BIODEGRADATION OF THE FLUORINATED NON-STEROIDAL ANTI-INFLAMMATORY PHARMACEUTICAL FLURBIPROFEN

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

KADİR YANAÇ

IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
ENVIRONMENTAL ENGINEERING

JUNE 2016

Approval of the Thesis:

BIODEGRADATION OF THE FLUORINATED NON-STEROIDAL ANTI-INFLAMMATORY PHARMACEUTICAL FLURBIPROFEN

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ABSTRACT

BIODEGRADATION OF THE FLUORINATED NON-STEROIDAL ANTI-INFLAMMATORY PHARMACEUTICAL FLURBIPROFEN

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June 2016, 147 pages

Flurbiprofen (FLB) is a fluorinated aromatic acid non-steroidal anti-inflammatory pharmaceutical which is widely consumed in Turkey. However, nothing is known regarding its environmental fate. The aim of this master thesis study was to contribute to the understanding of the biodegradation of flurbiprofen (FLB) by environmental bacteria and to gain understanding of the biological activities of fluorinated aromatics and their tendencies to result in toxic byproducts. FLB was spiked into aerobic sewage sludge from Ankara Municipal Treatment Plant. Metabolism of FLB by environmental bacteria resulted in accumulation of a highly persistent metabolite identified by LCMS as 4-(1-carboxyethyl)-2-fluorobenzoic acid. The production of this metabolite is consistent with described pathways for monochlorobiphenyl. Additionally, since FLB itself was quite recalcitrant, taking one week to 3 months to fully degrade, FLB and its metabolite are likely discharged into the environment from typical wastewater treatment plants. Aerobic sewage sludge from Ankara Municipal Treatment Plant was also enriched for FLB degraders. FLB degraders could not be isolated despite using different minimal salt medium (MSM) systems and including vitamins. On the other hand, enrichment for tolylacetic acids (TAA) and phenylacetic acid (PAA) degraders was successful, indicating that MSM system worked. This work suggests that FLB is very poorly degraded by aerobic bacteria, likely due to production of a dead-end fluorinated metabolite.

Keywords: Flurbiprofen, Microbial Biodegradation of Pharmaceuticals, Microbial Biodegradation of Flurbiprofen, Microbial Biodegradation of Fluorinated Aromatics

ÖZ

STEROİD YAPIDA OLMAYAN FLORLU ANTİENFLAMATUVAR FARMASÖTİK FLURBİPROFENİN BİYOBOZUNUMU

Yanaç, Kadir

Yüksek Lisans, Çevre Mühendisliği Bölümü
Tez Yöneticisi: Yardımcı Doç. Dr. Robert W. Murdoch

Haziran 2016, 147 sayfa

Flurbiprofen (FLB) Türkiye'de yaygın olarak kullanılan steroid yapıda olmayan antiemflamatuvar florlu bir aromatik asittir. Buna rağmen, çevresel akıbetine ilişkin hiçbir şey bilinmemektedir. Bu yüksek lisans tezinin amacı flurbiprofenin (FLB) çevresel bakteriler tarafından biyobozunumunun anlaşılmasına ve florlu aromatiklerin biyolojik aktivitelerinin ve bunların toksik yan ürünler üretme eğilimlerinin anlaşılmasına katkı sunmaktır. Flurbiprofen Ankara Atıksu Arıtma Tesisinden alınan aerobik arıtma çamuruna eklendi. FLB'nin çevresel bakteriler tarafından biyobozunumu, LCMS ile tanımlanan, güçlü bir şekilde kalıcı olan 4-(1-karboksietil)-2-florobenzoik asitin birikmesiyle sonuçlandı. Bu metabolitin üretimi tanımlanmış olan monoklorobifenillerin metabolik yollarıyla tutarlıydı. Ek olarak, FLB'nin kendisi bir haftadan üç aya kadar değişen bozunum süreleriyle oldukça kararlı olduğu için FLB'nin ve bozunum metabolitinin tipik atıksu arıtma tesislerinden çevreye salınması olasıdır. Aynı tesisden alınan aerobik arıtma çamuru FLB çürütücüleri için de zenginleştirildi. Vitaminler de içeren farklı minimal tuz medium (MTM) sistemleri kullanılmasına ragmen FLB çürütücüleri izole edilemedi. Öte yandan, minimal tuz medyumumuzun çalıştığını gösteren fenilasetik asit (FAA) ve tolilasetik asit (TAA) çürütücüleri zenginleştirilebildi. Bu çalışma FLB'nin, muhtemel bir kör uçlu flüorlu metabolit üretiminden dolayı aerobik bakteriler tarafından zayıf bir şekilde bozunduğunu göstermektedir.

Anahtar kelimeler: Flurbiprofen, Farmasötiklerin Mikrobiyal Biyobozunumu, Flurbiprofenin Mikrobiyal Biyobozunumu, Florlu Aromatiklerin Mikrobiyal Biyobozunumu

ACKNOWLEDGEMENTS

I would like to express my deepest gratitude to my supervisor Assist. Prof. Dr. Robert W. Murdoch for his valuable guidance, advice, trust, encouragement and patience throughout my thesis study. I am honored to be his first MSc student.

I wish to thank to my committee members Prof. Dr. F. Dilek Sanin, Assoc. Prof. Dr. Tuba Hande Ergüder Bayramoğlu, Assist. Prof. Dr. Barış Kaymak and Assist. Prof. Dr. Eda Çelik Akdur for their comments and contributions.

I would like to thank my friends Sarper Erinç Aktürk, Hüseyincan Eryılmaz, Murat Toprak, Servet Turan, Anja Martin, Onur Aksoy, Zeynep Yenen and Yiğitalp Ertem for their help, support, words of encouragement and sharing their experiences with me throughout my research.

I would like to thank Fadime Kara-Murdoch for her help about technical issues and encouragement during my thesis.

This study was supported by the Scientific and Technological Research Council of Turkey National Master Scholarship Program: 2211 (TÜBİTAK 2211 Yurt İçi Lisansüstü Burs Programı).

Finally, my deepest thanks are dedicated to my family for their endless patience and encouragement, support and confidence in me throughout my life.

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ABBREVIATIONS

3FPAA	3-fluorophenylacetic acid
BP	Biphenyl
FLB	Flurbiprofen
IBP	Ibuprofen
LB	Luria Bertoni Broth
MSM	Minimal Salt Medium
mTAA	*m*-tolylacetic acid
pTAA	*p*-tolylacetic acid
PAA	Phenylacetic acid
PCB	Polychlorinated biphenyl
PCR	Polymerase Chain Reaction
RFLP	Restriction Fragment Length Polymorphism
TAA	Tolylacetic acid

CHAPTER 1

INTRODUCTION

The global community has become very concerned about the environmental fate and effects of pharmaceutical compounds (Daughton & Ternes, 1999; Dietrich et al., 2002; Halling-Sørensen et al., 1998). For the vast majority of human pharmaceuticals, the potential metabolic strategies employed for bacterial biodegradation are largely unexplored. The possibility of dead-end or metabolic intermediates increases the complexity of the challenge in regards to recalcitrance and ecotoxicology (Chou, 2006; Flaherty & Dodson, 2005; Laetz et al., 2009).

Flurbiprofen (FLB) is a phenylacetic acid (PAA) based non-steroidal anti-inflammatory pharmaceutical. FLB is quite popular in Turkey, where it is sold under the brand name Majezik and is the standard analgesic used for menstrual pain. Approximately 22% of consumed FLB is released unmodified or as a readily-cleavable phase 2 metabolic conjugate (Risdall et al., 1978; Szpunar et al., 1987). Given the high dose (100-400mg per day) and likely high rate of consumption, FLB has the potential to reach sewage treatment systems and surface waters in significant concentrations.

The biodegradation of FLB has not been studied to any degree. FLB is a modified PAA. Until the last few years, bacterial metabolism of PAAs was poorly understood. Very recently, two new and potentially widespread pathways for aerobic bacterial biodegradation of PAAs have been described, the *paa* and *ipf* pathways (Murdoch & Hay, 2013; Teufel et al., 2010). This research is in its early stages and much remains to be learned regarding the substrate specificities and distributions of these newly characterized pathways.

Additionally, FLB is also a substituted biphenyl and contains a fluorine moiety. It is known that the bacterial metabolism of halogenated biphenyls mostly results in

accumulation of halogenated benzoates. Fluoro-aromatics are quite poorly studied. A certain fluorinated aromatic chemical, 3-fluorocatechol, is routinely employed as a metabolic poison in microbiology, raising the possibility that FLB biodegradation may yield a toxic byproduct that could adversely affect sewage treatment processes or have further ecological effects (Carvalho et al., 2006; Duque et al., 2012).

Based on current situation, the objectives of this thesis study are;

- Investigation of the activity of environmental bacteria towards FLB in terms of
    - Degradation rates of FLB
    - Toxic effects of FLB
- Investigation of biodegradation pathway of FLB
- Investigation of the biological activities of fluorinated aromatics

Most obviously, the activity of environmental bacteria towards FLB has yet to be studied and will offer a clear novel contribution. As a modified PAA and biphenyl, the study of FLB biodegradation pathway will make clear contributions to the current state of aromatic biodegradation research. Additionally, study of FLB contributes to the body of knowledge regarding the biological activities of fluorinated aromatics and in particular their tendencies to result in the toxic byproducts.

CHAPTER 2

LITERATURE REVIEW

2.1. Pharmaceuticals in the Environment

The pharmaceuticals sector has been growing due to new, simple and relatively cheap production techniques, deeper understanding of biological systems, increased governmental regulations on public health issues and increases in consumer purchasing power. Large amounts of pharmaceuticals are consumed annually around the world in order to cope with human and animal diseases (Jones et al., 2007; Jones et al., 2001; Uslu et al., 2013; Zhang et al., 2008). Alder et al. (2006) estimated the amount of annual consumption of drugs per capita as 15 g/person in world and 50-150 g/person in developed countries.

With an increasingly aging population, the consumption of pharmaceuticals will increase in the future (Daughton, 2003). According to IMS Institute (2011), medical spending will reach $1.1 trillion in 2015 with an annual growth rate of 3-6%. In Turkey, each person consumed nearly 26 packets of drugs in 2013 based on statistics from the Ministry of Health. The improvement in medical and pharmacy fields is not a question of debate but, on the other hand, potential and possible environmental impacts of pharmaceuticals from the growing pharmaceutical sector cannot be ignored even if they are discharged to environment at very low concentrations. As a natural consequence, the scientific community has been attracted by this issue (Carlsson et al., 2006a, 2006b; Christensen, 1998; Cleuvers, 2003; Dietrich et al., 2002; Fent et al., 2006; Heberer, 2002; Hirsch et al., 1999; Kümmerer, 2009).

Some precautions and regulations have entered into force in many regions and countries around the world. In 2001, European Council Directive 2001/83/EC stated that an environmental risk assessment (ERA) might be necessary before the approval of new medicinal products due to their potential effects on aquatic life forms and in 2012 it was decided to monitor concentrations of pharmaceuticals in surface waters (EC, 2012).

Most pharmaceuticals have cyclic or aromatic structures (Figure 2. 1). They have been used for many therapeutic purposes.

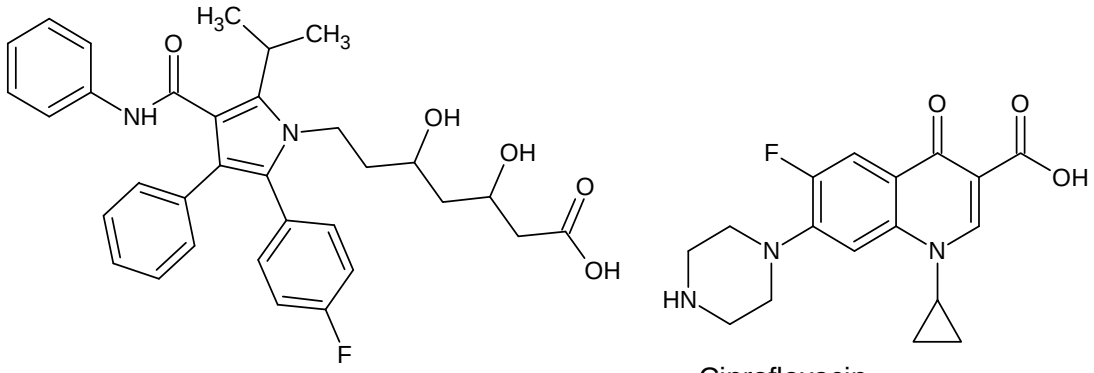


Figure 2. 1. Chemical structures of two common aromatic pharmaceuticals

Occurrence and Fate of Pharmaceuticals

Improvements in detection techniques for trace pharmaceutical residues have allowed researchers to demonstrate occurrence of pharmaceuticals in aquatic environments (Jiang et al., 2013; Jones et al., 2001; Peng et al., 2014; Uslu et al., 2013); Vulliet et al. (2011); Yang et al., 2011). Pharmaceuticals are considered emerging contaminants, also termed micropollutants. Micropollutants are observed in waters at ng/L and µg/L levels (Luo et al., 2014). Bolong et al., (2009) reported that precautions and monitoring actions in many WWTPs were inadequate despite improvements. Luo et al. (2014) also reported that there were no discharge guidelines or standards for most micropollutants and that the number of countries having adopted regulations for micropollutants is small. Thus, the effects and presence of micropollutants cannot be prevented or monitored in most countries. NSAIDs, analgesic drugs, beta-blockers, antidiabetics, steroid hormones, blood lipid lowering agents, antineoplastic drugs, neuroactive compounds and antibiotics are the most observed pharmaceutical classes in wastewater, surface water and groundwater and are even observed in drinking water (Valavanidis et al., 2014). Among pharmaceutical classes, NSAIDs and antibiotics are the most widely detected pharmaceutical classes in waters (Hughes et al., 2013).

The physical and chemical properties of pharmaceuticals are responsible for their widespread distribution in the environment (Rosal et al., 2010). Hydrophilicity and hydrophobicity of the pharmaceuticals determine their fate in the in natural and artificial ecosystems (Suárez et al., 2008). One of the most important properties is octanol-water partition coefficient. Octanol-water partition coefficient (K_{ow}) determines the hydrophobic sorption capacity of chemicals, particularly onto sludge or sediment. The sorption abilities of chemicals can be classified as:

i) If log K_{ow}<2.5, low sorption ability

ii) If 2.5<log K_{ow}<4.0, medium sorption ability

iii) If log K_{ow}>4.0, possible high sorption ability (Caliman & Gavrilescu, 2009; Jones-Lepp & Stevens, 2007; Rogers, 1996)

Not only K_{ow} but also acidity constant (pK_a) and the solid water distribution coefficient (K_d) are important determinants of sorption (Joss et al., 2005; Ternes et al., 2004).

Human pharmaceuticals are mostly excreted unchanged or only slightly transformed. Municipal wastewater is believed to be the main route by which pharmaceuticals reach aqueous environments (Jones et al., 2002; Kasprzyk-Hordern et al., 2009; Zhang et al., 2008). In Turkey, there have been only a few studies related to occurrence of pharmaceuticals in water environments and therefore, there is no clear information about the current situation (Aydin & Talinli, 2013; Komesli et al., 2015).

Unused and expired pharmaceuticals disposed as solid waste or discarded into sewer systems are another source of pharmaceuticals in the environment (Bound & Voulvoulis, 2005; Scheytt et al., 2006). In addition to these contributors, discharges from hospitals and pharmaceutical industries are other sources of pharmaceutical loads in domestic wastewater. Pharmaceuticals loads arising from hospitals and pharmaceutical industries are mostly negligible when mixed with domestic wastewater (Bondar et al., 1998; Fent et al., 2006; Santos et al., 2013; Saussereau et al., 2013; Verlicchi et al., 2010). Leachates and leakage from poorly designed sewer and landfills can be a pharmaceutical contamination source of groundwater (Fawell

& Ong, 2012). The main reason behind why conventional wastewater treatment plants are the main source of pharmaceutical pollution is that conventional wastewater treatment plants (WWTPs) are not capable of removing pharmaceuticals efficiently (Carballa et al., 2004; Joss et al., 2006; Lindqvist et al., 2005; Paxeus, 2004; Petrie et al., 2013; Petrovic et al., 2009; Repice et al., 2013; Sim et al., 2011; Vidal-Dorsch et al., 2012). Concentrations of pharmaceuticals in the environment depend on many factors, such as pharmaceutical production rate, excretion rate, sales and practices, water used per capita per day and treatment capacities of WWTPs (Jelić et al., 2012; Petrovic et al., 2009). Seasonal variation is another important factor affecting concentrations of pharmaceuticals due to alterations in the flow pattern of wastewater, especially in the case of combined sewer systems (Luo et al., 2014). For example, in dry seasons, the concentrations of pharmaceuticals are greater compared to rainy weather conditions (Kasprzyk-Hordern et al., 2009) and conversely, Wang et al. (2011) reported that lower concentrations of pharmaceuticals can be observed in summer due to promotion of biodegradation rates at higher temperatures and dilution during rainy summers. Moreover, concentrations of pharmaceuticals can actually increase during treatment. Concentrations of pharmaceuticals such as carbamazepine, erythromycin, and diclofenac in WWTPs can be greater than the influent concentrations due to transformation of secondary biotransformation metabolites into the original parent compounds (Celiz et al., 2009; Göbel et al., 2007; Kasprzyk-Hordern et al., 2009) and release of pharmaceuticals from fecal particles during treatment (Luo et al., 2014).

The main receivers of these pharmaceutical loads from effluents of wastewater treatment plants (WWTP) are coastal water, ground water, surface water and tap water/drinking water (Benotti et al., 2009; Bull et al., 2011; Fick et al., 2009; Jelic et al., 2011; Jelic et al., 2011; Kim et al., 2007; Lindqvist et al., 2005; Schriks et al., 2010; Uslu et al., 2013; Vidal-Dorsch et al., 2012; Vieno et al., 2007). The discharge from WWTPs into natural water environments such as rivers dilutes pollutants to some degree (Gros et al., 2007). Groundwater has been shown to be less polluted with pharmaceuticals and other micropollutants compared to surface water due to loss during transmission of pharmaceuticals into ground waters (Loos et al., 2010; Vulliet & Cren-Olive, 2011). However, the situation still poses a potential threat to

groundwater. It is known that pharmaceuticals adsorb onto sewage sludge which can then be used for agricultural purposes (Carrara et al., 2008). Emerging contaminants, including pharmaceuticals, can pollute soil and water through agricultural use of sewage sludge and sewage effluent (Kinney et al., 2008; Kinney et al., 2006; McClellan & Halden, 2010; Mohapatra et al., 2014; Tijani et al., 2013; Wu et al., 2009). The introduction of pharmaceuticals into food chains is made possible by irrigation of edible plants with reclaimed wastewater (Shenker et al., 2011; Tanoue et al., 2012; C. Wu et al., 2010). The various routes of release, transport, and fate of micropollutants are summarized in Figure 2. 2.

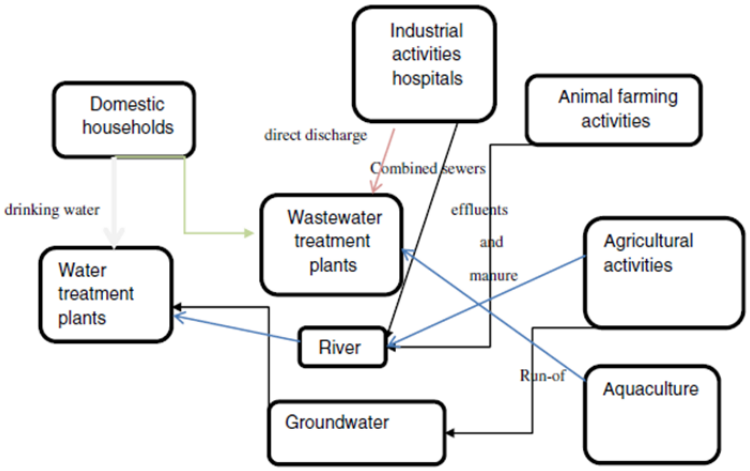


Figure 2. 2. Routes and sources of pharmaceutical loads (Petrović et al., 2003)

Environmental Effects of Pharmaceuticals

Pharmaceuticals and their metabolites at trace levels in waters carry health risks for aquatic life and human beings (Carlsson et al., 2006a; Corcoran et al., 2010; Farré et al., 2008; Fent et al., 2006; Liu et al., 2009; Moldovan, 2006; Pomati et al., 2006; Tamtam et al., 2008; Zuccato et al., 2006). Some specific compounds may interact with non-target living organisms and ecosystems even at environmentally relevant concentrations because the goal of pharmaceutical design is to achieve biological activity (Aydin & Talinli, 2013; Mohapatra et al., 2014). Neither the fate and behavior of pharmaceuticals and their metabolites nor their effects on living creatures

are well known in soil and water environments, although there are many studies related to these issues (Brausch et al., 2012; Corcoran et al., 2010).

Based on recent studies, the environmental bioaccumulation potential of pharmaceuticals and personal care products (PPCPs) is very high. This situation affects hormonal control and antibiotic resistance causing reoccurrence of hospitalization and increasing treatment costs (Tijani et al., 2013). Feminization of male fish (Corcoran et al., 2010; Fent et al., 2006), alterations in liver, kidney and gills in fish (Fent et al., 2006; Gagne et al., 2006) and pathogen antibiotic resistance (Witte, 1998; Zuccato et al., 2006) are some of the clearly proven effects of pharmaceuticals in the environment. Synthetic steroids can have long term adverse effects on fish at environmentally relevant concentrations or even at very low concentrations (Lange et al., 2001). Ethinyl estradiol, one of best known endocrine disruptors, has negative effects on zebrafish embryonic development at concentrations as low as 5 ng/L (Carlsson et al., 2006b; Kime & Nash, 1999). Many other micropollutants have endocrine disrupting effects (Figure 2. 3). Jones et al. (2002) classified acute toxicities of some types of pharmaceuticals. Antibiotics are classified as extremely toxic to microorganisms (EC50 < 0.1 mg/L) and very toxic to algae (EC50 = 0.1-1 mg/L), antidepressants and cardiovascular pharmaceuticals are classified as very toxic to crustaceans and analgesics are classified as toxic (EC50 = 1-10 mg/L) or harmful to crustaceans and fish (EC50 = 10-100 mg/L). Additionally, bioaccumulation of pharmaceuticals in earthworms has been reported with concentrations exceeding 1000 µg/kg (Kinney et al., 2008).

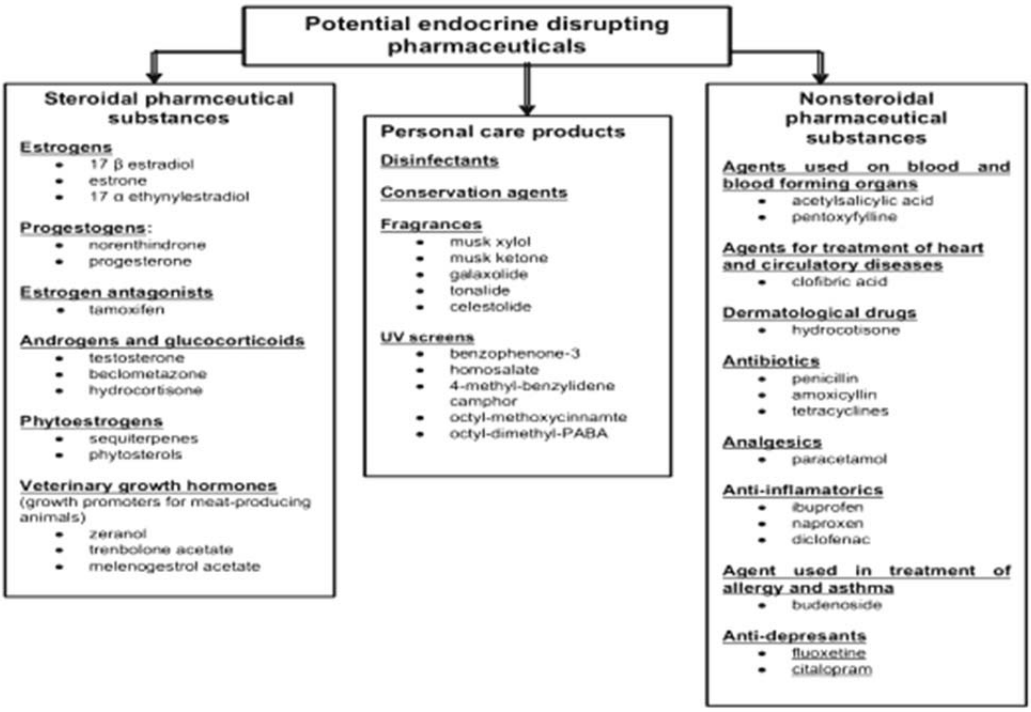


Figure 2. 3. Potential endocrine disrupting pharmaceuticals (Caliman & Gavrilescu, 2009)

Pharmaceuticals occur in the environment as complex mixtures rather than isolated chemicals, which may lead to greater toxic effects on living organisms (Backhaus, 2014; Cleuvers, 2003; Kolpin et al., 2002). A pharmaceutical mixture consisting of atenolol, furosemide, sulfamethoxazole, ciprofloxacin, and others at environmentally relevant concentrations was shown to inhibit the growth of human embryonic cells (Pomati et al., 2006). Zebra fish (*Danio rerio*) exhibited significantly decreased embryo production after 6 week exposure to a pharmaceutical mixture including acetaminophen, carbamazepine, gemfibrozil and venlafaxine at environmental concentrations (Galus et al., 2013). A mixture of diclofenac, ibuprofen and carbamazepine was associated with increase in mortality at 60 µg/L concentration for each and histopathological changes in the liver, kidney, skin and gill of tench (a fish, *Tinca tinca*) at lower concentrations (Stancova et al., 2014). A mixture of eleven pharmaceuticals from different therapeutic classes showed significant toxic effects on *Hydra* at environmentally relevant concentrations (Quinn et al., 2009).

The continuous flow of pharmaceuticals to the aquatic environment has created a chronic exposure problem in addition to acute (Crane et al., 2006). In a chronic toxicity study, carbamazepine caused impairments in evolutionarily conserved specific biochemical pathways of the Mediterranean mussel (*Mytilus galloprovincialis*) at environmentally relevant concentrations, 0.1 and 10 µg/L in a 7 day-exposure (Martin-Diaz et al., 2009).

Removal of Pharmaceuticals

Global climate change, rapid population increase, urbanization and technological advancement, bad agricultural practices and poor wastewater treatment plants have created water scarcity and pollution problems (Johnson et al., 2008; Mara, 2003; Montgomery & Elimelech, 2007; Moore et al., 2003). The UN reported that there are about 50 countries faced with water scarcity (UN, 2014), making alternative water resources increasingly attractive. Use of sustainable water sources, such as use of recycled water and reclaimed water for irrigational and environmental purposes, industrial applications, urban uses and potable reuse has become more important in arid and semi-arid areas in order to meet needs of growing populations (Asano et al., 2007; Chen et al., 2013; Council, 2012; Dodgen et al., 2013; Hamilton et al., 2007; Kinney et al., 2006; Plumlee et al., 2012). Before using reclaimed waters, the health of the water sources should be taken into consideration. Direct use of surface waters may also create problems; many studies have reported the occurrence of pharmaceuticals in surface waters at ng/L to µg/L levels (Castiglioni et al., 2005; Clara et al., 2005; Fernandez et al., 2010; Kleywegt et al., 2011; Vanderford et al., 2003).

In many countries, conventional wastewater treatment technologies with poor removal efficiencies for micropollutants are in use. By looking at the processes involved in conventional WWTPs, fate, toxicity and occurrence of pharmaceuticals during and after treatment may be understood. Primary treatment processes in which the main mechanism is sorption are not effective for removal of pharmaceuticals (Carballa et al., 2005; Ternes et al., 2004) and by considering that sorption onto solids is the main removal mechanism for most pharmaceuticals (Verlicchi et al., 2012), the magnitude of the risk caused by occurrence of pharmaceuticals in water

environments can be estimated. In secondary treatment, many mechanisms such as dispersion, biodegradation, sorption onto sludge, dilution and abiotic transformation take place and might provide better removal efficiency for pharmaceuticals (Jelic et al., 2011). However, in order to achieve good removal efficiencies, some treatment parameters should be taken into consideration. For example, extended sludge retention times (SRT) can achieve better removal efficiencies for pharmaceuticals by positively influencing microbial communities in terms of size and diversity (Fernandez-Fontaina et al., 2012; Suarez et al., 2010). Activated sludge processes are generally regarded as better for removal of pharmaceuticals than other low-cost treatment options (Camacho-Muñoz et al., 2012). pH and temperature characteristics of WWTPs may also affect the removal by influencing biodegradation capacity of micropollutants (Cirja et al., 2008).

Most WWTPs do not employ tertiary treatment processes which are good for removal of pharmaceuticals but are rather applied for reducing public and environmental health issues. Certain advanced treatment technologies have been investigated for more efficient removal of pharmaceuticals from wastewater (Klamerth et al., 2010; Martínez et al., 2013; Mestre et al., 2009; Rosal et al., 2010; Rossner et al., 2009; Sipma et al., 2010; Trinh et al., 2012). Treatment of PPCPs and EDCs by advanced oxidation processes (AOPs) have shown great effectiveness and almost complete mineralization of these compounds (Méndez-Arriaga et al., 2008). Toxic by-products may be observed during treatment of pharmaceuticals (Tijani et al., 2013). 1,4-Benzoquinone as a transformation product of acetaminophen during water chlorination or an intermediate product of photolysis of clofibric acid is extremely toxic (Bedner & MacCrehan, 2006; Nikolaou et al., 2007). When biological and chemical treatment methods are combined, higher treatment efficiencies with less by-product generation can be obtained (Zhang et al., 2012). For water reuse, advanced treatment technologies such as membrane bioreactor/reverse osmosis should be used (De la Torre et al., 2012)

Knowledge about fate, occurrence and removal of pharmaceuticals in the environment is limited. Most conventional wastewater treatment plants are not qualified to remove pharmaceuticals. Advanced treatment processes are costly and

not applicable in most cases. Even advanced treatment processes can lead to toxic by-products. To conclude, there is still much to be learned about the fate, occurrence and removal of pharmaceuticals in both natural and constructed environments.

2.1.1. NSAIDs in the Environment

NSAIDs are class of aromatic acidic drugs used for analgesic, antipyretic and anti-inflammatory purposes through their inhibition of cyclooxygenase-1 (COX-1) and cycloxygenase-2 (COX-2) isoenzymes, which prevents the formation of prostaglandins and thromboxane (Gagné et al., 2005; Gentili, 2007; Gierse et al., 1995). Many NSAIDs are substituted PAAs, a core structure which may be responsible for their activity. NSAIDs are one of the most consumed drug classes. When introduced into the environment, there may be impacts on human and environmental health (Almeida et al., 2013). The inclusion of the NSAID diclofenac in the European monitoring list in 2013 indicates the gravity of the situation (Union, 2013).

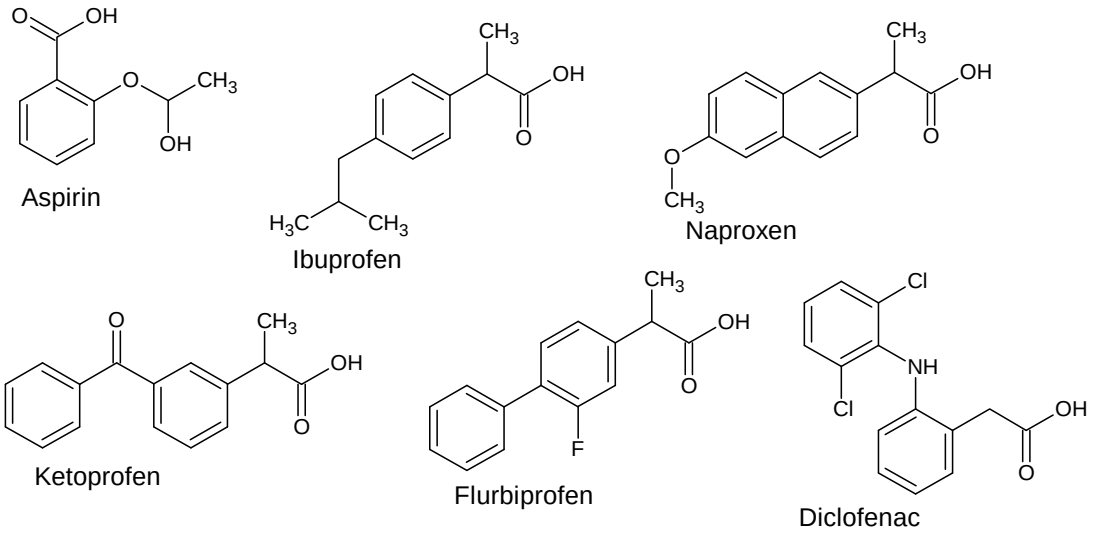


Figure 2. 4. Some common NSAIDs

NSAIDs are frequently detected in different environments, with concentrations ranging from ng/L to µg/L (Gavrilescu et al., 2015; Lapworth et al., 2012; Lloret et al., 2010; S. Wu et al., 2012). Diclofenac, IBP, ketoprofen, mefenamic acid, naproxen, salicylic acid are the most reported pharmaceuticals in WWTPs (Luo et al., 2014). In Turkey, Aydin and Talinli (2013) conducted a study related to

occurrence of pharmaceuticals in surface water; results indicated that the highest concentrations of naproxen, IBP and diclofenac in Büyükcekmece watershed were 12.3 µg/L, 263 ng/L and 52 ng/L, respectively. NSAIDs exhibit a rate of unmodified excretion varying between 0 and 39 % (Luo et al., 2014). Luo et al. (2014) reported concentrations of NSAIDs including IBP, naproxen, ketoprofen, diclofenac and mefenamic acid at ng/L levels in surface water and groundwater. Focazio et al. (2008) reported IBP concentration up to 29 ng/L in untreated drinking water in the USA. In Spain, IBP was one of the most detected pharmaceuticals in wastewater influents, with concentrations varying between 3.73 µg/L and 603 µg/L (J. L. Santos et al., 2009).

NSAIDs can cause serious problems in the environment. Meloxicam is used instead of diclofenac in India due to impacts of diclofenac on vulture populations (Oaks et al., 2004; Swarup et al., 2007). Chronic exposure to diclofenac at concentrations 1, 5 and 25 µg/L can lead to tissue damage and impairments of biochemical functions in rainbow trout (Mehinto et al., 2010). In Europe, diclofenac was considered for inclusion in the list of priority water contaminants considering its toxicity and recalitrance (Richardson & Ternes, 2011). A chronic toxicity test revealed that 0.2 µg/L exposure of IBP to zebra mussel can cause cyto-genotoxicity on hemocytes and in cases of higher exposure concentrations, serious genetic and cellular damage (Parolini et al., 2011). Diclofenac and IBP can cause endocrine disruption, transient oxidative stress, neurotoxic alterations and tissue damage in *Mytilus galloprovincialis* at environmental concentrations (Gonzalez-Rey & Bebianno, 2011, 2012; Gonzalez-Rey & Bebianno, 2014; Schmidt et al., 2011). Zebrafish was exposed to 320 µg/L diclofenac and fortunately no adverse effects were observed. This might offer comfort to researchers when considering lower diclofenac concentrations in many rivers (Memmert et al., 2013). However, 1 µg/L diclofenac exposure for 28 days caused cytological alterations in gills, kidneys and liver of rainbow trout (Triebskorn et al., 2004). Moreover, stability of NSAIDs makes their elimination difficult in treatment plants which may lead to unintentional consumption by humans (Gentili, 2007). For example, the presence of naproxen in groundwater and drinking water may lead to impacts on human health (Gentili, 2007; Juvancz et al., 2008). Bioaccumulation is another potential problem. IBP, naproxen

and diclofenac show a tendency to bioaccumulate in fish (Brozinski et al., 2011; Lahti & Oikari, 2011; Mehinto et al., 2010; Nallani et al., 2011). Pharmaceuticals can enter the environment not only in the form of the parent compound but also in the form of human phase I and II metabolites (Davies, 1998; Davies & Anderson, 1997; Larsson et al., 2014; Skordi et al., 2004; Vree et al., 1993). Additionally, transformation of NSAIDs into their corresponding human metabolites has been observed during treatment (Ferrando-Climent et al., 2012; Lahti & Oikari, 2011; Zwiener et al., 2002). It is known that IBP metabolites have less toxicity towards certain freshwater organisms (Lienert et al., 2007) but data related to the toxicity of metabolites of NSAIDs is not extensive enough to make larger generalizations. In another study, hydroxylated derivatives of IBP exhibited increased inhibition percentage of bioluminescence from *Vibrio fischeri*, indicative of general cytotoxicity (Méndez-Arriaga et al., 2008). Diclofenac photolysis by-products and naproxen phototransformation byproducts can be more toxic than parent compounds (Coelho et al., 2009; Diniz et al., 2015; Isidori et al., 2005).

Log k_{ow} values of NSAIDs are generally between 2.5 and 5 (Aydin & Talinli, 2013; Salgado et al., 2012; Trenholm et al., 2006). This shows their tendency to sorption onto solid particles and to bioaccumulate.

NSAIDs are not fully eliminated by activated sludge processes (Rodarte-Morales et al., 2011). IBP, naproxen and ketoprofen have moderate and high removal efficiencies in conventional WWTPs while diclofenac has poor removal efficiency (Luo et al., 2014). IBP and ketoprofen exhibited high biodegradation (>75%), while diclofenac biodegraded at a low rate (<25%) (Salgado et al., 2012). Removal efficiencies about 50% for naproxen were observed in biological treatment (Carballa et al., 2004). Diclofenac has been reported as the most persistent NSAID in terms of biodegradability in WWTPs under both aerobic and anaerobic conditions (A. Jelic et al., 2011; Lahti & Oikari, 2011; Xue et al., 2010; Zwiener & Frimmel, 2003). In primary treatment, diclofenac can be removed by up to 28% by sorption onto particles, which might be accepted as the main removal mechanism for diclofenac (Behera et al., 2011; Salgado et al., 2012). Membrane bioreactors (MBRs) have good NSAID removal efficiencies except for diclofenac (Beier et al., 2011; Bo et al., 2009;

Tadkaew et al., 2011; Trinh et al., 2012). The removal of NSAIDs by advanced treatment processes is a controversial topic. Diclofenac removal may be achieved to a greater extent by physical and chemical processes (Kovalova et al., 2012; Luo et al., 2014; Yang et al., 2011). Diclofenac removal was achieved at high rates with ultrasonic irradiation with TiO_2, SiO_2, SnO_2, TiO_2/SiO_2 and with fenton and photo-fenton treatment (Hartmann et al., 2008; Pérez-Estrada et al., 2005). UV/H_2O_2 treatment also provided high rates of removal of naproxen (Pereira et al., 2007). It can be said that membrane processes, ozonation and AOPs can achieve high removal of NSAIDs from wastewater (Luo et al., 2014). In some cases, physicochemical processes may generate secondary pollutants (Zhang et al., 2013). Another promising treatment option is attached growth technology, with removal efficiencies up to 100% (Falas et al., 2012; Reungoat et al., 2011). Among the treatment parameters, pH plays a significant role in the removal of acidic NSAIDs by affecting the affinity between the biosolids and NSAIDs in WWTPs (Kimura et al., 2010).

Conventional wastewater treatment technologies have poor NSAIDs removal efficiencies. In many aquatic environments, such as groundwater, surface water and drinking water, NSAIDs have been detected at concentrations ranging from μg/L to ng/L. Toxicological studies suggest that not only NSAIDs, but also metabolites of NSAIDs at these concentrations can cause serious problems in the environment. Occurrence, fate, toxicity and biodegradation of NSAIDs will continue to draw attention of researchers considering increasing consumption rates of NSAIDs, poor removal efficiencies of conventional wastewater treatment technologies and inapplicability of advanced wastewater treatment technologies.

2.1.2. Fluorinated Organics and Pharmaceuticals in the Environment

Fluorine substituents introduce many useful properties to pharmaceuticals. Fluorine is mostly used for increasing biological half-lives of compounds. On the other hand, introduction of fluorine into chemicals creates environmental problems due to increased lipophilicity and recalcitrance (Khetan & Collins, 2007). In the fluorination of organics, a flourine or trifluoro-methyl group is used in place of a hydrogen atom or hydroxyl group; often the moiety targeted for replacement is the site of human metabolic attack. By this replacement, compounds with identical therapeutic effect

but slightly different structure, called bioisosteres, are obtained, ideally with improved pharmacological properties (Olesen, 2001). Introduction of fluorine into pharmaceuticals improves pharmacodynamic and pharmacokinetic properties (Park et al., 2001) and leads to increased bioavailability, intrinsic activity and chemical and metabolic stability (Maienfisch & Hall, 2004). The stability of fluorinated pharmaceuticals is due to higher strength of the C-F bond than the C-H bond (Park et al., 2001). Fluorination of pharmaceuticals can also increase binding affinity of a drug to a target protein (Bohm et al., 2004). Anticancer drugs, drugs acting on the central nervous system, cardiovascular drugs, drugs for infectious diseases, eye care drugs, endocrine system drugs, NSAIDs and antidepressants are some common pharmaceutical classes that include fluorinated pharmaceuticals; currently, 25% of pharmaceuticals are fluorinated and it seems this percentile will increase (Figure 2. 5) (Park et al., 2001; Wang et al., 2014).

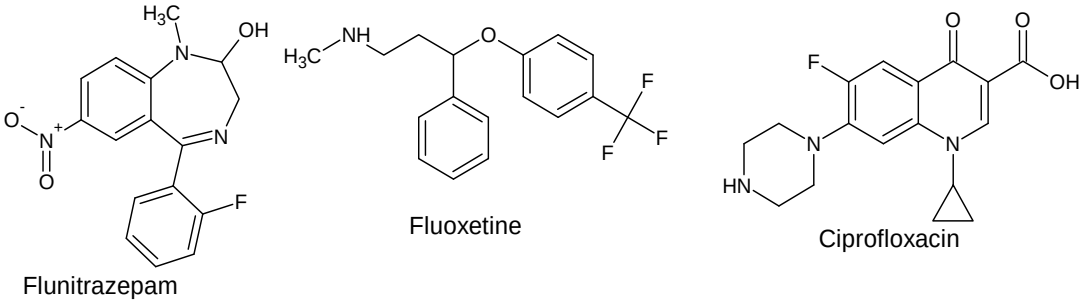


Figure 2. 5. Some common Fluorinated Drugs

Perfluorinated compounds (PFCs) have drawn significant attention, especially perfluorooctanesulfonic acid (PFOS) and perfluorooctanoic acid (PFOA). They are widely used in industry. Carbon-fluorine provides stronger physicochemical properties and high resistance against both biotic and abiotic degradation (Rayne & Forest, 2009). Therefore, their occurrence in the environment is inevitable. Concentrations of PFCs are generally at low ng/L levels and in some cases they can reach to µg/L (Arvaniti et al., 2012; Kunacheva et al., 2011). There have been some studies reporting toxicity of PFOA such as impacts on tumor formation in animals (Andersen et al., 2008; Kennedy et al., 2004; Lau et al., 2007).

Fluoroquinolones are one of the most detected fluorinated pharmaceutical groups at ng/L and µg/L in aqueous environments (Figure 2. 6). Addition of fluorine to quinolones provides an increased antibacterial spectrum (Van Doorslaer et al., 2014). They are known for their potential to generate antibiotic resistance.

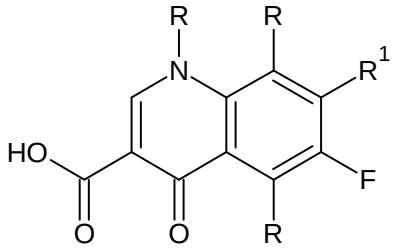


Figure 2. 6. General structure of fluoroquinolones, R1: generally piperazine

Many organic halogenated compounds are resistant to degradation mechanisms in the environment (Tijani et al., 2013). Antibiotics are generally resistant against biodegradation (Jia et al., 2012; Li & Zhang, 2010; Verlicchi et al., 2012). Some of them have half-lives over 1000 days in soil which increases their bioaccumulation potential (Walters et al., 2010). Moreover, the removal of fluoroquinolones by conventional activated sludge processes is very poor (Halling-Sørensen et al., 2000; Jia et al., 2012; Li & Zhang, 2010) although with MBRs the removal rates may approach 60% (Dorival-García et al., 2013; Senta et al., 2011). The poor removal rates increase their occurrence in the environment.

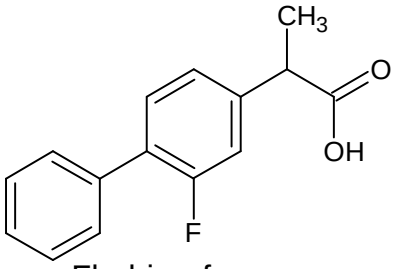
Each year, tons of fluoroquinolones are introduced into soil, representing high risk of development of bacterial resistance (McClellan & Halden, 2010). Many of the fluorinated pharmaceuticals such as ciprofloxacin, levofloxacin, atorvastatin, citalopram, fluvoxamine have toxic effects on alga, invertebrates, plants and fish based on chronic toxicity tests (Brain et al., 2004; Brooks et al., 2003; Ferrari et al., 2004; Ferrari et al., 2003; Henry et al., 2004; Pascoe et al., 2003). Santos et al. (2010) reported concentrations of citalopram, fluoxetine, norfluoxetine, fluvoxamine and paroxetine at ng/L levels and their effects on organisms at these concentrations based on 134 previous toxicity studies. Ciprofloxacin, norfloxacin and levofloxacin are classified as harmful to aquatic organisms in some cases based on UN acute toxicity classification (Ortiz de García et al., 2014). Ciprofloxacin and ofloxacin are

environmentally recalcitrant and have toxic effects on wastewater bacteria at environmentally relevant concentrations (Kümmerer et al., 2000). Fluoxetine is recalcitrant to many natural processes (Kwon & Armbrust, 2006). Fluoxetine exhibits high acute toxicity on algae based on EC_{50} (48h, 0.024 mg/L) and LC_{50} (48h, 2 mg/L) values (Brooks et al., 2003). Fluoxetine and norfluoxetine have bioaccumulation potential in fish tissues and are highly persistent (Meredith-Williams et al., 2012; Paterson & Metcalfe, 2008). Fluoxetine exhibited endocrine disruptor effect on M. galloprovincialis at 75 ng/L (Gonzalez-Rey & Bebianno, 2013). Flutamide has impacts on fish (Hutchinson et al., 2003). It is known that 3-fluorocatechols, which are potential degradation byproducts of fluorinated pharmaceuticals, have broad toxic effects. These impacts of fluorinated pharmaceuticals and chemicals might be not only due to fluorine but also complexity of their structure.

Despite the common usage of fluorinated organics in both the chemical and pharmaceutical industries, their fate, occurrence and removal are still poorly understood.

2.1.3. FLB in the Environment

FLB is a fluorinated NSAID sold under the brand names of Majezik, Ansaid, Algopet, Fiera, Flubimak, Flupen, Flurflex, Fortine, Frolix, Maxaljin, Maximus, Merdex, Netfen, Porjezil, Strefen, Unijezik and Zero-P in Turkey. Dosage per tablet or capsule is generally 100 mg. The excretion ratio of FLB as unmodified or slightly modified compound is about 22% (Risdall et al., 1978; Szpunar et al., 1987). FLB is a substituted phenylacetic acid or a substituted biphenyl (BP). Despite this popularity, there has been no study related to its occurrence in Turkish surface waters. New investigations suggest that FLB may be used for cancer treatment, especially prostate cancer, inhibition of colon tumors, anti-obesity purposes and for some other purposes in the future (Abdel-Aziz et al., 2012; Wechter et al., 2000).



Flurbiprofen

There are only few studies reporting the occurrence of FLB in water. 0.21 and 0.34 µg/L of FLB were detected in the WWTP effluents of France and Italy, respectively (Andreozzi et al., 2003). No FLB was detected in Swedish WWTP effluents (Bendz et al., 2005). There are no reports related to fate, toxicity and removal of FLB, likely because it is not popular in countries where scientific research is dense.

FLB probably has high sorption ability and bioaccumulation potential in the environment. Flurbirpofen is poorly soluble in water and has a K_{ow} of 4.2 (Abdel-Aziz et al., 2012). This may give an idea about its fate, occurrence and removal.

By looking at the fate, occurrence, toxicity and removal of other NSAIDs and fluorinated pharmaceuticals, a general idea about FLB in the environment can be obtained. The toxicity of FLB should be investigated and also the metabolites and byproducts during its degradation should be considered.

2.2. Bacterial Metabolism of Aromatics

Microorganisms have an extraordinary ability to degrade the vast majority of pollutants including recently introduced pollutants into the environment. The recalcitrance of aromatics and their impacts on human and environmental health make them problematic (Assessment, 2005; ATSDR, 2007). Aromatics are in the structures of many natural and anthropogenic chemicals. They have significant roles in biological activities. The ubiquitous presence of aromatics in nature leads to the conclusion that the bacteria able to degrade them should be common. Aromatics can be simply described as circular hydrocarbons and heterocycles with delocalized π-orbital electrons (Phale et al., 2007; Vaillancourt et al., 2006). The inaccessibility of the carbons and the negative resonance of the delocalized electrons make them resistant against chemical attacks (Phale et al., 2007; Vaillancourt et al., 2006). Since they are naturally found in the environment, energy rich and ubiquitous, there are

common bacterial pathways for metabolizing them. However, it is known that this metabolism is not easy and requires highly specialized enzymatic machinery.

The abundance and variety of aromatics has led to diverse degradation mechanisms in bacteria. One of these mechanisms is simply the addition of either one or two atoms of oxygen to the aromatic ring (Harayama et al., 1992) which leads to cleavage of the ring by destabilizing the aromatic structure (Fuchs, 2008; Ju & Parales, 2010; Masai et al., 2007; Phale et al., 2007; Zeyaullah et al., 2009). Aromatic xenobiotics, especially halogenated aromatics, with complex structures may be more resistant to biodegradation due to absence of specific enzymatic machinery responsible for their metabolism in bacteria (George & Hay, 2011). Understanding molecular mechanisms and bacterial strategies for biodegradation of aromatics improves our ability to predict and monitor their biodegradation *in situ*.

Generally, biodegradation of an aromatic ring occurs in two steps referred to as the upper pathway and lower pathway. *meta*-, *ortho*- and and gentisate cleavage are the main aerobic mechanisms for ring opening of aromatics (Figure 2. 7). Major intermediates in aerobic pathways of aromatic degradation are catechols, protocatechuates and gentisates. In the case of anaerobic biodegradation, the upper pathways converge to benzoyl-CoA. Dearomatizing processes of this benzoyl-CoA intermediate are catalyzed by special multi-component reductases in the presence of ATP as energy (Cao et al., 2009).

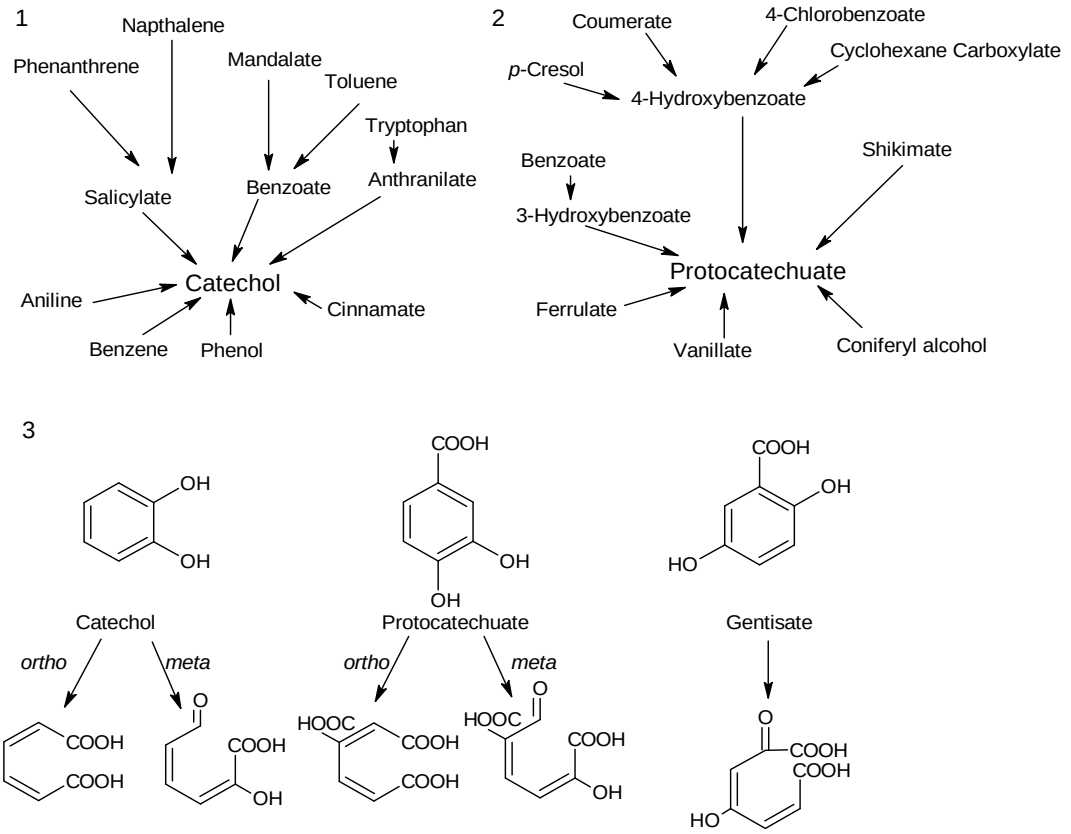


Figure 2. 7. Aerobic biodegradation mechanisms for (1) aromatics funneled to catechol, (2) Aromatics funneled to protocathecuate, (3) ortho-, meta- and gentisate cleavage (Cao et al., 2009; Harwood & Parales, 1996).

Organization and regulation of biodegradation genes

Aromatic degradation pathways are encoded by genes arranged in clusters or operons (Figure 2. 8). Clusters generally contain catabolic genes, transport genes and one or more regulatory genes. Catabolic genes, transport genes and regulatory genes are responsible for encoding degradative enzymes, encoding proteins enabling uptake of the compound and controlling total gene expression, respectively (Diaz, 2004; Khomenkov et al., 2008).

Regulatory proteins play a significant role in functioning of a pathway. Regulatory proteins appear to modulate gene expression when suitable substrate is present. There are many families of regulators for catabolic pathways (Tropel & van der Meer, 2004). For example, *LysR*-type regulators, the largest family, are involved in biodegradation of numerous aromatic compounds. Some other families are the *AraC/Xyl*S family, the *IclR* family and the *XylR*/*NtrC* family (Tropel & van der

Meer, 2004). Interestingly, different classes of regulators often regulate similar catabolic genes in various microorganisms (Cases & de Lorenzo, 2001; Shingler, 2003).

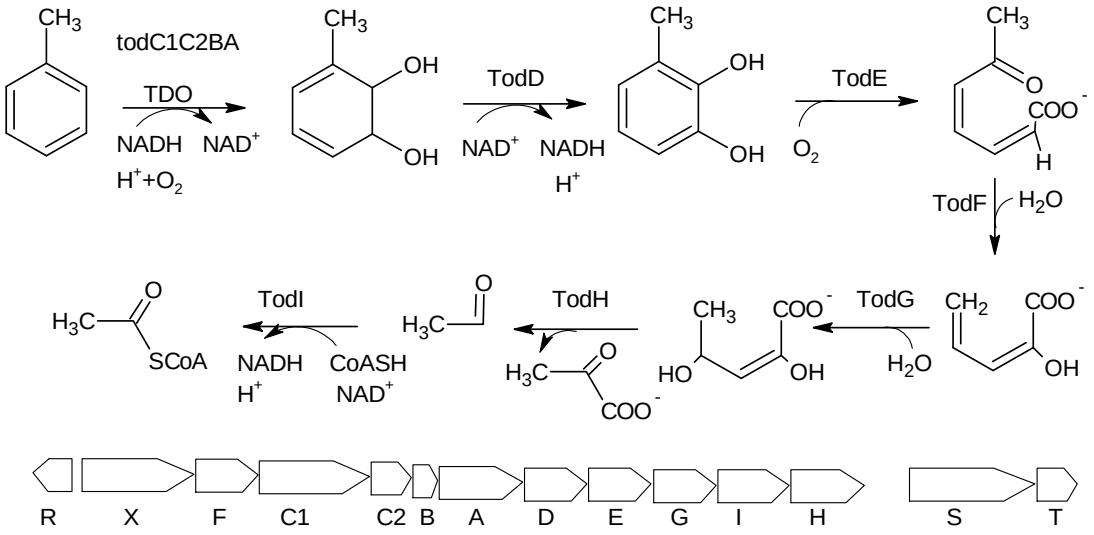


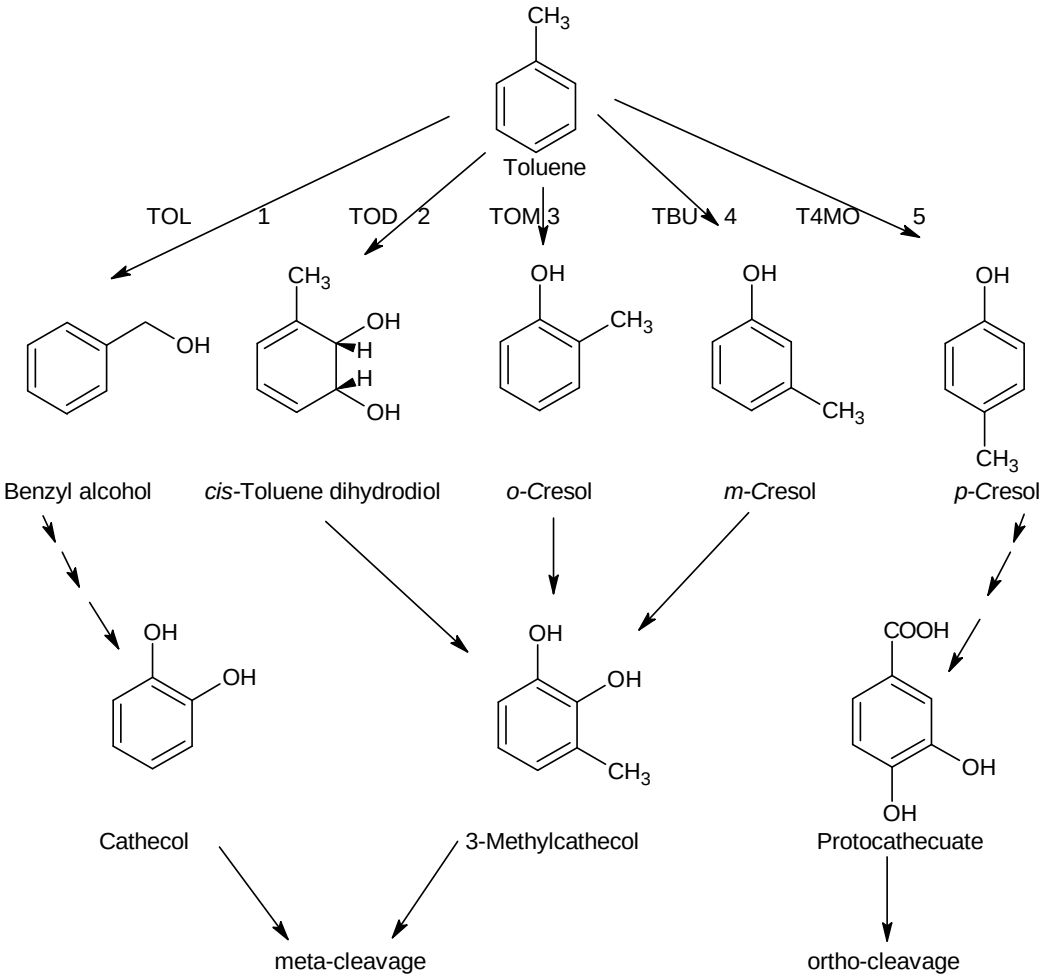
Figure 2. 8. The organization of the catabolic operon, encoding the tod pathway of Pseudomonas putida F1. X is transport gene. F, C1, C2, B, A, D, E, G, I and H are catabolic genes. S and T are regulatory genes. PtodX promoter transcribes the operon. TodS and TodT (Zylstra & Gibson, 1989; Zylstra et al., 1988).

2.2.1. Biodegradation of Simple Aromatic Hydrocarbons

The simplest aromatic hydrocarbons are monocyclic hydrocarbons such as phenol, toluene and benzene. They are common in environment and can be toxic at low concentrations. They have been studied extensively to understand their degradation mechanisms and to construct new bioremediation methods. Most research has been focused on biodegradation of the BTEX group (benzene, toluene, ethylbenzene, and xylene). Toluene (Figure 2. 9) is considered the most easily degraded compound of the BTEX group (Gülensoy & Alvarez, 1999).

The enzyme systems present in the microorganisms determine the metabolic pathways of degradation for the simple aromatics. For instance, the formation of catechol followed by *meta*- or *ortho*- aromatic ring cleavage is the main mechanism for biodegradation of phenol; the type of cleavage depends on the enzymatic machinery present (Ahamad & Kunhi, 1996; Herrmann et al., 1995). Another example is biodegradation of *o*-xylene by *Pseudomonas stutzeri* OX1. *o*-xylene is exposed to two monooxygenase attacks, which results in the formation of 3,4-

dimethyl catechol, which is then cleaved via *meta* cleavage (Baggi et al., 1987). In case of the biodegradation of toluene, different microorganisms exhibit different biodegradation pathways (Gülensoy & Alvarez, 1999) (Figure 2. 9).



1. Pseudomonas putida mt-2, 2. P.putida F1, 3. Burkholderia cepacia G4, 4. B. picketti PKO1, 5. P. mendocina KR1

Figure 2. 9. Different biodegradation pathways of toluene

Classical double-dioxygenation metabolism of aromatics

While aromatics can be degraded biologically by both aerobic and anaerobic mechanisms in the environment, the aerobic mechanism is mainly responsible for biodegradation (Cao et al., 2009) because aerobic processes are fast, substantive and thermodynamically favorable.

The classical double-dioxygenation metabolism proceeds via two steps, the upper and lower pathways (Diaz, 2004). In the upper pathway, the addition of two hydroxyl

groups to the mono- or polycyclic aromatics destabilizes the ring (Mason & Cammack, 1992). The lower pathway proceeds after formation of catechol or gentisate and hydroquinone in some cases (Corvini et al., 2006; Harayama et al., 1992; Harayama & Rekik, 1989; Vaillancourt et al., 2006). Following the cleavage of the ring, the metabolites are directed to the tricarboxylic acid cycle for biosynthesis and energy production (Figure 2. 10).

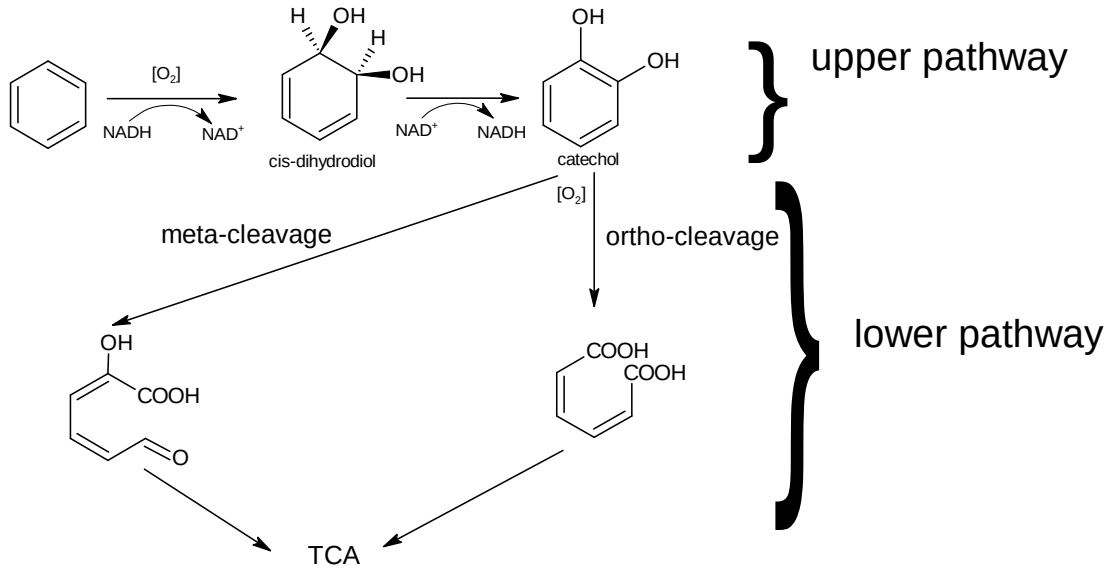


Figure 2. 10. Basic features of the double-dioxygenation metabolism of aromatics

There are some other details of upper and lower pathways worth mentioning. In the upper pathway, ring oxidation requires a reactive oxygen species because of the stability of molecular oxygen. Addition of the oxygen atoms to the aromatic ring is catalyzed via ring-hydroxylating oxygenases. Many of the best known oxygenases require transfer of electrons from NADPH to a terminal oxygenase via electron transport proteins (Butler & Mason, 1997; Gibson & Parales, 2000). The terminal oxygenase with its large (α) and small (β) subunits functions as an oxygen activation center and is responsible for substrate recognition and binding (Butler & Mason, 1997; Furusawa et al., 2004; Gibson & Parales, 2000). In the lower pathway, ring fissions through *ortho*- and *meta*-cleavage take place (Harayama & Rekik, 1989) (Figure 2. 11). Intradiol and extradiol oxygenases initiate *ortho*- and *meta*-cleavages using Fe(III) and Fe(II) at the active site, respectively (Harayama et al., 1992). Additionally, the ring fission product of *meta*-cleavage reaction exhibits a diagnostic

yellow color that disappears upon acidification. In the case of *ortho*-cleavage, coloration is not observed. Broadly speaking, extradiol oxygenases (catechol-2,3-dioxygenases, C23Os) are frequently observed in catabolic and biosynthetic pathways (Vaillancourt et al., 2006).

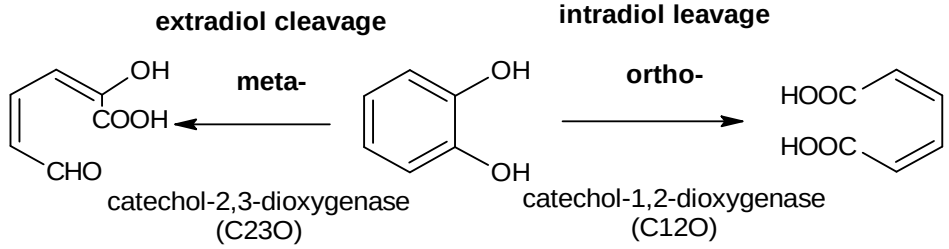


Figure 2. 11. meta- versus ortho- cleavage. meta- and ortho-cleavage take place at 2,3- and 1,2 position on the catechol, respectively. The catalyzers of the reactions are C23Os and C12Os, respectively.

In many cases, intermediates of aromatic metabolism are responsible for cellular toxicity (Chavez et al., 2006; Park et al., 2004; Perez-Pantoja et al., 2003; Pumphrey & Madsen, 2007), requiring specific bacterial adaptations for degradation. Catecholic intermediates can be problematic in that they cause inactivation of C23Os during catalysis (Bartels et al., 1984; Klecka & Gibson, 1981). It is known that some chlorocatechols and alkylcatechols are especially problematic in this regard (Vaillancourt et al., 2006). This situation is also called suicide inhibition, resulting in subsequent accumulation of catechol and limitation of the substrate range. Beyond suicide inhibition, catechols can cause toxicity by different molecular mechanisms such as production of reactive oxygen species and direct protein damage (Schweigert et al., 2001).

Metabolism of Aromatic Acids

Dioxygenations at the 1,2 or 2,3 position are the most-studied aromatic degradation processes to date. The TOL pathway of *Pseudomonas putida* mt-2 is an example of 1,2 dioxygenation. The genes responsible for biodegradation of xylenes and toluene are encoded by *TOL* operon. Toluene is sequentially oxidized at the methyl group to benzoate. *Cis*-dioxygenation of benzoate in the 1,2 position produces c*is*-benzoate dihydrodiol, which is then decarboxylated and dehydrogenated to form catechol (1,2-

dihydroxybenzene). Subsequent dioxygenation of catechol at the 2,3 position then cleaves the ring (Eaton, 1996, 1997).

4-isopropylbenzoate (cumate) is an aromatic acid with a branched aliphatic substituent in the para-position which is often cited as a model for alkyl-substituted aromatic acids. It is dioxygenated at the 2,3 position by *Pseudomonas putida* F1. In this case, the *cmt* operon encodes the enzymes for dioxygenation. 2,3-dihydroxy-4-isopropylbenzoate is then produced by dehydrogenation. This product is dioxygenated at the 3,4 positon to cleave the ring (Figure 2. 12). Because this is a *meta*-cleavage process, a diagnostic yellow color is observed (DeFrank & Ribbons, 1977a, 1977b; Eaton, 1996, 1997).

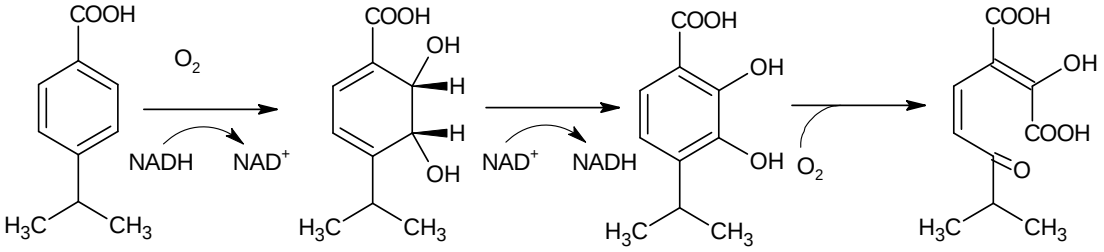


Figure 2. 12. General scheme of the 1,2 dioxygenation *cmt* pathway.

The biodegradation of phenylacetic acid

Until recently, it was believed that bacterial metabolism of phenylacetic acid is similar to those for simple aromatics, such as BTEX and benzoates. This misunderstanding was derived from knowledge of the bacterial pathways for degradation of hydroxyphenylacetic acids. In these pathways, either 3,4-hydroxyphenylacetic acid (homoprotocatechuate) or 2,5-hydroxyphenylacetic acid (homogentisate) are produced as intermediates via sequential monooxygenation (Arias-Barrau et al., 2004; Sparnins & Chapman, 1976; Sparnins et al., 1974; Wegst et al., 1981). A representation of the pathways for phenylacetic acids is presented in Figure 2. 13 (Luengo et al., 2007).

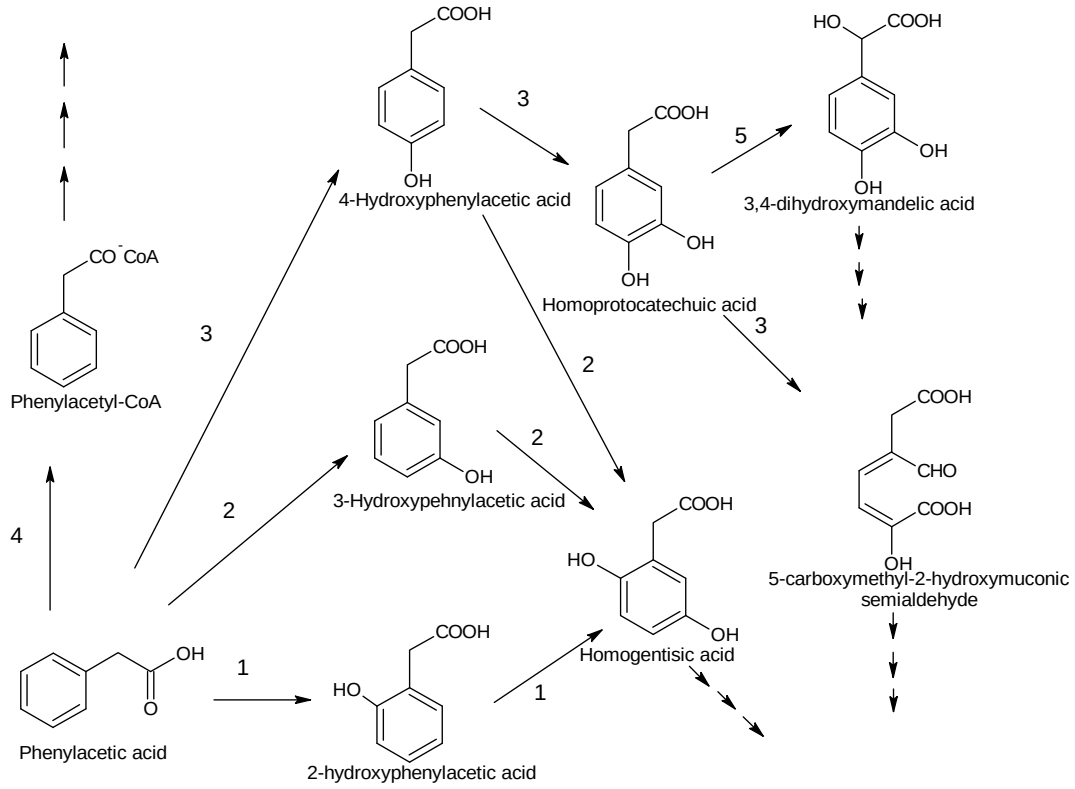


Figure 2. 13. Metabolism of phenylacetic acids by different microorganisms. 1. Nocardia salmonicolor 2. Trichosporon cutaneum and Flavobacterium sp. 3. Escherichia coli, Klebsiella pneumoniae. 4. P. putida U. 5. P. putida F6

It is known that phenylacetic acids are degraded under aerobic conditions by some bacteria, such as *E. coli* (Ferrandez et al., 1998), *P. putida* U (Arias-Barrau et al., 2004; Arias-Barrau et al., 2005), and *Nocardia salmonicolor* (Sariaslani et al., 1974).

Phenylacetyl coenzyme A ligase pathway (the paa pathway)

Molecular investigations into the pathway for bacterial metabolism of phenylacetic acid under aerobic conditions have offered a new perspective on aromatic metabolism. Interestingly, CoA derivatives are used as intermediates and no typical oxygenases are observed during aerobic metabolism of phenylacetic acid in most cases. This suggests an aerobic/anaerobic hybrid catabolism pathway including both oxygenation of aromatic ring (aerobic pathway) and CoA ligation and hydrolytic ring cleavage (anaerobic pathway) (Ferrandez et al., 1998; Fuchs, 2008). Coenzyme A (CoA) is a nucleotide-based cofactor utilized in a wide variety of metabolic systems throughout all branches of life (Leonardi et al., 2005; Spry et al., 2008; Villemur, 1995).

In the early 1990's, some studies showed that pseudomonads utilize phenylacetyl-coenzyme A under anaerobic conditions (Dangel et al., 1991; Mohamed et al., 1993; Mohamed & Fuchs, 1993; Seyfried et al., 1991). It is reported that phenylacetyl-coenzyme A ligases are also induced in *Alcaligenes*, *Acinetrobacter*, *E. coli* (Vitovski, 1993), *Thermus thermphilus* (Erb et al., 2008), *Silicibaacter* (Yan et al., 2009) and *Rhodococcus* (Navarro-Llorens et al., 2005).

Dr. Luengo and his research group described the generation of phenylacetyl-coenzyme A by *Pseudomonas putida* U under aerobic conditions (Martinez-Blanco et al., 1990). This situation was not expected considering the typical aerobic models for aromatic metabolism accepted until that day. The loss of ability to grow on phenylacetic acid with the loss of ability to generate phenylacetyl-coenzyme A made the situation clear (Schleissner et al., 1994).

Some other studies related to this issue made the uncertainty more clear. Several genes responsible from phenylacetic acid metabolism in *P. putida* U (Olivera et al., 1998) and the styrene-metabolizer *P. putida* Y2 (Alonso et al., 2003; Bartolome-Martin et al., 2004) were identified and sequenced. These genes were coenzyme A ligase (*phaE*), four genes associated with ring hydroxylation (*phaFGHI*) and a gene encoding a putative ring-opening enzyme (*phaL*). Very similar genes were also discovered in the aerobic phenylacetate-metabolizer *E. coli* W (Ferrandez et al., 1998; Olivera et al., 1998), *Azoarcus evansii, Escherichia coli*, *Rhodopseudomonas palustris* and *Bacillus stearothermophilus* (Mohamed Mel et al., 2002). Furthermore, a monooxygeantion mechanism is strongly suggested for oxygenation of phenylacetic acid (Fernandez et al., 2006; Teufel et al., 2010).

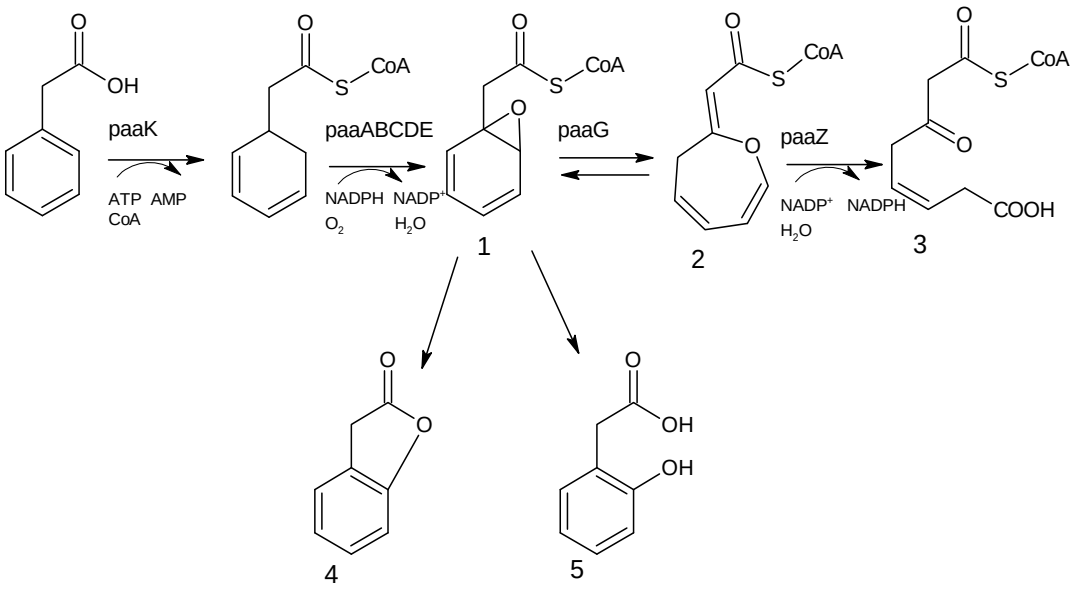


Figure 2. 14. The paa pathway for the aerobic metabolism of phenylacetic acid (Teufel et al., 2010).

The *paa*-like genes are present in the 16% of sequenced bacterial genomes. CoA-ligase hydrolytic ring-cleavage mechanism may be a central paradigm for the aerobic metabolism of aromatics (Teufel et al., 2010). A similar mechanism has also been observed for the metabolism of benzoate derivatives under anaerobic conditions (Fuchs, 2008). It is becoming clear that similar hybrid mechanisms are wide-spread and may be as common as typical aerobic pathways.

The metabolism of ibuprofen by the ipf pathway

Ibuprofen is a NSAID like FLB and a substituted phenylacetic acid. A newly described pathway for the degradation of substituted phenylacetic acids is the *ipf* pathway, which carries some similarities and some significant differences with the *paa* pathway (Figure 2. 15). *Sphingomonas* Ibu-2 has the ability to grow on ibuprofen by using it as carbon and energy source. Like FLB, ibuprofen also has substitutions on the 4th-position and it is known that bulky 4-substitutions require some unique metabolic strategies due to change in the behavior of aromatic oxygenase enzymes (Corvini et al., 2006). Unlike the *paa* pathway, coenzyme A ligation is followed by deacylating dioxygenation in the degradation of ibuprofen by *Sphingomonas* Ibu-2 (Murdoch & Hay, 2005). The mechanism behind the

degradation of ibuprofen may provide an insight for the degradation of other alpha-branched phenylacetic acids like FLB, ketoprofen, and naproxen.

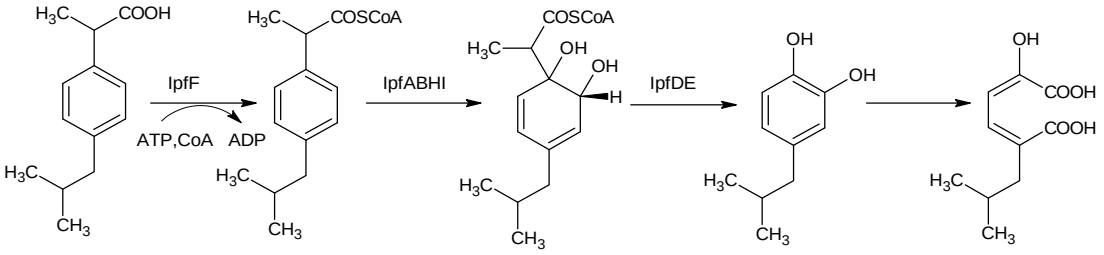


Figure 2. 15. The metabolism of ibuprofen by Sphingomonas Ibu-2 (Murdoch & Hay, 2005, 2013).

2.2.2. Biodegradation of Polycyclic Aromatic Hydrocarbons (PAHs)

PAHs are very common in the environment. High concentrations of PAHs with the existence of co-contaminants such as heavy metals and BTEX compounds creates problems in terms of biodegradability and recalcitrance (Bamforth & Singleton, 2005; Meckenstock et al., 2004). The scientific community has mainly focused on metabolism of PAHs with two or three aromatic rings. Especially, the pathways for degradation of substituted and halogenated PAHs will be important in determining metabolism of FLB.

They are mostly degraded by oxygenase enzymes like the degradation of many simple aromatics. For example, naphthalene is oxidized by mono- or dioxygenation leading to systematic breakdown of naphthalene (Bamforth & Singleton, 2005). PAHs can be oxidized by *Mycobacterium* sp. via a special monooxygenase enzyme (Kelley et al., 1990). *Sphingomonas* sp. LB126 can initially oxidize fluoranthene by monooxygenase. This strain is also capable of co-oxidizing some other PAHs (van Herwijnen et al., 2003). *Nocardia*, *Mycobacterium*, *Pseudomonas*, *Rhodococcus*, and *Sphingobium* species can metabolize anthracene via a pathway proceeding through 3-hydroxy-2-napthoic acid and 2,3-dihydroxynaphtalene (Cerniglia, 1992; Dean-Ross et al., 2001; Moody et al., 2001). Not only bacteria, but also fungi and algae can degrade PAHs. The lignolytic fungal degradation mechanism for PAHs proceeds through oxidation of ring by lignin and Mn-peroxidase enzymes, formation of PAH-quinones and ring fission (Haritash & Kaushik, 2009).

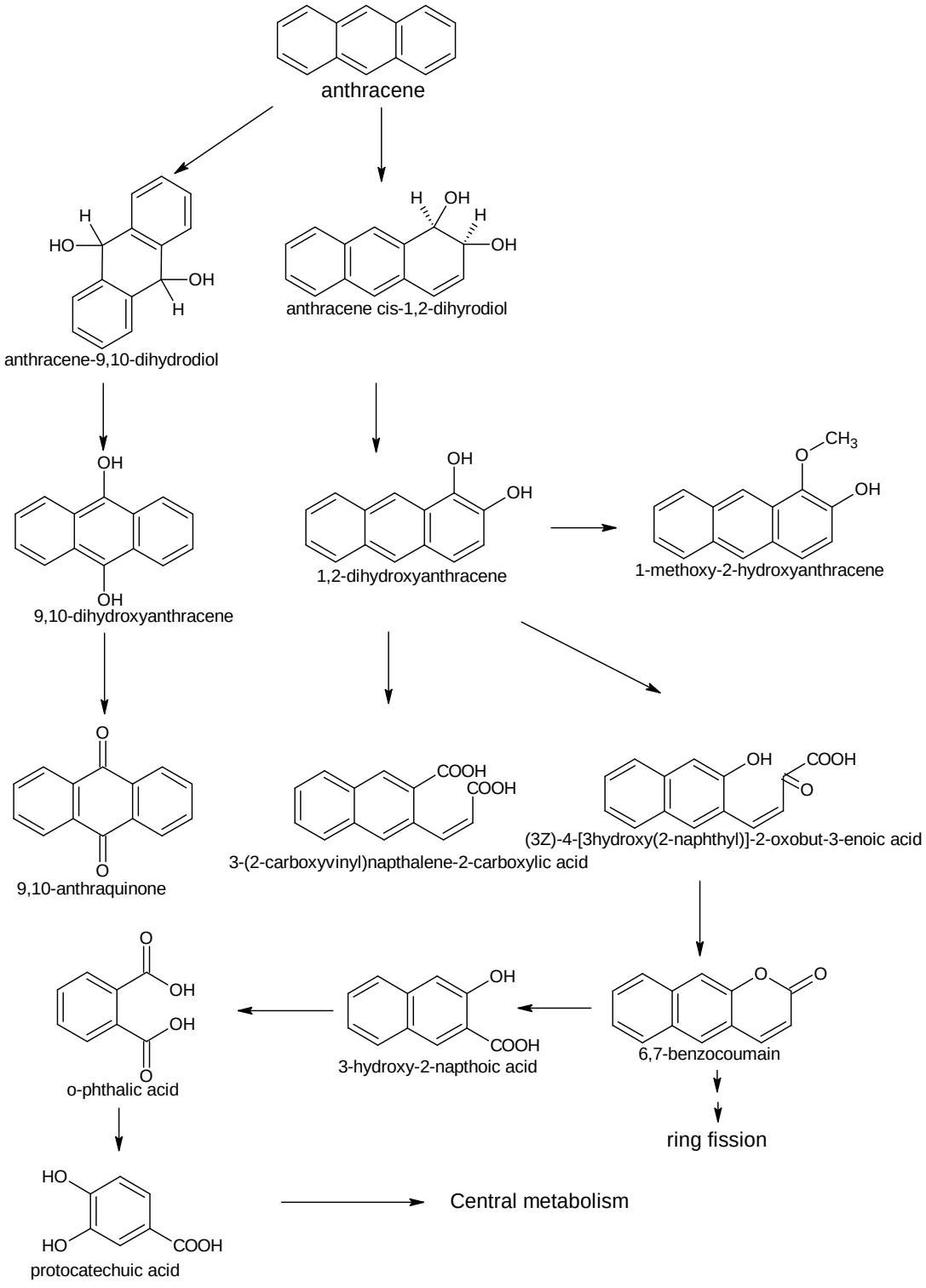


Figure 2. 16. Proposed pathways for degradation of anthracene by Mycobacterium sp. PYR-1 (Moody et al., 2001; René van Herwijnen et al., 2003)

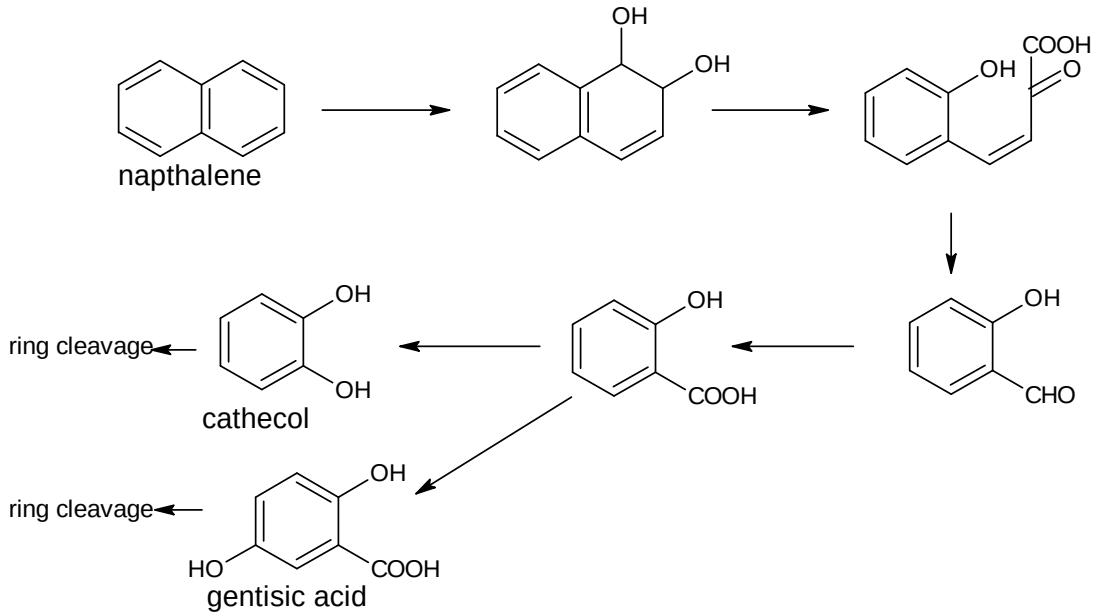


Figure 2. 17. Proposed pathway for napthalene degradation by some pseudomonas species (Mrozik et al., 2003)

While the aerobic degradation of PAHs has been studied very well, there is significant lack of knowledge on the anaerobic degradation of PAHs (Coates et al., 1996; Coates et al., 1996). It is known that some PAHs with two or three aromatic rings can be degraded anaerobically, although the mechanisms behind the degradation processes are not known (Bregnard et al., 1996; Coates et al., 1996; Langenhoff et al., 1996).

Metabolism of Biphenyl

FLB as a substituted BP may be a substrate for the *bph* pathway when aerobically degraded by microorganisms. Especially, the pathways for degradation of halogenated BPs may enlighten the mechanism behind the degradation of FLB. It has been reported that BP can be degraded by many bacteria such as *Sphingobium* sp. Strain PNB (Roy et al., 2013) and *Pseudomonas pseudalcigenes* KF707 (Furukawa et al., 1993) (Figure 2. 18). In the first step of metabolism of BP, *BphA1A2A3A4* is responsible for converting BP to a dihydrodiol via biphenyl dioxygenase. *BphB* dehydrogenates the dihydrodiol to 2,3-dihydroxybiphenyl. Then 2,3-dihydroxybiphenyl is ring-opened. This last product is converted into benzoic acid

and 2-hydroxy-pent-2,4-dienoic acid by 2-hydroxy-6-oxo-6-phenylhexa-2,4-dienoic acid hydrolase (Furukawa et al., 1993).

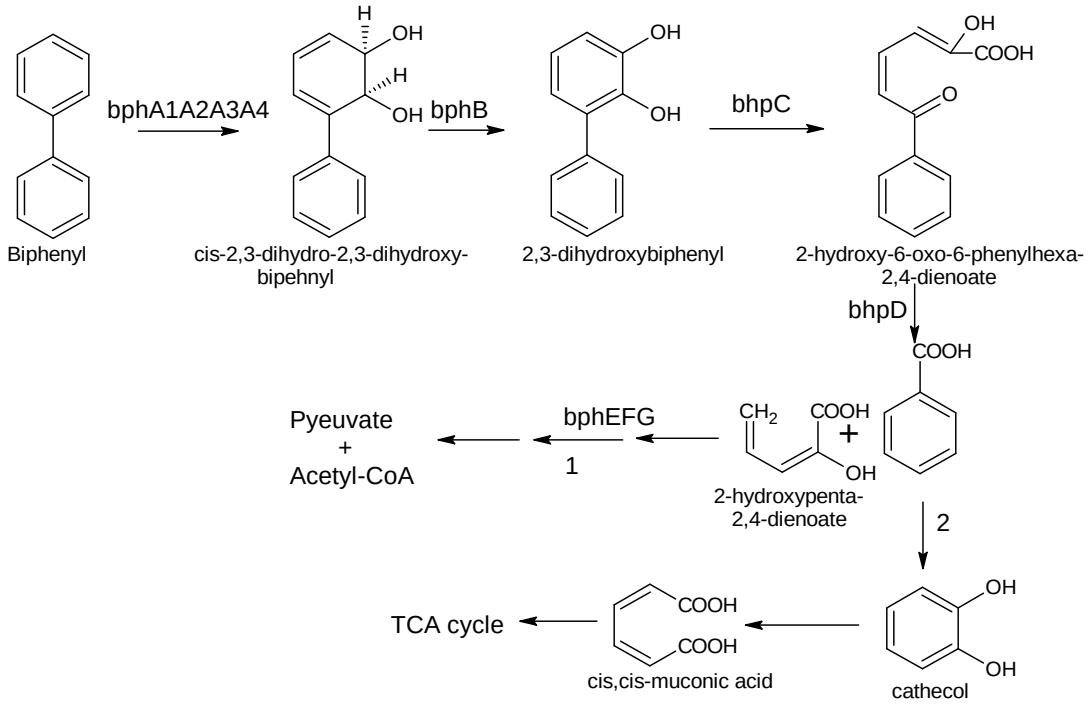


Figure 2. 18. BP degradation pathway. 1. (Ohtsubo et al., 2004) 2. (Roy et al., 2013)

2.2.3. Biodegradation of Halogenated Aromatics

Halogenated aromatics, especially chlorinated aromatics, have been used widely as pesticides, insecticides, pharmaceuticals, plasticizers and many other industrial purposes. In many different regions of world, many chlorinated aromatics are considered priority pollutants. Chlorinated aromatics have been more widely studied compared to other halogenated aromatics. Thus, their metabolisms are well known, especially the metabolism of polychlorinated biphenyls (PCBs).

Before considering the degradation of PCBs, understanding the degradation of chlorinated single aromatics may be useful for understanding the degradation, toxicity and inhibitory effects of PCBs and their degradation metabolites. 4-chlorophenol is degraded via either chlorocatechol or hydroquinone pathways (Bae et al., 1996). 2-chlorophenol is degraded via the formation of 3-chlorocatechol, while

3-chlorophenol is degraded either via the formation of 3-chlorocatechol or via the formation of 4-chlorocatechol (Farrell & Quilty, 1999; Solyanikova & Golovleva, 2004). In the next steps, 5-chloroformyl-2-hydroxypenta-2,4-dienoic acid as a product of *meta*-cleavage of 3-chlorocatechol, is a dead end product which inactivates catechol-2,3-dioxygenase. This results in accumulation of 3-chlorocatechol in the media (Figure 2. 19) (Bartels et al., 1984; Farrell & Quilty, 1999).

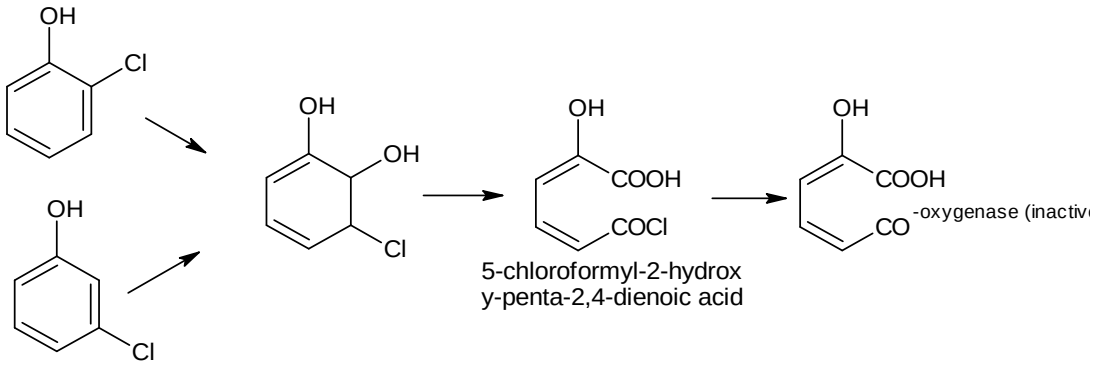


Figure 2. 19. Inactivation of chlorophenol metabolism and accumulation of 3-fluorocatachols

Both aerobic and anaerobic degradation of chlorophenols have been well studied. Various chlorophenols are degraded based on initial reductive dehalogenation as the initial step (Field & Sierra-Alvarez, 2008). Becker et al. (1999) described two pathways for anaerobic degradation of 2-chlorophenol in a sediment slurry reactor. The first pathway begins with an initial dehalogenation of 2-chlorophenol, then carboxylation to 4-hydroxybenzoate and lastly dehyroxylation to benzoate while the second pathway gives a dead-end compound, 3-chlorobenzoate. Mineralization of chlorophenols coupled with sulfate reduction was studied by (Häggblom & Young, 1990). In several other studies, the mineralization of 2-, 3- and 4-chlorophenols coupled with sulfate reduction was reported (Haggblom et al., 1993; Häggblom & Young, 1995). These anareboic chlorophenol degradation studies were based on microbial consortia.

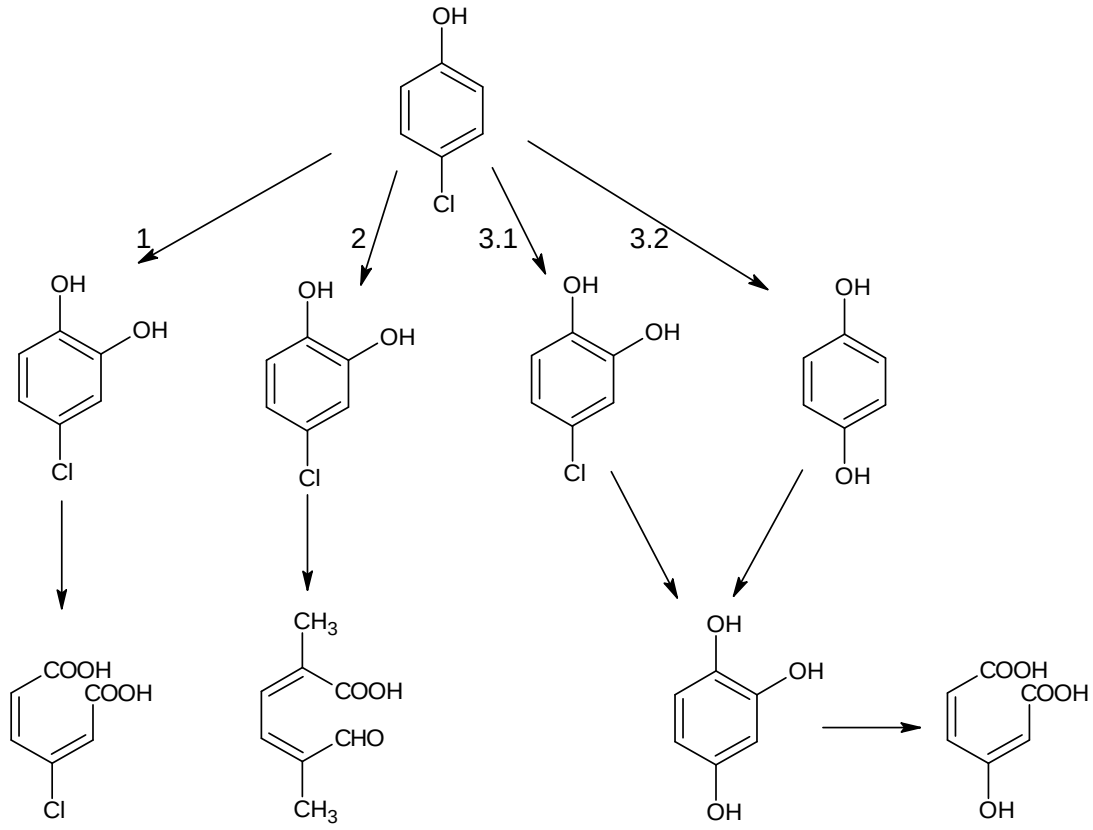


Figure 2. 20. Degradation of 4-chlorophenol via ortho-cleavage (1), via meta-cleavage (2), via 4-chlorocatechol-benzetriol pathway (3.1) and hydroquinone pathway (3.2) (Arora & Bae, 2014)

Adriaens and Focht (1990) pointed out the ability of BP-degrading bacteria to also metabolize PCBs. The enzymes having roles in the *bph* pathway are able to transform PCBs. BP degradation by bacteria is initiated by biphenyl 2,3-dioxygenase. However, toxic effects of certain dead-end metabolites of PCBs can inhibit the degradation of PCBs. It has been reported that PCBs can be transformed into chlorobenzoates and 2-hydroxypenta-2,4-dienoate which is a usable growth source for most bacteria (Pieper, 2005). The dehalogenation of PCBs generally occurs via biphenyl 2-3-dioxygenase. For example, in the degradation of 3-3'-dichlorobiphenyl via an initial step catalyzed by biphenyl 2,3-dioxygenase, Cl was removed from the aromatic ring, although in this case alterations in regioselectivity properties of biphenyl 2,3-dioxygenase was necessary (Suenaga et al., 2002) (Figure 2. 21).Without the alterations, dehalogenation was not observed at the biphenyl 2,3-dioxygenation stage (Haddock & Gibson, 1995; Seeger et al., 1995; Seeger et al., 1999).

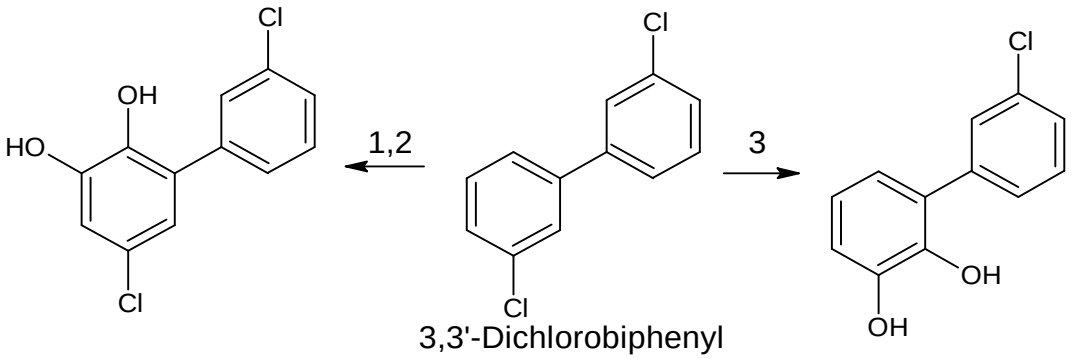


Figure 2. 21. Degradation of 3,3'-Dichlorobiphenyl by (1) Burkholderia sp. LB400, (2) Pseudomonas pseudoalcaligenes KF707 and (3) Phe227Val and Phe377Ala mutants of KF707 dioxygenase

Additionally, the degradation pathways for monochlorobiphenyls, in which the non-chlorinated ring is exposed to dioxygenation attack (Figure 2. 22), can suggest a model for the degradation pathway of FLB.

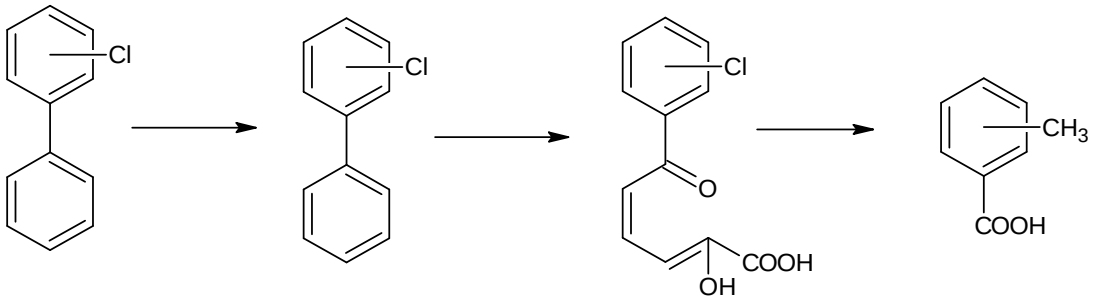


Figure 2. 22. The degradation pathway of monochlorobiphenyl in aerobic bacteria (Harkness et al., 1993)

Besides the toxic effects of chlorinated biphenyls, the formation of dihydroxybiphenyls as metabolites is potentially dangerous for bacteria, affecting bacterial performance (Camara et al., 2004).

Several studies demonstrated that the enzymes degrading fluorinated aromatics such as fluorophenols and fluorobenzoates are the same as those degrading the non-fluorinated versions of these chemicals (Boersma et al., 2004; Brooks et al., 2004; Ferreira et al., 2008). The degradation of 4-fluorobenzene by *Rhizobiales* strain F11 occurs predominantly via 4-fluorocatechol followed by *ortho* cleavage. It is also possible that an initial defluorination followed by catechol formation takes place in the degradation of 4-fluorobenzene (Figure 2. 23) (Carvalho et al., 2006). Another

study by Franco et al. (2014) demonstrated that 4-fluorobenzene had inhibitory effects towards the *ectomycorrhizal* fungi *Pisolithus tinctorius*, while 2- and 3-fluorobenzenes did not. Successful degradation of 2- and 4-fluorobenzoates have been reported many times, while 3-fluorobenzoates cannot be degraded efficiently due to accumulated toxic intermediates. 2-, 3- and 4- fluorobenzoates were successfully degraded by a FLB 300 strain (*Agrobacterium-Rhizobium* branch) without formation of toxic 3-fluorocatechol (Figure 2. 24). However, another study reported that the formation of 4-fluorocatechol in the degradation of 3-fluorobenzoate was because of regioselectivity of the initial dioxygenation process (Engesser et al., 1990). 3-fluorocatechol is strongly resistant against *ortho*-cleavage enzymes and has tendency to accumulate and has toxic effects on cells (Dorn & Knackmuss, 1978; Engesser et al., 1988; Schreiber et al., 1980). By one possible pathway for FLB degradation, toxic 3-fluorocatechol can be generated as intermediate that can inhibit the degradation.

There are few studies related to degradation of fluorinated phenylacetic acids. p-fluorophenylactic acid was reported to be metabolized by *Pseudomonas* sp.. A clear pathway for the metabolism was not reported although some fluorinated metabolites and free fluoride ions were observed (Harper & Blakley, 1971a, 1971b).

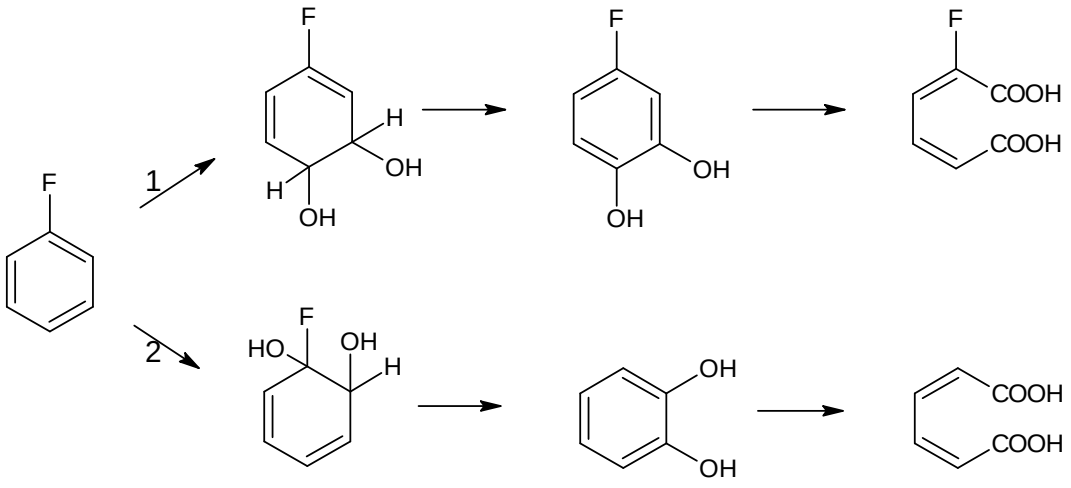


Figure 2. 23. The degradation pathway of 4-fluorobenzene. (1) 4-fluorocatechol pathway which predominantly occurs. (2) Catechol pathway

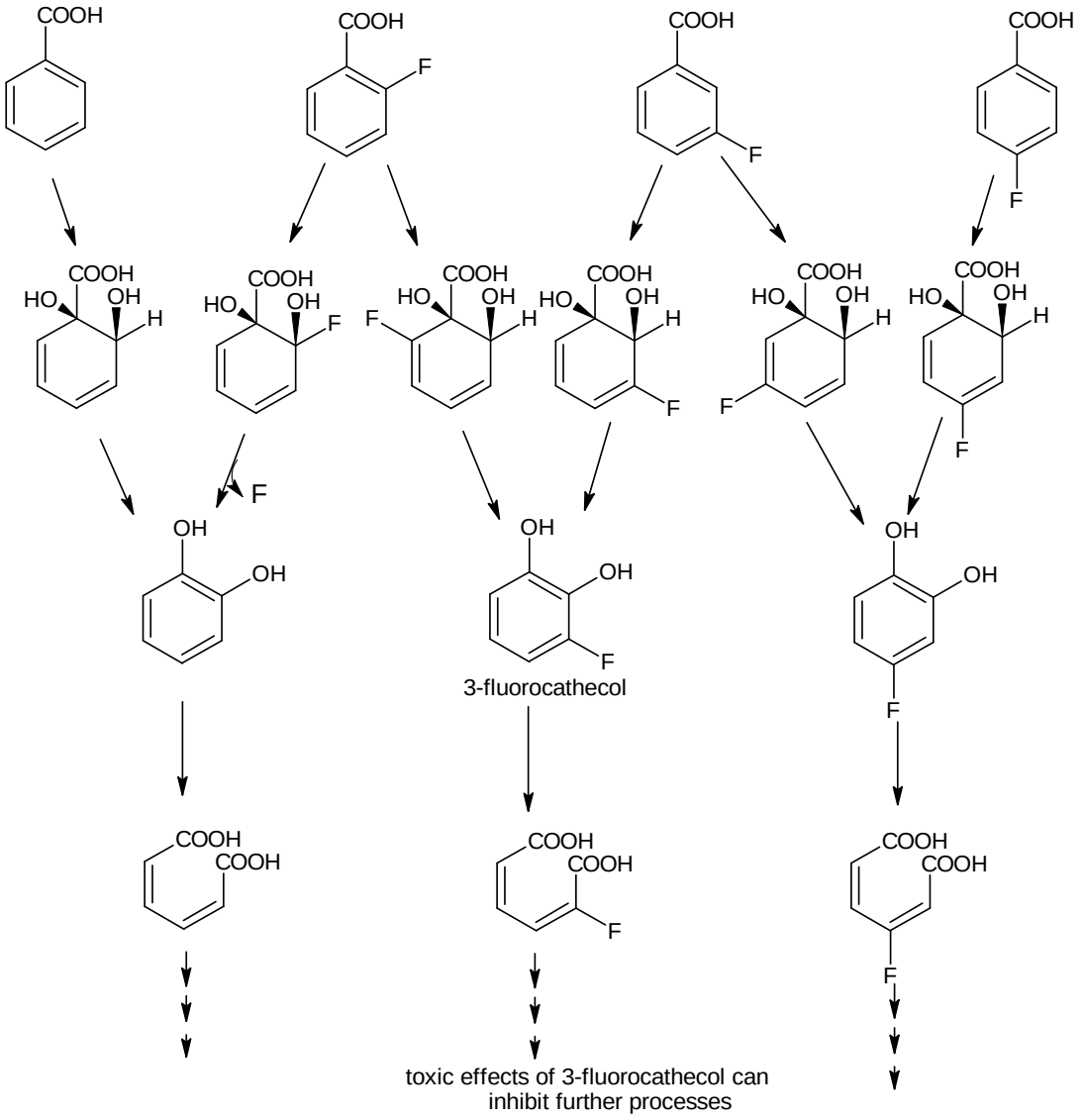


Figure 2. 24. The pathways for degradation of benzoate and fluorobenzoates by bacteria (Schreiber et al., 1980). Benzoate, 2-, 3- and 4-fluorobenzoate are located at the top respectively

The formation of 4-fluorocatechol instead of 3-fluorocatechol allows successful degradation for 3-fluorobenzoate. In a described pathway for 4-fluorobenzoate degradation, *Aureobacterium* sp. removes fluoride ion enzymatically in the initial step of degradation (Oltmanns et al., 1989). In the case of degradation of 2-fluorobenzoate, fluoride ion can be removed in the initial step by dioxygenation or toxic 3-fluorocatechol can be formed by dioxygenation (Engesser & Schulte, 1989; Vora et al., 1988).

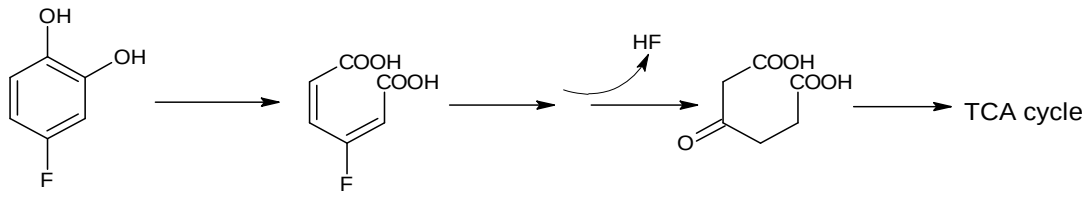


Figure 2. 25. The pathway after formation of 4-fluorocatechol

The trifluoromethyl group is involved in many compounds. Both the degradation of 3- and 4-trifluoromethyl benzoates and 2-trifluoromethylphenol by bacteria exhibit the formation of 2-hydroxy-6-oxo-7,7,7-trifluoro-hepta-2,4-dienoate which is a *meta*-cleavage product of the related catechols (Engesser et al., 1988; Engesser et al., 1988; Reinscheid et al., 1998) (Figure 2. 26). Pesticides and herbicides having a trifluoromethyl moiety can be degraded by bacteria (Bellinaso Mde et al., 2003) and fungi (Guha et al., 1995) without fluoride loss.

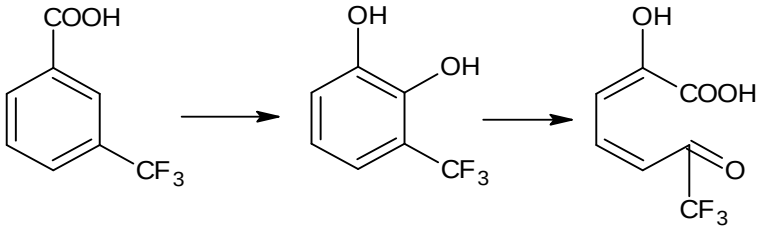


Figure 2. 26. The degradation pathway of 3-trifluoromethyl benzoate

The classical aromatic degradative pathways take part in the degradation of fluorobiphenyls by fungi and bacteria. The degradation 4-fluorobiphenyl by fungi results in conjugated and hydroxylated products, such as 4-fluoro-4'-hydroxybiphenyl, 4-fluorobiphenyl glucuronide and 4-fluorobiphenyl sulphate (Amadio & Murphy, 2010; Green et al., 1999). *Pseudomonas pseudoalcaigenes* KF707 degrades 2- and 4-fluorobiphenyl via biphenyl degradation pathway (Murphy et al., 2008) (Figure 2. 27). The end-products are 2- and 4-fluorobenzoate. The non-fluorinated ring is the initial site of dioxygenation, which is valid also for degradation of 2,3,4,5,6-pentafluorobiphenyl by KF707 and *Burkholderia* sp. LB400 resulted in a dead-end metabolite, pentafluorobenzoate (Hughes et al., 2011). In the case of fluorine substitution not confined to the one ring, both KF707 and *Burkholderia* sp. LB400 degraded 4,4'-difluorobiphenyl (Hughes et al., 2011) (Figure 2. 28). It was also demonstrated that 2,2'-difluorobiphenyl was transformed

to 2'-fluoro-2,3-dihydroxybiphenyl via *bphA* by *Burkholderia* sp. LB400 (Seeger et al., 2001). While it seems the enzymes responsible for the degradation non-fluorinated compounds are also responsible for degradation of fluorinated compounds, there are some studies reporting specialized enzymes employed for degradation of fluorinated compounds (Murphy et al., 2008). However, there is still much work to be done in order to enlighten the actual mechanisms of degradation in all its aspects.

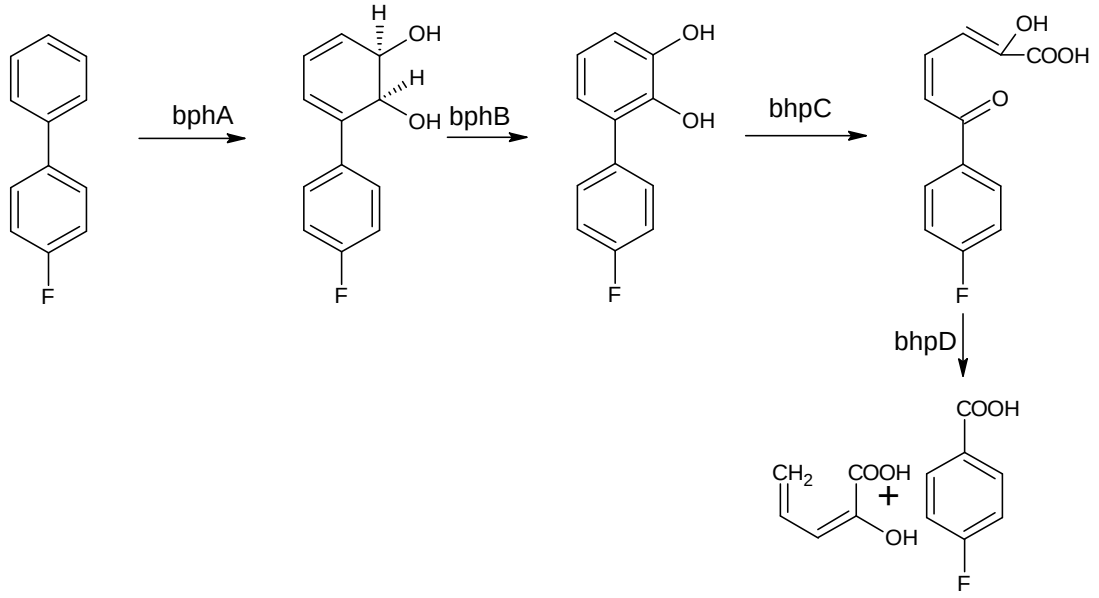


Figure 2. 27. The degradation pathway of 4-fluorobiphenyl (KF707 cannot mineralize fluorobenzoate)

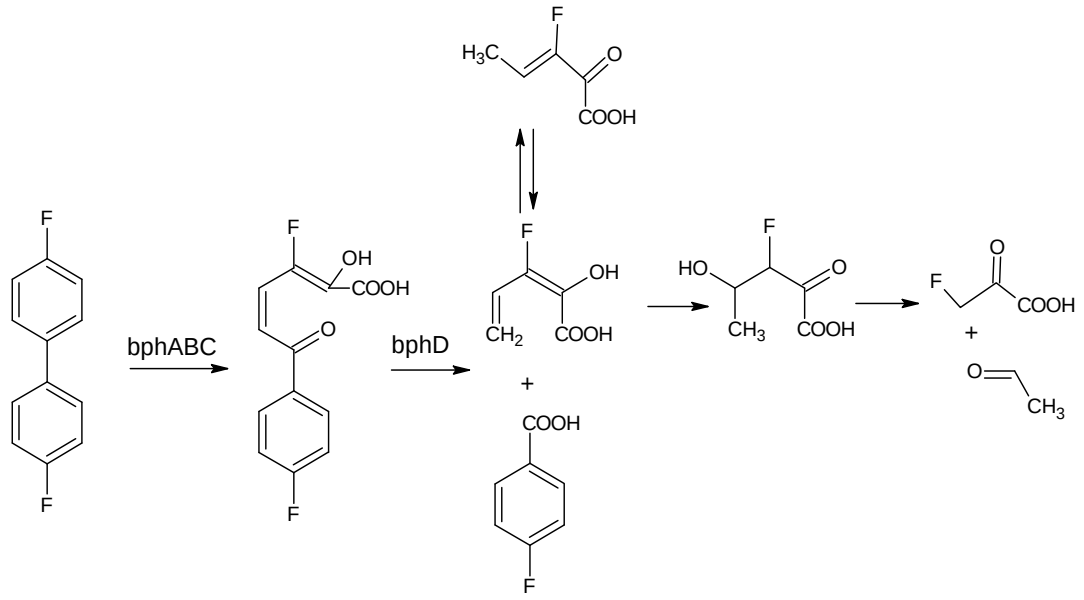


Figure 2. 28. The degradation pathway of 4,4'-difluorophenyl

The aerobic degradation of 4-fluorocinnamic acid by *Arthrobacter* sp. strain G1 and *Ralstonia* sp. strain H1 occurs via a pathway similar to the *paa* pathway. 4-fluorocinnamic acid was converted into 4-fluorobenzote by strain G1. A dead-end side product, 4-fluoroacetophenone yielded during the degradation by strain G1. Strain H1 degraded 4-fluorobenzoate via 4-fluorocatechol followed by *ortho*-cleavage (Hasan et al., 2011) (Figure 2. 29).

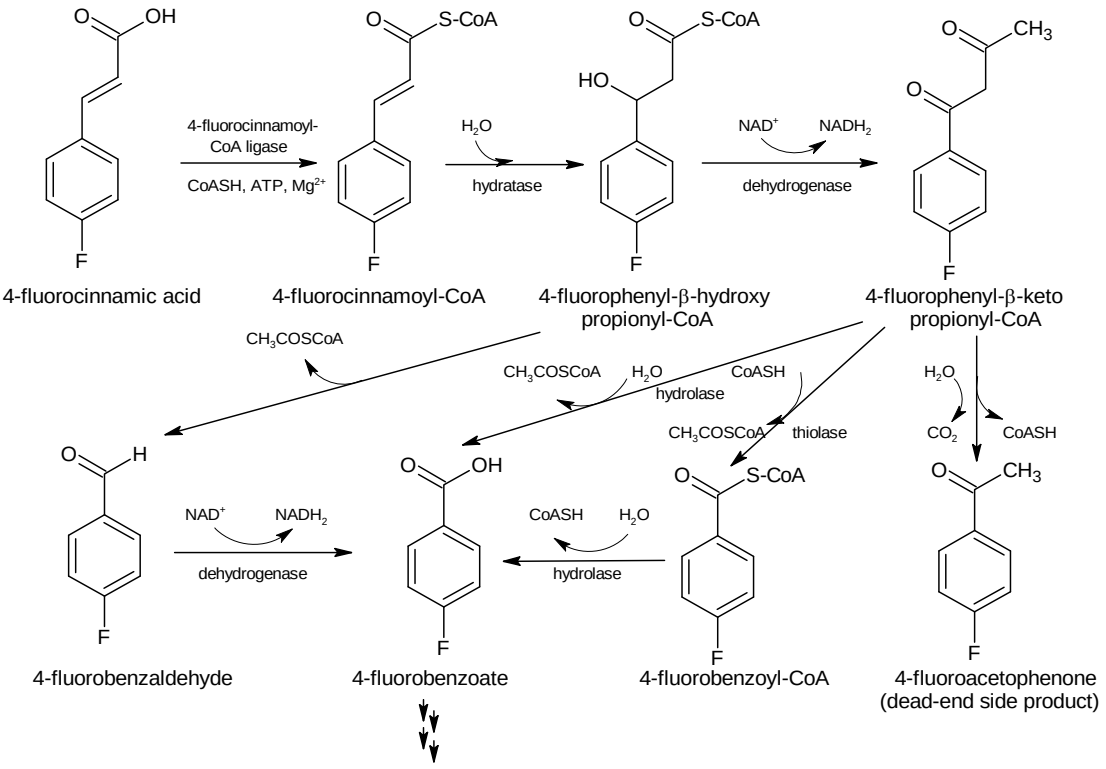


Figure 2. 29. The degradation pathway of 4-fluorocinnamic acid by Arthrobacter sp. Strain G1 (4-fluorobenzoate was degraded by strain H1)

Information about the anaerobic degradation of fluorinated aromatics is limited. 2- and 4-fluorobenzoate can be mineralized by denitrifying bacteria (Vargas et al., 2000). Sulfate reducing bacteria can use 2- and 4-fluorobenzoate as electron donors (Drzyzga et al., 1994). In another study, it was found that a sulfate-reducing enrichment culture, degrading BP, co-metabolized 4-fluorobiphenyl (Selesi & Meckenstock, 2009).

To sum up, it is expected that the bacterial aerobic degradation of FLB proceeds through either *bph* pathway or *paa* pathway. The degradation of FLB through chlorinated or fluorinated *bph* pathways is more likely to occur.

2.2.4. Biodegradation of NSAIDs

Both monocyclic and polycyclic NSAIDs have been detected in the environment. The degradation pathways for most of them have not been described. Taking a closer look at the degradation of NSAIDs can give a point of view for FLB degradation. In the environment, the degradation of NSAIDs is carried out most probably by fungi

and bacteria. Among the NSAIDs, naproxen, ibuprofen, diclofenac, ketoprofen, FLB, aspirin, acetaminophen and FLB are the most popular ones with polycyclic or monocyclic aromatic structures. The ibuprofen degradation pathway (*ipf*) is described in summary in the previous sections.

In case of biodegradation of polycyclic NSAIDs, studies related to their metabolism by fungi or bacteria is limited. Domaradzka et al. (2015) reported that there is no completely described degradation pathway for any polycyclic NSAID except olsalazine (Figure 2. 30).

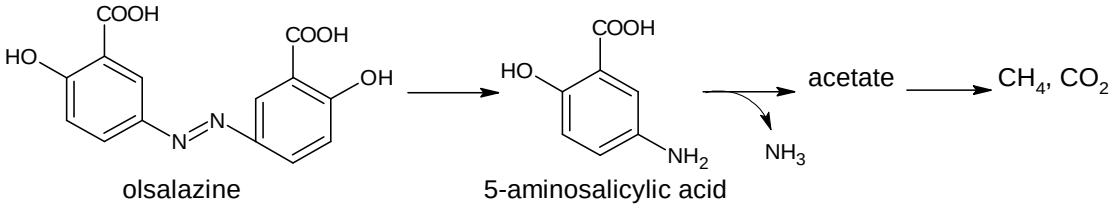


Figure 2. 30. Anaerobic degrdation of olsalazine by methagonenic consortium (Razo-Flores et al., 1997)

Another polycylic, ketoprofen, was degraded based on the *bph* pathway in an activated sludge. 2-(3-oxalophenyl)propanoic acid was yielded as a dead-end metabolite (Figure 2. 31).

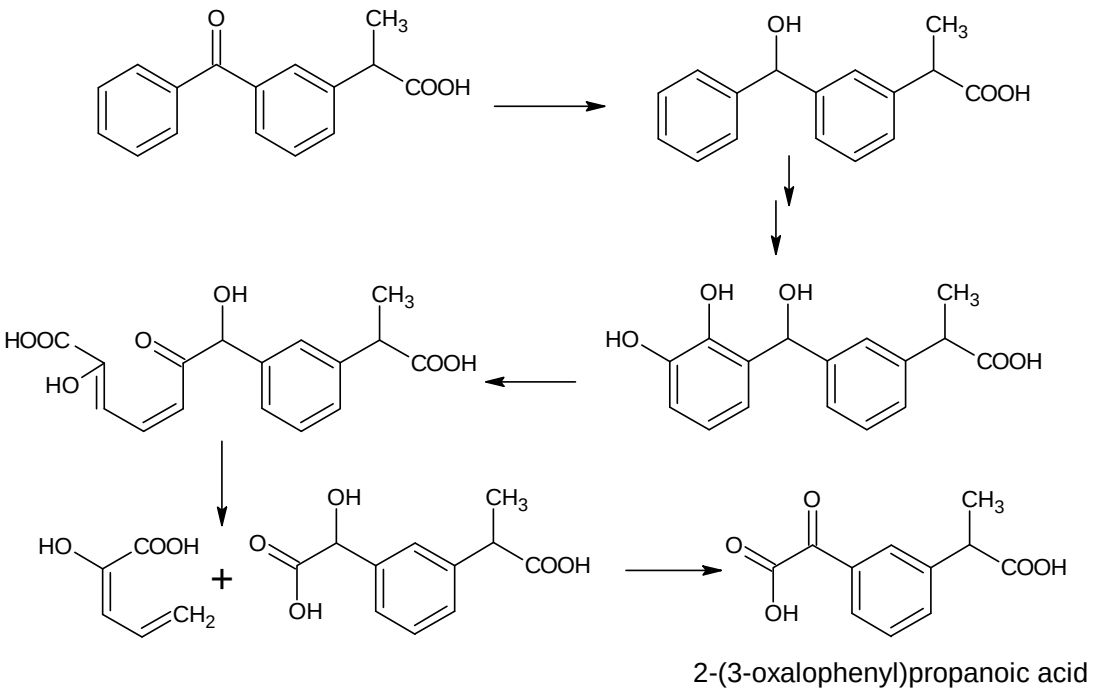


Figure 2. 31. The aerobic degradation pathway of ketoprofen

On the other hand, the degradation pathways of some monocyclic NSAIDs have been well studied. The monocyclic NSAIDs are mainly degraded via formation of catechol, gentisate, hydroquinone and protocatechuate followed by *ortho*- or *meta*-ring cleavage. For example, salicylates are degraded via formation of catechol and gentisate followed by *ortho*- or *meta*- ring cleavage (Marchlewicz et al., 2015).

CHAPTER 3

MATERIALS AND METHODS

The experimental design consisted of enrichment and isolation of microbial strains and analysis of both sludge and culture supernatants. Enrichment and isolation of cultures aimed to determine the strains degrading FLB and analysis of both sludge and culture supernatants aimed to determine biodegradation pathway of FLB and metabolites arising from the biodegradation.

Detailed information about the experimental design is provided in this section.

3.1. Chemicals

2-Fluoro-alpha-methyl-4-biphenylacetic acid (FLB), m-tolylacetic acid (mTAA), p-tolylacetic acid (pTAA), phenylacetic acid (PAA), biphenyl (BP), Ibuprofen (IBP) and 3-fluorophenylacetic acid (3FPAA) were purchased Alfa Aesar (Lancashire, U.K.).

3.2. Enrichment and Degradation Studies

pTAA, mTAA, PAA and FLB were separately spiked into sludge in flasks in order to enrich for bacteria able to use these chemicals as carbon and energy sources. mTAA, pTAA and PAA were control experiments which showed that the experimental system worked properly. Aerobic sewage sludge from the discharge of secondary sedimentation tank of Ankara Tatlar Municipal Wastewater Treatment Plant (ATMWTP) was enriched with the chemicals according to experimental setups in the next sections. Alternative mineral salt medium MSM recipes were used in order to stimulate growth because FLB strains did not grow well. All the prepared flasks and tubes during experiments were put into an incubated shaker at 120 rpm shaking speed at 30 ^{o}C.

Additionally, FLB was spiked into aerobic sludge from Yozgat and Eskişehir Municipal Wastewater Treatment Plants (YMWTP and EMWTP, respectively) as part of further attempts to obtain FLB-degrading isolates.

3.2.1. Enrichments of Aerobic Sewage Sludge for FLB Metabolism and Characterization of Metabolite Production

Aerobic sludge was taken from Ankara municipal sewage treatment plant. Fresh sludge was used directly and added volumetrically for all the experiments without any extra processes. Four enrichment treatments were prepared;

1. 500 parts per million (ppm) FLB in 250 mL of sludge
2. 500 ppm mTAA, a.k.a. 3-methylphenylacetic acid in 250 mL of sludge
3. 500 ppm pTAA, a.k.a. 4-methylphenylacetic acid in 250 mL of sludge
4. No addition into 250 mL of sludge

Treatments 2 and 3 were prepared as controls for the FLB enrichment. As they are both similar modified PAAs with much simpler structures, they provide good reference for reactions and changes that may occur with a simpler system. Treatments 2 and 3 are also positive controls for enrichment system and might be useful to show that enrichment system works.

The color change in the flasks was monitored for detection of metabolism and metabolic by-products of the degradation of the three chemicals.

Enrichment in MSM

The method of McCullar et al. (1994) MSM preparation was used for enrichments. The pH of MSM was checked to confirm the proper level of 7.4. The trace elements, $MnSO_4$, $NaMo.2H_2O$, $CuSO_4$, $CoSO_4.7H_2O$ and H_3BO_3, were not added to MSM initially. 100 µL of enriched sludge of each chemical was transferred to MSM + 500 ppm of test chemical.

3.2.2. FLB Disappearance Assay and Enrichment of Degraders

FLB Disappearance Assay

Initial observations suggested that toxicity might be a major factor within our working concentration range, 50 - 500ppm. An assay was started to address this possibility and at the same time, to obtain a sense of the rate of metabolism of FLB in sewage sludge. 100mL samples of sewage sludge were spiked with 50, 250, or

500ppm FLB in triplicate (nine flasks total). Additionally, color change was observed to characterize possible FLB metabolism.

Enrichment for FLB Degraders

Enrichment cultures took much longer to become established than anticipated. An initial enrichment was made by transferring a mature spiked sewage sludge system that had dropped to approximately 50% initial FLB concentration into MSM media with 500 ppm FLB. This initial enrichment failed to show notable growth or reduction of FLB concentration following transfer, lending weight to the aforementioned hypothesis that high concentrations of FLB have toxic effects.

A second round of enrichments was made. Firstly, 250 ppm of FLB, (pTAA) or mTAA were added 500 mL sludge. To increase the chance of obtaining an enriched culture and/or isolates, three different media were used; 100 ppm FLB, 100 ppm FLB +2 ppm yeast extract, and 500 ppm FLB. FLB concentration was lowered, in the 100 ppm treatments, in order to address toxicity issues. The yeast extract media was prepared so that isolates would ave access to micronutrients and vitamins, a standard procedure for minimal media systems.

Then after 3 transfers, the third generations were transferred onto 100 ppm FLB + 2 ppm yeast extract and 100 ppm FLB solid media composed of MSM and 17 g/L agar solidifying the media. Then, they were transferred onto Luria Bertoni Broth (Sambrook et al., 1989) with 17 g/L agar to make the colonies more obvious by promoting growth. The degraders were transferred many times on both solid MSM and LB to isolate the strains based on their colony shape and color.

Enrichment on Phenylacetic and Tolylacetic Acids

500 ppm mTAA, pTAA and PAA flasks with sludge were prepared. Then, they were transferred in series three times to MSM with 250 ppm of related chemical. They were streaked on 250 ppm mTAA, pTAA and PAA plates. After isolating the strains based on their colony appearance, they were again inoculated in MSM with 250 ppm of related chemical in order to confirm their growth and degradation abilities.

Finally, the strains that grew successfully were stored at -80 ^{o}C in 20 % glycerol solution.

Three different media were used for each chemical; 100 ppm mTAA, 100 ppm mTAA +2 ppm yeast extract, and 500 ppm mTAA and 100 ppm pTAA, 100 ppm pTAA +2 ppm yeast extract and 500 ppm pTAA and three transfers were made.

The mTAA and pTAA degraders from the third generation were streaked on solid MSM plates with 500 ppm mTAA and pTAA. Since a weak growth was observed on those plates, the concentration was lowered to 250 ppm in case of toxicity. After several streakings from one generation to another generation, the degraders were transferred onto Luria Bertoni Broth (LB) (Sambrook et al., 1989) with 17 g/L agar in order to obtain pure cultures and identify cultures based on colony shape and color.

Identification of Strains

Identification of strains were carried out by RFLP in order to find unique strains.

3.2.3. Confirmation of Putative FLB, mTAA, pTAA and PAA Degrading Strains

In order to confirm whether isolated strains really degraded the chemicals or not, the isolated strains were enriched again.

Confirmation of FLB degrading strains

Isolated FLB strains were inoculated in 100 ppm FLB flasks with standard MSM as described in the previous sections. Because FLB strains did not grow on 100 ppm FLB in liquid McCullar's MSM without trace elements, they were inoculated into media containing, in addition to 100 ppm FLB, 2% sodium acetate with MSM and 2mg yeast, 90% MSM+10% LB, or 0.2% glucose and 0.2% acetate in 100 mL of pure water in order to promote growth.

10 mL of 100 ppm FLB with 0.2% acetate + 2 mg yeast, 0.2% glucose + 2 mg yeast, 10% LB, or 0.2% glucose were prepared in triplicate and whole culture extraction was applied to these tubes in order to see whether FLB was really degraded or not.

Then, the strains were transferred onto LB plates and agar plates in order to determine strains have ability to grow on agar or not.

Additionally, a trial of using sterilized tap water instead of pure water was attempted in order to promote growth.

Finally, strains were stored at -80 ^{o}C in 20 % glycerol solution.

Confirmation of Phenylacetic and Tolylactic acids Degrading Strains

The strains were inoculated in 100 ppm pTAA and mTAA flasks. The strains that failed to grow on mTAA and pTAA only were inoculated in 10% LB and 90% MSM with the appropriate 100 ppm TAA. The growing strains were stored at -80 ^{o}C in 20 % glycerol solution.

To confirm that growth was concurrent with disappearance of the substrate, the mTAA and pTAA degrading strains were inoculated into liquid MSM media with 250 ppm of the respective substrate in triplicate in order to determine their degradation and growth rates. Growth was measured with spectrophotometer at 600 nm wavelength and substrate concentration was measured by HPLC.

3.2.4. Confirmation of Second Peak Representing Putative FLB Metabolite

Monitoring of FLB degradation in spiked sludge by HPLC revealed the appearance of a second, faster-eluting peak that appeared to be consistent with a FLB metabolite; faster elution implies oxygenation or lower molecular weight. In order to investigate the appearance and magnitude of the second peak with the presence and biodegradation of FLB, two flasks of 50 ppm FLB in sludge, one flask of 500 ppm FLB in sludge and one flask without FLB were prepared. One of the 50 ppm FLB flasks was autoclaved for use as a non-biological control. The flask without FLB and the autoclaved flask with 50 ppm FLB was prepared in order to determine whether the appearance of second peak was due to FLB degradation or biological activity.

3.2.5. Repeating the Experiment of Enrichment of Degraders and Confirmation of Second Peak

In order to confirm the results of previous experiment and to confirm that the second peak did not appear without FLB addition, the experiment was repeated with some changes. 50 ppm FLB flasks, 50 ppm autoclaved FLB flasks and 0 ppm FLB flasks were prepared in triplicate. The transfers were made from 50 ppm FLB flasks to the 50 ppm FLB flasks with the McCullar's MSM.

3.2.6. Co-metabolic Stimulation of FLB Degradation

Flasks with 250 ppm mTAA+50 ppm FLB, 250 ppm pTAA+50 ppm FLB, 250 ppm PAA+50 ppm FLB and 50 ppm FLB and alternatively 250 ppm BP + 50 ppm FLB, 3FPAA + 50 ppm FLB and 250 ppm IBP + 50 ppm FLB were prepared in fresh sludge. 3FPAA has the fluorine moiety at 3rd position on aromatic ring like FLB. FLB is a substituted BP. This experiment was conducted under the hypothesis that organisms capable of degrading these similar chemicals may also fortuitously degrade FLB.

3.2.7. Enrichments in Sludge from Other Cities

In a further attempt to obtain FLB degrading isolates, 500 ppm FLB was added to fresh sludge taken from Eskişehir and Yozgat. After the degradation started, sample from the sludge was transferred to MSM (McCullars' recipe without trace elements) with 500 ppm FLB. Since no clear FLB degradation was observed, 250 ppm IBP was added to MSM with 250 ppm FLB in order to stimulate FLB degradation.

3.2.8. Enrichments with Alternative MSMs

FLB degraders were enriched in aerobic sludge spiked with 100 ppm FLB. Then, transfers were made from sludge to MSM after degradation started in aerobic sludge. This time, three recipes were prepared with 100 ppm FLB because there was no clear growth of FLB degraders in McCullar's recipes with and without trace elements. The aim was to see whether different recipes and different water resources stimulate the growth of FLB degraders or not. The first recipe is the McCullar's recipe without trace elements (McCullar et al., 1994) in which FLB degraders did not exhibit a clear growth. The recipe was prepared all over again and 100 mL of McCullar's MSM spiked with 100 ppm FLB were prepared in triplicates. The second recipe was M9

mineral medium (Sambrook et al., 1989). 100 mL of M9 mineral medium spiked with 100 ppm FLB were prepared in triplicate. The last one was again McCullar's recipe but spring water (Saka, Sakarya, Turkey) was used instead of ultra-pure water.

3.2.9. Identification of Bacterial Strains

The 16S rRNA gene sequences were amplified and subjected to restriction fragment analysis using a 4-hitter restriction enzyme so as to identify unique strains degrading FLB, PAA, mTAA and pTAA. Duplicate strains were disposed of. The unique strains were stored at -80 ^{o}C in 20.0 % sterilized glycerol solution.

3.2.9.1. Amplification of the 16S rRNA Gene Sequences by PCR

3.2.9.1.1. Preparation of Bacterial Strains for PCR

25.0 µL of reaction volume consisting of 12.5 µL of 2X PCR *taq* master mix (Promega Corp, Madison, USA), 1.0 µL of 27F primer and 1.0 µL of 1492R primer with a final concentration 400 nM (Alpha DNA, Canada), 10.5 µL of nuclease-free water and template was prepared for each strain. Template DNA was added by touching a sterile pipette tip to a bacterial colony and placing into the reaction mixture.

3.2.9.1.2. Polymerase Chain Reaction (PCR)

A thermal cycler (Thermo Fisher Scientific Inc., USA) was used for the amplification. The DNA samples were stored at – 20 ^{o}C. The PCR program for this study was as shown in the Figure 3. 1.

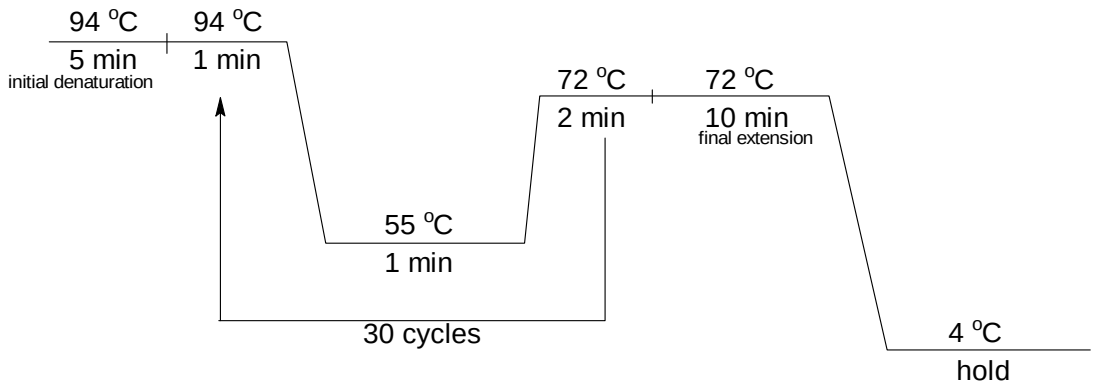


Figure 3. 1. PCR programming for amplification

3.2.9.1.3. Agarose Gel Electrophoresis

1.0 % agarose (Biotium Corp., USA) gel in TAE buffer (Thermo Fisher Scientific Inc., USA) was prepared and microwaved one minute to dissolve the agarose. Then 5.0 µL of GelRed (Biotium Corp., USA) was added to the 100 mL of 1X TAE buffer (Thermo Fisher Scientific Inc., USA). 1.0 µL of 6X DNA Loading Dye plus 4.0 µL of PCR product and 1.0 µL 6X DNA Loading Dye plus 1.5 µL of GeneRuler 100 bp DNA Ladder (Thermo Fisher Scientific Inc., USA) were loaded to agarose gel. An electric current, 80 V, was applied for about 1 hr by a power supply (Thermo Fisher Scientific Inc., EC 1000XL, USA).

3.2.9.1.4. Gel Visualization

Quantum ST-4 3000 Gel Image Acquisition Sytem (Montreal Bıotech Inc., Canada) was used in order to visualize the gels.

3.2.9.2. Identification of Unique Strains by Restriction Fragment Length Polymorphism (RFLP)

The PCR products were subjected to RFLP analysis to identify unique strains by using a 4-hitter restriction enzyme.

3.2.9.2.1. Preparation of PCR Products for RFLP analysis

20 µL of reaction volume consisting of 10.0 µL of PCR product, 1.0 µL of restriction enzyme HaeIII, 2 µL of Buffer C (Promega Corp., Madison, USA) and 7 µL of distilled water was prepared. Then the samples were put into a water bath at 37 ^{o}C for 4 hr.

3.2.9.2.2. Gel Electrophoresis and Visualization

For RLFP analysis, 2.0 % agarose gel was prepared. 1.5 µL of 6X DNA Loading Dye plus 4.0 µL of sample was prepared and loaded into agarose gel. All the remaining procedure for gel electrophoresis and gel visualization was the same as described above. Unique strains were identified after visualization.

3.3. Analytical Methods

In this section, methods, instruments, procedures for measurement and determination of degradation and degradation products of the chemicals are described.

3.3.1. Monitoring the Concentrations of the Chemicals by HPLC

The concentrations of FLB, mTAA, pTAA, PAA, 3FPAA, BP and IBP were monitored by HPLC. This HPLC device consisted of a system controller (SHIMADZU, SCL-10A VP, Kyoto, Japan) connected to a PC, a pump (SHIMADZU, LC-10AT VP, Kyoto, Japan), a low pressure gradient unit (SHIMADZU, FCV-10AL VP, Kyoto, Japan), a degasser (SHIMADZU, DGU-14A, Kyoto, Japan), a UV-VIS detector (SHIMADZU, SPD-10A VP, Kyoto, Japan), a column oven (SHIMADZU, CTO-10A VP, Kyoto, Japan) and a column (Macherey-Nagel, CC 250/4 NUCLEOSIL 50-5C 18ec., Düren, Germany).

3.3.1.1. Preparation of Samples for HPLC Analysis

Samples were centrifuged and the supernatants were filtered with syringe filters (ETO sterile, 28 mm diameter, hydrophilic, 0.2 μm pore size, surfactant-free cellulose acetate for membrane material, Meta acrylate butadiene styrene polymerisate for housing material, Minisart, 16534 and non-sterile, 15 mm diameter, hydrophilic, 0.2 μm pore size, regenerated cellulose for membrane filter, polypropylene for housing material, Minisart, 17761, Goettingen, Germany).

An extraction method was applied to extract FLB from sludge. During monitoring of FLB by HPLC, some of the added FLB was missing shortly after being added to the sludge. Adsorption of FLB onto sludge particles and improper syringe filters for FLB filtration were potential causes for the missing FLB. Thus, an extraction method was developed (APPENDIX B). After applying extraction, it was observed that syringe filters could also be a potential cause for missing FLB, thus, Minisart 17761 syringe filters were used instead of Minisart 16534 syringe filters.

3.3.1.2. Measuring Concentrations of the Chemicals and Fractionation of Aromatic Metabolic Byproducts

An appropriate HPLC method was developed for separation and quantification of each chemical and for detection and fractionation of a second peak which appeared during FLB degradation in aerobic sludge in flasks. A second peak representing a putative FLB metabolite was firstly observed with a detection wavelength 247 nm.

Table 3. 1. HPLC methods for FLB, mTAA, pTAA, PAA, 3FPAA, IBP, BP and 2nd Peak

Chemical	Solution		Detection Wavelength (nm)	Oven Temperature (^{o}C)	Elution Time (min)
	40 mM Acetic acid in water (%)	Methanol (%)			
FLB	40	60	247	60	6.0
mTAA	60	40	212	60	5.2
pTAA	60	40	212	60	5.2
PAA	60	40	206	60	4.5
3FPAA	60	40	210	60	4.2
IBP	35	65	214	60	5.0
BP	25	75	254	60	5.3
2nd Peak	70	30	210	60	4.8

Standard curves were formulated in order to convert HPLC peak areas of FLB, mTAA and pTAA into concentration as ppm (APPENDIX A).

3.3.2. Characterization of FLB Degradation by LCMS

LCMS analyses of a blank sample, a 500 ppm FLB standard sample and an aerobic sludge sample spiked with 500 ppm FLB and taken after FLB degradation started were carried out using a Waters (Milford, MA, USA) Acquity UPLC connected to Waters Synapt G1 MS (Milford, MA, USA) mass spectrometer in negative mode. The LCMS studies were carried out in Central Laboratory, METU. HPLC and MS methods are given in Table 3. 2, Table 3. 3 and Table 3. 4.

Table 3. 2. HPLC method. A: Methanol. B: 40 mM Acetic acid in water.

Time (min)	Flow rate (mL/min)	% A	% B
0	0.030	70	30
15	0.030	40	60
18	0.030	40	60
19	0.030	0	100
20	0.030	70	30

Table 3. 3. HPLC properties

Column (Reverse phase)	ACQUITY UPLC BEH C18 (Milford, MA, USA) 1.7 µm 1.0*100 mm Column
Mobile phase A	Methanol
Mobile phase B	40 mM Acetic acid in water
Column Temperature	35 ^{o}C
Sample temperature	4 ^{o}C
Flow profile	Gradient

Table 3. 4. MS method

MS System	Waters SYNAPT G1 MS (Milford, MA, USA)
Mode	ESI -
Capillary Voltage	3 Kv
Source Temperature	80 ^{o}C
Desolvation Temperature	350 ^{o}C
Parent Survey High Collision Energy	15 V
Parent Survey Low Collision Energy	6 V
Mass Interval	50 – 600 Da

3.3.3. Characterization of FLB Degradation by Color Appearance

Yellow color appearance during degradation is consistent with meta-cleavage of catecholic metabolites. The yellow color of a meta-cleavage product is acid labile; it disappears when acidified and reappears when returned to neutral pH. Spectral scan analysis of the supernatant was expected to reveal an absorbance maximum in the 360-380nm range as is usually found with meta-cleavage products. For the spectral scan analysis and measurement of color intensities of FLB supernatants, a spectrophotometer (HACH LANGE, DR 3900, Colorado, USA) was used. In this study, absorbance wavelength of yellow FLB supernatant was chosen as 370 nm (Figure 3. 2).

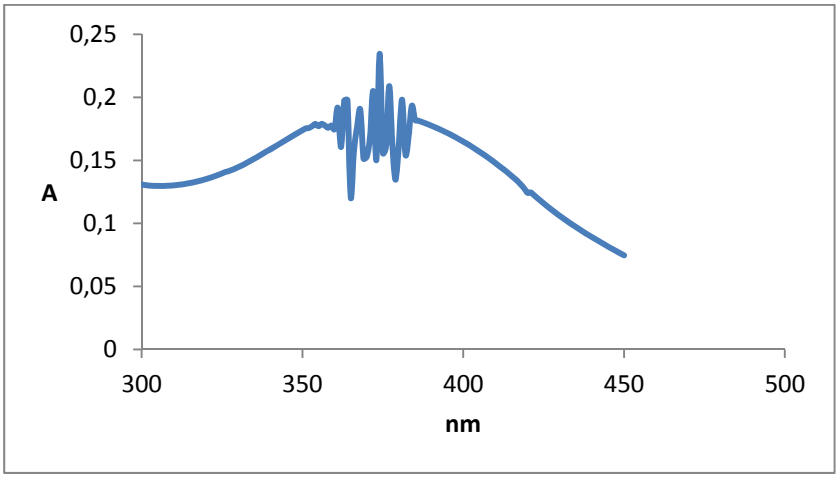


Figure 3. 2. UV-Vis absorbance spectrum from 300-500nm of yellow FLB enrichment supernatant.

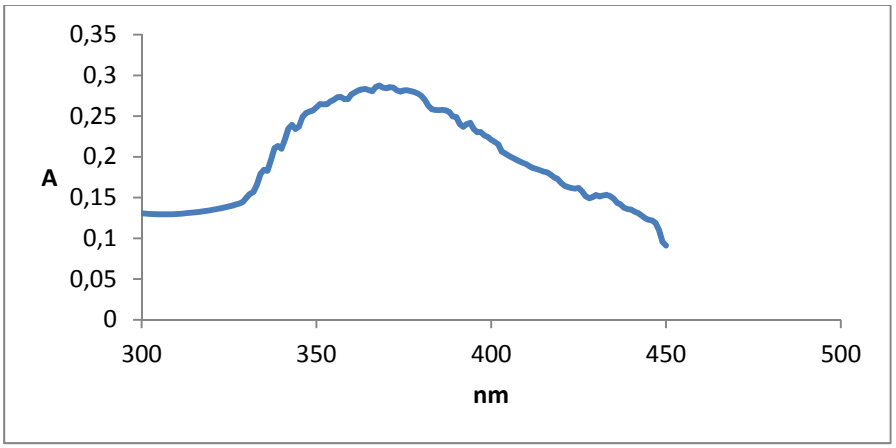


Figure 3. 3. UV-Vis absorbance spectrum from 300-500nm of yellow FLB enrichment supernatant with UV lamp turned off

Brown coloration can indicate many things. Within the field of aromatic biochemistry, it is regarded as a sign of catechol polymerization. The accumulation of catecholic metabolites was analyzed by mixture of culture samples with ferric chloride, which encourages the polymerization and visualization of catechols. Catechols turn black and brown when exposed to ferric iron (Murdoch & Hay, 2013).

3.3.4. Free Fluoride Detection

During degradation, fluorine ions can be released as a result of dehalogenation or complete mineralization. In this study, microdiffusion cell method described by WHO (ORGANIZATION, 2003) was modified in order to determine whether or not isolated byproducts contain fluorine.

In the modified method, 0.25 mL of cerous nitrate, 0.25 mL alizarine complexone and 0.5 mL of sample are directly mixed and allowed to stand for 1 hr at room temperature. In order to confirm the method, some standards were prepared and tested. In case of existence of fluoride, the mix gives a blue or light lilac color (Figure 3. 5).

Absorbance wavelength of fluoride was detected as 625 nm by spectrophotometer. A standard curve showing the relationship between fluoride concentration and absorbance of the solution was obtained.

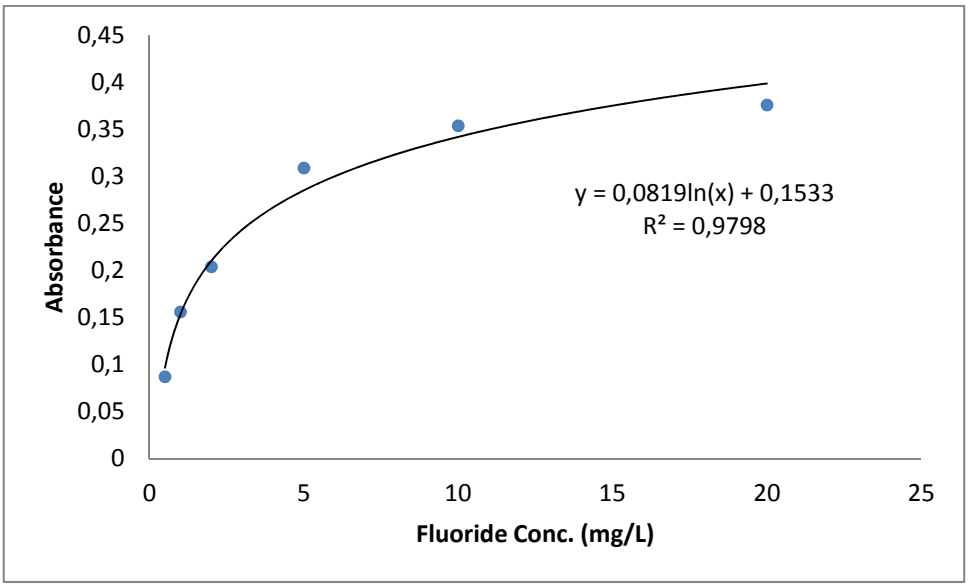


Figure 3. 4. Standard Curve: Fluoride Conc. vs. absorbance

Figure 3. 5. The color appearance of 1, 2 and 10 mg/L of NaF added fluoride standards tested by the modified microdiffusion cell method. A purple/lilac color was observed in three of the samples

3.3.5. Dissappearance Assay and Growth Analysis

The mTAA and pTAA degrading strains were inoculated into liquid MSM media with 250 ppm mTAA and 250 ppm pTAA in triplicates, respectively, in order to determine their degradation and growth rates. Growth was measured with spectrophotometer at 600 nm wavelength.

CHAPTER 4

RESULTS AND DISCUSSION

4.1. Enrichments of Aerobic Sewage Sludge for FLB Metabolism and Characterization of Metabolite Production.

250 mL aerobic sludge was gathered from Ankara municipal sewage treatment plant. Four enrichment treatments were prepared; 500 ppm FLB, 500 ppm mTAA, 500 ppm pTAA and no addition.

Treatments 2 and 3 were prepared as controls for the FLB enrichment. As they are both similar modified PAAs with much simpler structures, they provide good reference for reactions and changes that may occur with a simpler system. In these treatments, mTAA and pTAA were degraded successfully.

Figure 4. 1. Four enrichments immediately following amendment with, respectively, FLB, pTAA, mTAA, and no amendment.

Immediately following the start of the enrichments, no remarkable differences are visible. Some slight cloudiness in the FLB enrichment is consistent with the slow dissolution of the FLB (cloudiness disappeared quickly afterwards) (Figure 4. 1).

After two weeks, the FLB enrichment became a bright yellow color (Figure 4. 2). None of the other enrichments showed any color changes or unusual activity:

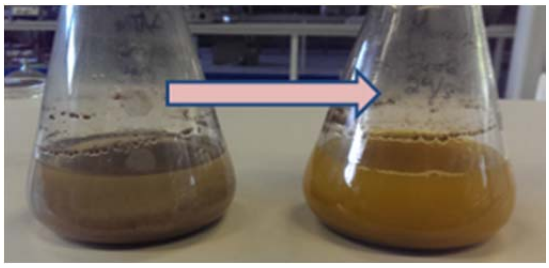


Figure 4. 2. Yellow color in FLB enrichment compared to control enrichment.

The yellow supernatant, removed from the cell mass and solid materials (centrifuged), is shown in Figure 4. 3.

Figure 4. 3. Supernatant of yellow FLB enrichment

The yellow color was acid labile (disappeared when acidified and reappeared when returned to neutral pH), which is consistent with a ring meta-cleavage product. Spectral scan analysis of the supernatant was expected to reveal an absorbance maximum in the 360-380nm range as is usually found with meta-cleavage products. However, upon attempting to analyze with two different pieces of equipment, very unusual results were revealed. A broad absorbance maximum focused on 360-380nm was observed, but within the range, "noise" was also observed, possibly representing both absorbance and transmission/emission (Figure 4. 4).

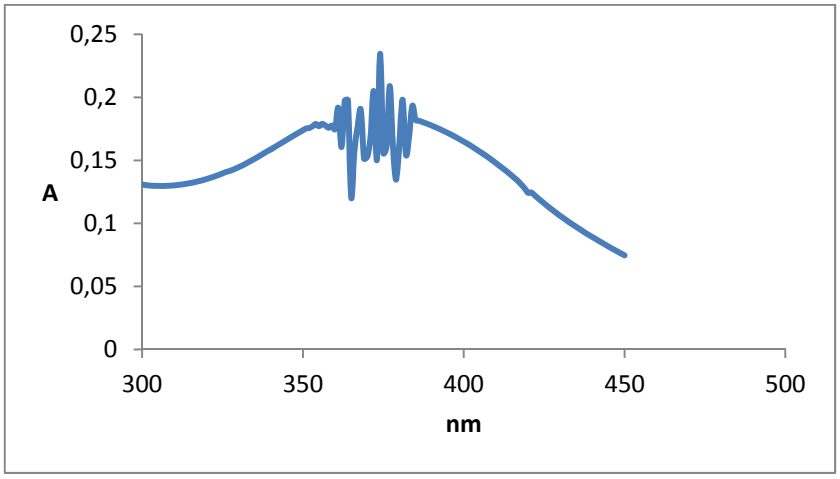


Figure 4. 4. UV-Vis absorbance spectrum from 300-500nm of yellow FLB enrichment supernatant.

This is consistent with a fluorescent chemical to some degree. No similar reports of fluorescent meta-cleavage products or any similar phenomena can be identified in the literature. Somewhat consistent with the fluorescence hypothesis, when the UV lamp of the spectrophotometer was turned off (leaving only the visible spectrum lamp activated), the noise began to disappear, although the peak remained somewhat noisy (Figure 4. 5):

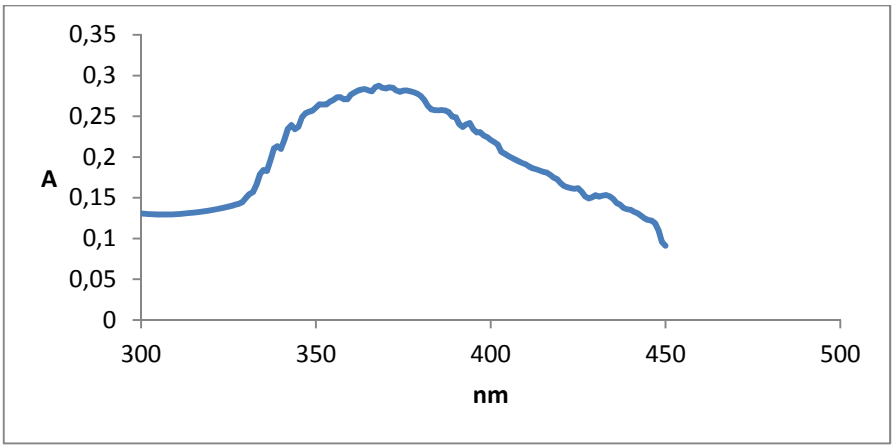


Figure 4. 5. UV-Vis absorbance spectrum from 300-500nm of yellow FLB enrichment supernatant with UV lamp turned off.

This exploratory research offers three pieces of evidence for the accumulation of a meta-cleavage product in the supernatant of FLB-amended aerobic sewage sludge

1. pH dependence of the color
2. Appearance only with addition of a particular aromatic chemical (FLB)

3. Absorbance maximum around 370nm

The accumulation of a meta-cleavage product does occur in rapidly-metabolizing aromatic degradation systems. After another week of incubation, the yellow disappeared and some other chemical or chemicals accumulated creating a brown color (Figure 4. 6):

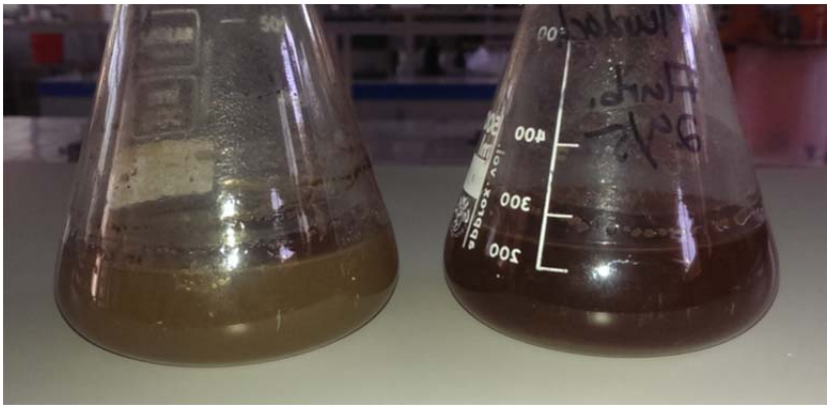


Figure 4. 6. Brown color appearance in FLB enrichment compared to control enrichment

This brown coloration can indicate many things. Within the field of aromatic chemistry, it is regarded as a sign of catechol polymerization, again consistent with bacterial aromatic metabolism. While extracellular accumulation of meta-cleavage intermediates is typical, accumulation of catecholic metabolites in natural systems is very unusual. Catechols are very reactive and organisms typically dispose of them quickly due to their toxic effects, i.e. their ability to react non-specifically with biomolecules.

The supernatant from the enrichment at a later date, when there was not a strong yellow or brown color apparent, was harvested with the goal of adding ferric iron (final concentration ~1mM), which is a standard reagent for visualizing catechols. Catechols turn black and brown when exposed to ferric iron. The supernatant of the FLB was still colored with yellowish brown when recovered.

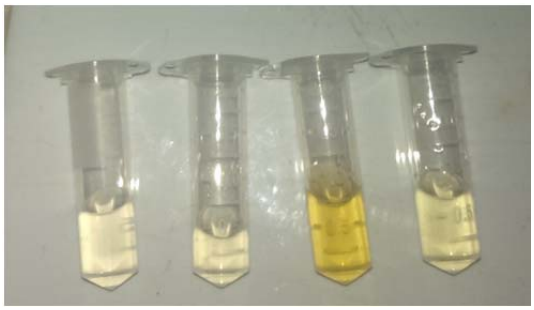


Figure 4. 7. Supernatants of six week old enrichments, sample order is negative control, pTAA, FLB, mTAA.

Addition of ferric chloride caused a flocculation in the supernatants of all enrichments. When centrifuged, the FLB pellet was brownish red while the others were neutral colored (Figure 4. 8):

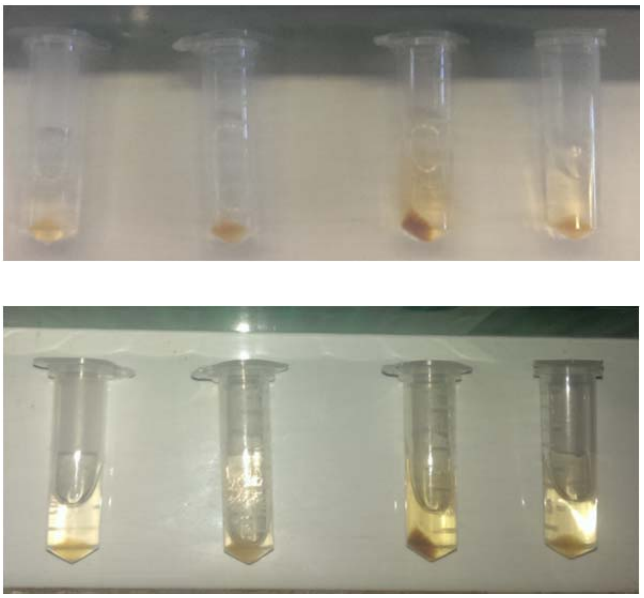


Figure 4. 8. Supernatants pictured in Figure 4. 7, with 1mM ferric iron and centrifuged. Sample order is negative control, pTAA, FLB, mTAA. No camera flash above, flash used below.

This enhancement of dark color is consistent with free catecholic metabolites, but is not definitive proof. The slight possible color generation in the pTAA sample was observed. This is consistent with coloration found in later transfer cultures. However, it does definitely indicate the presence of some unique metabolite accumulating in the enrichment. The presence of an extracellular accumulating catechol would be highly unusual and interesting.

Enrichments underwent transfers to minimal medium systems (mineral salts + FLB only) in order to work towards isolating pure cultures. Initial data appeared promising; the first FLB transfer showed signs of growth, although with significant cell lysis. As the other transfers (mTAA and pTAA controls) were also showing signs of lysis, this likely represented a mistake with the medium, possibly the wrong osmolarity. This was addressed by creation of new media and new transfers. Nevertheless, some growth did occur. Initial HPLC analysis of FLB concentration in the FLB transfer indicated approximately 50% loss of FLB, from 500ppm to 250ppm, despite the likely media problems. This loss might cause by filter material which was changed later with another filter material that is suitable for FLB filtration. Of additional interest, dark brown/black coloration concentrated in the lysed cell materials in the FLB culture and the pTAA culture, but not the mTAA culture (Figure 4. 9).

Figure 4. 9. Centrifuged lysed cell material in enrichment transfers with indicated parent chemical. Note the black coloration in the pTAA and FLB cultures.

While non-definitive, this again suggests the presence of catecholic metabolite accumulation. It is occurrence in the pTAA culture also is a bit puzzling, although factors such as iron concentration and other food sources could affect the rate of polymerization and/or rate of catechol accumulation. As tolylacetic acids can be metabolized via catechols also, their accumulation extra-cellularly is possible, though not expected due to their simple chemical nature (they would be expected to be rapidly metabolized). This data is regarded as subjective and qualitative but may provide future guidance.

As mentioned, the mineral salts media enrichment transfers were repeated with a focus on proper media preparation. They were monitored for color generation and more importantly, were expected to yield pure cultures. Enrichment cultures took much longer to become established than anticipated. An initial enrichment was begun by transferring a mature spiked sewage sludge system that had dropped to approximately 50% initial FLB concentration. This initial enrichment failed to show notable growth or reduction of FLB concentration following transfer, lending weight to the aforementioned hypothesis that high concentrations of FLB have toxic effects.

4.2. FLB Disappearance Rate from Sludge, the Effect of Initial Concentration and Enrichment and Identification of Degraders

Initial observations suggested that toxicity might be a factor within the working concentration range, 50 - 500ppm. An assay was started to address this possibility and to at the same time obtain a sense of the rate of metabolism of FLB in sewage sludge. 100mL samples of sewage sludge were spiked with 50, 250, or 500ppm FLB in triplicate (nine flasks total). Samples were taken on a bi-weekly basis. During periodic analysis of the samples by HPLC, it was observed that FLB disappearance was much slower than expected and sorption to the solid phase was a major factor in the system; HPLC analyses of t=0 samples revealed that roughly 50% of the added FLB was unaccounted for (Figure 4. 10). Later, it was observed that this sudden decrease in the concentration of FLB measured by HPLC might not be just due to sorption to the solid phase but also due to syringe filters used before HPLC analysis. Thus, an extraction method was developed and syringe filters were replaced with new syringe filters more suitable for FLB filtration. There was still 30-40 % FLB misseing despite these all attempts.

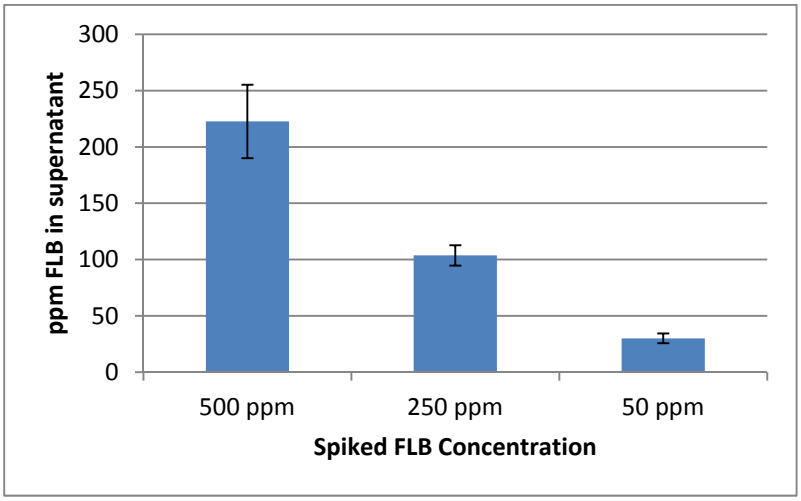


Figure 4. 10. Concentration of FLB remaining in supernatant determined by HPLC shortly following spiking of the concentration of FLB indicated on the x-axis.

One important observation during monitoring of the FLB sludge disappearance assay was the appearance of a secondary peak in the samples that eluted from the HPLC column faster than FLB, indicating a lower molecular weight and/or polar residues consistent with oxidation (Figure 4. 22). The concentration of this secondary peak was roughly equivalent to the starting concentration of FLB, suggesting that it is a metabolite.

4.2.1. HPLC Analysis of Disappearance Essay and Colored Metabolite Appearance

The disappearance kinetics and production of metabolites were explored by spiking high concentrations of FLB into aerobic sewage sludge. Casual observations during the enrichments and previous observations with other aromatic acids suggested that higher concentrations of FLB might have toxic effects. Therefore, this experiment was conducted using a range of FLB concentrations. 500 ppm, 250 ppm and 50 ppm FLB flasks were prepared by adding FLB to flasks containing 100 mL aerobic sewage sludge. For each concentration, three flasks were prepared. 500 ppm FLB flasks were encoded as T1-500, T2-500, T3-500, 250 ppm FLB flasks were encoded as T1-250, T2-250, T3-250 and 50 ppm FLB flasks were encoded as T1-50, T2-50, T3-50.

Yellow color is indicative of appearance of meta-cleavage products. Observation of yellow color in T1-500 and T2-500 on day 6 indicated that FLB was degraded

(Figure 4. 12). The samples taken on those days lost their color when they were acidified. This strongly suggested that there were meta-cleavage products present. Later, a brownish color appeared. Yellowish color in T1-50 and T3-50 was observed on later days (Figure 4. 11 & Figure 4. 13). Yellowish color in T2-50 was not strong. Color change was observed in 250 ppm FLB flasks lastly.

Figure 4. 11. Observation of yellowish color in T1-50.

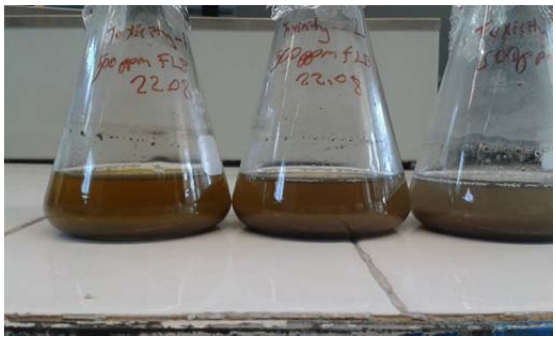


Figure 4. 12. Observation of yellowish color in T1-500 and T2-500.

Figure 4. 13. Observation of a dark brownish color in T3-50.

The samples were analyzed by HPLC in order to determine FLB disappearance. Additionally, spectrophotometric analyses of the samples were carried out in order to see relationship between FLB disappearance and color appearance. UV-Vis spectral scan of yellow supernatant revealed a slight peak at 370nm, consistent with typical meta-cleavage products. Absorbance at 370nm was monitored in the samples in order to explore its correlation with FLB concentration.

During analysis of the samples, a second peak was observed using 247nm detection wavelength (Figure 4. 14 & Figure 4. 15). The magnitude of this new peak appeared to be proportional to the amount of FLB disappearance, indicating that it might be a FLB metabolite. FLB concentration is given in ppm and the second peak concentration is given in area in the tables and graphs. In order to clearly separate the second peak and to insure the absence of any additional peaks, a solvent composed of 30% methanol and 70% 40 mM acetic acid was used in HPLC analysis under a more general detection wavelength, 210 nm and with 60^{O}C oven temperature. Under these conditions, the second peak eluted at 4.8 minutes. This peak was collected from the waste-line (fractionated), i.e. when the peak was observed, the waste was diverted into a collection tube.

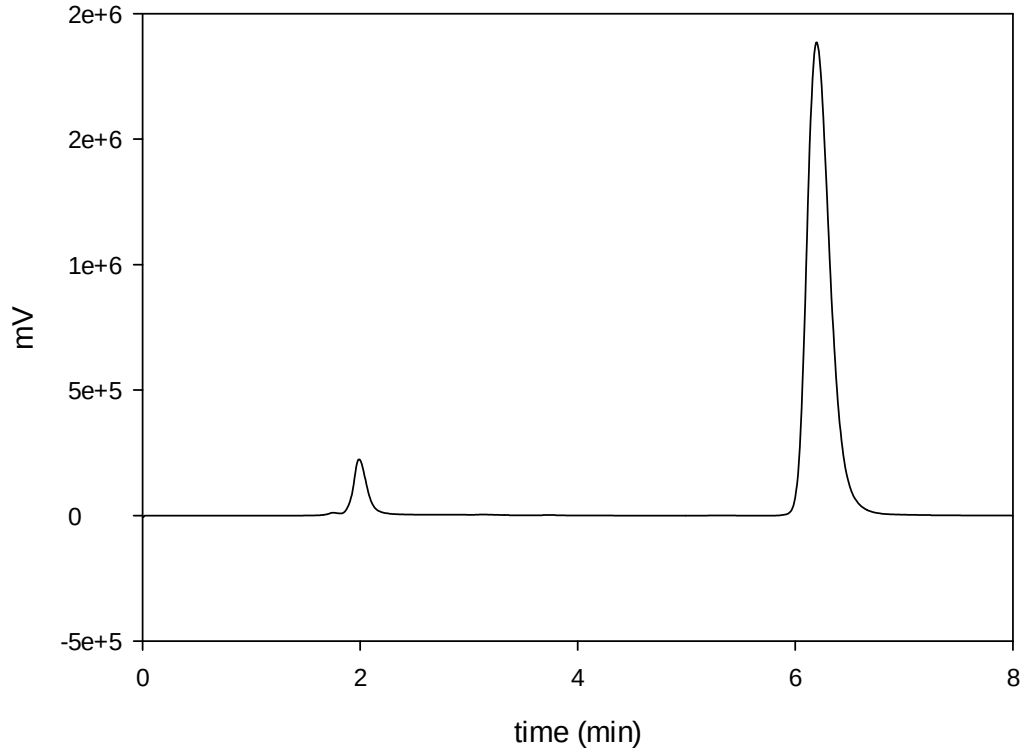


Figure 4. 14. HPLC chromatogram result of supernatant of T1-500 at day 1.

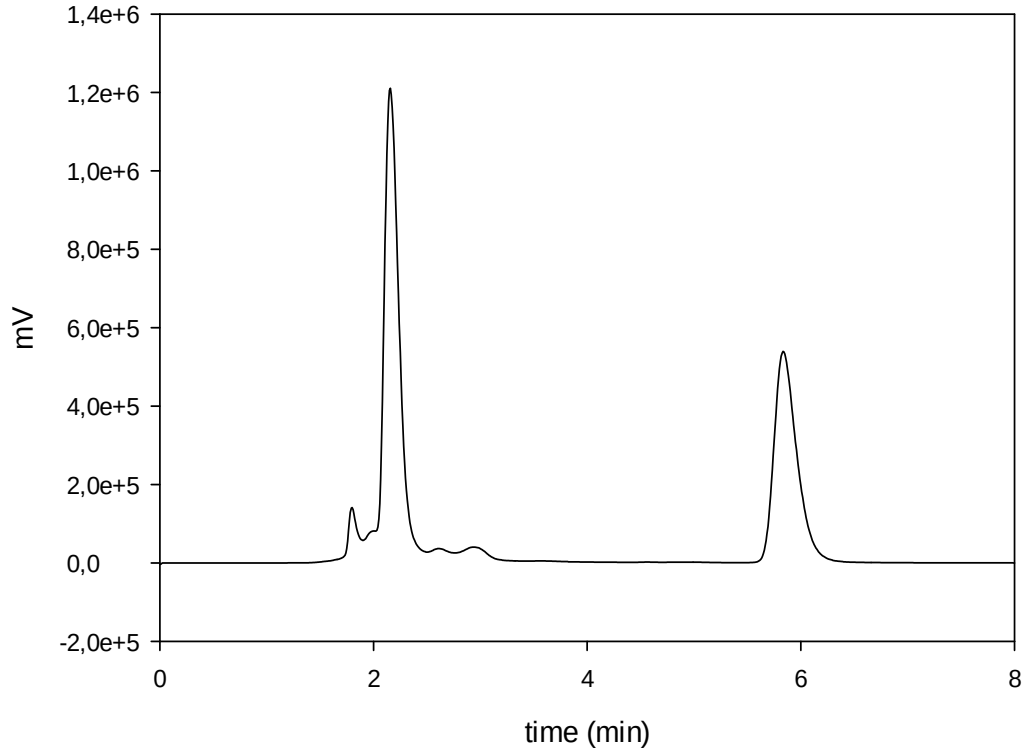


Figure 4. 15. HPLC chromatogram result of supernatant of T1-500 at day 8 showing the appearance of a novel peak at 2.1 minutes.

As seen in Figure 4. 14 and Figure 4. 15, there was observation of a new peak with 2.1 minute retention time at day 8. The area of FLB peak at 6th minute decreased, while a second peak appeared at 2.1 minute.

Table 4. 1. FLB disappearance as ppm in 500 ppm FLB flasks

Days	T1-500	T2-500	T3-500	Average	Standard deviation
1	351	322	327	333	15
8	82	242	464	263	192
16	69	272	421	254	177
34	66	203	346	205	140
46	30	292	307	210	156
59	68	287	356	237	150
76	32	318	368	239	182

Table 4. 2. Second peak appearance as area in 500 ppm FLB flasks.

Days	T1-500 (2^{nd} Peak)	T2-500 (2^{nd} Peak)	T3-500 (2^{nd} Peak)	Average	Standard deviation
1	0	0	0	0	0
8	9,021,041	2,513,446	1,035,840	4,190,109	4,248,442
16	10,757,106	2,064,520	768,425	4,530,017	5,431,615
34	6,577,527	2,570,394	983,242	3,377,054	2,883,059
46	4,941,159	4,224,714	1,097,996	3,421290	2,043,672
59	9,066,642	3,147,449	776,709	4,330,267	4,269,665
76	9,669,752	3,339,718	1,359,836	4,789,769	4,340,583

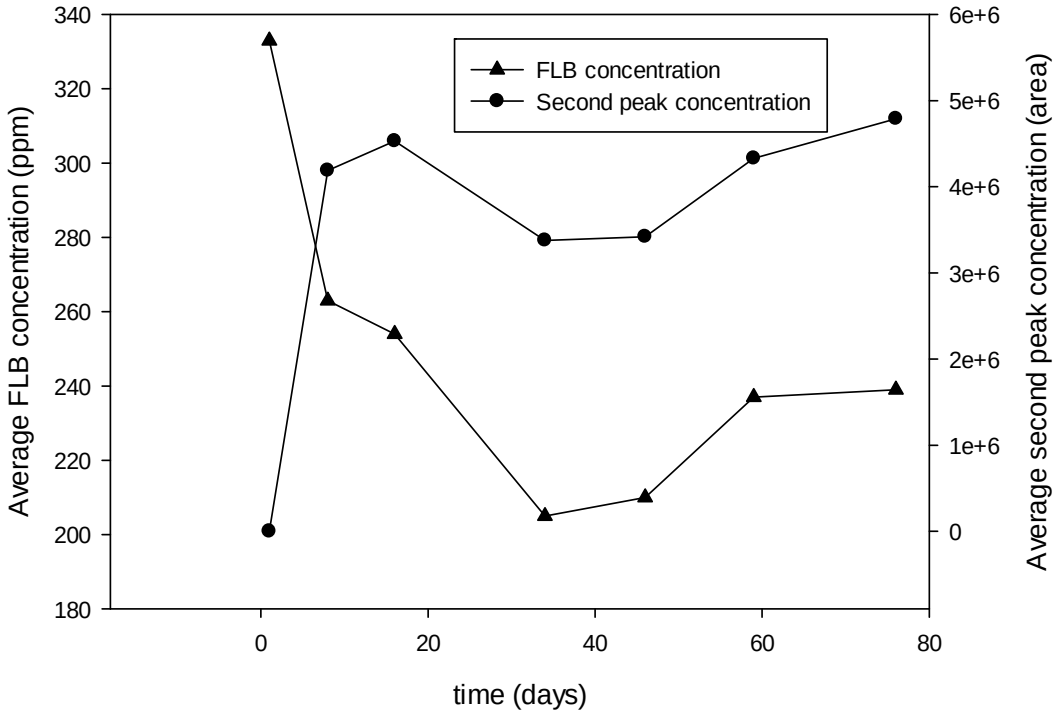


Figure 4. 16. Average FLB concentration versus average second peak area in sludge with 500 ppm FLB flasks.

As indicated in the Table 4. 1, Table 4. 2 and Figure 4. 16, as FLB concentration decreased, the second peak concentration increased. Linear regression indicated a negative correlation between FLB concentration and second peak area (R=0.87). The lowest FLB concentration and the highest second peak concentration were observed in T1-500 flask. In T1-500 flask, FLB concentration dropped to 82 ppm by day 8. Despite observation of yellowish color in T2-500, FLB concentration did not decrease after a point and second peak concentration did not increase. This might be due to acidification or inhibition of FLB degraders by other chemicals in the flask. T3-500 might exhibit the same manner with T2-500.

There was no strong correlation between FLB disappearance and absorbance (R=0.26). All flasks yielded almost the same color intensities over time, ultimately in a manner not correlated to FLB concentration (Figure 4. 17 &Table 4. 3)

Table 4. 3. Absorbance @ 370nm of 500 ppm FLB samples

Days	T1-500 (OD)	T2-500 (OD)	T3-500 (OD)	Average	Standard deviation
1	0.088	0.064	0.056	0.069	0.017
8	0.296	0.320	0.136	0.251	0.100
16	0.306	0.268	0.216	0.263	0.045
34	0.266	0.336	0.21	0.271	0.063
46	0.424	0.406	0.322	0.384	0.054
59	0.408	0.44	0.436	0.428	0.017
76	0.088	0.064	0.056	0.069	0.017

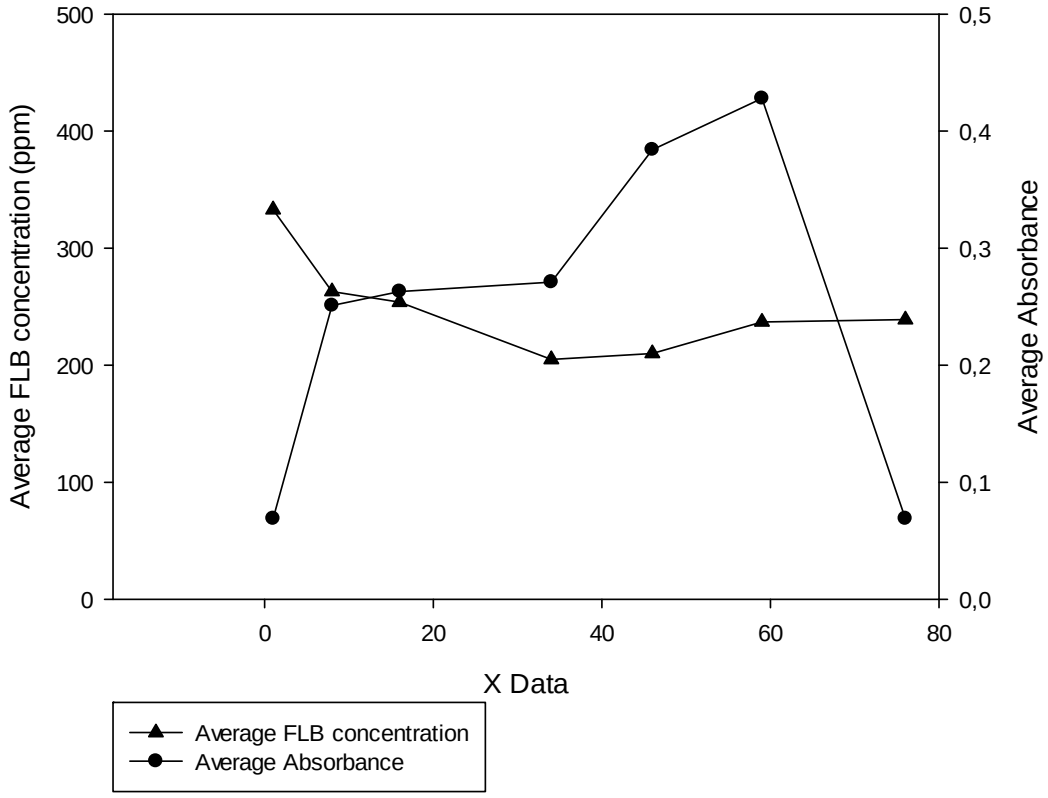


Figure 4. 17. Average FLB concentration versus average absorbance at 370 nm of 500 ppm FLB flasks.

Table 4. 4. FLB disappearance as ppm in 250 ppm FLB flasks.

Days	T1-250	T2-250	T3-250	Average	Standard Deviation
1	192	343	182	239	90
8	248	103	155	169	73
16	203	186	153	180	26
34	163	162	172	166	5
46	217	195	181	198	18
59	252	205	176	211	38
76	238	238	203	226	20

Table 4. 5. Second peak appearance as area in 250 ppm FLB flasks.

Days	T1-250 (2nd PEAK)	T2-250 (2nd PEAK)	T3-250 (2nd PEAK)	Average	Standard Deviation
1	0	0	0	0	0
8	0	206,700	548,290	251,663	276,897
16	251,001	289,484	751,410	430,632	278,468
34	290,686	297,140	245,069	277,632	28,384
46	309,369	362,496	550,327	407,397	126,599
59	446,578	384,145	640,581	490,435	133,725
76	730,053	498,415	840,808	689,759	174,717

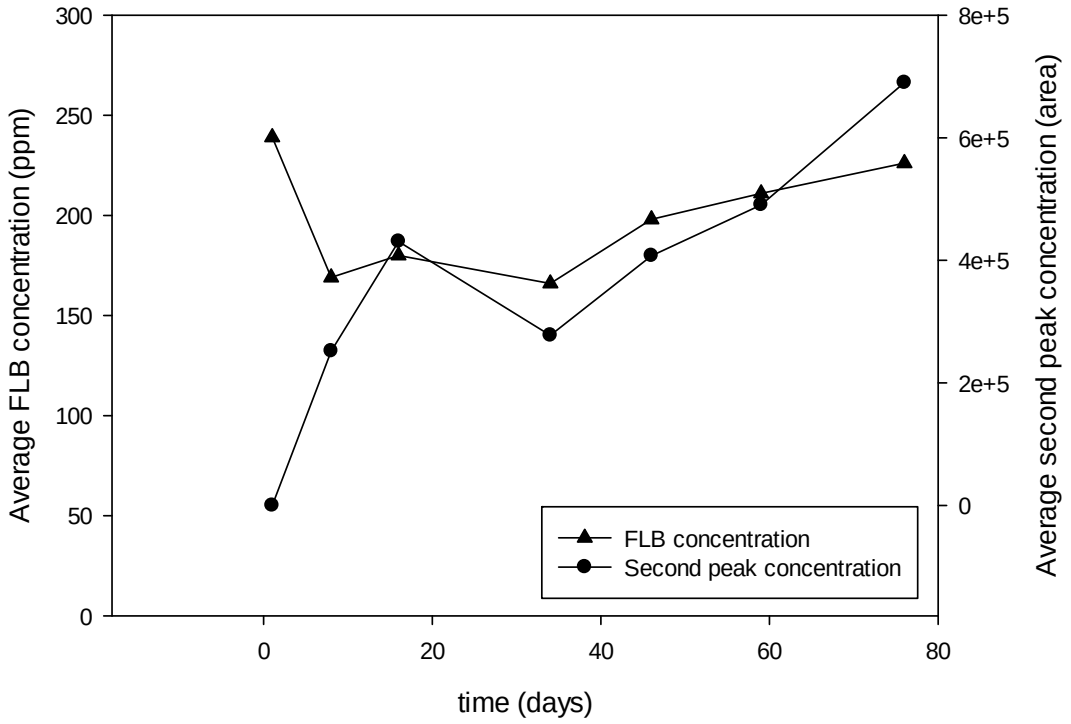


Figure 4. 18. Average FLB concentration versus average second peak area in 250 ppm FLB flasks.

There was no clear indication of FLB disappearance in 250 ppm flasks (Table 4. 4, Table 4. 5 & Figure 4. 18). The reason for the high concentration of FLB in T2-250 flask at first day might be that FLB was not uniformly distributed in T2-250 flask. The low concentrations of second peak area might also indicate that there was no degradation in 250 ppm FLB flasks; while the second peak area reached over 9,000,000 in the 500 ppm treatments, it reached only 700,000 in the 250 ppm flasks. Inhibition of FLB degraders, competition between bacterial cultures, non-uniformly distributed FLB and physical and chemical conditions of the sludge might be the reasons for lack of FLB disappearance.

Average absorbance of 250 ppm FLB flasks was higher than that of 500 ppm FLB flasks. Increase in absorbance with time was observed although there was no clear indication of FLB degradation in the flasks (Figure 4. 19 & Table 4. 6). Absorbance change might be due to some other chemical and physical changes in the flasks and might not depend on FLB disappearance.

Table 4. 6. Absorbance of 250 ppm FLB samples

Days	T1-250 (OD)	T2-250 (OD)	T3-250 (OD)	Average	Standard Deviation
1	0.048	0.04	0.052	0.047	0.006
8	0.032	0.04	0.03	0.034	0.005
16	0.144	0.126	0.132	0.134	0.009
34	0.210	0.204	0.176	0.197	0.018
46	0.236	0.324	0.36	0.307	0.064
59	0.572	0.52	0.4	0.497	0.088
76	1.06	0.652	0.572	0.761	0.262

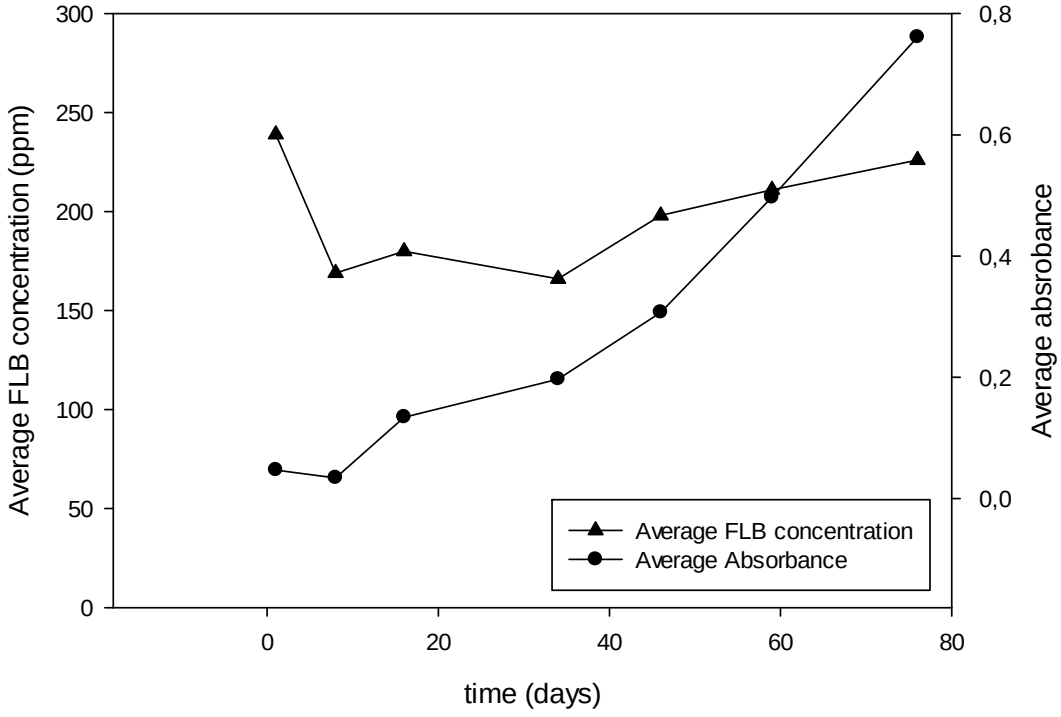


Figure 4. 19. Average FLB concentration versus average absorbance of 250 ppm FLB flasks.

Table 4. 7. FLB disappearance as ppm in 50 ppm FLB flasks.

Days	T1-50	T2-50	T3-50	Average	Standard Deviation
1	49	45	64	52	10
8	18	43	32	31	13
16	11	40	6	19	18
34	3	24	3	10	12
46	10	23	2	11	11
59	11	24	3	13	11
76	10	34	6	17	15

Table 4. 8. Second peak appearance as area in 50 ppm FLB flasks.

Days	T1-50 (2nd PEAK)	T2-50 (2nd PEAK)	T3-50 (2nd PEAK)	Average	Standard Deviation
1	0	0	0	0	0
8	1,237,630	417,339	1,971,833	1,208,934	777,644
16	1,745,080	342,871	2,802,919	1,630,290	1,234,035
34	1,378,752	802,580	2,456,639	1,545,990	839,616
46	820,789	414,868	1,436,641	890,766	514,468
59	1,356,361	481,781	2,104,338	1,314,160	812,101
76	1,456,412	512,929	2,605,053	1,524,798	1,047,737

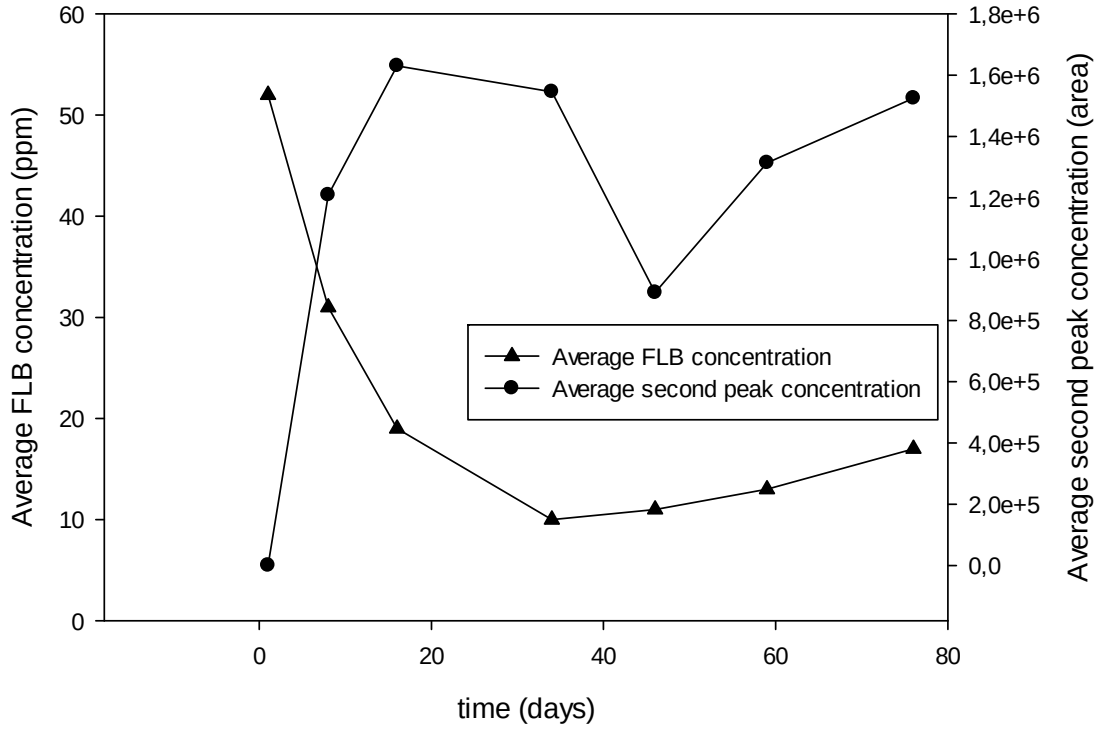


Figure 4. 20. Average FLB concentration versus average second peak area in 50 ppm FLB flasks.

The highest concentration of second peak and lowest concentration of FLB were observed in T3-50 flask (Figure 4. 20, Table 4. 7 & Table 4. 8). A strong negative correlation (R=0.78) between FLB concentration and the second peak was detected by linear regression analysis. An average of 68% of the FLB was degraded after 76 days. It is clear that FLB degradation on a percentage basis was better for lower concentrations of FLB. This suggests there might be toxic effects of high-dose FLB.

50 ppm FLB flasks had the lowest absorbance values (Table 4. 9). Decreases in FLB concentrations of 50 ppm FLB flasks were observed around day 34 day 46. These deccreases might indicate improper filtration due to syringe filters. At the same time, decreases in absorbance and second peak concentrations were observed. This is interesting because when FLB concentration decreased, second peak concentration decreased and when second peak concentration decreased, absorbance also decreased. This situation shows a direct correlation between them. There exists no clear explanation for this sudden decrease in FLB concentration and decrease in second peak concentration and absorbance. This might be due to dissolution of particulate FLB or improper filtration.

Table 4. 9. Absorbance at 370 nm of 50 ppm FLB samples.

Days	T1-50 (OD)	T2-50 (OD)	T3-50 (OD)	Average	Standard Deviation
1	0.032	0.048	0.064	0.048	0.016
8	0.027	0.042	0.032	0.034	0.008
16	0.204	0.216	0.228	0.216	0.012
34	0.148	0.092	0.16	0.133	0.036
46	0.147	0.145	0.115	0.136	0.018
59	0.18	0.092	0.124	0.132	0.045
76	0.224	0.164	0.184	0.191	0.031

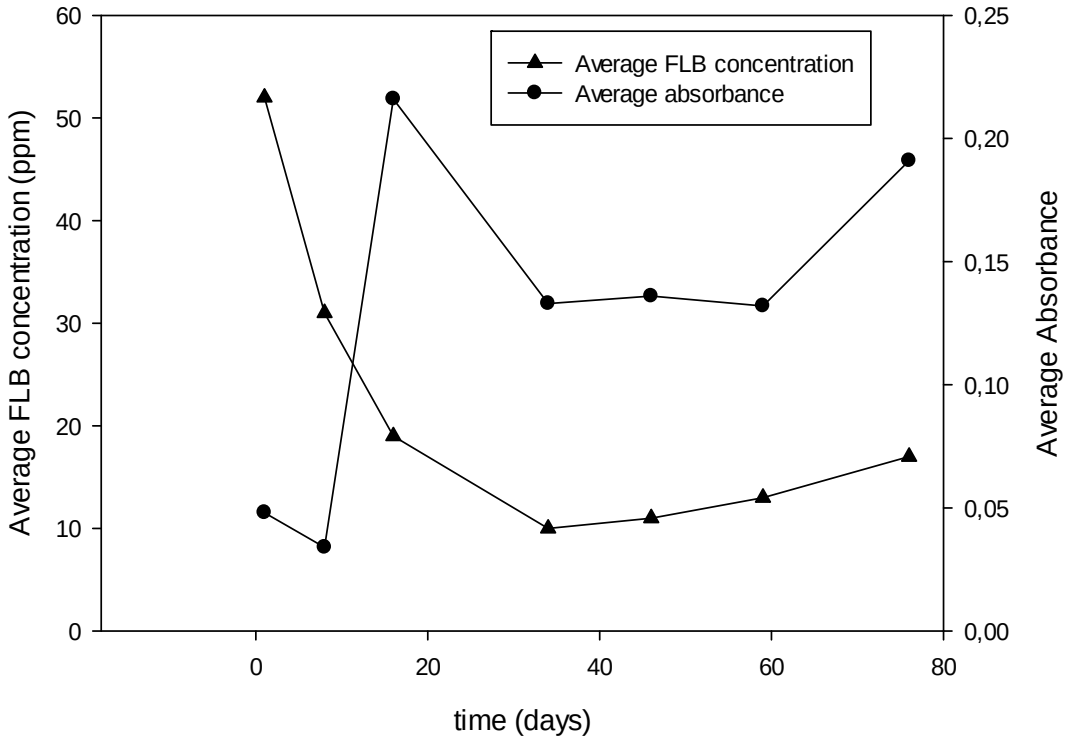


Figure 4. 21. Average FLB concentration versus average absorbance at 370 nm of 50 ppm FLB flasks.

For 250 ppm FLB flasks, no clear FLB disappearance was observed but they had highest absorbance. In T1-500, T2-500, T1-50 and T3-50 flasks, there was a clear disappearance of FLB and most of the FLB was degraded in 16 days in these flasks. The degradation might be stochastic or not depend on FLB concentration.

There was a strong negative correlation between FLB concentration and second peak (metabolite) abundance based on test for the significance of the Pearson product-moment ($R=0.847$, $p<0.0000001$) (Figure 4. 22).On the other hand, the correlation between absorbance and FLB disappearance was weak.

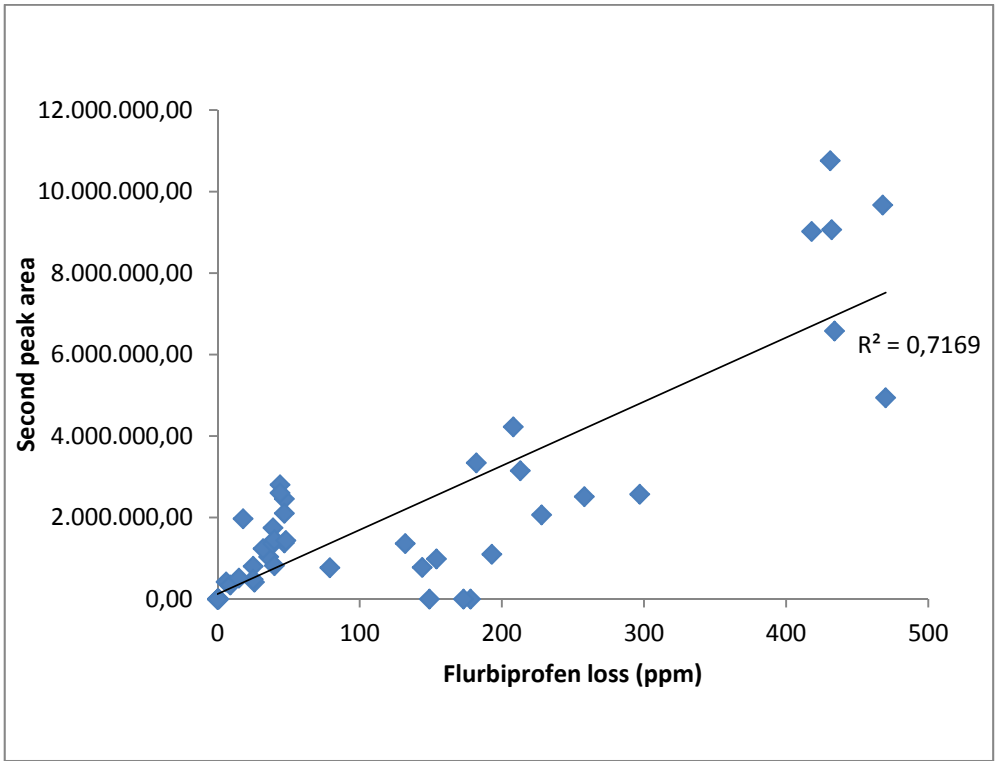


Figure 4. 22. FLB loss versus second peak area. This plot was derived from the data of FLB dissapperance rate

4.2.2. Enrichment for FLB Degraders

The previous enrichment for FLB degraders was not successful. Firstly, enrichment for FLB degraders was carried out by preparing 250 ppm FLB flasks in fresh aerobic sludge from Ankara municipal wastewater treatment plant. FLB was degraded in about two weeks. To increase the chance of obtaining an enriched culture and/or isolates, three different media were used for the subsequent enrichment phases; 100 ppm FLB, 100 ppm FLB +2 ppm yeast extract, and 500 ppm FLB. FLB concentration was lowered, in the 100 ppm treatments, in order to address toxicity issues. The yeast extract media was prepared so that isolates would have access to micronutrients and vitamins, a standard procedure for minimal media systems. After approximately one month, the initial enrichment showed signs of growth and were promptly transferred to identical media types. As predicted, the 500 ppm FLB enrichment took much longer to begin to grow. The second enrichment cultures developed granule-like structures after approximately 3 weeks (Figure 4. 23).

Figure 4. 23. Depiction of granule-like structures present in second 100 ppm FLB + yeast extract enrichment.

The third generation of 100 ppm FLB and 100 ppm FLB + yeast enrichments grew turbid within three days, although clear signs of FLB degradation for all three generations were not observed based on HPLC analysis. Then they were streaked onto both LB and 100 ppm FLB + yeast extract solid media. The yeast – supplemented media enrichment culture appeared to be dominated by a pink-colored bacterium, a proportional representation of the enrichment culture that appeared to have increased with each subsequent enrichment cycle (Figure 4. 24). This pink-colored bacterium is absent from LB streaks of the other two enrichments.

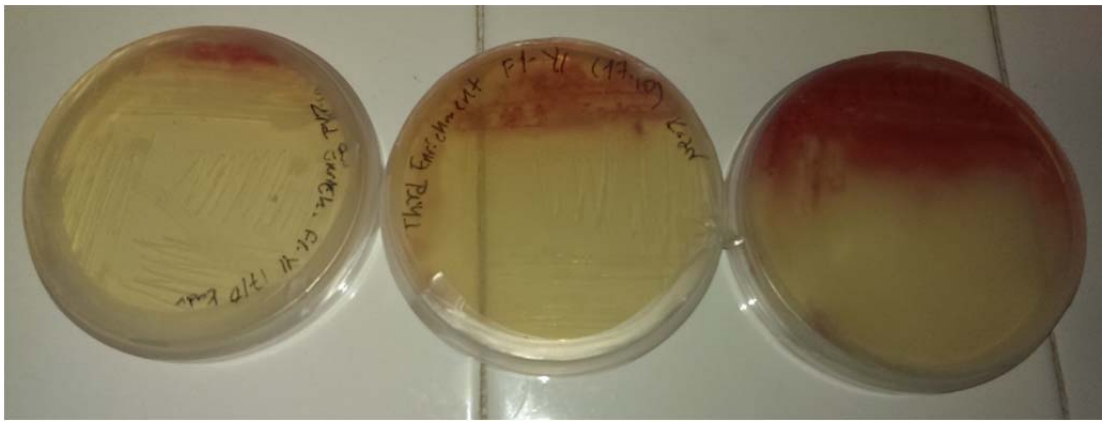
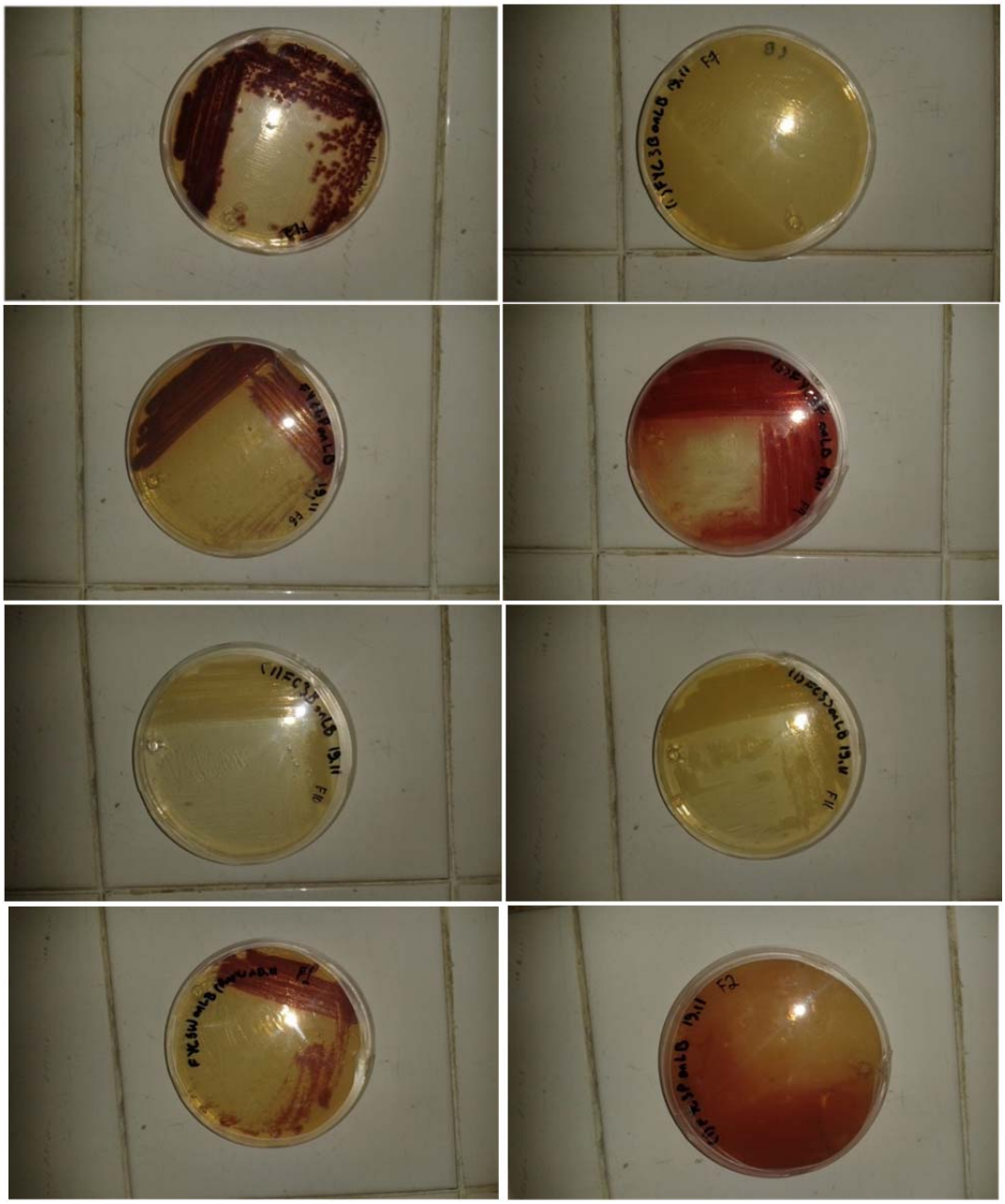


Figure 4. 24. Three generations, pictured left to right, of 100 ppm FLB + 2 ppm yeast extract enrichment cultures plated onto LB media. Note the proportional increase of the reddish bacterium with subsequent generations.

The third generations of all three enrichments showed signs of growth on 100 ppm FLB + 2 ppm yeast extract solid media; colonies were visible after 1 week of growth. It appeared that isolation was successful. After larger colonies were obtained, they were re-isolated on FLB media and then subjected to 16S sequencing and RFLP.



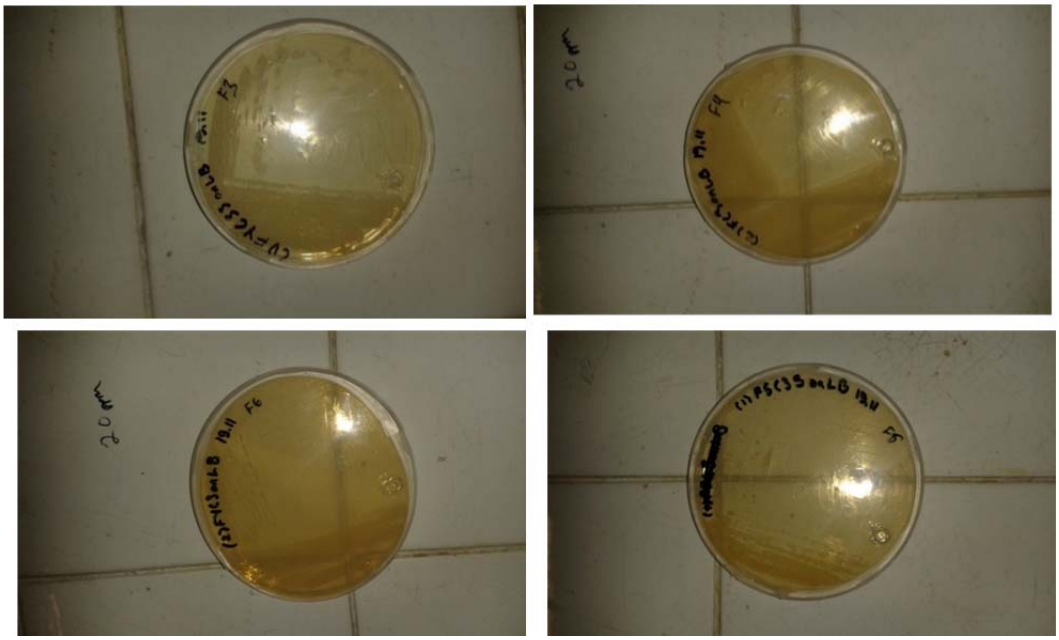


Figure 4. 25. Photographs of putative FLB degraders on LB solid media after one week. Note they were firstly isolated and identified based on colony appearance.

4.2.3. Enrichment for Tolylacetic Acids Degraders

Enrichments for pTAA and mTAA were successful and isolates capable of growth on the appropriate minimal media were obtained and stored at -80ºC.

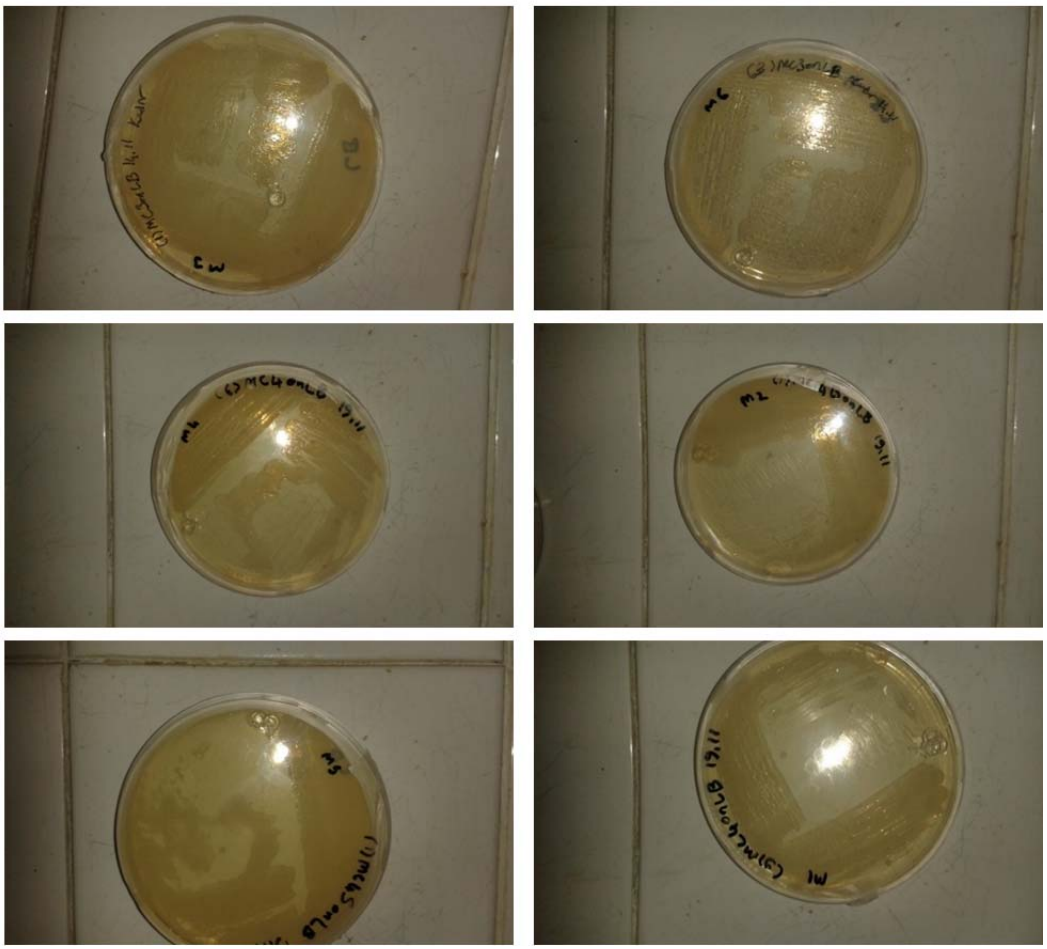
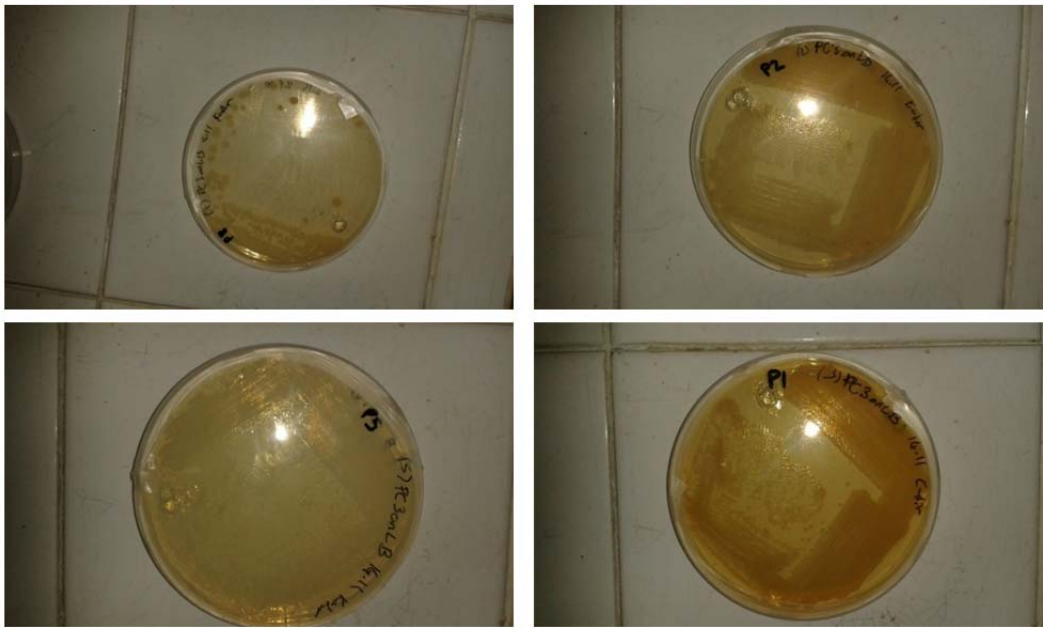


Figure 4. 26. Photographs of putative mTAA degraders



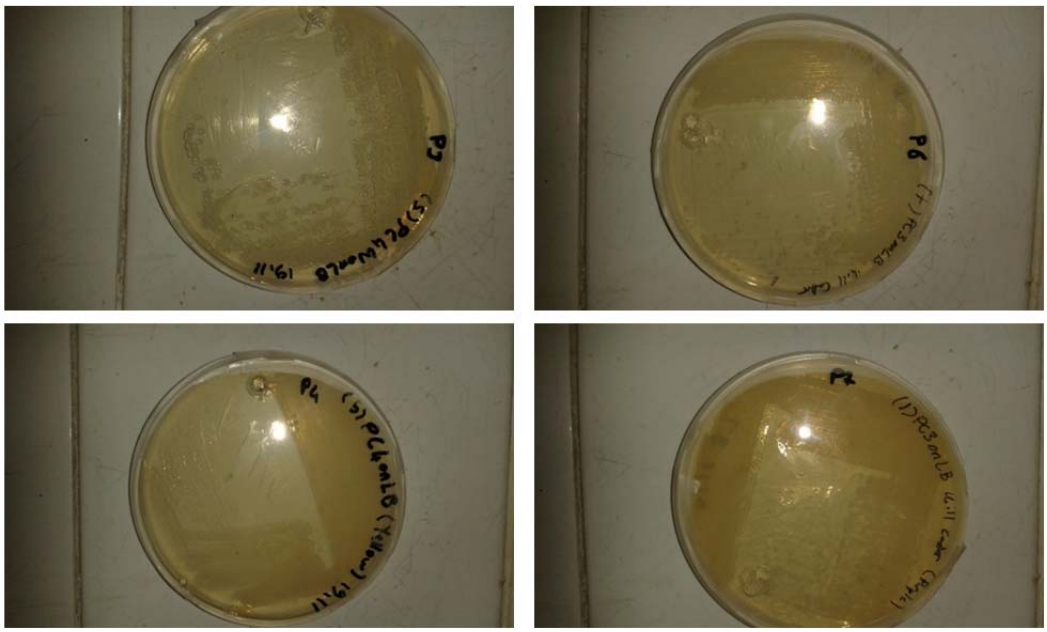


Figure 4. 27. Photographs of putative pTAA degraders.

4.2.4. On the Catechol Metabolism Indicators Present During Enrichment Studies

None of the enrichments cultures produced any of the notable yellow coloration that had been observed in the initial FLB-spiked sewage sludges as reported previously. However, third-generation 100 ppm FLB + yeast extract cultures produced a dark black precipitate, which may indicate polymerized catechols, though there are other explanations. Later, analysis of isolates provided more definitive answers.

4.2.5. Identification of Unique Degrader Strains

16S rRNA gene sequences of isolated cultures were amplified via polymerase chain reaction (PCR) and amplicons were subjected to restriction fragment analysis (RFLP) by using a 4-hitter restriction enzyme (HaeIII) in order to identify unique strains.

4.2.5.1. Identification of Putative FLB Degraders

16S rRNA gene sequences of 12 FLB degraders isolated based on colony appearance were amplified by PCR and their amplicons were analyzed by the standard method (gel electrophoresis + UV visualization).

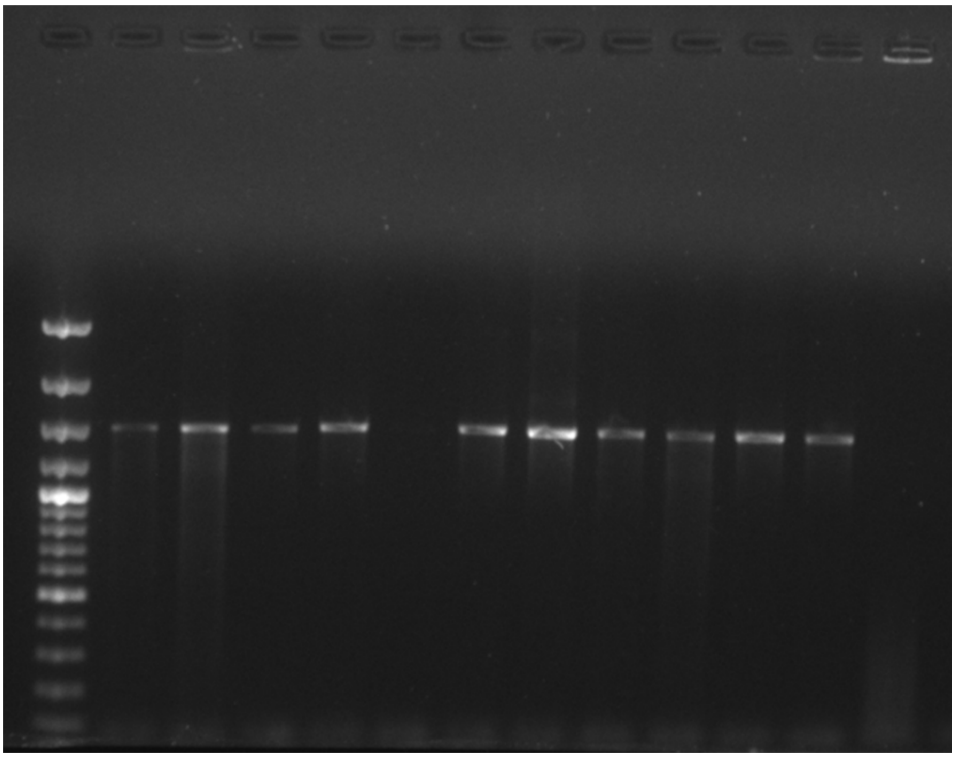


Figure 4. 28. Confirmation of PCR products of FLB isolates. Note three of the isolates were not confirmed and a second run were carried out for them.

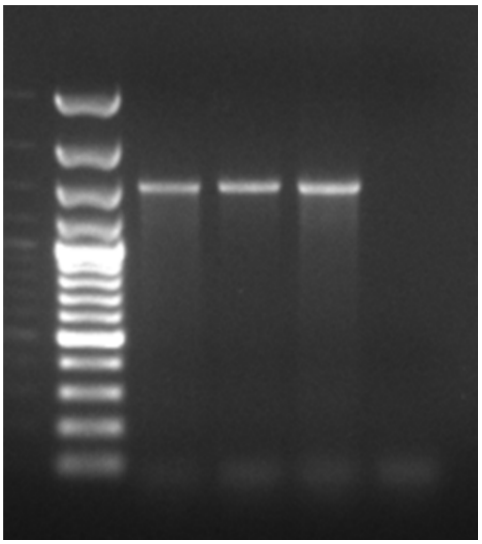


Figure 4. 29. Confirmation of PCR products of remained FLB isolates.

Finally, all FLB degraders were amplified successfully. Then, they were identified by RFLP analysis and 5 unique FLB degrading isolates were obtained (Figure 4. 30).

Figure 4. 30. The RFLP analysis of FLB degraders. The second strain have the same colony shape and color with 12th strain and they were treated as the same strain Each unique strain was signed with a specific letter.

4.2.5.2. Identification of Putative Tolylacetic Acid Degraders

5 mTAA and 7 pTAA degraders were amplified and their PCR products were confirmed. Then they were subjected to RFLP analysis. As a result, 2 unique mTAA degrading strains and 5 unique pTAA degrading strains were obtained.

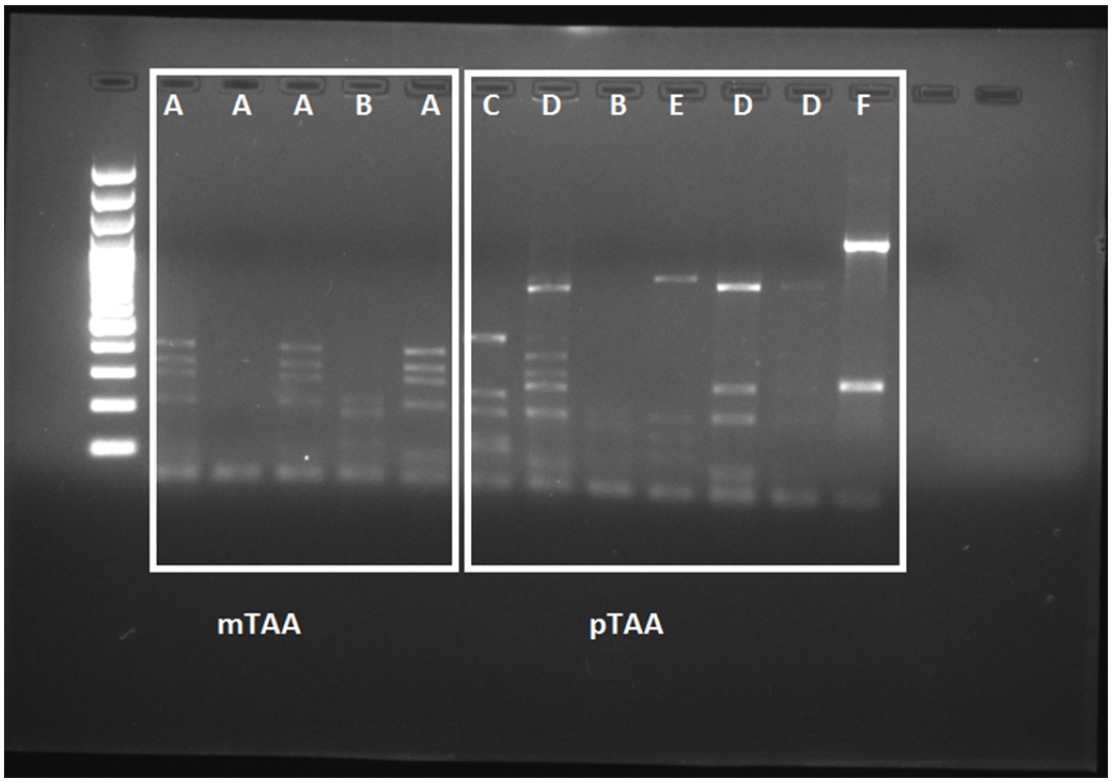


Figure 4. 31. The RFLP analysis of mTAA and pTAA degraders. Each unique strain was signed with a specific letter.

4.3. Confirmation of Putative FLB, mTAA, pTAA and PAA Degrading Strains

In order to confirm whether FLB degrading strains had ability to degrade FLB or not, the experiments described in section 3.2.3 were carried out. It was observed that FLB degrading strains did not actually degrade FLB as a result of experiments run for at least 20 days. Surprisingly, it was observed that some of the strains had ability to grow on agar, forming microcolonies after long incubation periods. The strains did not degrade FLB, but they grew on the other easily metabolized chemicals, such as glucose and sodium acetate. However, they were stored at -80^{o}C in glycerol solution.

One mTAA degrading strain and one pTAA degrading strain had the ability to grow on mTAA and pTAA, respectively. Other strains had growth in the presence of 10 % of LB. The strains were stored at -80 ^{o}C in glycerol solution. Additionally, PAA degraders were also stored at -80 ^{o}C in glycerol solution after confirmation.

The mTAA and pTAA degrading strains were inoculated into MSM with 250 ppm mTAA + 50 ppm FLB and MSM with 250 ppm pTAA + 50 ppm FLB, respectively, in order to investigate whether these strains have the ability to degrade FLB. There was no FLB degradation at the end of three weeks.

4.4. mTAA and pTAA Disappearance Essay and Growth Analysis

The disappearance and growth for mTAA and pTAA were investigated for the strain degrading mTAA and the strain degrading pTAA. mTAA was completely degraded in about 120 hours in all flasks while pTAA was degraded in about 72 hours in all flasks. By measuring the turbidity via optical density at 600nm, the growth rates of bacteria were determined. As the degradation percentages increased, the turbidity in the flasks was also increased. This is consistent with the correlation between degradation rate and growth rate (Figure 4. 32 & Figure 4. 33). These results also indicated that the enrichment system worked properly.

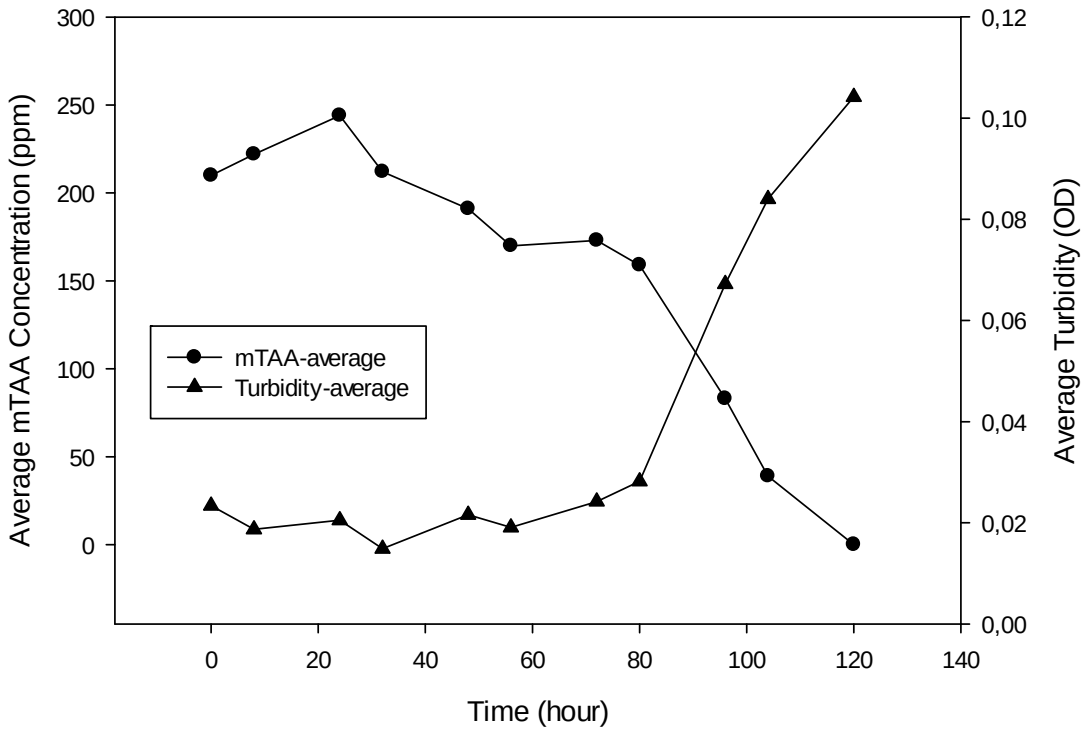


Figure 4. 32. mTAA disappearance versus growth as turbidity.

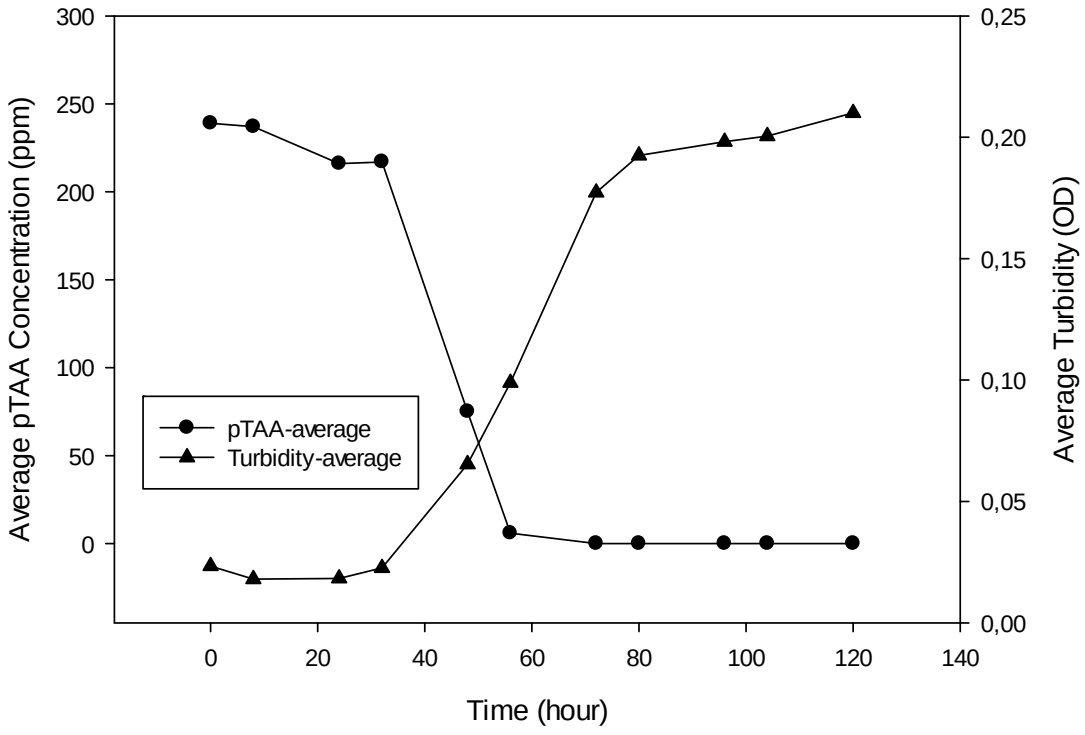


Figure 4. 33. pTAA disappearance versus growth as turbidity (OD).

4.5. Confirmation of Second Peak Represanting Putative FLB Metabolite

For the examination of second peak production, two flasks of 50 ppm FLB in sludge, one flask of 500 ppm FLB in sludge and one flask without FLB (negative FLB control) were prepared and one of the 50 ppm FLB flasks was autoclaved (biological control). The rationale of this experiment was that if the second peak truly represents a FLB metabolite, it would not appear in the absence of biological activity (autoclaved control) or in the absence of FLB. 50 ppm FLB and 500 ppm FLB were degraded in 8 days and 13 days, respectively and a slight yellow coloration was observed. Second peak and third peaks were observed in the 50 ppm FLB and 500 ppm FLB flasks as disappearance of FLB progressed. The autoclaved samples and those without FLB did not yield a second peak, but they did yield a third peak at week 4. It was clear that second peak was due to degradation of FLB while the third peak was actually unrelated.

Table 4. 10. Disappearance of FLB in 500 ppm FLB (F-500), 50 ppm FLB (F-50) and 50 ppm autoclaved FLB (AF-50) flasks.

time (day)	F-500 (ppm)	F-50 (ppm)	AF-50 (ppm)
0	238	21	27
2	218	18	24
4	246	24	29
6	202	25	23
8	234	2	25
10	198	0	21
13	13	0	27

4.6. Repeating the Experiment of Enrichment of FLB Degraders and Confirmation of Second Peak

50 ppm FLB flasks, 50 ppm autoclaved FLB flasks and 0 ppm FLB flasks were prepared in triplicate. 50 ppm FLB was degraded in 2 weeks with slight yellow coloration and second peak was observed with disappearance of FLB and fractionated for further analyses. No FLB and autoclaved FLB flasks did not yield second peak but they yielded third peak at the week 4.

Table 4. 11. Disappearance of FLB in 50 ppm FLB (F-50) and 50 ppm autoclaved FLB (AF-50) flasks.

time (day)	F-50 average	Standard Deviation	AF-50 average	Standard Deviation
0	21	2	26	4
2	22	5	22	2
5	21	2	23	4
7	22	4	22	3
9	22	3	24	3
11	20	6	25	5
14	1	1	23	3

The MSM media composition was changed in order to see the effects of MSM composition. Transfers were made from 50 ppm FLB flasks to the new flasks with 50 ppm FLB and MSM prepared according to McCullar's formula. At the end of 4 weeks, there was no FLB degradation.

4.7. Co-metabolic Stimulation of FLB Degradation

Treatments with 250 ppm mTAA+50 ppm FLB (25M5F), 250 ppm pTAA+50 ppm FLB (25P5F), 250 ppm PAA+50 ppm FLB (25PA5F), 50 ppm FLB (F50), 250 ppm BP + 50 ppm FLB (25B5F), 3FPAA + 50 ppm FLB (25FP5F) and 250 ppm IBP + 50 ppm FLB (25I5F) were prepared separately in fresh aerobic sludge in order to stimulate co-metabolic activity and see the effect of co-metabolism on FLB degradation. They were prepared in triplicates.

FLB has both BP and PAA moieties. 3FPAA has a fluorine moiety at the same position with the FLB. Altogether, it was expected that at least one of these similar chemicals would have stimulatory effects on FLB degradation.

Table 4. 12. Concentrations of pTAA and FLB in 250 ppm pTAA + 50 ppm FLB flasks.

Time (day)	25P5F-average (pTAA area)	Standard Deviation	25P5F-average (FLB conc.)	Standard Deviation
0	136	2	37	3
1	137	3	37	4
4	165	4	37	3
5	170	10	36	1
8	166	9	33	2
11	164	12	32	3
12	151	20	34	4
13	69	60	34	1
14	2	2	34	3
17	0	0	27	11
18	0	0	23	17
19	0	0	23	20
20	0	0	23	20
21	0	0	21	18
22	0	0	21	19
29	0	0	12	20
30	0	0	12	21
38	0	0	10	17
48	0	0	11	19
52	0	0	11	20

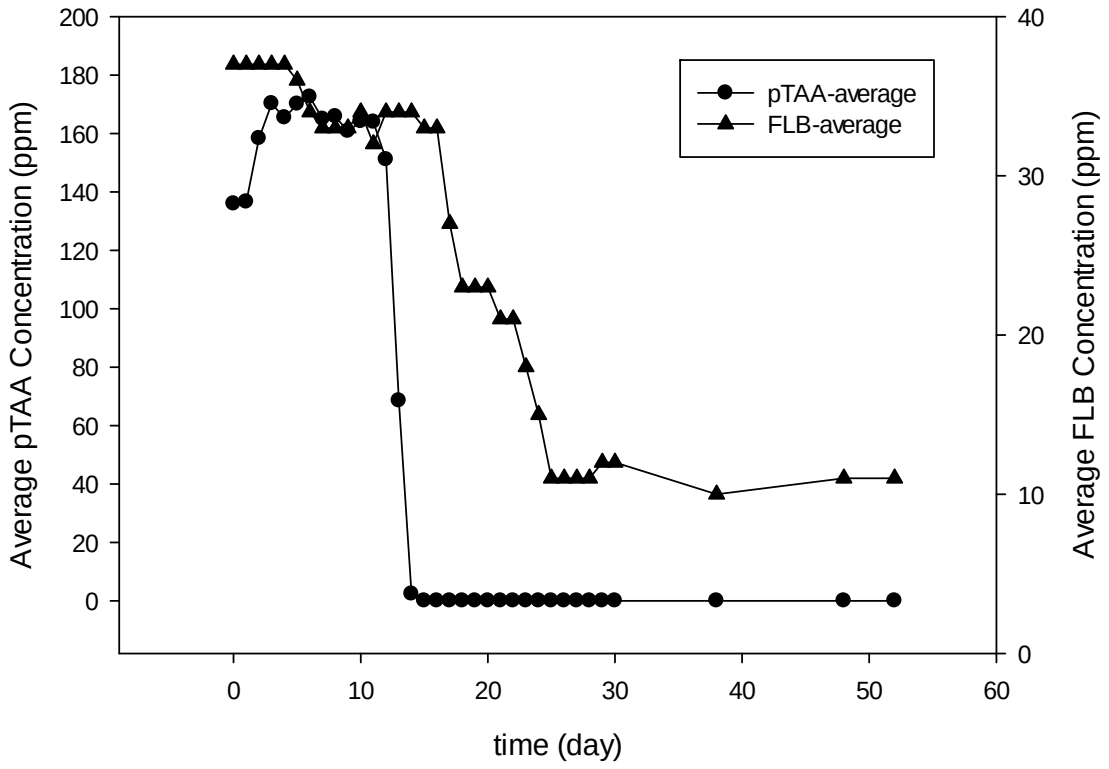


Figure 4. 34. pTAA concentration change versus FLB concentration change in 250 ppm pTAA + 50 ppm FLB flasks.

FLB degradation started after degradation of pTAA. There was no clear indication of the stimulation of FLB degradation by pTAA degradation (Figure 4. 33 & Table 4. 12).

Table 4. 13. Concentrations of mTAA and FLB in 250 ppm mTAA + 50 ppm FLB flasks

time (day)	25M5F-average (mTAA conc.)	Standard Deviation	25M5F-average (FLB conc.)	Standard Deviation
0	135	8	35	3
5	156	5	36	2
6	153	3	34	1
10	153	3	35	3
14	108	55	33	3
20	38	46	32	5
21	4	7	33	3
38	0	0	33	5
48	0	0	32	3
52	0	0	32	5

mTAA was degraded in three weeks in all flasks while there was no FLB degradation at the end of 52 days. Additionally, there might have been be inhibitory effects of mTAA on FLB degradation (Table 4. 13).

Table 4. 14. Concentrations of PAA and FLB in 250 ppm PAA + 50 ppm FLB flasks.

time (day)	25PA5F-average (PAA area)	Standard Deviation	25PA5F-average (FLB conc.)	Standard Deviation
0	12,441,368	265,755	34	4
1	0	0	33	2
2	0	0	34	1
3	0	0	35	1
4	0	0	34	2
5	0	0	35	2
6	0	0	32	1
7	0	0	32	2
8	0	0	34	2
9	0	0	31	2
10	0	0	33	1
11	0	0	32	3
12	0	0	28	9
13	0	0	23	16
14	0	0	23	16
19	0	0	22	16
20	0	0	19	16
22	0	0	18	16
23	0	0	20	17
24	0	0	19	17
25	0	0	19	16
26	0	0	17	15
27	0	0	12	12
28	0	0	5	9
29	0	0	0	0
30	0	0	0	0

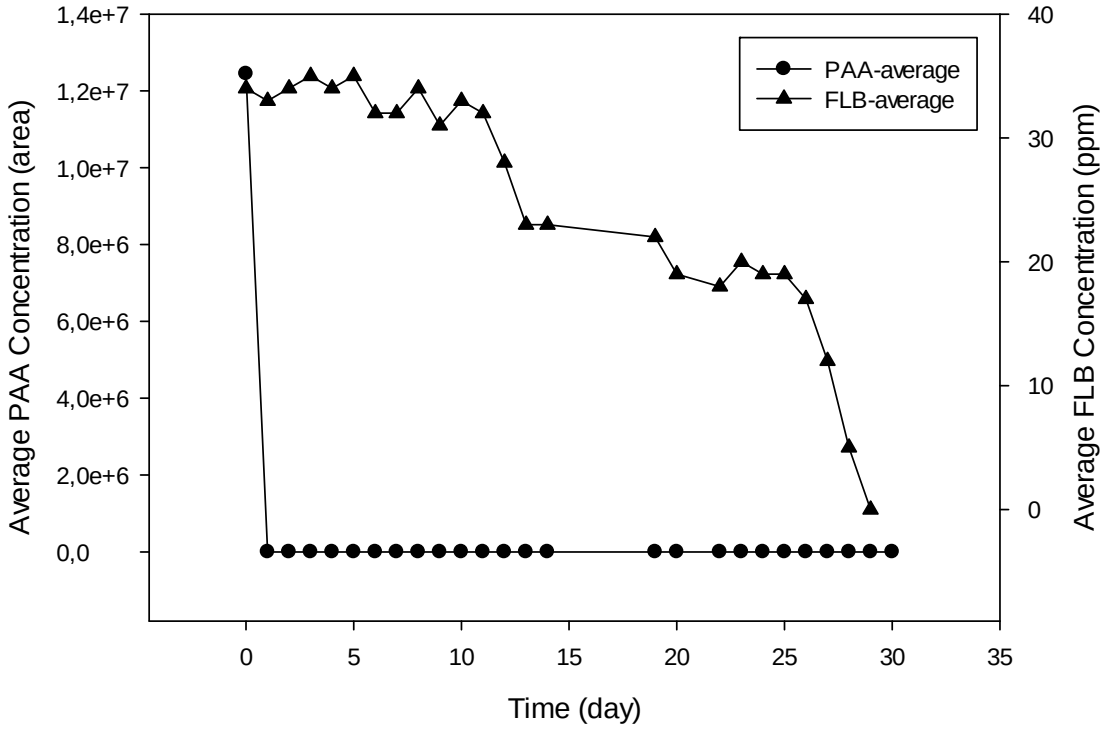


Figure 4. 35. FLB concentration change in 250 ppm PAA + 50 ppm FLB flasks.

PAA was degraded in all flasks in one day while FLB was degraded in four weeks. It did not clearly indicate co-metabolic activity (Figure 4. 35 & Table 4. 14).

Table 4. 15. Concentration of FLB in 50 ppm FLB flasks.

time (day)	5F-average (FLB conc.)	Standard Deviation
0	32	3
1	34	2
3	34	2
5	34	3
6	34	1
7	33	1
8	31	2
14	35	2
20	31	2
21	33	2
22	32	5
23	16	5
24	1	2
25	0	0

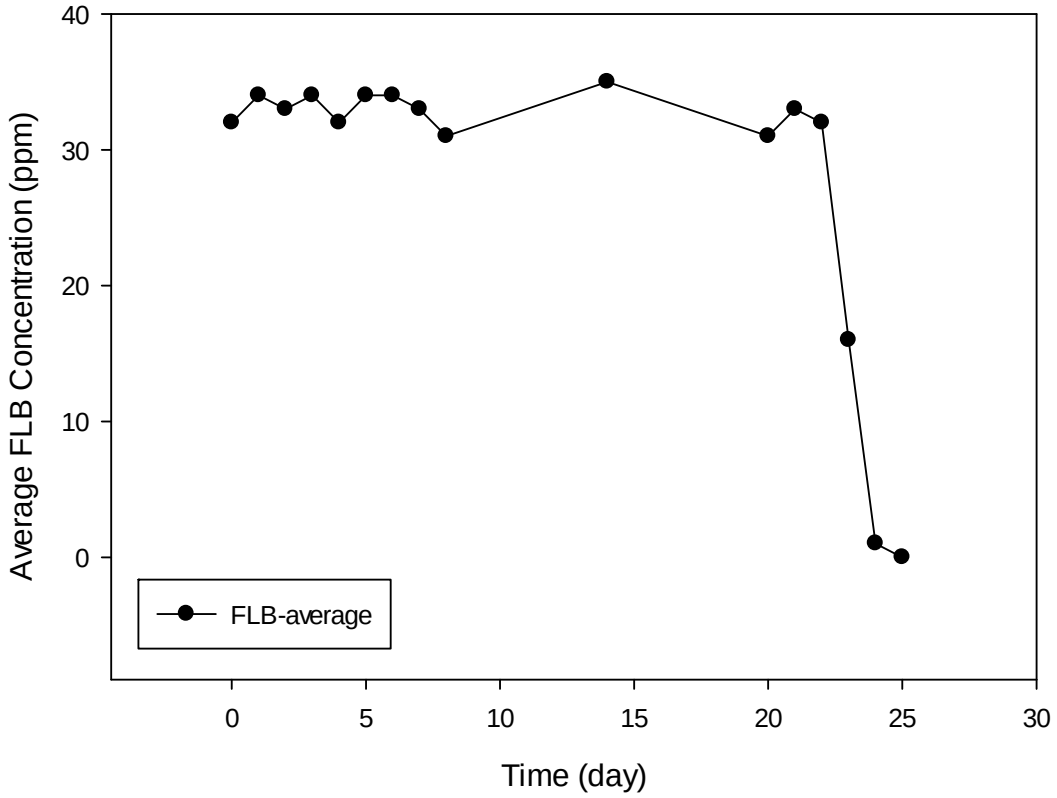


Figure 4. 36. FLB concentration change 50 ppm FLB flasks with respect to time.

FLB was degraded in all 5F flasks in 24 days with a small standard deviation (Table 4. 15 & Figure 4. 36).

Table 4. 16. FLB concentration in 250 ppm BP + 50 ppm FLB flasks. BP concentration could not be measured with HPLC because BP is very hydrophobic.

Time (day)	25B5F-average (FLB conc.)	Standard Deviation
0	31	3
1	29	1
3	30	1
5	31	1
7	30	1
8	29	1
14	30	1
20	28	3
22	31	1
23	23	7
24	6	7
25	0	0

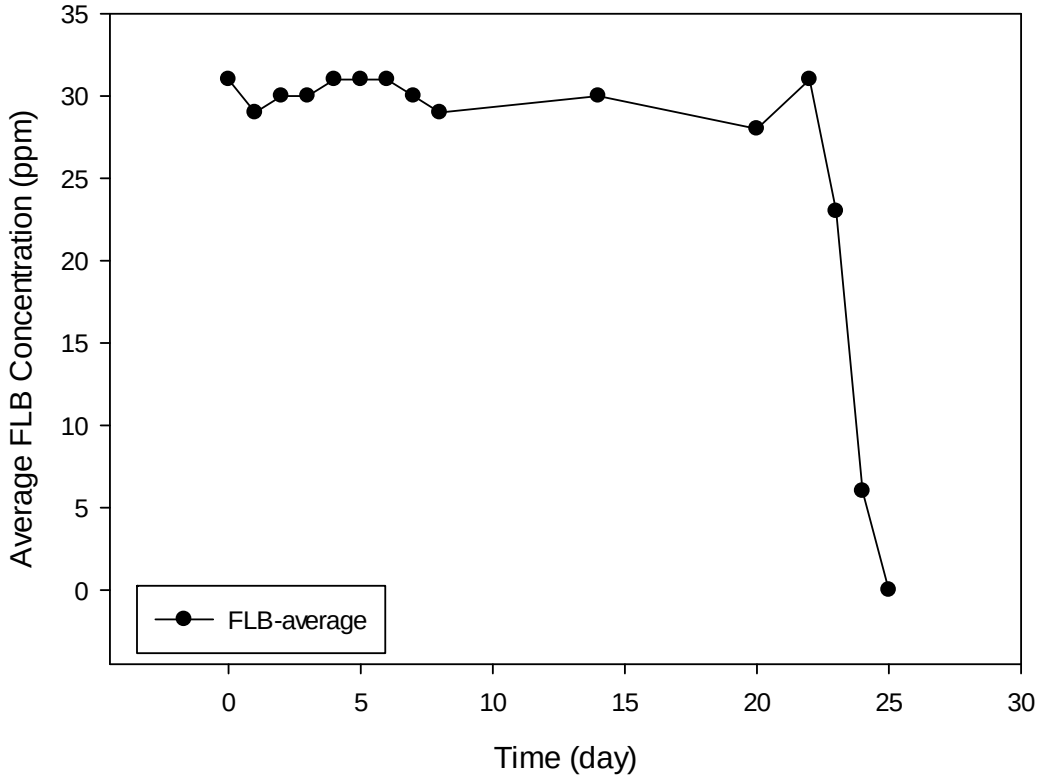


Figure 4. 37. FLB concentration change in 250 ppm BP + 50 ppm FLB flasks.

FLB was degraded in all flasks in 24 days and actually the degradation trend was very similar to trend of FLB degradation in 50 ppm FLB flasks. Thus, it is not clear that whether the presence of BP had effects on FLB degradation or not (Figure 4. 37 & Table 4. 16).

Table 4. 17. Concentrations of 3FPAA (as area) and FLB in 250 ppm 3FPAA + 50 ppm FLB flasks.

time (day)	25FP5F-average (FP conc.)	Standard Deviation	25FP5F-average (FLB conc.)	Standard Deviation
0	8,778,868	246,518	30	2
1	8,541,173	352,718	31	4
3	9,015,623	44,035	33	2
5	8,975,783	78,451	31	2
8	9,070,253	249,418	30	3
14	9,036,509	459,364	31	3
20	8,962,599	556,755	32	1
28	9,094,870	946,436	28	1
38	8,821,134	414,709	31	3
48	9,000,983	660,525	32	4
52	9,011,461	498,925	34	1

3FPAA was not degraded to any degree and there might be inhibitory effects of 3FPAA on FLB degradation. Neither 3FPAA nor FLB was degraded at the end of 52 days (Table 4. 17).

Table 4. 18. Concentrations of IBP (as area) and FLB in 250 ppm IBP + 50 ppm FLB flasks.

Time (day)	25I5F-average (IBP area)	25I5F-2 (IBP area)	25I5F-average (FLB conc.)	Standard Deviation
0	9,675,116	381,726	31	2
1	9,624,709	225,887	34	1
3	10,591,425	383,516	33	2
5	10,208,652	423,340	33	2
8	10,358,314	594,242	29	1
14	8,269,921	1,445,916	32	4
16	6,736,561	1,167,247	30	3
17	6,575,460	2,732,400	27	7
18	3,978,666	3,552,058	16	16
19	2,489,555	2,156,178	10	18
20	0	0	8	13
21	0	0	0	0
22	0	0	0	0

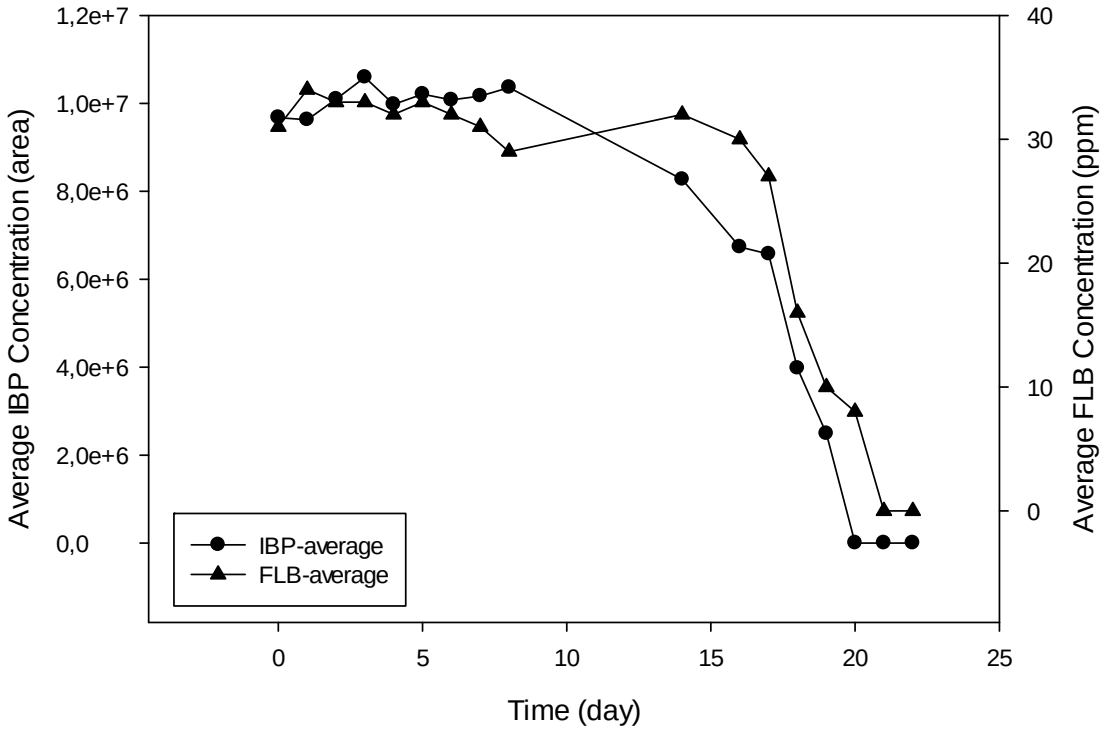


Figure 4. 38. IBP concentration change versus FLB concentration change in 250 ppm IBP + 50 ppm FLB flasks.

The rate of FLB disappearance in 25I5F flasks was slightly faster than under the other co-metabolic stimulants or without any stimulation, although the variation of the system made the result statistically insignificant (Figure 4. 39). Additionally, FLB degradation was clearly inhibited by 3FPAA judging by the fact that FLB was never removed below 10 ppm under this condition (Table 4. 17 & Figure 4. 39). mTAA might have had an inhibitory effect on FLB degradation, because FLB was ultimately not removed under this condition.

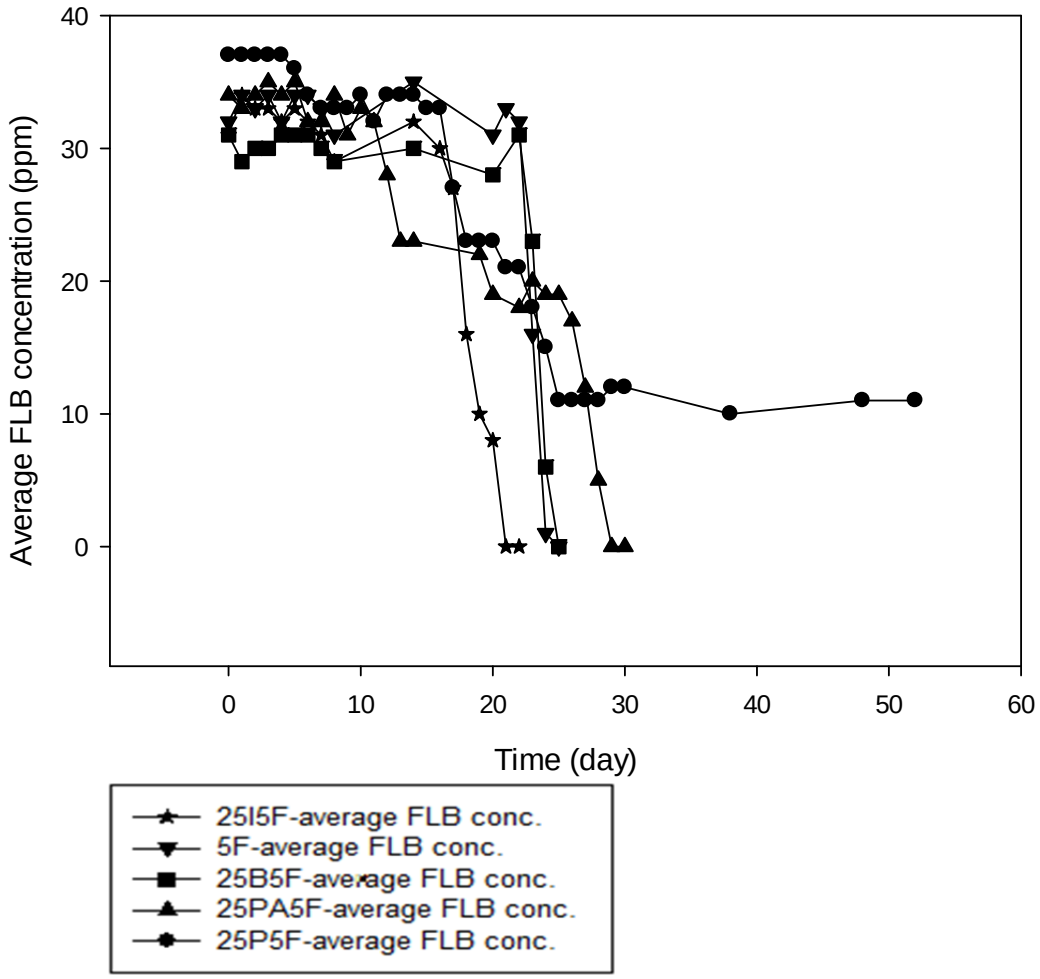


Figure 4. 39. Average FLB concentration in the treatments in which FLB was degraded in one of the flasks at least.

4.8. Enrichments in Sludge Samples from Other Cities

FLB was degraded in 11 days in the sludge taken from Yozgat and after degradation started and yellow color appeared it was transferred to liquid MSM. In about 6 weeks, there was no FLB degradation and second peak appearance in liquid MSM. At the end of 8 weeks there was no FLB degradation in the sludge taken from Eskişehir. Thus, FLB degrading isolates were not obtained from either of these sludges either.

4.9. Enrichments with Alternative MSMs

FLB degradation started in aerobic sludge in 18 days. Transfers were made from aerobic sludge to different MSM recipes. There was no clear sign of FLB degradation in McCullar's MSM with 100 ppm FLB (O-1, O-2, O-3) after three weeks in all of the flasks (Figure 4. 40).

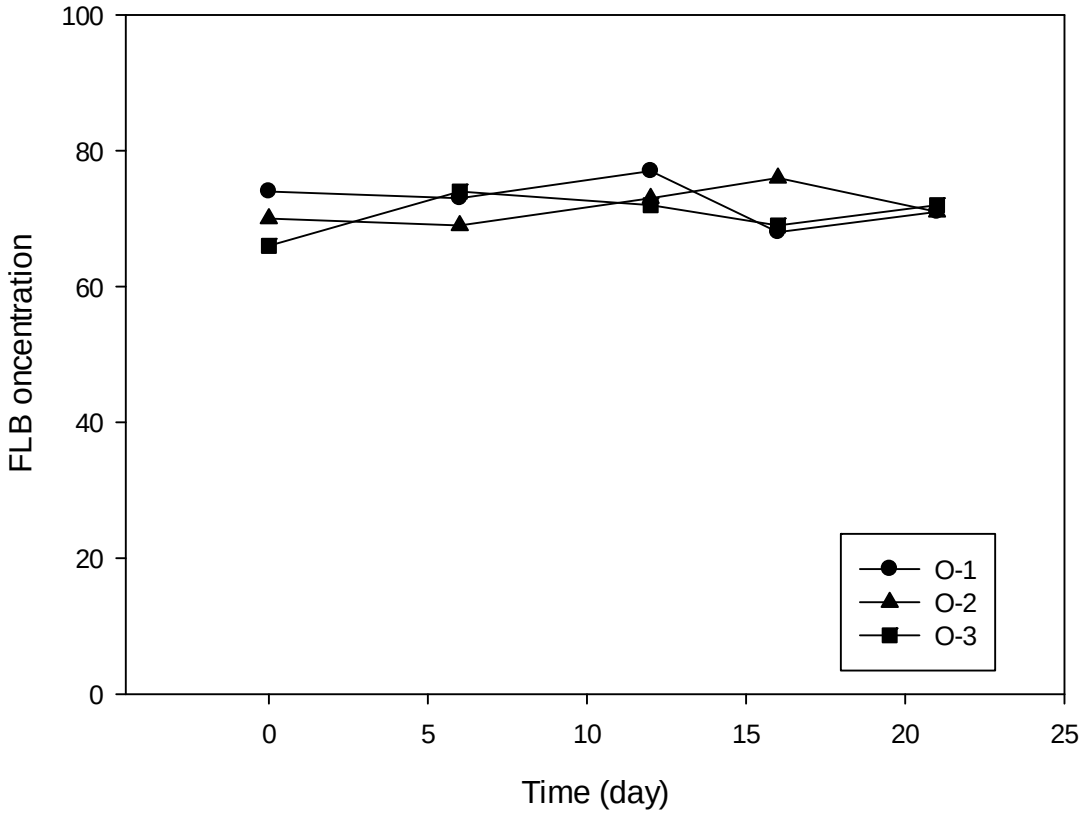


Figure 4. 40. FLB disappearance in McCullar's MSM vs time. FLB concentrations was lower than 100 ppm which might be caused by low solubility of FLB or filtration material.

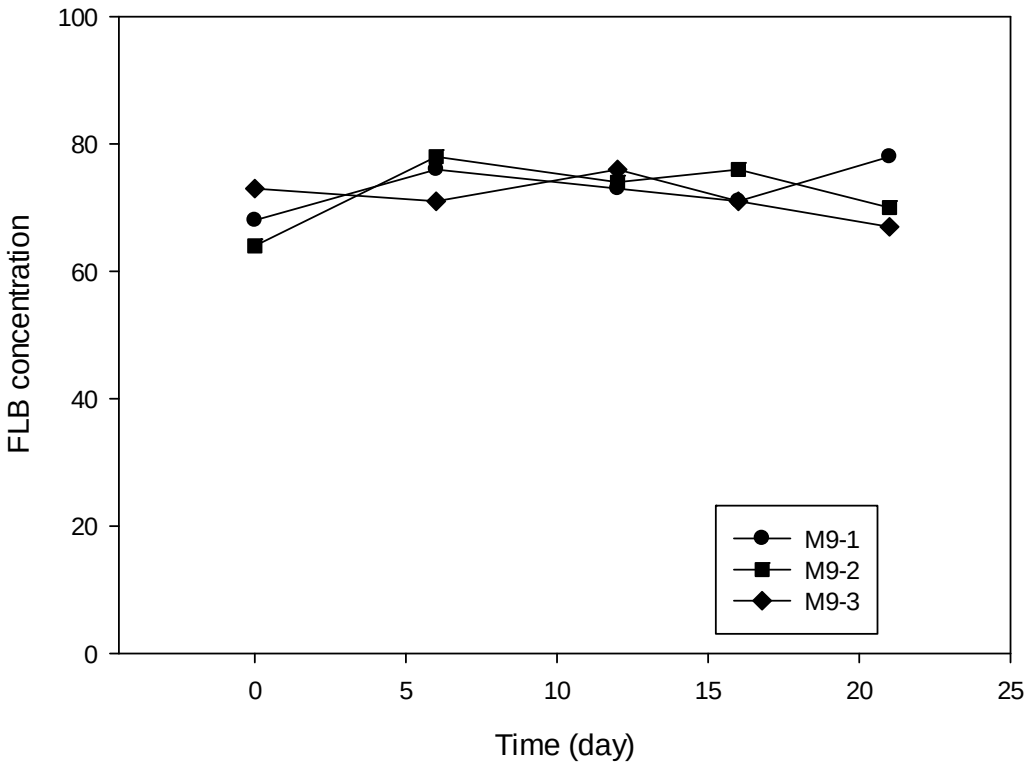


Figure 4. 41. FLB disappearance in M9 recipe vs. time. FLB concentrations were lower than 100 ppm which might be caused by low solubility of FLB or filtration material.

There was also no clear sign of growth and FLB disappearance in McCullar's MSM with spring water (S) (Figure 4. 42) and M9 MSM (M-1, M-2, M-3) (Figure 4. 41). It seems different water source and alternative mineral medium did not also stimulate the growth.

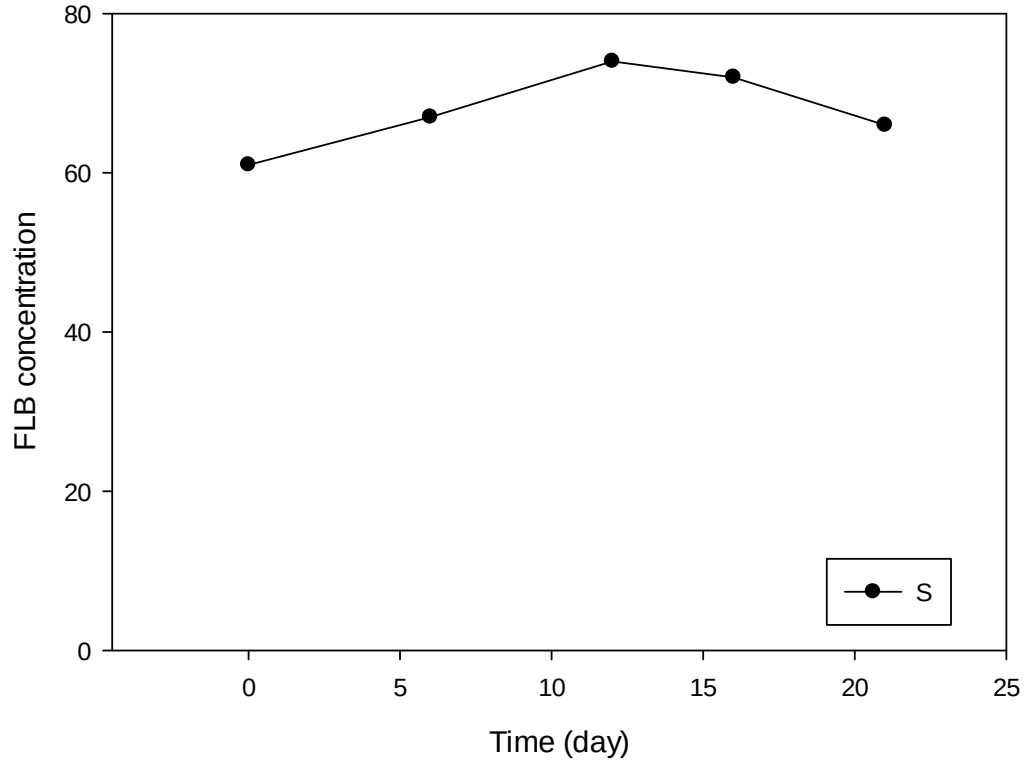


Figure 4. 42. FLB disappearance in McCullar's recipe with spring water vs. time. FLB concentrations were lower than 100 ppm which might be caused by low solubility of FLB or filtration material.

4.10. Detection of Fluoride

The available method, which is targeted towards analysis of blood samples, was modified for this system. The new method was comprised of direct mixing of 0.25 mL of alizarin reagent, 0.25 mL cerous nitrate and 0.5 ml of sample. In case of existence of fluoride the mix gives a blue or light lilac color as shown in the section 3.3.4.. This approach will only detect fluoride that is in free ion form, not organic fluoride, thus it serves as an indicator for defluorination. The system was confirmed by testing standard solutions of sodium fluoride and a standard curve was drawn based on color generation generated by the standard solutions.

Samples from previous FLB enrichments in which degradation was observed were tested with this method and no blue or light lilac coloration was observed while there was FLB degradation, suggesting that the fluoride is not liberated from the parent compound and thus likely accumulation of a fluorinated metabolite.

4.11. Characterization of FLB Degradation by LCMS

UPLC was applied to separate analytes from 500 ppm FLB in aerobic sludge. Relative abundances were calculated based on 500 ppm FLB standard sample for the analytes. The peak with the retention time of 2.72 observed both in blank sample and sludge sample spiked with FLB (Figure 4. 43-a & Figure 4. 43-b). Therefore, this peak was confirmed as caused by chemicals already available in the sludge, not by FLB degradation. Both the peak of the retention time of 12.92 observed during the UPLC analysis of 500 ppm FLB standard and the peak of the retention time of 4.63 observed during the UPLC analysis of sludge sample spiked with FLB were later subjected to ESI-TOF-MS in negative mode.

Two ions are generally dominating the mass spectra of FLB: m/z: 199 and m/z 243 and the relative abundances of product ions depend on the level of collision energy and the configuration of MS or MS-MS (Abdel-Aziz et al., 2012; Déglon et al., 2011; Lee et al., 2010; Vinci et al., 2006). During mass spectra analyses of NSAIDs, parent compounds generally loses CO_2 group having m/z: 44 (Lacey et al., 2008; Vinci et al., 2006) which is consistent with the results. The configuration of Waters Synapt G1 for FLB mass spectrum produced m/z: 199 as the precursor ion (Figure 4. 44).Similar cases were reported by Lee et al. (2010); Vinci et al. (2006). The results were compared to those of Competitive Fragmentation Modeling for Metabolite Identification CFM-ID, an online program predicting the spectra, assigning peaks and identifying compounds generated by ESI-MS/MS for confirmation (Table 4. 19) (Allen et al., 2015; Allen et al., 2014). CFM-ID calculates the relative abundances of product ions based on collision energy. The higher collision energy produces parent compounds with the lower relative abundance.

Table 4. 19. Comparison of results of FLB spectrum generated by Waters Synapt G1 and CFM-ID.

Ions (m/z)	TOF-MS ES- Relative Abundance	CFM-ID prediction Relative Abundance		
	Low Collision Energy	10V of Collision Energy	20V of Collision Energy	40V of Collision Energy
171	-	1	11	100
199	100	36	100	35
200	19	-	-	-
243	2	100	72	6

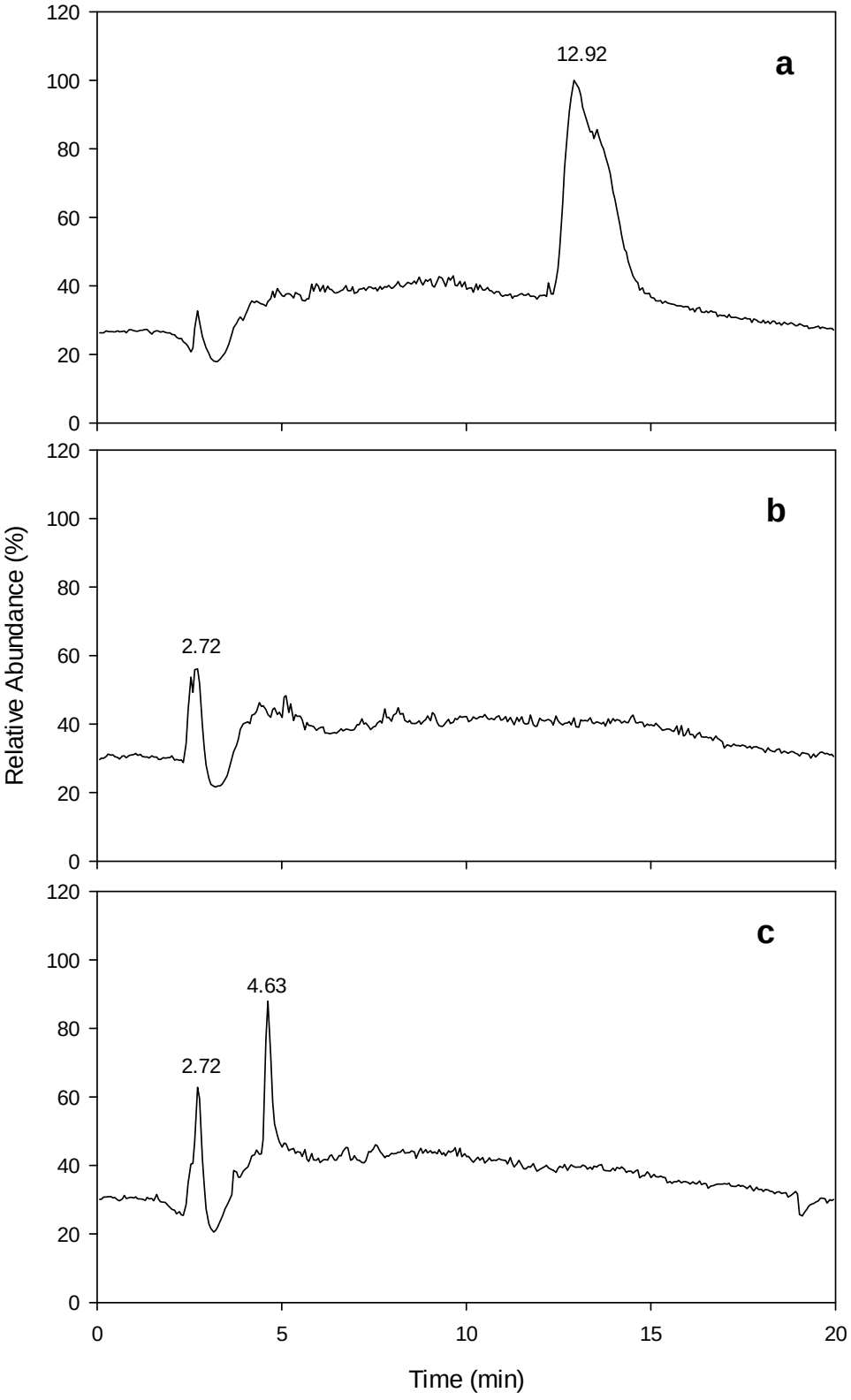


Figure 4. 43. LC/MS Chromatograms of 500 ppm FLB in methanol (a), sludge blank sample (b) and sludge sample spiked with FLB (c).

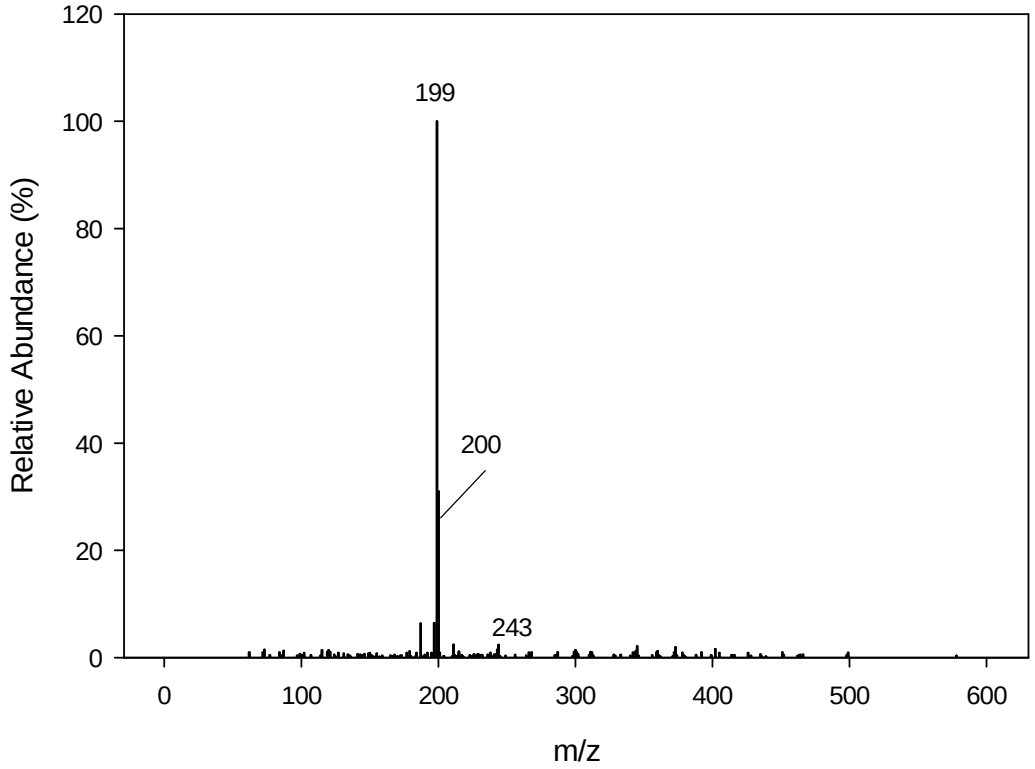


Figure 4. 44. TOF MS ES- spectrum of 500 ppm FLB in methanol (12.92 minute peak).

Two different levels of collision energy were applied for MS analysis of FLB degradation metabolites and blank sample metabolites: 6V (low collision energy) and 15V (high collision energy). Mass spectra of FLB degradation metabolites did not give strong proofs for parent compound. m/z: 167 ion was observed as the precursor ion in MS analysis at low collision energy (Figure 4. 46). At high collision energy, more fragmentation was observed and m/z: 119 ion became precursor ion (Figure 4. 47). By using a guide, some predictions were produced and it was decided that parent compound lost a carboxylic group, which was resulted in product ion m/z: 167. Two hypothesizes support the loss of carboxylic group from parent compound: predicted degradation pathway of FLB based on degradation pathway of monochlorinated biphenyls (Figure 4. 45) and tendency of carboxylic acids to lose carboxyl group first during MS (Sparkman; Vinci et al., 2006). CFM-ID fragmentation predictions also support this situation (Table 4. 20). It was clear that m/z: 211 ion was the parent compound which is end-product of FLB degradation. By using the guide for mass

spectral prediction (Sparkman) and CFM-ID program, predictions for fragmentation of m/z: 211 were made (Figure 4. 48 & Figure 4. 49). The fragmentation patterns strongly supported that the m/z: 211 ion is parent compound and end-product of FLB degradation.

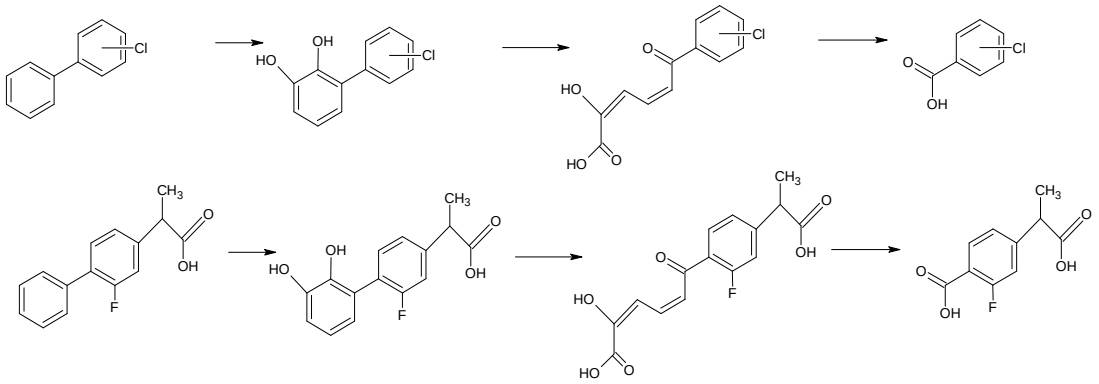


Figure 4. 45. Predicted degradation pathway for FLB based on degradation pathway for monochlorinated biphenyl. The top pathway is the monochlorinated biphenyl pathway. The bottom pathway is a predicted pathway for FLB degradation based on monochlorinated biphenyl pathway

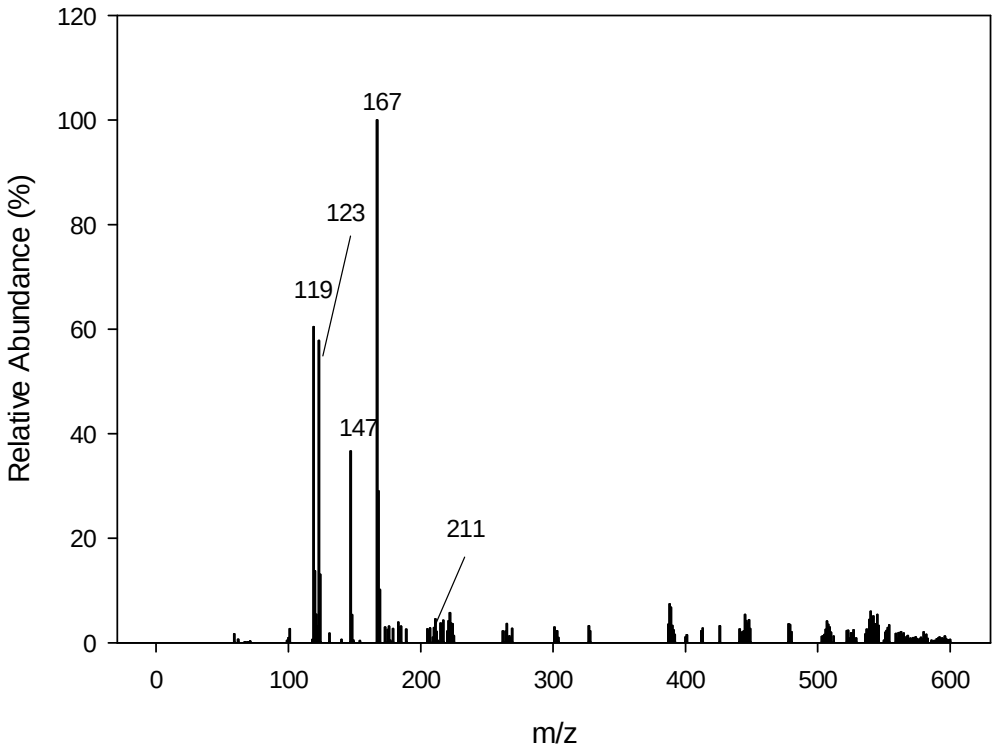


Figure 4. 46. TOF MS ES- spectrum of 4.6 minute peak (sludge sample spiked with FLB) (low collision energy: 6V).

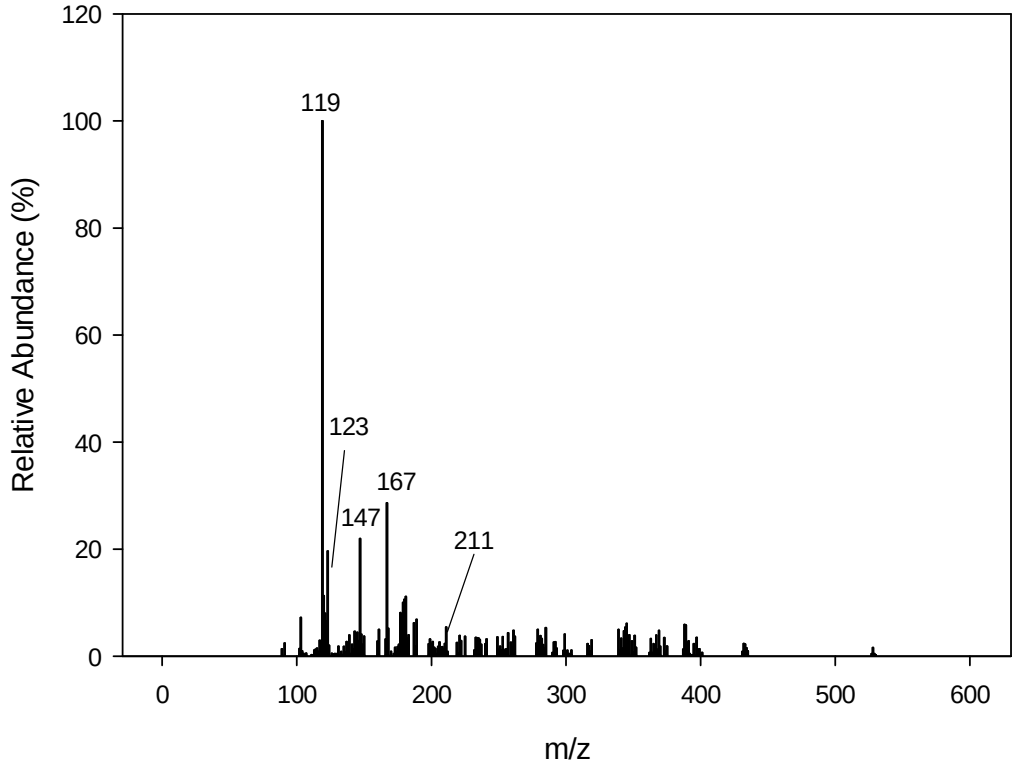


Figure 4. 47. TOF MS ES- spectrum of 4.6 minute peak (sludge sample spiked with FLB) (high collision energy: 15 V).

CFM-ID makes fragmentation predictions based on ESI-MS/MS system while Waters Synapt G1 is a ESI-TOF-MS system. Different systems and operation parameters can explain fragmentation patterns produced by these two systems. It is clear that relative abundances of product ions depend on systems and system configurations.

Table 4. 20. Comparison of results of FLB metabolites spectra generated by Waters Synapt G1 and CFM-ID.

Ions (m/z)	TOF-MS ES- Relative Abundance (%)		CFM-ID Relative Abundance (%)		
	Low Collision Energy (6V)	High Collision Energy (15V)	10V of Collision Energy	20V of Collision Energy	40V of Collision Energy
119	60	100	1	6	6
123	58	20	6	35	44
139	-	-	0	9	100
147	37	22	6	7	48
167	100	29	57	100	52
211	5	6	100	67	10

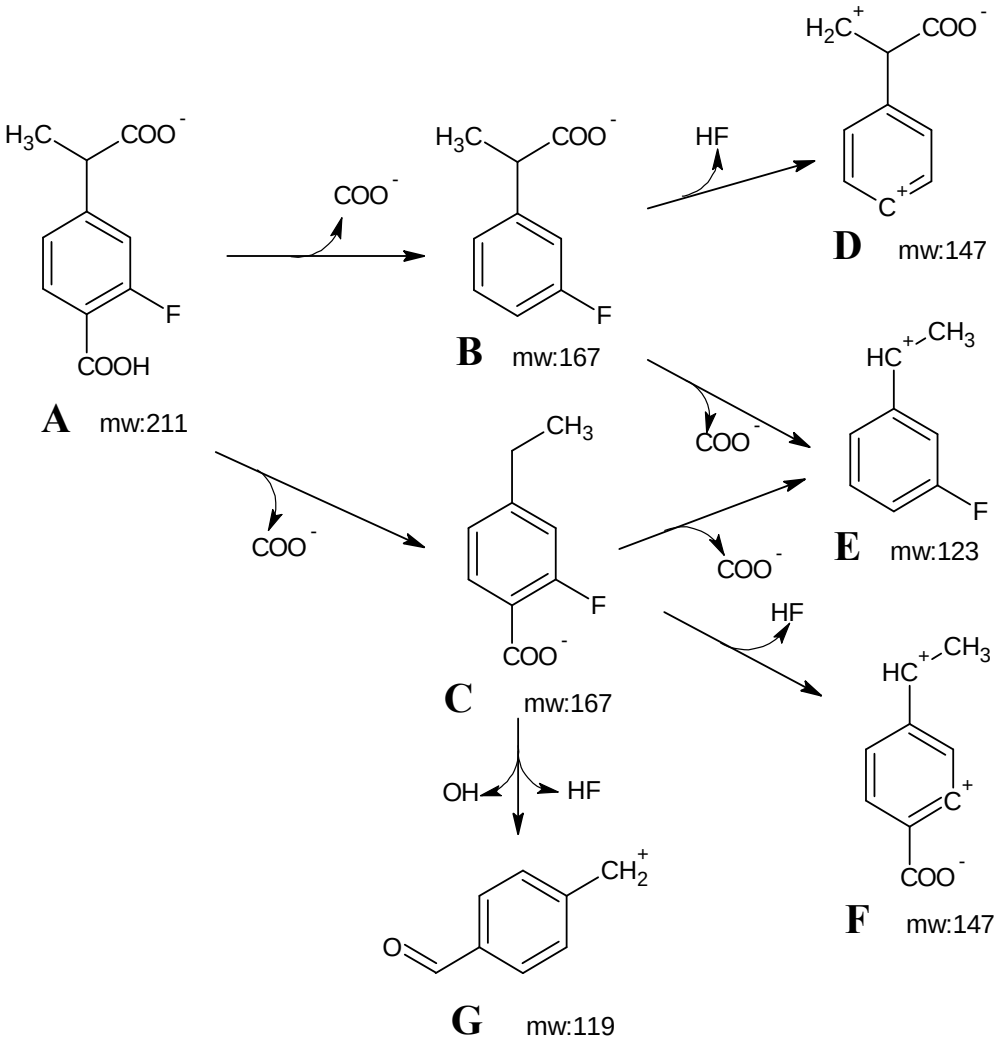


Figure 4. 48. Interpretation of the fragments observed for 4-(1-carboxyethyl)-2-fluorobenzoic acid (m/z: 211) based on guide for mass spectral interpretation.

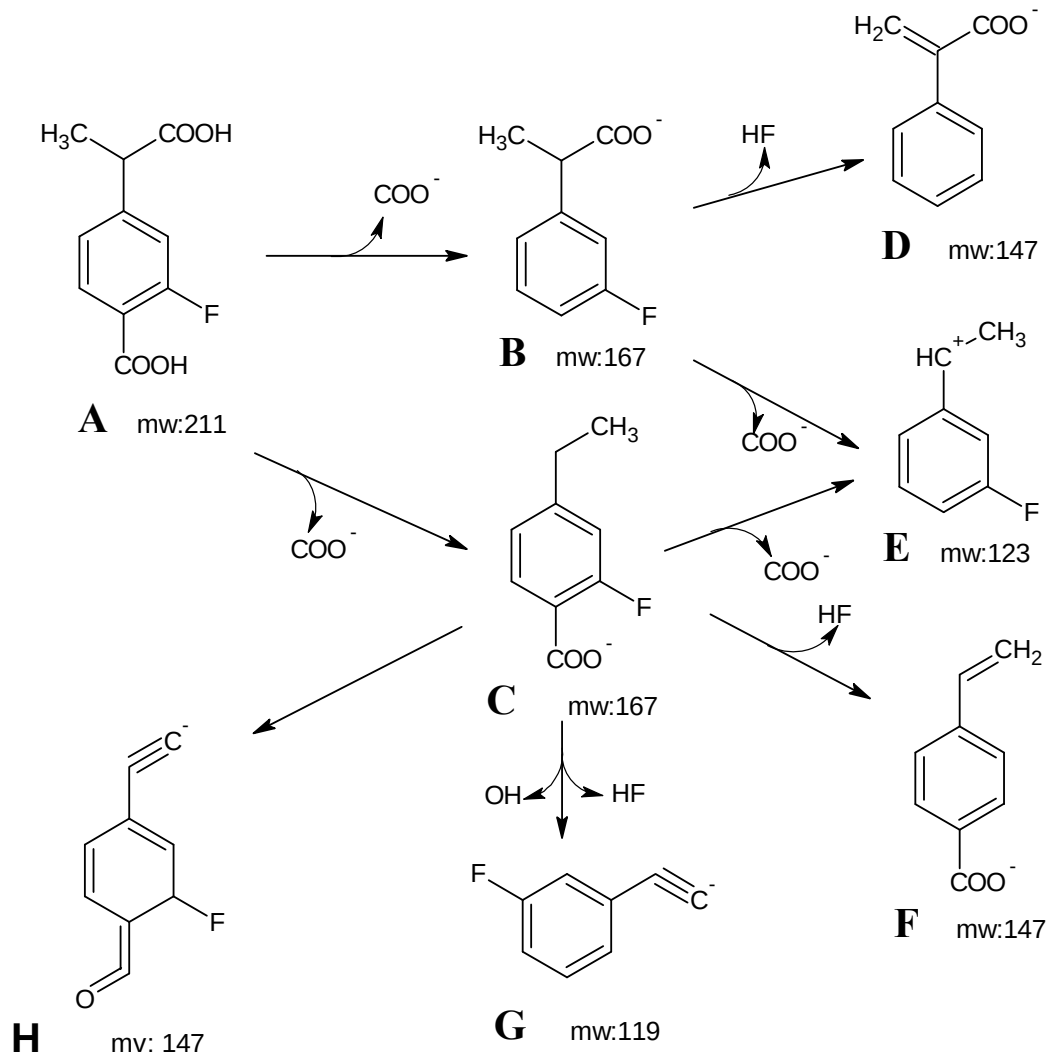


Figure 4. 49. Fragmentation pattern of m/z: 211 based on guide for mass spectral interpretation and CFM-ID program which makes computational predictions.

4.12. Prediction of FLB Degradation Pathway

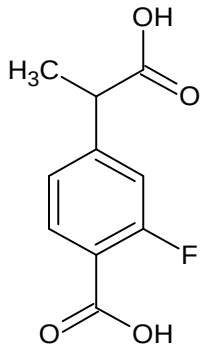
Initially, three degradation pathways were suggested for FLB degradation. The first one is *paa* pathway because FLB is a substituted PAA. This similarity may lead to degradation of FLB with a similar pathway to *paa* pathway. Secondly, *ipf* pathway was suggested because FLB has structural similarities with IBP and the mechanism behind the degradation of IBP might provide an insight for the degradation of other alpha-branched PAAs like FLB, ketoprofen, naproxen. The third one is *bph* pathway. The *bph* pathway takes active role in degradation of most of the halogenated

biphenyls (Adriaens & Focht, 1990; Harkness et al., 1993; Hughes et al., 2011; Murphy et al., 2008).

Observation of yellow coloration during FLB degradation and MS results of FLB degradation metabolites provided strong evidences for a *bph* pathway being active during FLB degradation. MS results suggested that the parent ion is m/z: 211 which is consistent with the molecular weight of the end-product of FLB degradation via a pathway similar to that of monochlorinated biphenyl pathway (Figure 4. 45). Therefore, the degradation pathways for monohalogenated biphenyls, in which the non-halogenated ring is exposed to dioxygenation attack can suggest a model for the degradation pathway of FLB. Several studies demonstrated that the enzymes degrading fluorinated aromatics, such as fluorophenols, fluorobiphenyls and fluorobenzoates are the same as those degrading the non-fluorinated versions of these chemicals. The enzymes having roles in the bph pathway are able to transform monohalogenated biphenyls. BP degradation by bacteria is initiated by biphenyl 2,3-dioxygenase and in case of monohalogenated biphenyls, the degradation ends up with halogenated benzoates (Boersma et al., 2004; Brooks et al., 2004; Ferreira et al., 2008; Murphy et al., 2008). Similarly, the end-product of FLB is also a substituted benzoate. Additionally, there are some studies reporting specialized enzymes employed for degradation of fluorinated compounds. However, there is still much work to be done in order to enlighten the actual mechanisms of degradation in all its aspects (Murphy et al., 2008).

During experiments, FLB was degraded in aerobic sludge and samples taken from sludge were subjected to MS. The end-product was likely a substituted benzoate with a fluorine moiety, which is consistent with predicted pathway for FLB degradation based on monochlorinated biphenyl degradation pathway, MS results and fluoride detection test, and there was no clear indication of further degradation. Therefore, understanding the degradation of halogenated single aromatics may be useful for understanding the degradation, toxicity and inhibitory effects of halogenated biphenyls and their degradation metabolites and may elucidate why there was no further degradation during FLB metabolism.

Degradation of monohalogenated biphenyls and the biphenyls which have halogens on one ring usually result in substituted halobenzoates (Harkness et al., 1993; Hughes et al., 2011). Therefore, understanding the degradation of halogenated benzoates, phenols and benzenes may provide clues for why FLB degradation stops after formation of 4-(1-carboxyethyl)-2-fluorobenzoic acid.



4-(1-carboxyethyl)-2-fluorobenzoic acid

The end product can be described as a substituted 2-fluorobenzoate. K. Engesser et al. (1988) reported that degradation of 2-fluorobenzoate can result in accumulation of toxic 3-fluorocatechol. Additionally, several other studies also reported that in the case of degradation of 2-fluorobenzoate, fluoride ion can be removed in the initial step by dioxygenation or toxic 3-fluorocatechol can be formed by dioxygenation (Engesser & Schulte, 1989; Vora et al., 1988). Successful degradation of 2- and 4-fluorobenzoates has been reported many times, while 3-fluorobenzoates cannot be degraded efficiently due to accumulated toxic intermediates. 2-, 3- and 4-fluorobenzoates were successfully degraded by a FLB 300 strain (*Agrobacterium-Rhizobium* branch) without formation of toxic 3-fluorocatechol. 3-fluorocatechol is strongly resistant against ortho-cleavage enzymes and has tendency to accumulate and has toxic effects on cells (Dorn & Knackmuss, 1978; Engesser et al., 1988; Schreiber et al., 1980). Observation of dark-brownish color can also be an evidence for the accumulation of catecholic intermediates (Vora et al., 1988) which was observed during FLB degradation in both sludge amd MSM. For example, in *Pseudomonas* (spp), 2-FB is metabolized via catechol, which is then further catabolized to *β*-ketoadipate, following the ortho fission pathway. An intermediate in the conversion of 2-FB to 3-fluorocatechol is 6-fluoro DHB; however, since the organism did not have the machinery to tackle halocatechols, they accumulated in the

medium, giving it a brown color (Vora et al., 1988). It is clear that fluorobenzoates exhibit strong resistance against degradation.

To sum up, degradation of FLB resulted in the formation of 4-(1-carboxyethyl)-2-fluorobenzoic acid as a dead-end product. The formation of halogenated benzoates as end-products of monohalogenated biphenyls was reported by many studies. It is possible that degradation of 4-(1-carboxyethyl)-2-fluorobenzoic acid resulted in formation of toxic intermediates such as 3-fluorocatechols and inhibited the degradation process.

CHAPTER 5

CONCLUSIONS

The aim of the study was to contribute to the understanding of the biodegradation of FLB by environmental bacteria. Additionally, there is the possibility to gain understanding of the biological activities of fluorinated aromatics, their fate in the environment and wastewater treatment plants and their tendencies to result in toxic byproducts. It can be concluded from the results:

- FLB disappearance rates were very slow and highly variable between sampling sessions and even within replicates of the same samples.
- FLB degraders could not be isolated. Firstly, McCullar's recipe without trace elements was used as MSM and weak growths were observed on FLB/MSM plates but not in FLB/MSM liquid medium; later it was revealed that the isolates were able to grow using only agar as carbon and energy source, not FLB. In further attempts to obtain FLB-degrading isolates, different minimal medium systems were used and McCullar's recipe was supported by nutrients. These alternative mineral medium systems did not stimulate the growth of FLB degraders. Finally, a co-metabolic stimulation approach was developed with similar chemicals but there was no clear indication of stimulation of FLB degradation. On the other hand, enrichment of TAA and PAA degraders was successful, indicating that McCullar's recipe works.
- Some chemical changes that supported FLB degradation were observed following FLB spike. Firstly, yellow color indicating meta-cleavage of the ring was observed in the sludge spiked with FLB. After appearance of yellow color in sludge, a brownish color indicating accumulation of catecholic compounds was observed.

  A fluoride detection test was applied in order to understand release of fluoride ion during degradation. Test results did not indicate an accumulation of fluoride ion. This might indicate that defluorination did not occur but rather that a fluorinated metabolite accumulated.

During HPLC analysis of FLB degradation, a metabolite was observed with the appearance of a second peak. This metabolite appeared as FLB disappeared, only was present when FLB was added, and was not produced in abiotic systems. The size of metabolite peak depended on FLB concentrations and did not decrease over time periods up to 76 days. This strongly suggested accumulation of a metabolite. Separation and analysis of the peak by LC/MS yielded a mass spectrum consistent with a substituted fluorobenzoate, 4-(1-carboxyethyl)-2-fluorobenzoic acid.

- Altogether, fluoride test and appearance of yellow color and the metabolite allows for a prediction for the pathway. It seems the ring on which there is no fluoride underwent metacleavage. Metacleavage of the ring was supported with appearance of acid-labile yellow color during FLB disappearance and this yellow color got weaker with time. Although there was no strong correlation between FLB dose and optical density of yellow color, metacleavage of the ring was suggested. The accumulation of 4-(1-carboxyethyl)-2-fluorobenzoic acid was consistent with a predicted pathway based on monochlorobiphenyl degradation and with lack of fluoride ion. 4-(1-carboxyethyl)-2-fluorobenzoic acid may lead to formation of 3-fluorocatechols which are known as metabolic poisons. This could also explain why 4-(1-carboxyethyl)-2-fluorobenzoic acid accumulated.

Based on the results, it can be concluded that metabolism of FLB by environmental bacteria resulted in accumulation of 4-(1-carboxyethyl)-2-fluorobenzoic acid dead-end metabolite by a pathway similar to that of monochlorobiphenyl. 4-(1-carboxyethyl)-2-fluorobenzoic acid seems to be persistent and inhibits the degradation process. Additionally, since the FLB degradation rates vary dramatically, FLB and the dead-end metabolite can be discharged into environment from wastewater treatment plants with short sludge retention times.

Future Work

- FLB degradation was studied at very high concentrations. The fate of FLB at environmentally relevant concentrations should be studied.
- FLB degradation should be investigated under anaerobic conditions.

- Although the formation of 4-(1-carboxyethyl)-2-fluorobenzoic acid is consistent with *bph-like* pathway and is supported by the data, an NMR analysis of the metabolite should be carried out to definitively characterize the structure.
- 4-(1-carboxyethyl)-2-fluorobenzoic acid was highly persistent in the aerobic sludge systems tested. Concentrations and fate of this metabolite in sewage treatment systems and the environment should be investigated.
- Toxicological studies of FLB and 4-(1-carboxyethyl)-2-fluorobenzoic acid should be carried out.

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APPENDIX A

Standard Curves

Standard curves were constructed in order to find concentrations of FLB, mTAA and pTAA in ppm with respect to their area.

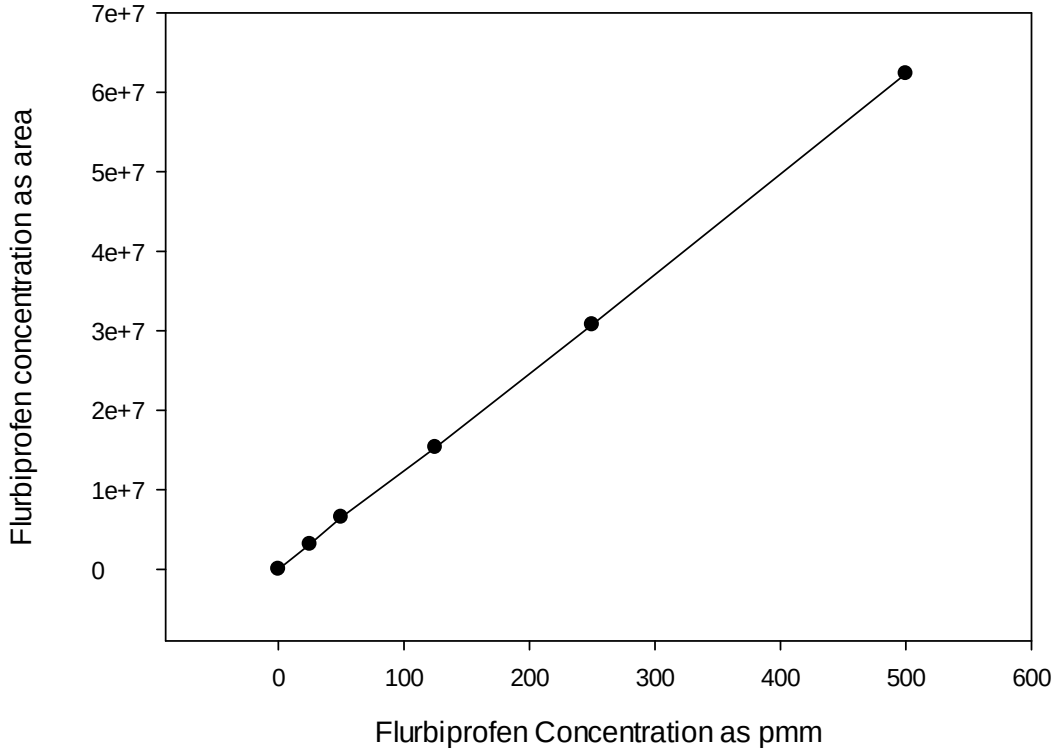

Figure A. 1. HPLC Standard Curve: FLB concentration vs. area (y=124280x, R2=0.999).

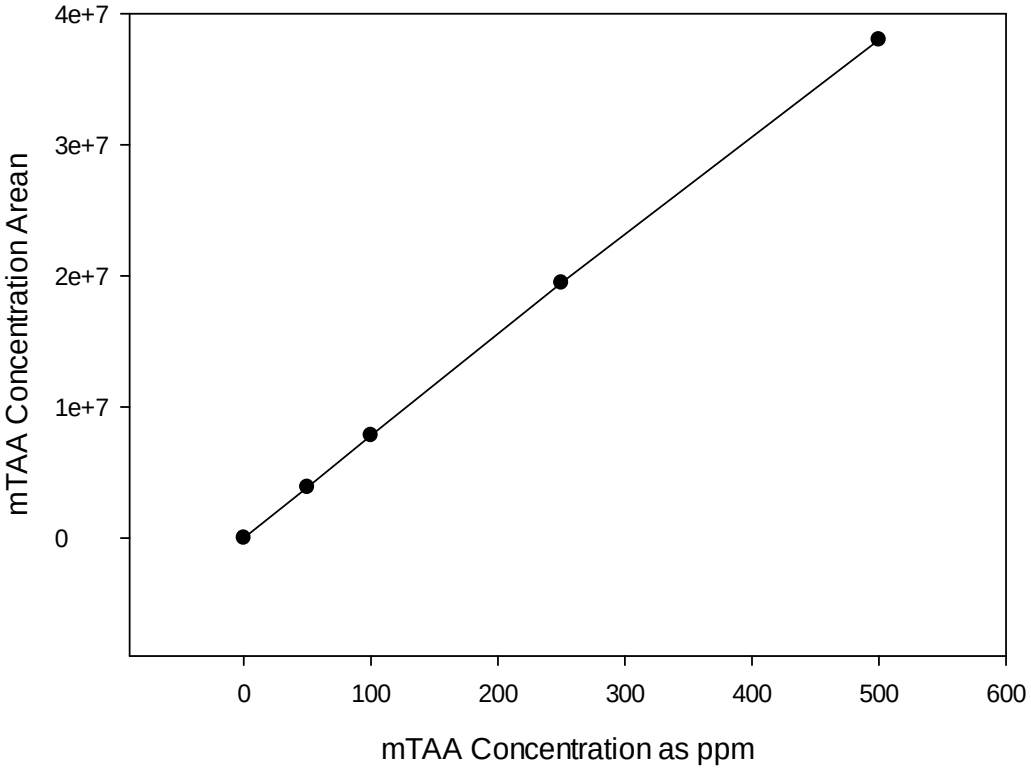

Figure A. 2. HPLC Standard Curve: mTAA concentration vs. area (y=76436x, R2=0.999).

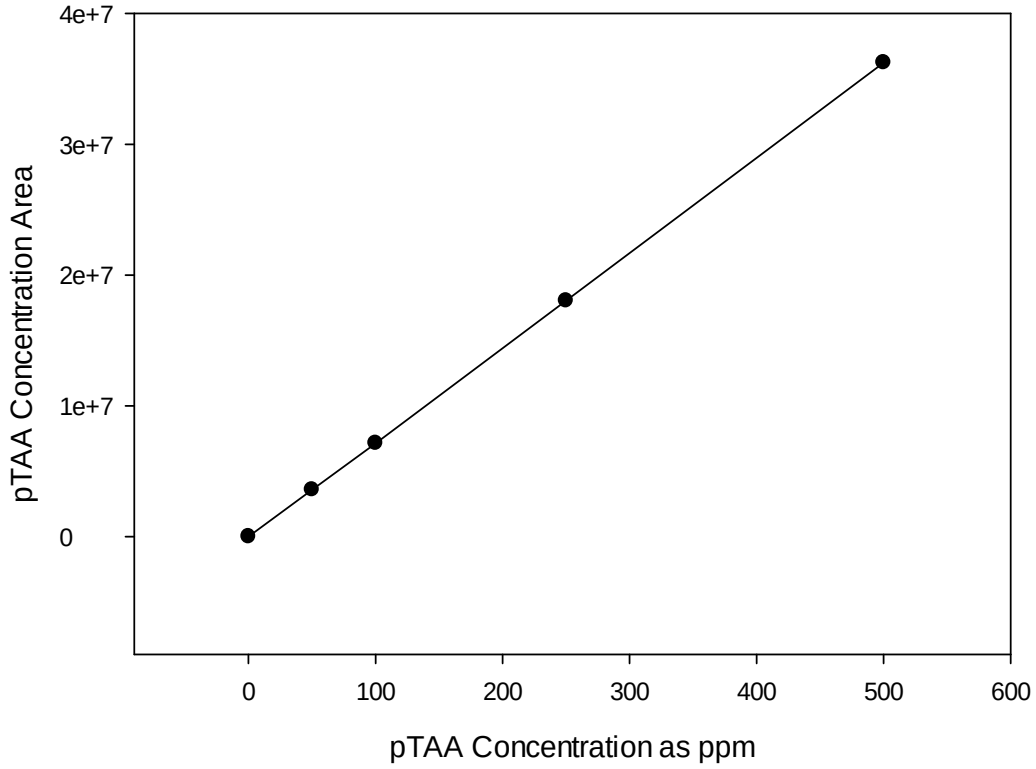

Figure A. 3. HPLC Standard Curve: pTAA concentration vs. area (y=72357x, R2=0.999).

APPENDIX B

Extraction of FLB from Sludge

An extraction method was developed after many trials with different configurations and solvents such as acetone, methanol and ethyl acetate. After centrifugation and separation of sludge supernatant, the following extraction method was applied to the solid:

- Lyophilize the solid phase at -55 ^{o}C of ice condenser temperature, +20 ^{o}C of shelf temperature (CHRIST ALPHA 1-4 LOC-1, Germany)
- Add acetone as much as the original volume of the sample
- Vortex for 10 minutes
- Sonicate for 40 minutes at room temperature in cold water (Voltage line: 230 V, Frequency Line: 50-60 Hz, Power Line US: 80-180 W, Power Line Heating: 100W, Frequency US : 28-34 kHz) (Ultrasonic FALC, Treviglio, Italy)
- Centrifuge and take the supernatant
- Analyze by HPLC

An extraction efficiency of 67 % was determined by addition of set concentrations of FLB to sludge samples followed by immediate extraction. Adding together the concentrations obtained by HPLC from the two phases (supernatant and solid) yields the total concentration when total measured concentration is divided by 0.67. The supernatant of sludge was responsible of 65 ± 2 % of total FLB while the solid phase was responsible of 2 ± 2 % of total FLB. On the other hand, recovery of FLB from solid phase was never greater than 12 % of missing FLB. Low recovery of FLB from solid phase indicated that syringe filters could be responsible for most of the missing FLB.