent.

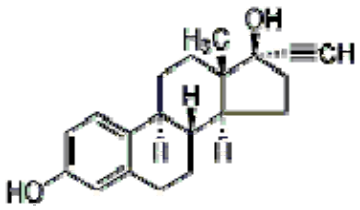

Figure 1: Molecular structure of 17α-ethinylestradiol (Ying et al., 2002)**.**

The annual production of EE2 is estimated to be a couple of hundreds kilograms per year in Europe (Fent et al., 2006), while the annual prescription of EE2 in Germany is approximated to be 50 kg/year (Ternes et al., 1999b). *In vivo* tests have demonstrated that EE2 is approximately 11-27 times more potent than the natural female sex hormone E2 (Huber et al., 2004) leading to suggestions that EE2 may be the most important endocrine disruptor. EE2's poor solubility in water is 4.8 mg/L (Ying et al., 2002), which is three times less soluble than natural estrogens.

2.3 Biodegradation of Pharmaceuticals

Biodegradation is defined as the breakdown of organic material by biological activity. The material is decomposed into carbon dioxide and water or other simpler components than the original compound. This technique has been applied in the removal of several environmental pollutants, such as plasticizers (Nalli et al., 2002), emulsifiers, wetting agents (Chen et al., 2005) and crude oil (Palittapongarnpim et al., 1998). However, not many studies have been conducted on the biodegradation of pharmaceuticals. Those who have explored biodegradation as a means to remove pharmaceuticals during wastewater treatment have done so using activated sludge. Activated sludge is a process in sewage treatment that is employed to remove and consume organic waste material. The sludge is typically composed of a mixed community of microorganisms able to degrade organics. Bacteria constitute the majority of microorganisms in activated sludge, but fungi, rotifers, and protozoans are also present. Some relevant studies investigating the use of activated sludge for biodegrading pharmaceuticals, in particular estrogens are reviewed in this section.

2.3.1 Biodegradation with Activated Sludge

Most papers investigating the degradation of pharmaceuticals and estrogens using activated sludge conducted batch experiments using some type of reactor under aerobic or anaerobic conditions. Urase and Kikuta (2005) examined the degradation of E2, E1, EE2, bisphenol A (BPA), benzophenone (BZP), clofibric acid (CA), gemfibrozil (GFZ), ibuprofen (IBP), fenoprofen (FEP), ketoprofen (KEP), naproxen (NPX), diclofenac (DCF), indomethacin (IDM), propyphenazone (PPZ) and carbamazepine (CBZ) by activated sludge through batch experiments using a lab scale reactor. The authors also sought to distinguish whether the pharmaceuticals and estrogens were being degraded or adsorbed by the microorganisms in the activated sludge. This was accomplished through the development of a reaction kinetic model (a two-phase fate model) in which the transfer of the target compounds between the water phase and the activated sludge phase and the degradation kinetics in the sludge phase were considered. Initial concentration of the target compounds was 100 μg/L.

E2, IDM and IBP were rapidly removed in comparison to the other compounds. EE2 remained relatively stable, however other pharmaceuticals exhibited more persistent behaviour, such as CA, CBZ and DCF (Urase and Kikuta, 2005). The developed two-phase fate model was then used to determine the contribution of adsorption and degradation by analyzing the separate concentrations of the target compounds in the water phase and sludge phase. EE2 exhibited the highest adsorption rate (more than 60%) after 20 min, while the majority of the pharmaceuticals had low adsorption tendencies (Urase and Kikuta, 2005). The authors concluded that most likely EE2's removal from treatment plants is more to do with excess sludge extraction than actual degradation.

Aerobic batch experiments were conducted to determine the biodegradability of natural and synthetic estrogens using a slurry of activated sludge from a STP diluted in drinking water (Ternes et al., 1999b). Estrogens were spiked to levels of 1 μg/mL and 1 ng/mL. After a period of 1 – 3 h, over 95 % of E2 had been removed, but the concentration of E1 had increased up to 95% with regard to E2's initial concentration (Ternes et al., 1999b). This was due to the oxidation of E2 which yields E1. Regarding experiments with E1, its concentration decreased by about 50% after 24 h. No degradation products were observed. While E2 and E1 were shown to degrade appreciably, the concentration of EE2 remained relatively stable. Approximately 20% of EE2 degraded after 24 h at an initial concentration of 1 ng/mL (Ternes et al., 1999b). Further confirming the persistence of EE2 in activated sludge, mestranol was degraded by 80% after 24 h, producing EE2 as a metabolite which in turn showed no signs of degradation up to 72 h. A study performed by Weber et al. (2005) supported the results obtained by Ternes et al. (1999b) regarding the degradation of EE2 and E2. Using activated sludge from a membrane bioreactor and from a conventional STP, batch experiments demonstrated that EE2 remained persistent after 70 h, while E2 was rapidly oxidized to E1 (E1 was then undetectable after 72 h) (Weber et al., 2005).

Another study investigated the biodegradation of E1 and E2 by also utilizing aerobic batch reactors with return activated sludge (RAS). Results demonstrated a removal of

more than 95% of both estrogens within 24 h (Servos et al., 2005). Despite these positive results, traces of E1 remained in the reactors after 26 days, possibly because E2 was being oxidized to E1. An additional study using aerobic and anaerobic reactors with activated sludge concluded similar findings observing a rapid degradation of E2 to E1 with no other metabolites being detected (Lee and Liu, 2002).

Besides estrogens, antibiotics have also been the subject of studies regarding biodegradation using activated sludge. A series of tests simulating biodegradation in activated sludge was performed with 12 different sulfonamides (Ingerslev and Halling-Sorensen, 2000). Once biodegradation had occurred in unadapted sludge, the sludge was considered sulfonamide acclimated. It was then respiked with the same drug as used for adaptation or with other sulfonamides than those used for adaptation purpose of describing the kinetics of the degradation pattern before and after adaptation of the sludge. Based on screening experiments, sulfonamides were classified as not readily biodegradable. However, following the adaptation period in aerated reactors, sulfonamides were observed to degrade. Degradation was completed in 2 to 4 days after a lag period of 6 to 12 days at 20^{o}C (Ingerslev and Halling-Sorensen, 2000).

2.3.2 Biodegradation with Nitrifying Activated Sludge

Contrary to Ternes et al. (1999b), Urase and Kikuta (2005), and Weber et al. (2005) studies performed by Shi et al. (2004) and Vader et al. (2000) demonstrated that EE2 could be removed using nitrifying activated sludge (NAS). Approximately 90% of EE2 was degraded after 96 h, while E2 was almost completely degraded within 2 h (Shi et al., 2004). An unknown degradation intermediate of EE2 was produced, however it was further degraded. Additionally, experiments were conducted to determine the effects of ammonia oxidation inhibition during estrogen degradation experiments. The results showed that ammonia oxidizing bacteria combined with other microorganisms in NAS were responsible for the degradation of the estrogens (Shi et al., 2004). NAS was also shown to degrade EE2 within 6 days in a study

where activated sludge was enriched with nitrifying microorganisms (Vader et al., 2000). Based on the results from these two studies, the most probable reason for why the studies completed by Ternes et al. (1999b), Weber et al. (2005) and Urase and Kikuta (2005) did not effectively remove EE2 is the use of NAS.

2.3.3 Biodegradation of Estrogens using Enzymes

Another study focused on removing the estrogenic activity of E2 and EE2 using manganese peroxidase (MnP) and a laccase-mediator system with 1-hydroxybenzotriazole (HBT) as mediator. This approach lowered the estrogenic activities of E2 and EE2 by over 80% in 1 h and completely removed the activities after 8h (Suzuki et al., 2003). Metabolites were not detected by HPLC analysis.

2.4 Biodegradation of EE2 using Pure Cultures

There have not been many studies focused on the biodegradation of estrogens in wastewater, specifically EE2, using pure microorganisms. The advantage of using pure cultures versus activated sludge is that since activated sludge is composed of numerous bacteria it is unclear as to what is degrading the target compound. Shi et al. (2002) isolated *Fusarium proliferatum* from a cowshed sample and identified it as a microorganism capable of degrading EE2. With an initial concentration of 25 mg/L, 97% of EE2 was removed in 15 days (Shi et al., 2002). Degradation products were shown by RP-HPLC analysis, however they were not identified.

Ammonia-oxidizing bacterium *Nitrosomonas europaea* was able to degrade natural and synthetic estrogens during a 187 h sample period (Shi et al., 2004). E1, E2, E3 and EE2 had similar biodegradation rate constants of 0.0022 mg/L h, 0.0020 mg/L h, 0.0016 mg/L h and 0.0019 mg/L h, respectively. Degradation products were observed from an EE2 sample after 120 h. An HPLC chromatogram shows a large peak at 4 – 7 min presumably an unknown degradation product of EE2 (Shi et al., 2004) whose retention time is 14.5 min. The authors suggested that this degradation product is

more polar than EE2 and has a phenolic group (Shi et al., 2004). The final study that was examined where pure cultures were used for the purpose of biodegrading estrogens identified four strains of microorganisms from an enrichment culture of activated sludge from wastewater treatment plants capable of degrading E1, E2, E3 and EE2 (Yoshimoto et al., 2004). Of the four strains, three were of *Rhodococcus equi*, Y50155, Y50156 and Y50157 and one belonged to *Rhodococcus zopfii*, Y50158. Each estrogen had an initial concentration of 100 mg/L in a solution of water/methanol solution. *R. equi* degraded EE2 by 70% in 8 h and by 80% in 24 h (Yoshimoto et al., 2004). Additionally, *R. equi* degraded E2 by 80% in 5 h and 99% in 24 h (Yoshimoto et al., 2004), with similar findings for the degradation of E1 and E3. *R. zopfii* was particularly efficient in degrading all four estrogens with results demonstrating a degradation of 70% in 6 h for EE2, 81% in 2 h for E2, E1 by 91% in 3 h and E3 by 96% in 4 h (Yoshimoto et al., 2004). After 24 h, all four estrogens were completely degraded. No degradation products were determined by GC-MS analysis.

In each of the aforementioned studies, when degradation products were produced and detected they were not identified, as well as no possible degradation pathways have been proposed for the degradation of EE2. One step further would be to determine the toxicity of the known metabolites to see if in fact the degradation products are more harmful than the original EE2 compound. Also common to the studies reviewed was the lack of a carbon source during cultivation of the bacteria and degradation of estrogens. The addition of a carbon source during degradation would be interesting to investigate given that it would better simulate waste water treatment conditions. These steps are significant to the understanding and improvement of wastewater treatment in terms of removing EE2 from ground and surface waters and in studying the fate of EE2 in the environment when exposed to common soil microorganisms.

The following section examines the four bacteria selected for attempting to degrade EE2 based on the literature reviewed.

2.5 Selected Microorganisms

Five bacteria were chosen for the biodegradation experiments, *Mycobacterium fortuitum*, *Rhodococcus zopfii*, *Rhodococcus rhodochrous*, *Rhodococcus equi* and *Rhodococcus erythropolis*. A general description of each microorganism along with its known or expected potential for degrading EE2 is presented in the following sections. Table 1 provides a brief overview of some differences between the genera *Mycobacterium* and *Rhodococcus*.

Table 2: Comparison of Selected Bacteria Genera for Degradation Experiments

Characteristics	*Mycobacterium*	*Rhodococcus*
Morphology	Rods, occasionally branched filaments.	Scanty mycelium, fragmenting into irregular rods and cocci.
Growth Rate	2 – 40 days	1 – 3 days

(Sneath, 1986)

2.5.1 Mycobacterium fortuitum

M. fortuitum is commonly found in soil, rivers and lakes. It appears as rods 1 – 3 μm in length with occasional beaded or swollen cells having nonacid-fast ovoid bodies at one end (Sneath, 1986). M. fortuitum is known as a rapidly growing species of the genus *Mycobacterium* and can cause various clinical syndromes. With regards to *M. fortuitum*'s biodegradation ability, it has been successfully used to degrade cholesterol (Yoshimoto et al., 2004). Steroid hormones in general are synthesized from cholesterol (Figure 2) and have in common a cyclopentan-o-perhydrophenanthrene ring hence there is speculation that *M. fortuitum* could possibly biodegrade EE2.

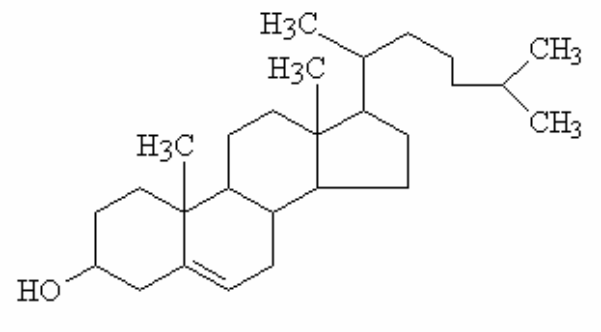

Figure 2: Molecular structure of cholesterol

2.5.2 Rhodococcus rhodochrous

R. rhodochrous is isolated from soil and grows as branched filaments which can fragment into rods and cocci. This microorganism has been shown to degrade or partially degrade various compounds, for example plasticizers such as bis 2-ethylhexyl adipate (BEHA), dioctyl phthalate (DOP) or dioctyl terephthalate (DOTP) (Nalli et al., 2002).

2.5.3 Rhodococcus erythropolis

R. erythropolis is also isolated from soil. It is common for this microorganism's morphology to vary. Colonies are usually circular to slightly irregular in form and are opaque and pale orange in colour. *R. erythropolis* is increasingly being used in biotechnological applications as its cells contain a large number of enzymes that allow them to carry out numerous bioconversions and degradations (Carla and Fonseca, 2005). Yoshimoto et al. (2004) reported that similar to *M. fortuitum*, *R. erythropolis* is capable of degrading cholesterol and hence might be capable of degrading EE2.

2.5.4 Rhodococcus zopfii

R. zopfii colonies are described as circular and pulvinate in shape and pale-orange in colour. The microorganism has been isolated from activated sludge in wastewater treatment plants using bioreactors and has been shown to degrade EE2 up to 70 % in

6 h (Yoshimoto et al., 2004). *R. zopfii* has also been found to degrade toluene, chlorobenzene and dichlorobenzene.

2.5.5 Rhodococcus equi

R. equi is an aerobic bacterium found in soil, herbivore dung and in the intestinal tract of some animals such as cows and pigs. *R. equi* is described as a facultative intracellular bacterium with rod to coccus pleomorphism. Yoshimoto et al. (2004) demonstrated that *R. equi* can degrade EE2, up to 80% of its initial concentration after 24 h.

III. Materials and Methods

3.1 Preconcentration of Samples using Solid Phase Extraction

Considering the low concentration of EE2 and its metabolites, a method to preconcentrate the samples was required prior to high performance liquid chromatography (HPLC) analysis. Different solid phase extraction (SPE) procedures have been developed over the last years for preparing environmental samples. However, these methods use very expensive materials such as Oasis HLB cartridges and isotopes for surrogates. Due to the large amount of samples involved in this project, a more cost effective method was needed to perform reliable SPE.

In developing the current SPE procedure, several variations to the solid phase extraction steps were tested. For example, the brand of cartridge, the volume of eluent, the type of filter paper and the nature of the performance surrogate were investigated and optimized in order to maximize and obtain reproducible analyte recoveries for neutral or basic pharmaceuticals such as EE2. The optimized procedure is presented in section 4.1 of the results section, while the full detailed procedure can be found in Appendix I.

3.2 Measurement of Estrogen Concentrations by HPLC

The concentration of EE2 was measured using an Agilent 1100 Series HPLC equipped with a Zorbax SB-C18 column (3.5mm, 4.6 x 75mm) and guard cartridge SB-C18 (5mm, 4.6 x 12.5mm). The detailed operating conditions and parameters for the method developed to analyze EE2 are listed in Table 3.

The calibration curves for EE2 and E2 can be found in Appendix II. These curves demonstrate the relationship between the peak areas of the pharmaceuticals for known concentrations. The peaks for the EE2 and E2 using the developed HPLC

method are shown in figure 3. Retention times for EE2 and E2 are 8.5 min and 6.9 min, respectively.

Table 3: HPLC Parameters

Parameters	EE2
Mobile Phase	A = 20mM NaH_2PO_4,, pH 2.0
	pH adjusted with H_2SO4
	B = acetonitrile
Flow Rate (ml/min)	1.0
Gradient	50% acetonitrile
	50% 20 mM NaH_2PO_4
Column Wash	16min = 10% B
Detector	UV 279 nm
Temperature (°C)	28
Stop Time (min)	10
Post Time (min)	5
Injection Volume (µl)	10

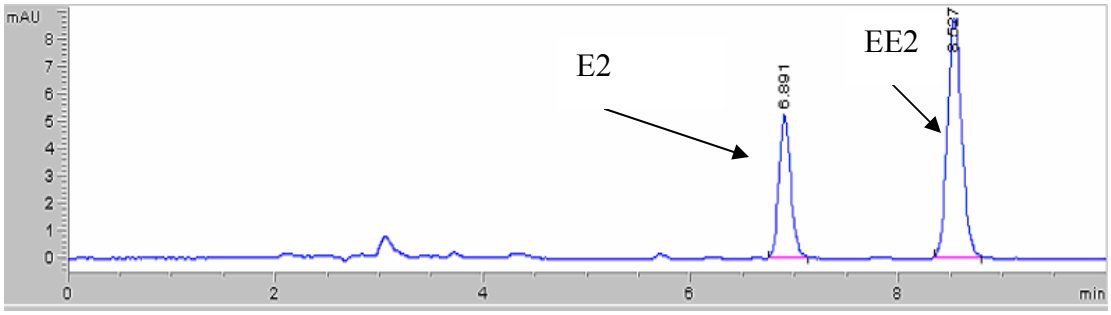

Figure 3: HPLC Chromatogram

The limit of detection (LOD) was determined to be 0.58 ppm, which yielded a limit of analysis of 1.73 ppm (three times the LOD). The limit of detection was determined by analyzing ten samples of a mixed solution 80 wt. % HPLC water and 20 wt. % ethanol and calculating the standard deviation of the height of the peaks at the retention time for EE2. By using the height of peaks of a known concentration one can associate a concentration to the calculate standard deviation. From a concentration 1.73 mg/L, which is the limit of analysis, the precision of the method is evaluated at 1.15 %. This value was evaluated by using relative standard deviation. Ten samples of a known concentration were analyzed. The standard deviation of concentrations obtained divided by the mean gives the relative standard deviation. The accuracy of the method was also verified by analyzing one standard sample before each run of experiments.

3.3 Identification of Degradation Products

3.3.1 Volatile Organic Compound

An experimental procedure was designed with the purpose of collecting any volatile organic compounds (VOCs) that were released during the biodegradation of EE2. *R. erythropolis* was selected for this experiment given the favourable results obtained by *R. erythropolis* for EE2 degradation (see section 4.3). The experimental setup is shown in Figure 4. Following the procedures in section 3.4, *R. erythropolis* was cultivated in one 500 ml Erlenmeyer flask. EE2 was added during the stationary phase at an initial concentration of 1.4 ppm. The flask was placed in the incubator shaker and two plastic tubes were inserted into the foam top of the flask. One tube supplied nitrogen to the flask, while the second tube carried the VOCs to a collection test tube placed in another flask inside the incubator. The collection tube was packed with silanized glass wool (supplied by Chromatographic Specialties Ltd) and 1 g of silicon oxide Chromosorb WHP (lot # R23484, supplied by Supelco, Inc). The nitrogen tank was opened and a flow of 1 ml/min was supplied to the EE2 solution for 24 h. The silicon oxide beads were extracted from the collection tube and placed in a headspace vial. The vial was set in an ice water bath at 0^{o}C while the oxidized

environment inside the vial was extracted and replaced with helium. The vial was removed from the water bath and brought to room temperature. Using a DigiPrep Jr. graphite heating block coupled with a built in thermocouple, the vial was heated to 120^{o}C and remained at that temperature for one hour. A 10 µl aliquot was injected into the GC MS.

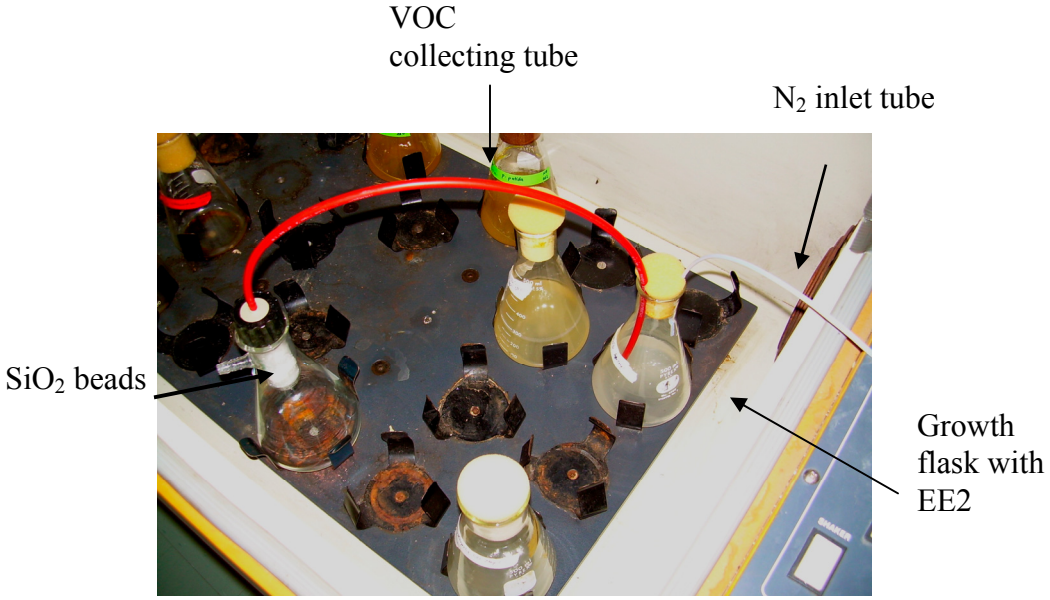

Figure 4: VOC Experimental Setup

3.3.2 Gas Chromatography – Mass Spectrometry (GCMS)

Gas chromatography & mass spectrometry were used to identify some of the degradation products. All experiments were performed on a Thermo (Trace GC 2000) gas chromatograph equipped with a RTX-5MS column (length: 30m, film thickness: 0.25 µm, I.D.: 0.25 mm) and a Thermo (GCQ/Polaris MS) mass spectrometer. GC-MS parameters needed to carry out analyses are presented in Appendix III. Helium was used as carrier gas at a constant flow of 1.0 mL/min. A blank and matrix correction was completed for the injected sample. MSn^1 analysis was then completed on the sample.

3.3.3 Gas Chromatography

Based on EE2's chemical structure, some assumptions regarding potential degradation products formed during biodegradation can be made. Expected degradation products were methanol, ethanol, and phenol. Gas chromatography was used to confirm the presence of those compounds after the degradation step. All analyses were performed on an Agilent 5890 gas chromatograph equipped with a Stabilwax (Length: 30 meters, ID: 0.32 mm, bonded with phase thickness of 0.25 μm) column. Helium (He) was used as carrier gas. GC operating conditions as well as a calibration curve for phenol may be found in Appendix IV.

3.4 Biodegradation Experiments

In this section, the procedures for populating, cultivating and manipulating the microorganisms are described. Additionally, the procedure for the biodegradation experiments and the setup is reviewed.

3.4.1 Cultivation of Bacteria

All bacteria, *Rhodococcus equi* (ATCC# 13557), *Rhodococcus erythropolis* (ATCC# 4277) *Rhodococcus zopfii* (ATCC # 51349), *Rhodococcus rhodochrous* (ATCC # 13808) and *Mycobacterium fortuitum* (ATCC # 6841) were purchased from ATCC.

In order to cultivate a strain of a bacterium a specific growth or culture medium was first prepared. This medium was sterilized using an autoclave (see Appendix V for the autoclave procedure) and the bacterium was inoculated in that medium. Each growth medium was composed of several nutrients that are key for a microorganism's successful cultivation. Table 4 summarizes the pertinent information for the preparation of each growth media as well as the Becton Dickinson (BD) reference number and which bacteria it is used to promote.

Table 4: Culture Media Characteristics

Culture Medium	BD Reference Number	Cultivate which Bacteria	Preparation
Brain Heart Infusion	237500	*R. rhodochrous*	Dissolved 37 g in 1 L of deionized water.
Nutrient Broth	234000	*R. equi*	Dissolved 8 g in 1 L of deionized water.
Pancreatic Digest of Casein and Yeast Extract	211705 and 212750	*R. erythropolis* and *R. zopfii*	Dissolved 5 g of Pancreatic Digest of Casein and 3 g of yeast extract in 1 L of deionized water.
Middlebrook 7H9 Broth and Middlebrook ADC Enrichment	271310 and 212352	*M. fortuitum*	Dissolved 4.7 g of 7H9 Broth in 900 ml of deionized water. Added 2 ml of glycerol and mixed well. Autoclaved the solution. When cooled, aseptically added 100 ml of ADC Enrichment to the solution.

Bacteria inoculation occurred in the laminar fumehood (Model VBM600, The Baker Company) in order to maintain a sterile environment. In fact, any further manipulations involving live bacteria were completed in the laminar fumehood. The microorganism was inoculated in the sterile culture medium. The inoculated flask was placed in an incubator, set at 150 RPM (New Brunswick Scientific Company), under the growth conditions advised by the microorganism's product sheet provided by ATCC. These conditions are listed in Table 5.

Table 5: Bacteria Growth Conditions

Bacteria	Incubator Temperature (^{o}C)	Approximate Cultivation Time (days)
R. rhodochrous	26	1 - 2
R. equi	26	2 – 3
R. erythropolis	26	2
R. zopfii	30	2 – 3
M. fortuitum	37	3 – 4

3.4.2 Bacterial Growth Monitoring

Bacterial growth was monitored through the sampling of the cultivation flasks followed by sample centrifugation and absorbance analysis of the biomass. 10 ml samples were extracted from the cultivation flasks and subsequently centrifuged at 10 000 rpm for 10 minutes (centrifuge: IEC Multi centrifuge provided by Thermo). The supernatant was discarded and the remaining biomass was rinsed with 10 ml of distilled water and vortexed. Approximately 2 ml of the dissolved biomass sample was injected in the UV – visible spectrophotometer at a wavelength of 500 nm (Cary 100 Bio provided by Varian) for absorbance analysis.

3.4.3 Preparation of Secondary Media

Once the bacterium has reached the exponential phase in the culture medium, it can be transferred to a generic growth medium referred to as Modified Mineral Salt Media (MMSM). MMSM is prepared by dissolving several salts in distilled water. Table 6 lists the various salts with the supplier and CAS number, as well as the concentration measurements for the desired volume of media.

Table 6: Required Salts for MMSM

Salt	Supplier	CAS #	Concentration (g/L)
NH_4NO_3	Anachemia	6484-52-2	4
KH_2PO_4	Fisher	7778-77-0	4
Na_2HPO_4	Fisher	7558-79-4	6
$MgSO_4 \cdot 7H_2O$	American Chemicals	10034-99-8	0.2
$CaCl_2 \cdot 2H_2O$	Sigma	10035-04-8	0.01
$FeSO_4 \cdot 7H_2O$	Anachemia	7782-63-0	0.01
Na_2EDTA	Anachemia	6381-92-6	0.014

With the exception of some experiments described in section 3.4.4.1 and 3.4.4.2, a co-substrate was added to the MMSM in order to provide the bacteria with a carbon source for growth. Adipic acid (CAS# 124-04-9) purchased from Fisher Scientific and was used as a co-substrate for *R. erythropolis*, *R. zopfii*, *R. rhodochrous* and *M. fortuitum* at a concentration of 2.5 g/L of MMSM, while glucose (CAS# 50-99-7) purchased from American Chemicals was used as a co-substrate for *R. equi* and *M. fortuitum* at the same concentration as adipic acid (refer to Appendix for the detailed procedure). Yeast extract (CAS# 8013-01-3), purchased from Fisher Scientific, was also added to the MMSM at a concentration of 0.1 g/L to supply nutrients. In the case where adipic acid was used as the co-substrate, the pH of the MMSM was adjusted to 6.8 – 7.0 using 1.0 N sodium hydroxide solution (CAS# 1310-73-2) purchased from Sigma.

3.4.4 Degradation Experiments Conditions

Degradation experiments were conducted in 500 ml sterilized Erlenmeyer shake flasks. Unless stated otherwise, each set of experiments involved 11 flasks. Figure 5 gives a general overview of the steps involved in the biodegradation experiments while table 7 provides the details of the conditions used in the shake flasks to investigate the degradation of EE2. The 3 high and low flasks were used to study degradation of EE2 at two levels of concentration of EE2. Due to findings reported by

Urase and Kikuta (2005) in section 2.3.1, 3 dead biomass control flasks were used to measure the contribution of adsorption on biomass in reducing the EE2 concentration in solution. Bacteria were not added to the 2 control flasks in order to observe if EE2 degraded naturally. 100 ml of EE2 stock solution was added to each flask, resulting in a solution volume of 200 ml of EE2 concentration listed in the following table.

Table 7: Experimental Flasks

Flasks	EE2 concentration (ppm)	Description of Flask Contents
3 High	1.4	MMSM, bacteria, high concentration of EE2
3 Low	0.5	MMSM, bacteria, low concentration of EE2
3 Dead	1.4	MMSM, dead biomass, high concentration of EE2.
2 Controls	1.4	MMSM, EE2

Following the initial cultivation of the bacteria in the culture media, 10 ml of the bacteria was transferred to fresh culture media. Once the bacteria entered the exponential growth phase 10 ml of bacteria was extracted from the new culture media flask and inoculated in the MMSM flasks. The advantages of using MMSM versus culture media to conduct the degradation experiments are that the components of MMSM are known simple salts, the culture media is too rich in nutrients and therefore often the bacteria will not use the pharmaceutical as a substrate and lastly the analysis of the degradation products becomes difficult in a complex solution such as with culture media. Frequent samples of bacteria were taken to observe the growth (as described in 3.4.2) and to determine when the stationary phase had been attained. Prior to the injection of EE2, the designated dead flasks were autoclaved to terminate bacterial growth. EE2 was then introduced into the MMSM flasks when the stationary phase was reached. 40 ml samples were extracted from each flask. Flasks were returned to the incubator shaker and further samples were taken at time intervals over 24 h to 80 h. The sampling procedure described in 3.4.2 was followed with the

exceptions that the supernatant was stored in 40 ml glass amber vials (Fisher Scientific) and 40 ml of distilled water was added to the biomass. The supernatant samples containing live bacteria were acidified using a few drops of 5.0 M HCl (Fisher Scientific, CAS# 7647-01-0) to lower the pH such that bacterial growth would cease. Collected samples were stored in the fridge at 4 – 8^{o}C for no more than 72 hrs prior to preparation for analysis. Any deviations from the above procedure are discussed for each bacterium in the following sections.

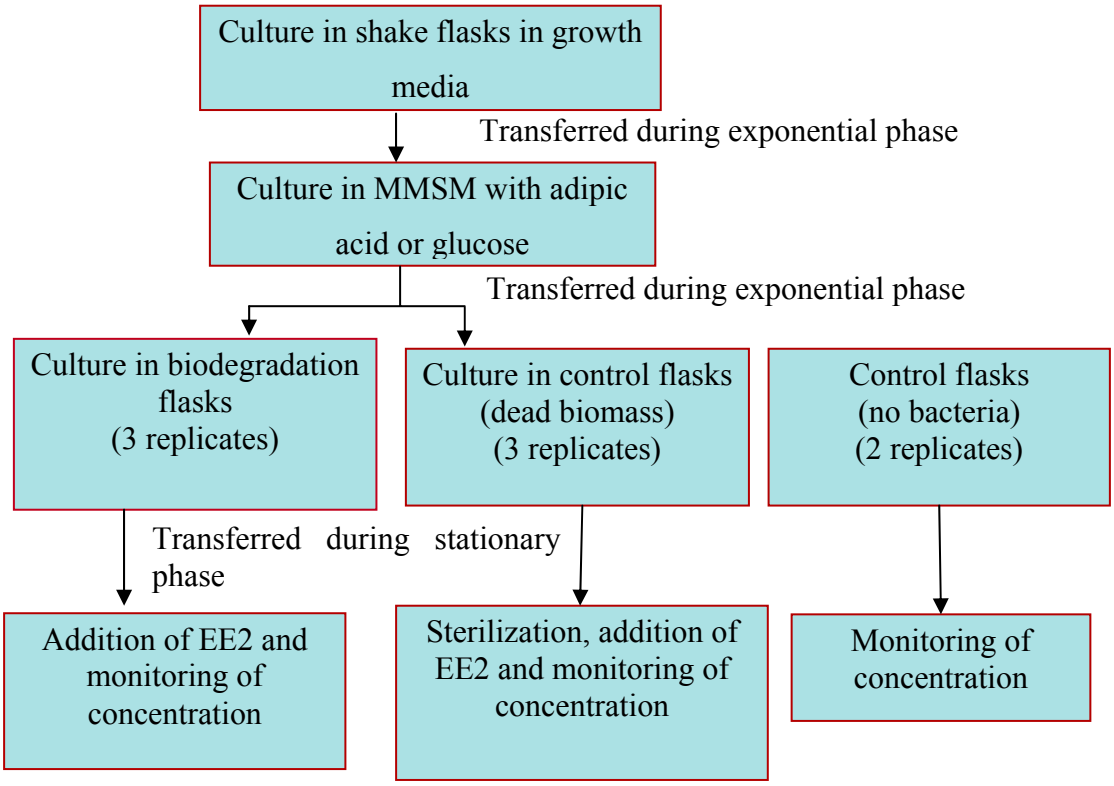

Figure 5: Biodegradation Experimental Procedure

3.4.4.1 Additional Experimental Conditions for *R.zopfii*

The procedure described in 3.4.4 was followed for one set of experiments. However, a second set of experiments was conducted in which only 2 high and 2 control flasks were employed. In this case, the biodegradation of EE2 was being investigated in the absence of a co-substrate, i.e. no adipic acid or glucose was added to MMSM. Therefore EE2 was the sole carbon source and was introduced following inoculation

of the bacteria in the 2 high flasks (and in the control flasks, without bacteria). The remaining parts of the procedure were performed the same as in section 3.4.4.

3.4.4.2 *R. equi*

Two brief sets of degradation experiments were conducted using *R. equi*: one with glucose as a co-substrate and the other in the absence of a co-substrate. For the experiments involving the co-substrate, the procedure described in section 3.4.4 was followed, the only exception being the reduced number of flasks composed of 2 high flasks, 1 dead biomass flask and 1 control flask. The experiment without a co-substrate was accomplished with 3 high flasks, where EE2 was introduced following inoculation of the bacteria.

IV. Results & Discussion

4.1 Developed Solid Phase Extraction Procedure

As previously stated in section 3.1, an efficient, cost effective SPE method needed to be developed and optimized in order to preconcentrate the EE2 samples prior to HPLC analysis. For this purpose two different SPE cartridges were examined – the Oasis HLB (purchased from Waters) and the Bond Elut C18 (purchased from Varian). Oasis HLB cartridges are commonly used in environmental sampling, however they cost almost 4 times more than the Bond Elut C18 cartrdiges. The C18 cartriges cost $2.52 per cartridge, while the HLB are $9.83 per cartridge. All experiments completed to developed the method were performed in triplicates.

Table 8 shows the results from trials conducted to evaluate the potential of C18 cartridges compared to OASIS cartridges. For these experiments EE2 samples were pre-filtered using 0.20 μm paper filters (supplied by Millipore). 40 ml of a 10 ppm EE2 solution was passed through both types of cartridges. Samples were eluted with 6 ml of ethanol or 4 ml to evaluate the impact of the volume of eluent on the recovery. Ethanol was selected as the eluent considering that EE2 is soluble in ethanol (50 mg/ml). In both cases, the HLB cartridge yielded EE2 recoveries greater than 100 %. An explanation of these results could be that some component of the packing material in the HLB cartridge was eluting during extraction and subsequently interfering with the EE2 signal on the HPLC. Regarding the C18 cartridge, the recoveries were significantly increased when the eluent volume was increased from 4 ml to 6 ml, yielding recoveries of 20% and 50%, respectively.

Table 8: EE2 Recovery Rates with Different Cartridges

SPE Conditions: Eluent Volume	Type of Cartridge	EE2 Recovery (average of triplicates)
6 ml eluent volume	Oasis HLB	> 100%
	Bond Elut C18	50%
4 ml eluent volume	Oasis HLB	> 100%
	Bond Elut C18	< 20%

Considering the potential to improve recoveries through the optimization of the operating conditions, alterations were made in an attempt to optimize the SPE method using the C18 cartridge. To further increase the concentration of EE2 in the sample, a nitrogen drying step was added following the elution. At this point, it was also questioned as to whether the paper filters were retaining some EE2 particles. To examine this query, 1 μm glass fiber filters (supplied by Millipore) were tested. Table 9 provides the EE2 average recoveries with the implementation of the nitrogen drying step using two different types filters for EE2 sample filtration. Recoveries ranged from 54 – 60% when the EE2 samples were filtered using 0.20 μm paper filters while EE2 recoveries were vastly improved to 94 – 100 % when employing the 1 μm glass fiber filters. The same experimental conditions were repeated and the recoveries were verified.

Table 9: Comparison of EE2 Recoveries using Bond Elut C18 Cartridges with Different Types of Filters

Type of Filter	EE2 Recovery (average of triplicates)
0.20 μm paper filters	54 – 60 %
1 μm glass fiber filters	94 – 100 %

The final step was to determine an adequate performance surrogate. 17β-estradiol (E2) was selected as a potential candidate based on its similar properties and molecular structure to EE2. Experiments showed that E2 demonstrates similar recoveries to those of EE2 when using the developed SPE method.

The full SPE method is outlined in detail in Appendix III. In recalling that the limit of analysis (LOA) using the HPLC was 1.73 ppm, the LOA was lowered to 0.173 ppm using the optimized SPE method in combination with the HPLC method. The combination of the developed SPE and HPLC methods offers a significant advantage in being able to monitor, at low cost, lower concentrations of EE2 during biodegradation experiments.

4.2 Biodegradation Using Bacteria Known to Degrade EE2

As previously stated in section 2.4, microorganisms *R. equi* and *R. zopfii* were shown to effectively degrade EE2 by Yoshimoto et al (2004). The growth curves for *R. zopfii* and *R. equi* are displayed in figures 6 and 7, respectively. In figure 6, the bacterial growth phases are clearly demonstrated as well as the injection point of EE2 during the stationary phase. Immediately following the addition of 100 ml of EE2 to the flasks, the biomass absorbance readings decreased due to dilution of the bacterial solution. In contrast, following the addition of EE2 and the subsequent dilution factor, *R. equi* in figure 7 continued its growth demonstrating that the EE2 solution may not have been added during the stationary phase, but rather during the exponential phase.

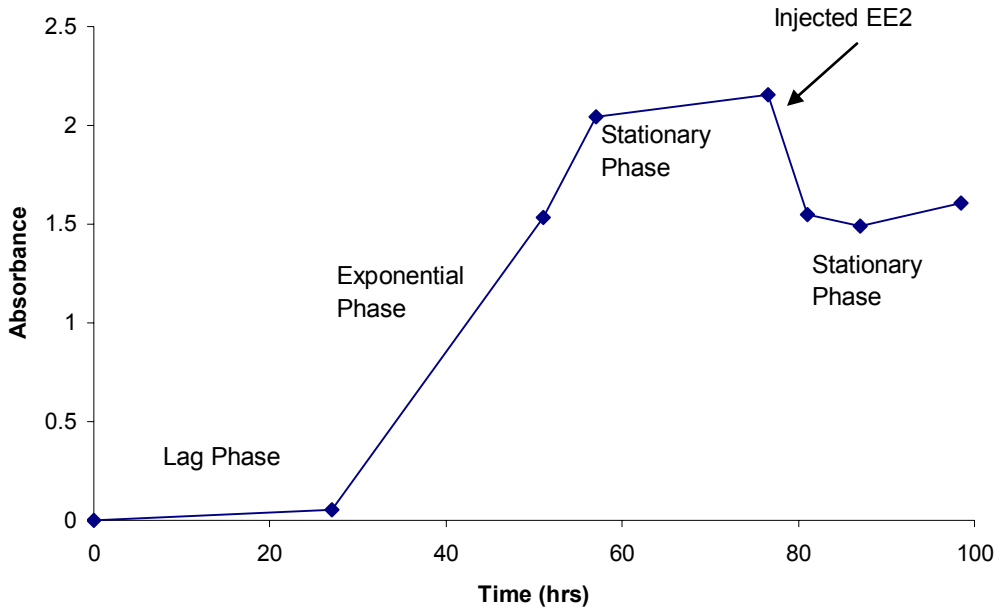

Figure 6: *R. zopfii* growth curve. EE2 added to flasks at 77.5 h

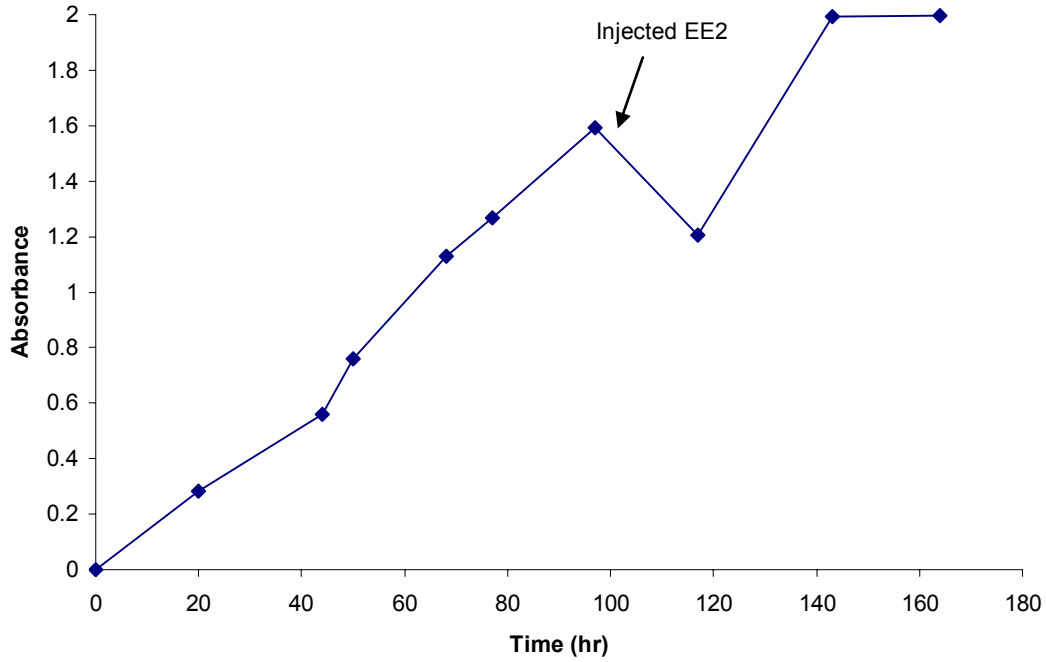

Figure 7: *R. equi* growth curve. EE2 added to flasks at 98 h.

Results for the degradation of EE2 using *R. equi* and *R. zopfii* with a co-substrate are presented in figures 8 and 9, respectively. At time 0 h, EE2 was added to the bacteria during the stationary phase. Figure 8 shows *R. zopfii* degraded EE2 up to 25% of its initial 0.5 ppm concentration in 22 h. However, the actual degradation percentage is more realistically around 10 % due to the removal of EE2 onto the dead biomass. Adsorption onto the dead biomass cannot be stated as the sole means of EE2 removal considering that during sterilization of the biomass cells can lyse and consequently can release active enzymes that in turn degrade EE2. Therefore enzyme degradation has to be included as a potential removal agent of EE2. Conversely the degradation of EE2 from an initial concentration of 1.4 ppm was practically negligible. Yoshimoto et al. (2004) had demonstrated *R. zopfii* capable of degrading a 100 ppm solution of EE2 completely within 24 h and therefore it was expected that a higher degradation of EE2 would be observed from experimental results. However, Yoshimoto et al. (2004) did not investigate the possibility of EE2 adsorbing to the biomass. Additionally, the bacteria strains were isolated from an enrichment culture of an activated sludge from a wastewater treatment plant likely meaning that the bacteria were well acclimatized to pharmaceuticals and other contaminants. This could have been the reason for the increased degradation of EE2 observed by Yoshimoto et al. (2004).

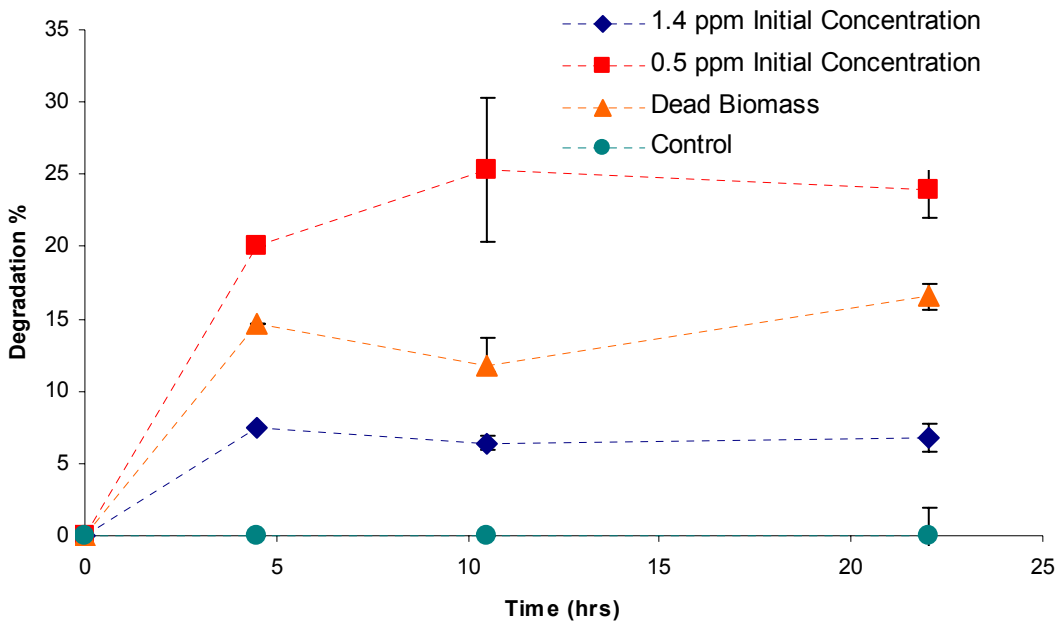

Figure 8: Degradation of EE2 using *R. zopfii* with a co-substrate (standard deviation based on triplicates).

As observed in figure 9, after 65 h, *R. equi* had degraded EE2 up to 39% of its initial concentration of 1.4 ppm. The dead biomass control flask showed little degradation demonstrating that biodegradation, not sorption, governed the removal of EE2. By comparison, R. equi had degraded 80% of the initial 100 ppm solution of EE2 in 24 h (Yoshimoto et al., 2004). Again the fact that the bacteria were acclimatized to wastewater conditions likely would assist in degrading EE2. It is also conceivable that different strains result in different degradation abilities as the bacteria strains used in the study conducted by Yoshimoto et al. (2004) were not identical to the ones used in the present study.

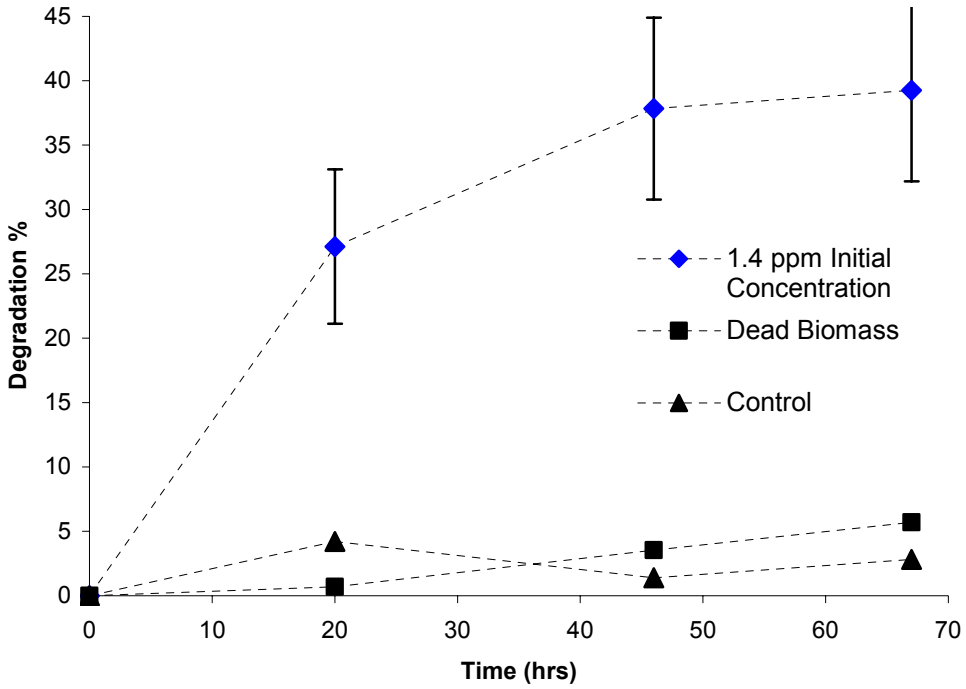

Figure 9: Degradation of EE2 by *R. equi* with a co-substrate (standard deviation based on duplicates).

Another factor to consider regarding the degradation of EE2 using *R. zopfii* and *R. equi* is the lower degradation of EE2 obtained in the presence of a co-substrate in comparison to the results observed by Yoshimoto et al. (2004) where no co-substrate was present. It had been hypothesized that with a co-substrate present in solution, efficient degradation of EE2 would have been achieved through co-metabolism. This however was not the result observed. Most likely the microorganisms degraded the co-substrate because it requires more energy to degrade EE2 than say adipic acid or glucose. Additionally, the concentration of EE2 was too low for continuous metabolism and thus the plateau effect seen in all the degradation figures resulted. The acclimation of the bacteria and the low EE2 concentration used also seemed to play an important role here considering that no EE2 degradation was observed when degradation experiments were performed without a co-substrate (data not shown).

4.3 Bacteria Screening for EE2 Degrading Microorganisms

Using the methods described in section 3.4, *R. rhodochrous*, *R. erythropolis* and *M. fortuitum* were examined to determine their EE2 degrading capability. Bacterial growth curves for *R. rhodochrous* and *R. erythropolis* are shown in figure 10. No growth curves are presented for *M. fortuitum* as once the microorganism was transferred from the culture medium to the MMSM it did not cultivate properly and remained in the lag phase. Therefore EE2 degradation could not be evaluated using that particular microorganism. Similar to figures 6 and 7, following the addition of EE2 dilution occurred resulting in decreased absorbance readings for the bacteria as seen in figure 10.

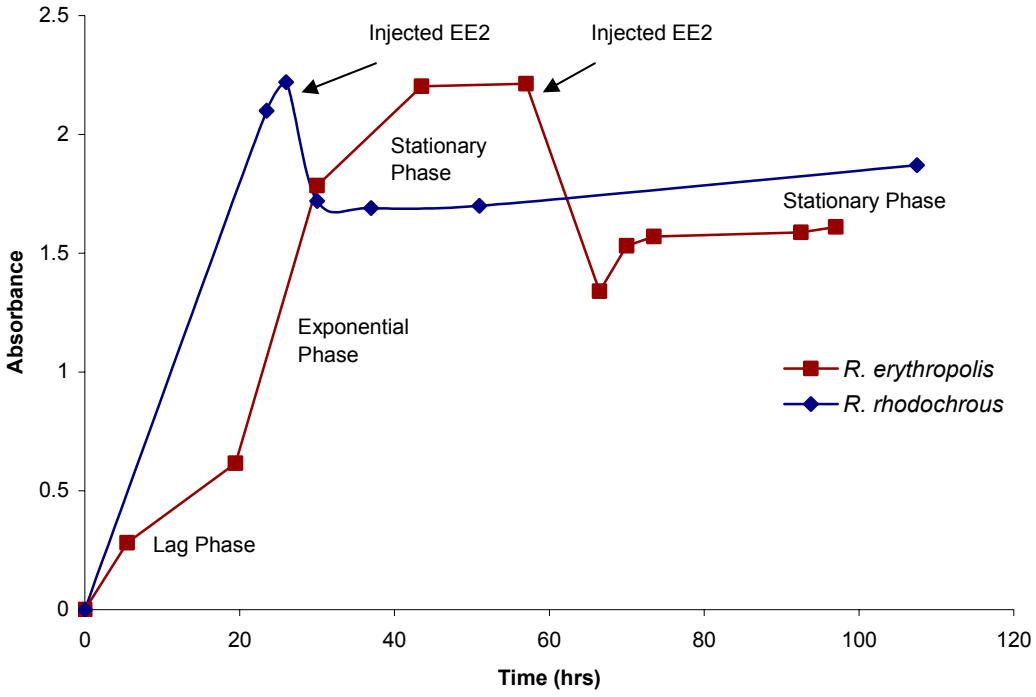

Figure 10: *R. rhodochrous* and *R. erythropolis* growth curves. EE2 added to R. rhodochrous and R. erythropolis at 27 h and 58 h, respectively.

R. rhodochrous was not particularly effective in reducing the concentration of EE2. As can be observed in figure 11, after 25 h, approximately 10% of the initial concentration of 1.4 ppm EE2 had been degraded while EE2 did not undergo any degradation for an initial concentration of 0.5 ppm. No significant degradation was further realized after 81.5 h. In the case of *R. rhodochrous*, it was very difficult to differentiate between adsorption and degradation considering that a decrease ofin concentration of 10% was also observed in the dead biomass control. The decrease in EE2 concentration can again be caused by adsorption on the biomass or enzymatic degradation.

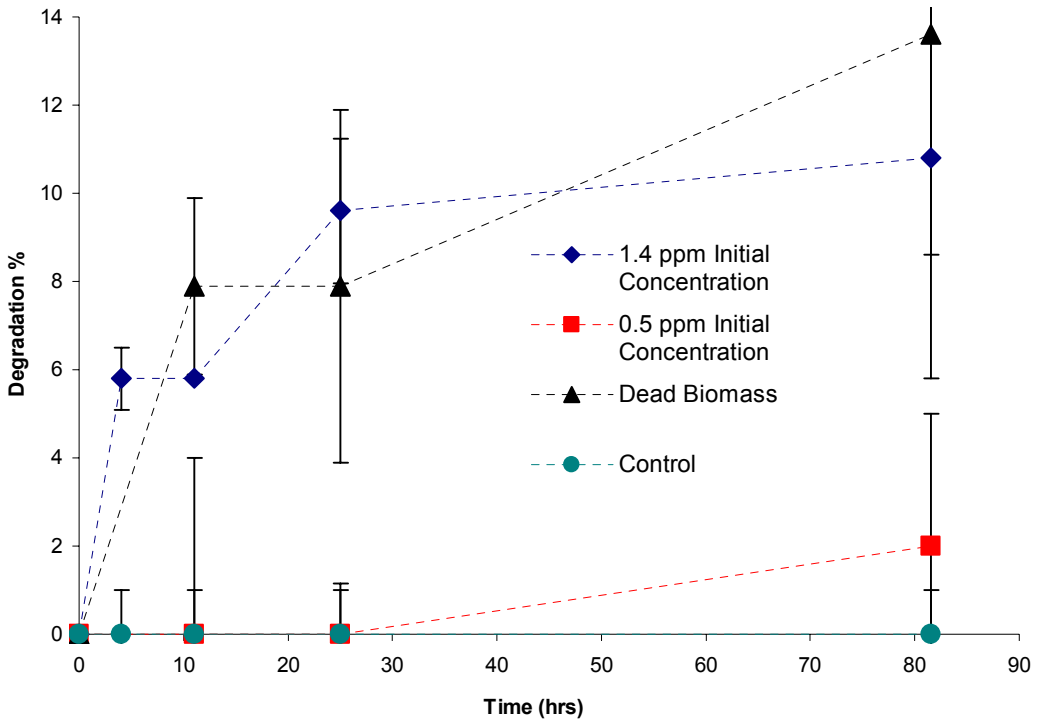

Figure 11: Degradation of EE2 by *R. rhodochrous* with a co-substrate (standard deviation based on triplicates).

Figure 12 shows the degradation results of EE2 by *R. erythropolis*. For an initial EE2 concentration of 1.4 ppm, *R. erythropolis* was capable of degrading EE2 up to 35 % in 9.5 h and 47% in 13 h. *R. erythropolis* also successfully degraded the 0.5 ppm

concentrated EE2 solution, eliminating 30% in 9.5 h and 48 % in 13 h. Despite these promising results in a relatively short time period, longer exposure times to the microorganism did not yield higher EE2 removal percentages. In the case of both the 1.4 ppm and 0.5 ppm initial concentrated solutions, approximately 47 – 48 % of EE2 was degraded in 35 h. This result can be attributed to the microorganism not receiving enough energy from degrading the low concentrated EE2 to continue in its healthy state. It can also be observed from figure 13 that sorption or enzymatic degradation through dead biomass was not responsible for significant EE2 removal. Based on these results, among the bacteria screened *R. erythropolis* has shown the greatest capability of degrading EE2; however it is limited.

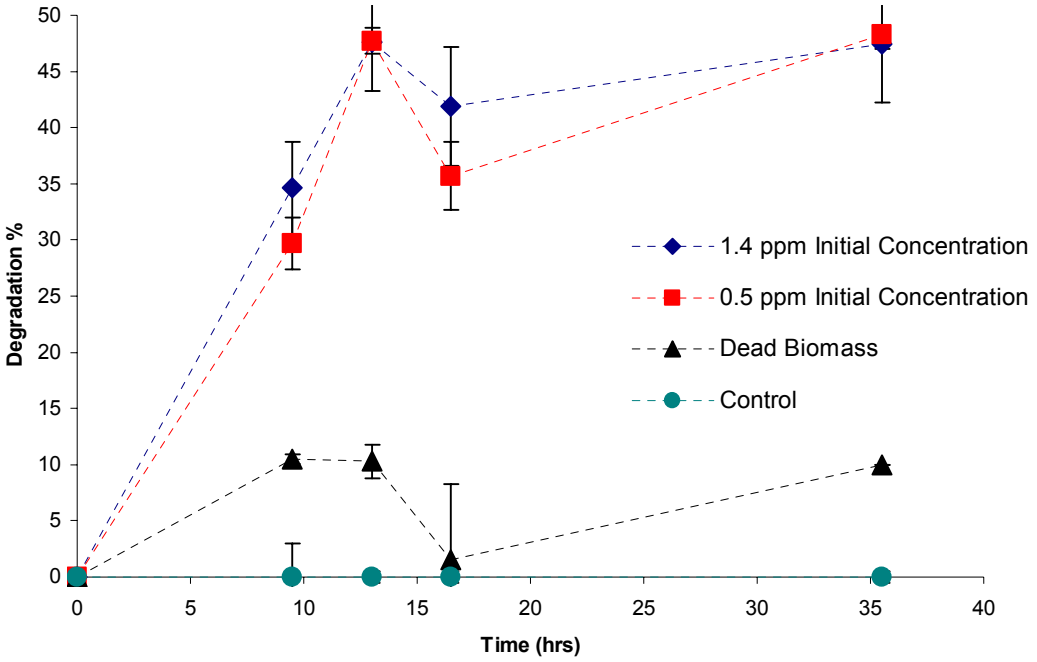

Figure 12: Degradation of EE2 by *R. erythropolis* with a co-substrate (standard deviation based on triplicates).

4.4 Identification of Degradation Products

Using the GC conditions stated in section 3.3.3 and listed in Table 11 in Appendix IV, phenol was detected in an EE2 degradation sample using *R. erythropolis* after 24

h and 44 h. MS was then used to examine any EE2 degradation products in the VOC samples. As previously mentioned blank and matrix corrections were done on the sample in order to eliminate outside interference. Figure 13 displays the GC-MS chromatogram and accompanying spectrum from the VOC samples taken at 24 h. Looking at the chromatogram, the prominent retention time peaks from 7.20 min to 16.01 min were studied to compare their individual spectrums in order to locate common fragmentation patterns. Based on this approach the peak at 9.51 min was selected to further investigate preliminary metabolites. Figure 14 shows the chromatogram at 9.51 min and the accompanying spectrum with the mass fragments. The next step involved selecting the most likely pathway of fragmentation of the EE2 compound.

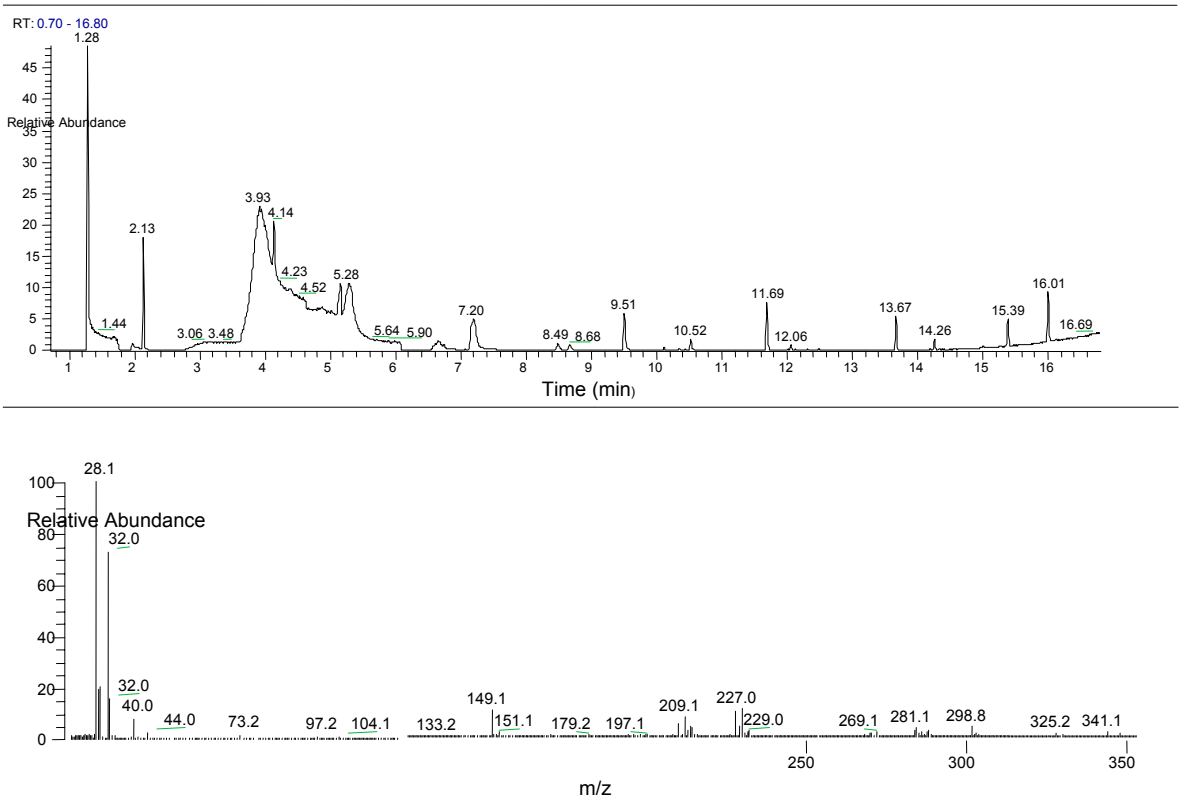

Figure 13: GC MS Chromatogram and Spectrum from VOC sample after 24 h. Degradation of EE2 using *R. erythropolis*.

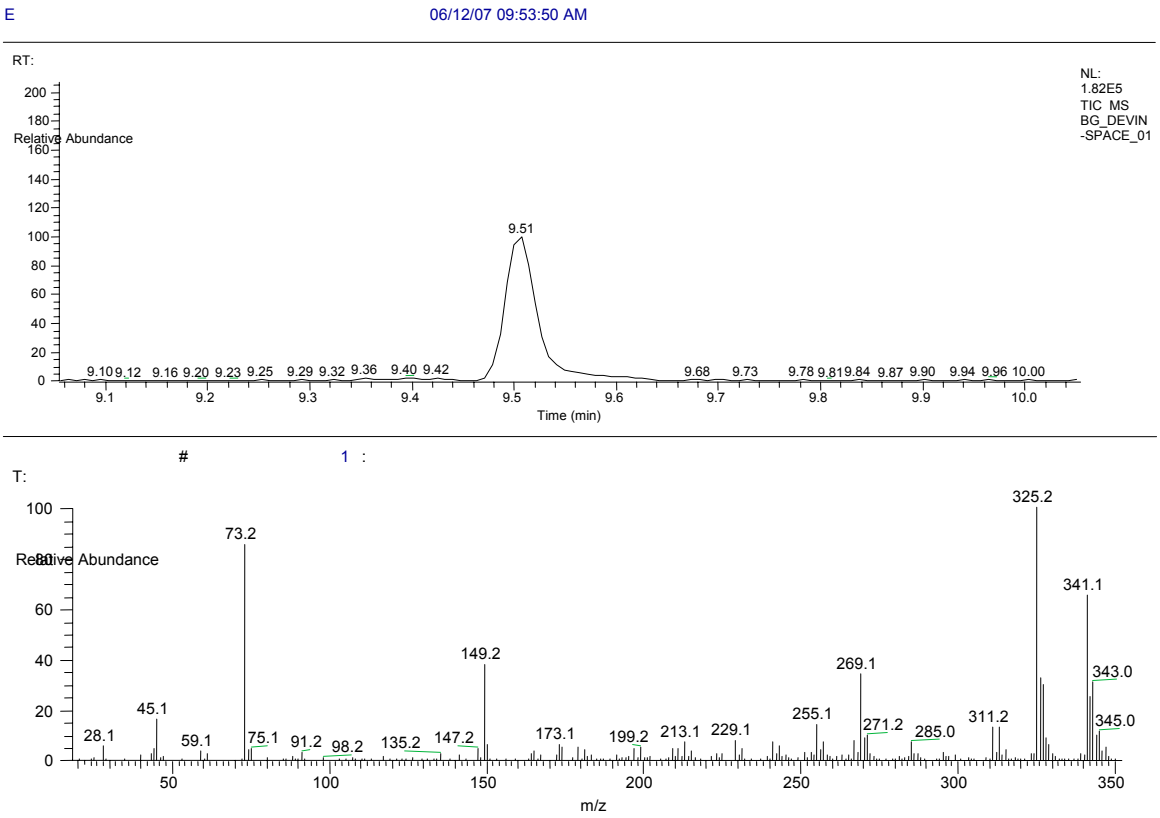

Figure 14: Chromatogram and Spectrum at 9.51 min.

Recalling the EE2 structure, an aromatic ring with an attached OH group is present. The m/z value of 91.2, seen in figure 15, is equivalent to phenol confirming the GC results that phenol was a degradation product of EE2 by *R. erythropolis*. One other metabolite was identified. The structure is shown in figure 16 and the MS spectrum is displayed in figure 17. Essentially, the triple carbon bond of EE2 was broken and two alcohol groups joined. One of the alcohol groups was displaced resulting in the formation of a carboxylic acid group. The total mass of the metabolite is 331, its peak observed in figure 17. This structure is conceivable given that mass fragments were observed for various segregations of EE2 at 9.51 min (the spectrums for these fragmentations can be viewed in Appendix VI). Additionally, analysis of the fragmentation of an ion, for example the phenol, using MSn^2 would be required to confirm the compound observed in figure 16.

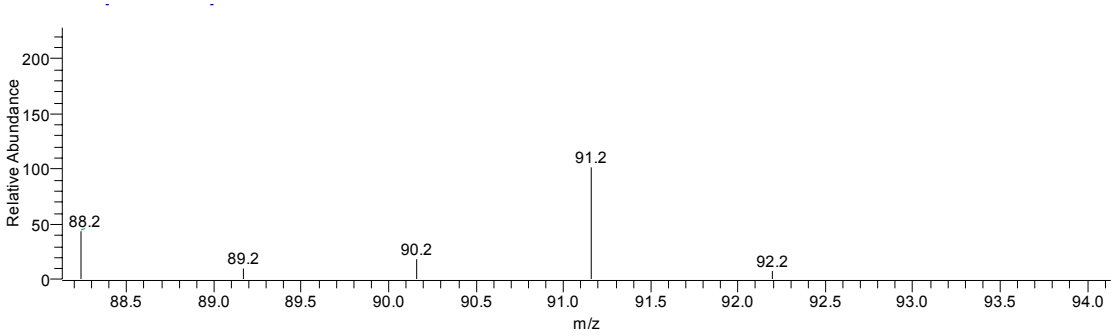

Figure 15: 9.51 min Spectrum showing 91.2 m/z

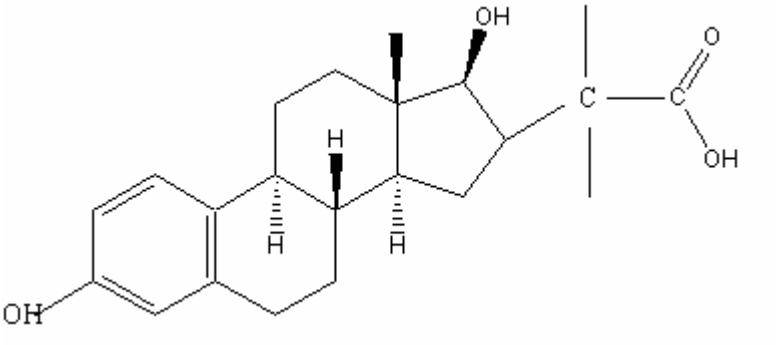

Figure 16: Identified Metabolite of EE2: EE2 plus a carboxylic acid group.

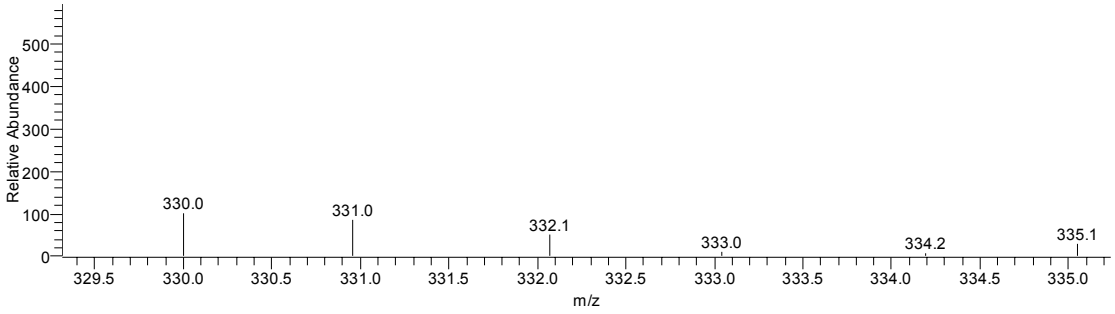

Figure 17: Spectrum of Identified Metabolite with a m/z of 331.

Results from early experiments indicated that metabolites from EE2 degradation could have potentially formed in the liquid phase. Table 10 provides the names of these degradation products and their associated probability as well as their molecular weights. The structures can be viewed in Appendix VI. Further HPLC-MS and GC-MS are required to identify the soluble degradation products.

Table 10: Other Potential Degradation Products of EE2

Metabolite	Probability	Molecular weight (g/mol)
Testosteron-11-ol 17-acetate	11%	346
Ergosta-7, 24 (28)-dien-3-ol, 4-methyl	40.3 %	412
Benzene, 1-chloro-3, 5-bis (1, 1-dimethylethyl)-2-(2-propenyloxy)	59 %	280

V. Limitations of this Study

5.1 Analytical Techniques

Despite a SPE method being developed to reduce the limit of analysis (LOA) on the HPLC to 0.173 mg/L, this value is still a limitation considering the low concentration of estrogens, including EE2, measured in STP influents. Baronti et al. (2000) measured an average of 3 ng/L of EE2 during a five month period in six STPs. This value is approximately 57 times smaller than the current LOA. However, the initial EE2 concentration of 1.4 ppm used here was more realistic in terms of wastewater influent concentration than the initial 100 ppm (Yoshimoto et al., 2004) or 25 ppm (Shi et al., 2002) used in other studies.

5.2 Sewage Treatment Plant Conditions

During wastewater treatment numerous species of microorganisms are present. Consequently the same degradation capabilities shown by certain microorganisms observed in laboratory conditions might not be replicated in a STP. Additionally, the opportunity exists, in an STP, for other microorganisms to degrade any metabolites that are produced during biodegradation. The use of a co-substrate here was again more realistic in terms of wastewater conditions than the sole carbon source approach used in other studies.

VI. Conclusions

It has been demonstrated in several studies that pharmaceuticals and hormones are often not eliminated during wastewater treatment or biodegraded in the environment. 17α-ethinylestradiol is one such hormone that has been detected in Canadian wastewater effluents. Biodegradation of EE2 using pure cultures has rarely been studied in order to investigate the degradation of EE2 and idenitfy subsequent metabolites that are produced. The present work examined certain microorganisms to determine their degrading capabilities of EE2 as well as conducting some preliminary identification of metabolites that were formed.

Several analytical methods required development in order to monitor the low concentration of EE2 during degradation and to identify the degradation products. Most significant was the development and optimization of a SPE method in order to pre-concentrate the EE2 samples prior to HPLC analysis. A cost effective, efficient SPE method was developed yielding analyte recoveries between 94 – 100%. The LOA was greatly improved from 1.73 ppm to 0.173 ppm using the combined SPE/HPLC procedure.

Five bacteria were screened to investigate their potential for degrading EE2. *R. erythropolis* and *R. equi* demonstrated the greatest ability to degrade EE2, removing up to 47% and 39% of the initial 1.4 ppm in 13 h and 65 h, respectively. Results demonstrated no significant EE2 degradation using *R. rhodochrous* and *R. zopfii*. Finally, *M. fortuitum* remained in the lag phase and was thereby not able to have its degradation potential tested. Based on literature, *R. zopfii* and *R. equi* were expected to quantitatively degrade EE2. However, the present study did not replicate the exact experimental conditions from literature. The primary differences were a much lower initial EE2 concentration, the use of a co-substrate to promote bacterial growth and cultivating bacteria from a frozen sample rather than isolation from activated sludge were characteristics of the present study and may explain the differences observed.

Preliminary analysis of the degradation products produced during the degradation of EE2 by *R. erythropolis* was completed. GC results identified phenol as a metabolite and MS was used to confirm its existence. Another EE2 metabolite was also identified through GC MS as the structure of EE2 with a carboxylic acid group attached. Further analysis is required in order to confirm and identify other volatile and soluble degradation products as well as to elucidate a possible degradation mechanism.

VII. Recommendations for Future Work

7.1 Biodegradation Experiments

Other microorganisms should be screened to determine their potential for degrading EE2 and producing less toxic metabolites. Some examples of other possible bacteria include: *Pseudomonas fluorescens* which has been linked to high estrogen degrading capacity in municipal sludge (Khanal et al., 2006), *Novosphingobium tardaugens* sp. nov has been isolated from activated sludge as an E2-degrading microorganism (Fujii et al., 2002), *Nitrosomonas europaea* was shown to quantitatively degrade EE2 (Shi et al., 2004) and *Fusarium proliferatum* has been isolated from cowshed samples as an EE2-degrading microorganism (Shi et al., 2002).

An acclimation step for the bacteria prior to degradation experiments could result in improved degradation capacities. Recall Ingerslev and Halling-Sorensen's (2000) study, previously mentioned in section 2.3.1, where following the adaptation period of the sludge to the antibiotics, degradation rates were drastically higher than experiments performed without an acclimation period.

7.2 Analysis of Degradation Products

Further analysis of the metabolites is needed in an effort to determine a potential degradation mechanism of EE2. Experiments should also be repeated under the same conditions in order to perform MSn^2 analysis on the retention peaks of interest in order to better confirm the identity of the degradation products.

Microtox® analysis could be performed to do a preliminary analysis of the relative toxicity of the degradation products compared to the parent compound. The toxicity of the metabolites showing higher relative toxicity could then be further investigated using mammalian cells.

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Appendices

Appendix II: SPE Procedure

Sample Preparation prior to SPE

10 ml of a 5 ppm 21.5 wt. % E2 solution , acting as the surrogate, was added to the 40 ml EE2 samples. Samples were filtered using 1 μm glass fiber filters (supplied by Millipore). The pH of all samples was adjusted to 7.5 (or higher) with the addition of 1.0 M NaOH. Additionally, the extraction equipment was cleaned (tubing, connectors) prior to extraction by passing acetone and HPLC water through the tubing.

SPE Cartridge Preconditioning

SPE cartridges were placed on top of the SPE manifold system (Supelco, Visiprep^{TM}) and were conditioned sequentially with 6 ml acetone, 6 ml ethanol, and 6 ml HPLC grade water adjusted to pH 7.5. During this process it was very important not to let the cartridges go dry. Once the cartridges were conditioned, the samples were passed through.

Extraction

The samples were pumped through the cartridges at a rate of approximately 10 ml/min using a vacuum pump (BioRad^{TM}). Once completed the cartridges were rinsed with 10 ml of pH 7.5 distilled water. The solid phase packing material was kept wet by trapping the last few millimeters of rinse in the cartridge until all the cartridges contained approximately the same amount of rinse water at which point they were emptied. The cartridges were dried for a minute or two in order to remove any excess water. The manifold cover was removed and 10 ml glass vials are placed inside to collect the eluent. As previously mentioned, ethanol was selected as the eluent and here, 3 ml of was added to the cartridges. Following 10 minutes the ethanol was passed through the cartridges and this step was repeated with another 3 ml of ethanol.

Drying and reconstitution of the samples

The EE2 samples dissolved in 6 ml of ethanol following SPE were placed on a heating plate under a nitrogen sparging apparatus. The flow of nitrogen was adjusted such that small bubbles appeared on the surface of the samples. The samples were also heated at low heat to aid in the drying process, which overall took approximately one hour.

Once the ethanol had evaporated, 4 ml of HPLC water and 1 ml of ethanol was added to reconstitute the remaining organics. This new sample was filtered using 13 mm syringe filter (PVDF, 0.22 μm, Fisherbrand) and stored in 2.0 mL-amber HPLC vials.

Appendix II: Calibration Curves

Figure 18 shows the calibration curve used to determine the concentration of EE2 by HPLC. The equation of the line is:

Concentration (ppm) = 0.2706 • Area (mAU) + 0.8915 $R^2 = 0.9985$

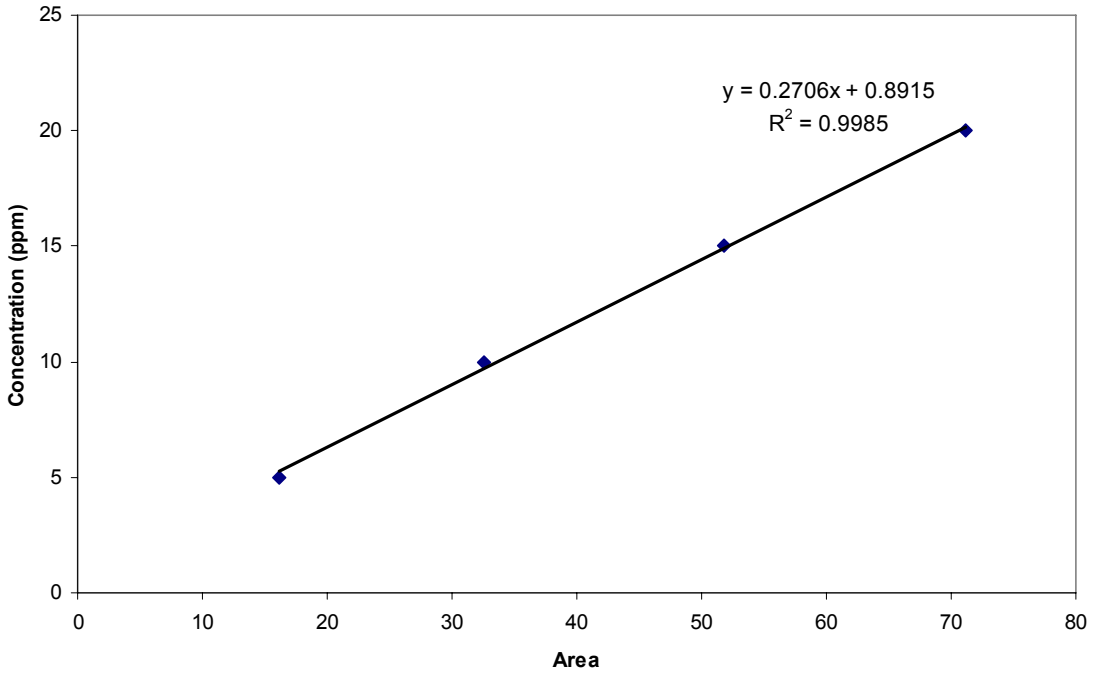


Figure 18: EE2 Calibration Curve for the HPLC

Similarly, figure 19 shows the calibration curve used to determine the concentration of E2 by HPLC. The corresponding line equation is:

Concentration (ppm) = 0.2568 • Area (mAU) + 0.3982 $R^2 = 1$

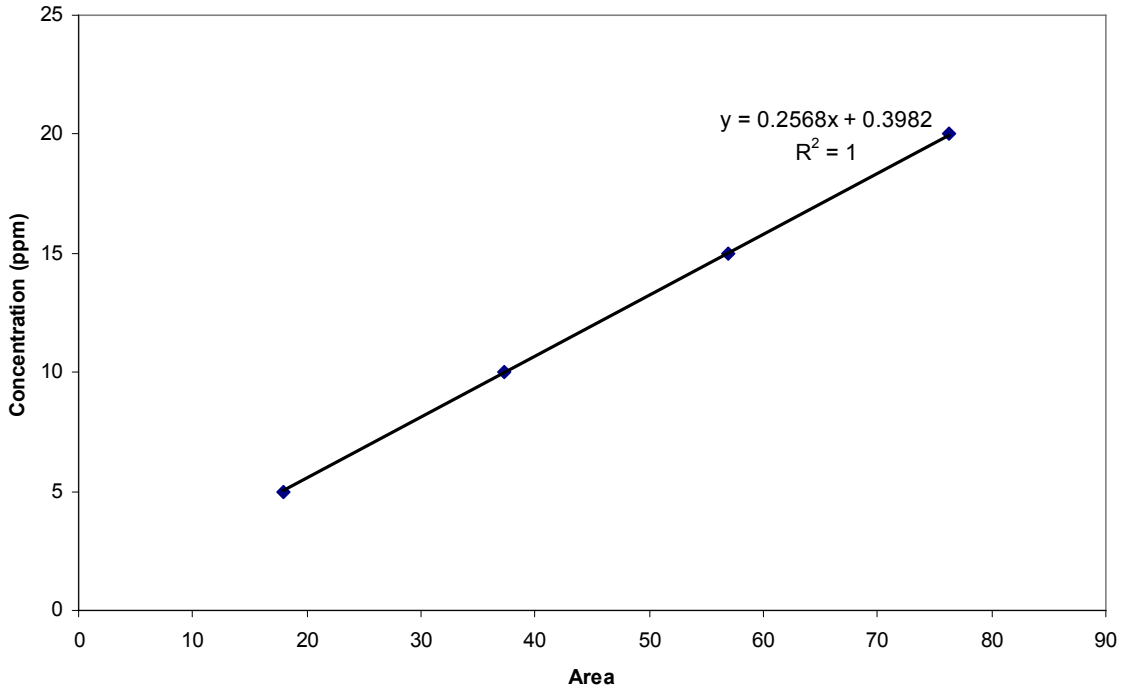


Figure 19: E2 Calibration Curve for the HPLC

Appendix III: GC MS Parameters

MS Method

Acquisition Time:	GC Run Time
Acquire Cal Gas:	No
Acquire Profile:	No
Source Temp:	240 C
Transfer Line:	300 C

Segment 1

Start Time:	0.10 minutes
Polarity:	POS
Mult Offset:	+0 volts
Tune File:	cl140605a

Scan Event 1

Micro Scans:	3
Max Ion Time:	50
Mass Defect:	0.00
Scan Mode:	Full Scan
First Mass:	10.0
Last Mass:	650.0

TRACE GC 2000 Method

Oven Method

Initial Temperature (C):	55
Initial Time (min):	3.00
Number of Ramps:	3
Rate #1 (deg/min):	10.0
Final Temperature #1 (C):	260
Hold Time #1 (min):	10.00
Rate #2 (deg/min):	5.0
Final Temperature #2 (C):	275
Hold Time #2 (min):	15.00
Rate #3 (deg/min):	0.1
Final Temperature #3 (C):	275
Hold Time #3 (min):	0.10
Post Run Temperature:	On
Post Run Temperature (C):	0
Post Run Time (min):	0.00
Left Inlet (kPa):	0.00
Right Inlet (kPa):	0.00
Auto Prep Run:	On
Oven Sub-ambient:	Off
Maximum Temperature (C):	350
Equilibration Time (min):	0.50

Right PTV Method

Base Temperature:	On
Base Temperature (C):	150
Mode:	PTV Solvent Split
Split Flow:	On
Split Flow Flow (ml/min):	150
Splitless Time (min):	1.50
Solvent Valve Temperature:	Off
Solvent Valve Temperature (C):	100
Surge Pressure (kPa):	3.00
Surge Duration (min):	0.00
Constant Purge:	On
Stop Purge At: (min):	1.50
Evaporation Phase:	On
Cleaning Phase:	On
Ramped Pressure:	Off
Sub-ambient:	Off
Inject Time (min):	0.2
Evaporation Rate (deg/min):	10.0
Evaporation Temperature (C):	60
Evaporation Time (min):	1.0
Transfer Rate (deg/min):	10.0
Transfer Temperature (C):	275
Transfer Time (min):	45.0
Clean Rate (deg/min):	10.0

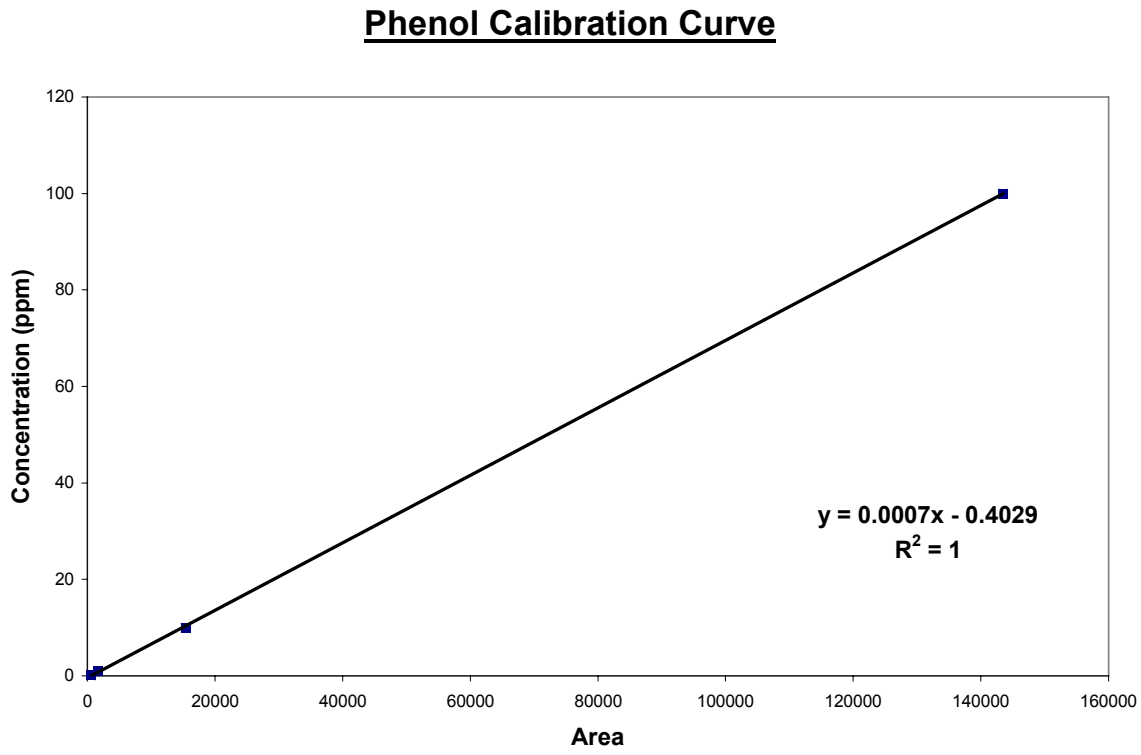
```
TRACE GC 2000 Method

   Clean Temperature (C):                  300
   Clean Time (min):                       10.0
Right Carrier Method
   Mode:                                   Constant Flow
   Initial Value:                          On
   Initial Value (ml/min):                 1.00
   Initial Time:                           1.00
   Gas Saver:                              Off
   Gas Saver Flow (ml/min):                20
   Gas Saver Time:                         2.00
   Vacuum Compensation:                    On
-------------------------------------------------------------
No Left Inlet
-------------------------------------------------------------
No Right Detector
-------------------------------------------------------------
No Left Detector
-------------------------------------------------------------
No Aux Detector
-------------------------------------------------------------
Aux Zones
   Aux Temperature 0:                      Off
   Aux Temperature 0 (C):                  0
   Aux Temperature 1:                      Off
   Aux Temperature 1 (C):                  0
   Aux Pressure 0:                         Off
   Aux Pressure 0 (kPa):                   0.00
   Aux Pressure 1:                         Off
   Aux Pressure 1 (kPa):                   0.00
   Aux Pressure 2:                         Off
   Aux Pressure 2 (kPa):                   0.00
-------------------------------------------------------------
Run Table
   External Event #1 Prep-Run Default:     Off
   External Event #2 Prep-Run Default:     Off
   External Event #3 Prep-Run Default:     Off
   External Event #4 Prep-Run Default:     Off
   External Event #5 Prep-Run Default:     Off
   External Event #6 Prep-Run Default:     Off
   External Event #7 Prep-Run Default:     Off
   External Event #8 Prep-Run Default:     Off
-------------------------------------------------------------
```

Appendix IV: GC Parameters and Calibration curve for phenol

Table 11: GC Parameters for Identifying Metabolites

Oven Temperature (^{o}C)	85
Initial Temperature (^{o}C)	85
Injection Temperature (^{o}C)	95
Rate (^{o}C/min)	5
Final Temperature (^{o}C)	150
Detector (^{o}C)	150
Initial Time (min)	1
Final time (min)	0.5



Appendix V: Operation of the Autoclave

The purpose of the autoclave (3021-S, AMSCO) is to sterilize medium or destroy microorganisms before disposal. The standard settings used for sterilization were 121 ^{o}C, 10 psig, for 15 minutes. Flasks were cooled to room temperature before inoculating with bacteria cultures or disposing of contents.

Appendix VI: Degradation Product Information

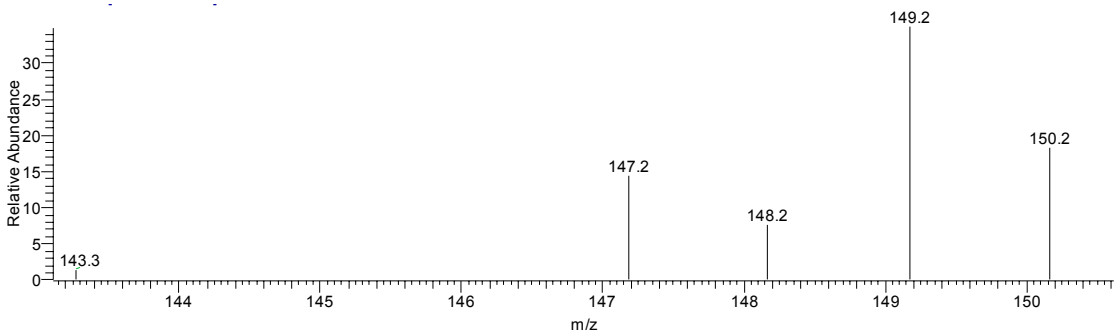


Figure 20: Mass Fragmentation of 147 at 9.51 min

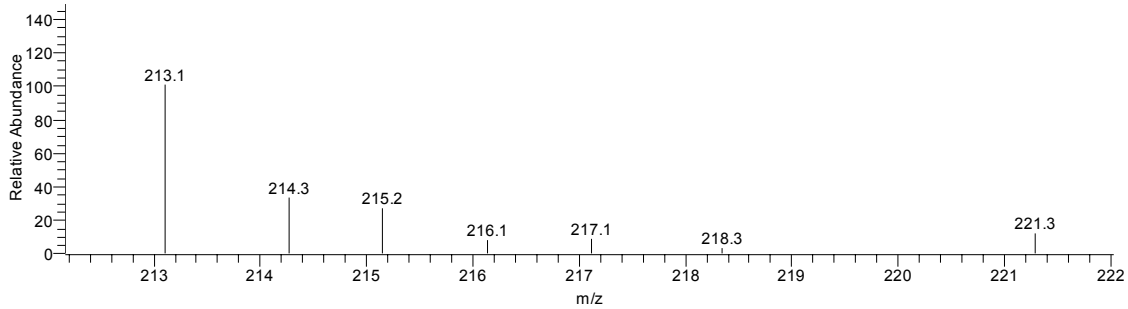


Figure 21: Mass Fragmentation of 215 at 9.51 min

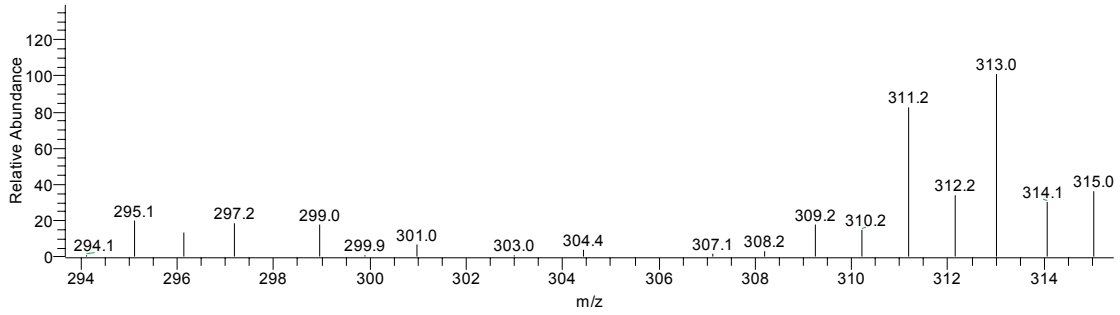


Figure 22: Mass Fragmentation of 297 at 9.51 min

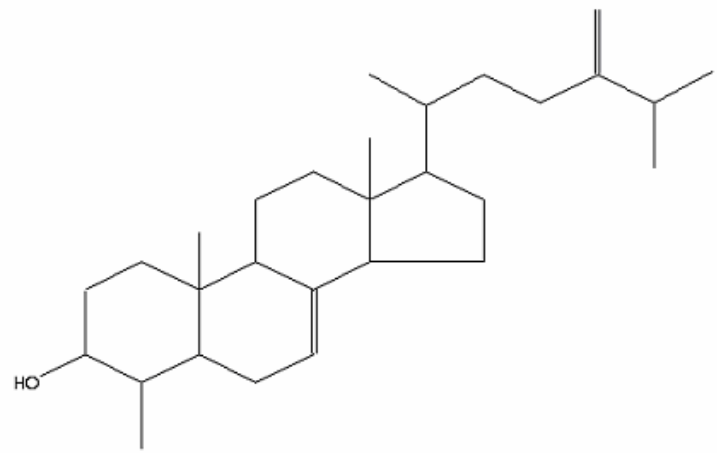


Figure 23: Ergosta-7, 24 (28)-dien-3-ol, 4-methyl

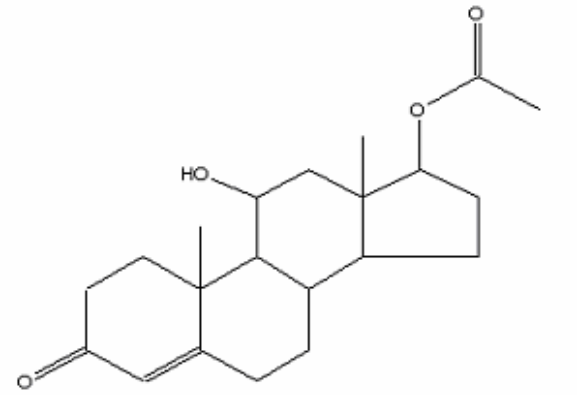


Figure 24: Testosteron-11-ol 17-acetate

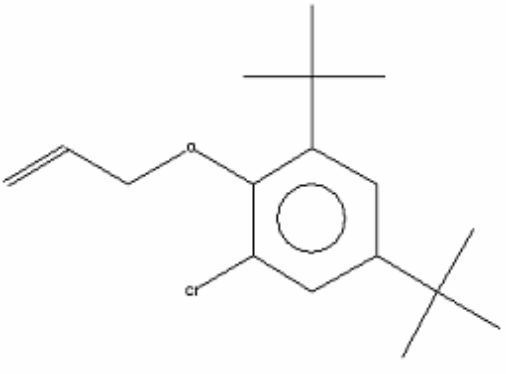


Figure 25: Benzene, 1-chloro-3, 5-bis (1, 1-dimethylethyl)-2-(2-propenyloxy)