Biodegradation of the Steroid Progesterone in Surface Waters

A Thesis Submitted for the Degree of

Doctor of Philosophy

By

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Abstract

Many studies measuring the occurrence of pharmaceuticals, understanding their environmental fate and the risk they pose to surface water resources have been published. However, very little is known about the relevant transformation products which result from the wide range of biotic and abiotic degradation processes that these compounds undergo in sewers, storage tanks, during engineered treatment and in the environment. Thus, the present study primarily investigated the degradation of the steroid progesterone (P4) in natural systems (rivers), with a focus on the identification and characterisation of transformation products.

Initial work focussed on assessing the removal of selected compounds (Diclofenac, Fluoxetine, Propranolol and P4) from reed beds, with identification of transformation products in a field site being attempted. However, it was determined that concentrations of parent compounds and products would be too low to work with in the field, and a laboratory study was designed which focussed on P4. Focus on P4 was based on literature evidence of its rapid biodegradability relative to the other model compounds and its usage patterns globally. River water sampling for the laboratory-based degradation study was carried out at 1 km downstream of four south east England sewage works (Blackbirds, Chesham, High Wycombe and Maple Lodge) effluent discharge points. Suspected P4 transformation products were initially identified from predictions by the EAWAG Biocatalysis Biodegradation Database (EAWAG BBD) and from a literature review. At a later stage of the present work, a replacement model for EAWAG BBD (enviPath) which became available, was used to predict P4 degradation and results were compared. Samples were analysed using low resolution and accurate-mass time-of-flight mass spectrometers. Three degradation studies were conducted. Sampling for all studies was carried out at the same time in the year to minimize temporal variability in conditions and allow for effective comparison of results. Androgenic and progesterone yeast screens were carried out to assess the biological activity of transformation products.

Overall, the present study indicates that P4 degradation is relatively rapid in surface waters. Degradation followed the first order degradation kinetic and spiked P4 concentration (1,000 µg/L) was degraded below instrument detection limit (0.32 µg/L) in 72 h (3 days) in all locations except High Wycombe where 90% degradation occurred. Degradation half-life ($t_{½}$) was approximately 7 hours in all sites except High Wycombe (29 hours) where some residual P4 (100 µg/L) was present after 72 hours. Overall, degradation rate was faster (0.1 hours) in the present study than those reported for algae (0.02-0.04 hours), thus, indicating that a more diverse microbial community was responsible for the breakdown of P4 observed in the present study. A number of putative degradation products were identified, including boldenone and (with less certainty) testosterone. The results highlighted that the transformation of progesterone can potentially create other classes of steroids, some of which may still be potent, and possess other types of biological activity. Although progesterone degraded to an androgen (boldenone) as shown by chemical analysis, the concentrations in the environment are unlikely to represent any immediate threat to aquatic resources, as results of the androgen screen did not show any androgenic activity when working at elevated (1,000 µg/L) initial progesterone concentrations.

Declaration

I hereby declare that the work described in this thesis were carried out independently by the author between 2014 and 2017 and has not been submitted anywhere for any award. Where other sources of information have been used, they have been appropriately referenced and all support received have been duly acknowledged.

Jasper Oreva OJOGHORO

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Dedication

This work is dedicated to Jehovah God for His abundant mercies, wisdom and grace upon me throughout my research. Also, to my lovely wife Mrs Victoria Oghenekaro Jasper-Ojoghoro and my adorable son Master Jason Oghenekome Jasper-Ojoghoro for love, devotion, support and encouragement.

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Table of Content

List of Figures

List of Tables

List of Abbreviations

APCI	Atmospheric Pressure Chemical Ionisation
BDP	Beclomethasone Dipropionate
BMP	Beclomethasone Monopropionate
bt	biotransformation
CAD	Collision Gas
CE	Collision Energy
CE	Curtain Gas
cells/mL	cells per millitres
CID	Collision Induced Dissociation
cps	count per second
CRPG	Chlorophenol red-β-D-galactopyranoside
CT	Control Tank
CXP	Collision Cell Exit Potential
DBE	Double Bond Equivalent
DCF	Diclofenac
ddH_2O	double deionised water
DHT	Dihydrotestosterone
DO	Dissolved Oxygen
DP	Declustering Potential

DT	Degradation Tank
E2	17β-estradiol
EAWAG BBD	EAWAG Biocatalysis/Biodegradation Database
EE2	Ethinyl Estradiol
enviPath	Environmental Contaminant Biotransformation Pathway
EP	Entrance Potential
ESI	Electrospray Ionisation
FLX	Fluoxetine
FP	Focussing Potential
GR	Glucocorticoid Receptor
hAR	human Androgen Receptor
HMRS	High Resolution Mass Spectrometer
HPLC	High Performance Liquid Chromatography
hPR	human Progesterone Receptor
hrs	hours
IS	Internal Standard
IV	IonSpray Voltage
KEGG	Kyoto Encyclopaedia of Gene and Genomes
kg/yr	kilogram per year
L/min	litres per minute

lac-Z	lactose Z
LC	Liquid Chromatography
LOD	Limit of Detection
LOQ	Limit of Quantification
LTQ	Linear Ion Trap Quadrupole
m/z	mass-to-charge-ratio
M1	Parent mass
M2	Fragment mass
MeOH	Methanol
MFG	Molecular Formula Generator
mg/L	milligram per litre
MS	Mass Spectrometer
NEB	Nebulizer Gas
ng/L	nanogram per litre
NSAIDs	Nonsteroidal Anti-Inflammatory Drug
OECD	Organisation for Economic Co-operation and Development
ONPG	2-Nitrophenyl β-D-galactopyranoside
P4	Progesterone
$P4_{d9}$	Progesterone d9
pKa	dissociation constant

ppm	parts per million
PPS	Pathway Prediction System
PRPL	Propranolol
QLIT	Quadrupole Linear Ion Trap
QToF	Quadrupole Time-of-Flight
R/RT	Relative Retention Time
rpm	revolution per minute
RSD	Relative Standard Deviation
RT	Retention Time
SD	Standard Deviation
SDS	Sodium Dodocyl Sulphate
SMILES	Simplified Molecular Input Line Entry System
SPE	Solid Phase Extraction
STW	Sewage Treatment Work
$t_{1/2}$	Half-life
TEM	Temperature
ToF	Time-of-Flight
TP-ALG	Algal related transformation products
TP-BBD	EAWAG Biocatalysis/Biodegradation Database predicted transformation product
TPs	Transformation Products

UK	United Kingdom
UV	Ultraviolet
v/v	volume per volume
w/v	weight per volume
WwTPs	Wastewater treatment works
µL/min	microliter per minute
µm	micrometres
$(NH4)_2SO_4$	Ammonium Sulphate
µg/L	microgram per litre
2-ME	2-Mecaptoethanol

List of Publications

Ojoghoro, J. O., Chaudhary, A. J., Campo, P., Sumpter, J. P., & Scrimshaw, M. D. (2017). Progesterone potentially degrades to potent androgens in surface waters. Science of the Total Environment, 579, 1876-1884.

List of Equations

Chapter One: Introduction

1.0 Introduction

Globally, there is an increasing demand for clean water supply. Water is a significant natural resource and an essential component of biological tissues, providing a medium for vital metabolic reactions. Surface and ground waters represent not only important potable supply sources worldwide, but also ecologically, surface waters that are fit for purpose, are major drivers for the efficient functioning of aquatic ecosystems. Thus, the emerging evidence of the presence of pharmacologically active compounds in the aquatic environment is a serious concern (López-Sernaet al. 2012).

The term 'pharmaceuticals' as used in environmental science, refers to a group of biologically active chemical compounds representing a broad range of therapeutic classes of drugs and that are designed to interact with specific organ sites (receptors) to elicit a desired therapeutic response in the target organism (Guo et al. 2014; Boxall et al. 2012; Pérez & Barceló 2007). Worldwide, over 4,000 different pharmaceuticals are used in human medicine (Guo et al. 2014; Boxall et al. 2012), and additionally, some specialised products are used in veterinary practice (Liu et al., 2015a; Liu et al., 2015b). Globally, annual average consumption of pharmaceuticals varies from 15 g per capita in less industrialised countries to 50-150 g per capita in industrialised countries (López-Serna et al. 2010). In the European Union alone, about 3,000 different substances are approved for use as pharmaceuticals (Al Aukidy et al. 2012; Sándor et al. 2012). Reports of a recently completed UK Water Industry chemical investigation program shows that over 1,754,488 kg/yr of common analgesics (paracetamol, ibuprofen, naproxen and aspirin) were prescribed in primary health care in Britain (Guo et al. 2014). The annual consumption of progesterone and its derivatives, progestins, by a 60 million population each in France and the United Kingdom was estimated to be 12,800 and 1,700 kg respectively, while in the Czech Republic, 2,400 kg/yr for a 10 million population (Table 1.1) (Besse & Garric, 2009; Kumar et al., 2015). These represent an approximately 213.3, 28.3, and 240 mg/yr per individual for progesterone alone in France, United Kingdom and the Czech Republic respectively. In the United Kingdom, progesterone use represents a higher usage relative to the combined use (706 kg/yr) of androgens and estrogens (Fayad et al. 2013;

Runnalls et al. 2010). In Hungary, 7,746 kg of diclofenac was sold in 2004 (Sándor et al. 2012). With human population predicted to increase into the future (Liu et al., 2015c) and the increased emphasis on improving the quality of human and animal life, pharmaceutical usage is expected to increase consistently into the future as new and more effective drugs are discovered and produced (Petrović et al. 2014).

Table 1.1: Annual Consumption of Progesterone and its Derivatives, Progestins

Country	Population (million)	Consumption (kg/yr)	Per capita use (mg/yr)
Czech Republic	10	2,400	240
France	60	12,800	213
United Kingdom	60	1,700	28.3

The use of pharmaceuticals has benefited society greatly as they are taken to protect, preserve and improve the quality of human health. However, these compounds have become emerging contaminants of scientific and public concern largely due to their widespread detection in the water cycle - especially in surface water. The first report of the presence of pharmaceuticals in the water environment was in 1965 by Stumm-Zollinger & Fair who showed that human steroids were not completely degraded during wastewater treatment (Stumm-Zollinger and Fair, 1965). Although further reports in the 1980s demonstrated the presence of these compounds in surface waters (Tabak et al. 1981; Aherne et al. 1985; Aherne and Briggs, 1989), very little interest was focused on them, perhaps because of their trace environmental levels. It was not until the 1990s when biological screening methods were developed and the toxicity of environmentally relevant concentrations of hormones on fish was demonstrated (Desbrow et al. 1998; Routledge et al. 1998; Jobling et al. 1998, 2002, 2006) that the interest of the scientific community was focussed on the significance of these microcontaminants. The first evidence of the presence of other pharmaceuticals in surface and wastewater were

published in the 1970s (Garrison et al., 1976; Hignite & Azarnoff 1977). Subsequently, studies in the 20th and the 21st century have measured a wide range of pharmaceuticals residues including their human metabolites in a range of environmental compartments , including water, sediment and soil (Haddad & Kümmerer, 2014; J. Liu et al., 2015; Peng et al., 2014; Runnalls et al., 2010; Ternes & Joss, 2006; Ying et al. 2002).

Concentrations of pharmaceutical compounds vary widely for different compounds and between different water types; wastewater, surface and groundwater, with hospital, pharmaceutical manufacturers and municipal wastewater having the highest recorded concentrations of all types of pharmaceuticals (Al Aukidy et al. 2012; Verlicchi et al. 2012; 2014). Taking diclofenac as an example, concentrations of 6.9 to 2,031 µg/L were reported by Sim et al. (2011), for hospital and pharmaceutical wastewater in South Korea. In municipal wastewater, Vieno & Sillanpää 2014 reported concentrations of 7.1 µg/L. Generally, reported concentrations of diclofenac in rivers have been generally <100 ng/L. However, concentrations of 1,200 and 8,500 ng/L were separately reported in Germany and Pakistan (Vieno, 2007; Scheurell *et al*., 2009; Ternes, 1998). High concentrations of diclofenac in drinking water were reported in some parts of Europe. For example, in Spain and Germany, 380 ng/L concentrations were reported (Heberer 2002; López-Serna et al. 2010; 2012). For other compounds, concentrations of 15.4 to 384.5 ng/L were reported by Liu *et al*., 2015 for antibiotics, Nonsteroidal anti-inflammatory drug (NSAIDs), β – blockers, antiepileptic and steroid hormones in surface waters that were downstream of sewage works in China. Reported groundwater concentrations of steroid hormones ranged from 9 to 115 ng/L for steroids in groundwater (Lopez, et al. 2015), with P4 and androstenedione ranking highest. Concentrations of other pharmaceuticals in Lopez et al. (2015) study ranged from 3 to 360 ng/L with non-prescription drugs recording highest. Concentrations of the common analgesics, ibuprofen, diclofenac and acetaminophen ranged from 19 - 34 ng/L. More information on the published environmental concentration of compounds selected for the present study are presented in section 2.4.

1.1 Sources of Pharmaceuticals in the Water Environment

The ubiquity of pharmaceuticals in the water environment is related to their constant excretion with urine and faeces following therapeutic use. Pereira et al. 2015 noted that human excretion represented the largest single pathway of pharmaceutical contamination of the water cycle. Pharmaceutical compounds are applied topically as gels, inhaled, ingested as tablets/capsules or as injections. Gels are absorbed up to 6 to 7% (Davies & Anderson, 1997) and the remaining residues are directly washed off, passing to wastewater treatment plants (WwTPs). Whatever the mode of administration, upon achieving systemic circulation, drugs undergo a set of biochemical reactions (metabolism) in the liver, transforming them to different extents depending on the individual drug (Pérez & Barceló 2007; 2008; Cwiertny et al. 2014). They are however not completely destroyed in the body, but are excreted as a mixture of metabolites, conjugates and unchanged parent compound with urine and faeces (Sammartino et al, 2008; Heberer 2002). Hepatic metabolism of drugs occurs in two main phases, phase I and II. The first phase involves oxidation/reduction and hydrolytic cleavage, leading to more polar molecules than the parent compounds (López-Serna et al. 2012). Depending on the pharmaceutical compound, the second phase (aka conjugation phase) consist of the process of glucuronidation, acetylation and sulphation, that allow metabolites to become hydrophobic and water soluble enough to be eliminated through urine and/or faeces (López-Serna et al. 2012). Thus, human metabolites of drugs can be phase I or phase II metabolites depending on the biochemical reaction resulting in their formation (Evgenidou et al. 2015). Phase I metabolites result from the oxidation, reduction and hydrolysis that some parent compounds, e.g. ibuprofen, diclofenac, propranolol, etc., undergo *in vivo* to increase their aqueous solubility, a process that aids their rapid elimination from the body (Pérez & Barceló, 2007). Phase II metabolites, also referred to as conjugated metabolites, are mostly products of glucuronidation and less often sulfation, acetylation and methylation depending on the parent compound (Pérez & Barceló 2007). Phase II reactions add a polar molecule e.g. glucuronic acid, sulfate, acetyl or methyl functional group to a parent compound or to a phase I product (Evgenidou et al., 2015; Liu et al. 2011). Like phase I reactions, glucuronidation increases the

polarity of parent compounds, thus facilitating their rapid elimination with urine. To illustrate, conjugation of steroids (e.g. natural estrogen) adds a glucuronide functional group to the molecule, a process that makes natural estrogen conjugates more polar and less estrogenic than their natural parent compounds (Liu et al. 2015d). The latter points thus suggest that conjugation is a detoxification reaction that helps protect cells from the toxic effects of parent compounds. Phase II metabolites can be deconjugated back to their respective parent compound by the enzymes *β*-glucuronidase (in the case of glucuronidation) or arylsufatase for sulfation, both secreted by microorganisms or specific organs in higher organisms (Evgenidou et al., 2015; Liu et al., 2015d; Gomez et al. 2009). For example, Gomez et al. (2009) reported the preferential deconjugation of glucuronides conjugates of estrogens relative to sulfate conjugates in crude sewage. The authors work, demonstrated that deconjugation is a biotic rather than abiotic process, requiring the metabolic activities of microorganisms in cleaving associated conjugate moiety. Furthermore, there are literature evidence of the ready deconjugation of glucuronated estrogens in sewer, due to the large amounts of the β-glucuronidase enzyme produced by faecal bacteria (*Escherichia coli*) (D'ascenzo et al. 2003; Heberer, 2002; Baronti et al. 2000). Based on the daily human excretion of conjugated estrogens, Baronti et al. (2000) noted that deconjugation of estrogens preferentially occurs in sewers. Additionally, two glucuronides of 17β-estradiol (17β-estradiol-17-glucuronide and 17β-estradiol-3-glucuronide) were reported to be cleaved when in contact with the diluted activated sludge solution resulting in release of 17β-estradiol (Ternes et al. 1999a; b). Ternes et al. (1999a; b) noted that depending on the processing time conjugates like glucuronides, are cleaved during WwTPs. It is logical therefore to assume that the deconjugation reaction occurring in sewer, wastewater treatment (activated sludge process) and in the environment, may account for some of the parent pharmaceuticals regularly detected in wastewater and surface waters (Ferrer and Thurman, 2010; D'Ascenzo et al., 2003).

Following excretion, parent compounds, their metabolites including conjugates enter sewers en-route to wastewater treatment facilities. Conventional wastewater treatment processes

(WwTPs) do not completely eliminate these compounds (Haddad et al., 2015; Verlicchi et al., 2012; Verlicchi & Zambello, 2014). Some compounds undergo structural reorganisation or transformation in sewers (Jelic et al. 2015), during wastewater treatment (Evgenidou et al., 2015) and in the environment (Pico et al. 2015) by a variety of biotic degradation (by bacteria and fungi) and abiotic processes (hydrolysis and photolysis) occurring in such systems. Incomplete breakdown by WwTPs and transformation reactions, thus results in the constant input of pharmaceutical residues with effluents into surface waters (Fig.1.1). Since there is a growing emphasis on water reuse, some of the fresh water bodies that receive effluents from WwTPs effluent are also sources of water used for irrigation purposes, fish farming and provision of municipal drinking water. Thus, constant ingestion of these micropollutants with drinking water is possible, although potable water treatment is likely to further remove these compounds (Escher et al., 2014; Fatta-Kassinos et al. 2011; Janna et al. 2011; Petrović et al., 2014).

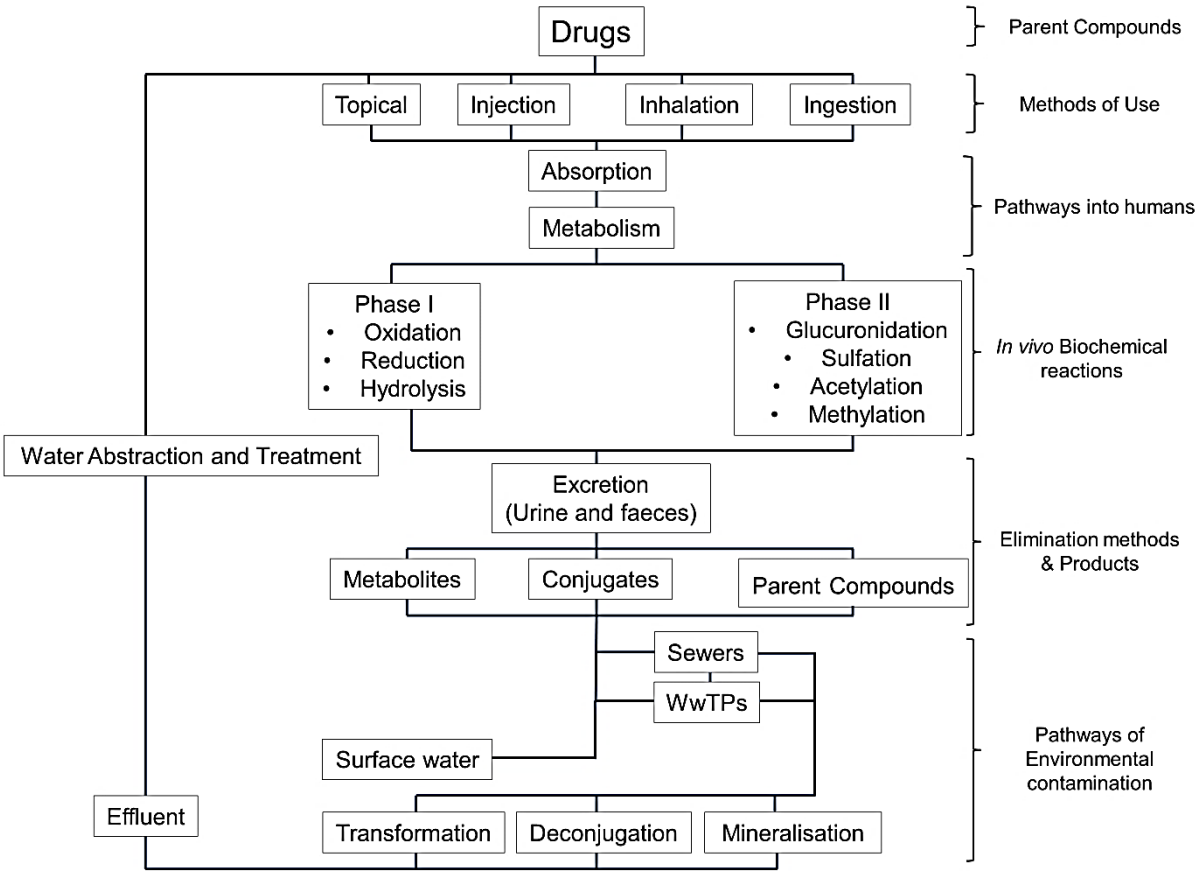


Figure 1.1: Pathways of environmental occurrence of pharmaceuticals (Evgenidou et al., 2015; Liu et al. 2011 Pérez & Barceló 2007)

Many studies measuring pharmaceuticals occurrence, understanding their environmental fate and the risk they pose to environmental resources have been published (Haman et al. 2015; Pereira et al. 2015; Altenburger et al. 2015; Escher et al. 2014; Li et al. 2016; Zhang et al. 2014; Agunbiade & Moodley 2014; Al Aukidy et al. 2012; Tölgyesi et al. 2010; Besse & Garric 2008). Substantial amounts of information are now available on the occurrence of parent drugs in the environment (Boxall et al., 2012; López-Serna et al., 2012), their physicochemical and toxicological properties (Kosma et al. 2014; 2015) as well as their behaviour and environmental fate (Evgenidou et al., 2015). However, less is known about the relevant transformation products (TPs) that result from the wide range of biotic and abiotic degradation processes that these compounds undergo in sewers, storage tanks, during engineered

treatment and in the environment (Evgenidou et al., 2015; Jelic et al., 2015; Johnson & Williams, 2004; López-Serna et al., 2012).

Since pharmaceuticals are contaminants of environmental concern, it is important to understand their transformation pathways in the environment and during wastewater treatment. To illustrate, TPs of some commonly used drugs (e.g. antibiotics, analgesics and steroids), have been reported to retain the structural backbone of the biologically active parent compounds and have exhibited equal or enhanced biological activity relative to the parent compound (Diniz et al. 2015; Haddad & Kümmerer 2014; Cwiertny et al. 2014). Furthermore, a reflection on lessons learnt from the adverse environmental impacts of organochlorine pesticides due to the prioritization of their economic benefits over and above understanding their environmental fate (Carson 2002; Krebs et al. 1999), stresses the need for information on the identity and toxicity of potential TPs resulting from natural and engineered degradation of pharmaceuticals. Thus, a detailed assessment of the fate of parent compounds in the environment will not be complete without holistic information on the formation of their TPs (Li et al., 2016). Such knowledge is especially relevant as transformation is often not detoxification and TPs may be persistent and/or biologically active and capable of eliciting a toxicological effect on non-target organisms (Li et al., 2016; Pico et al., 2015). An important consideration is that, TPs of some commonly used drugs (e.g. antibiotics, analgesics and steroids), have been reported to retain the structural backbone of the biologically active parent compounds and have exhibited equal or enhanced biological activity relative to the parent compound (Cwiertny et al., 2014; Diniz et al., 2015; Haddad & Kümmerer, 2014). Transformation of acyclovir, an antiviral drug produced two TPs that were reported to have adverse effects on *Daphnia magna* (reduction in reproduction levels) and inhibition of algae growth (Schlüter-Vorberg et al. 2015). Wang & Lin 2012 reported the increased potency that UV irradiation of surface water containing cephalosporin antibiotics had on algae. Back transformation of TPs to their corresponding parent molecules has been reported (Su et al. 2016), a process that increases the exposure to parent pharmaceuticals downstream of point of initial transformation and/or the point of effluent discharge (Qu et al., 2013). The extent of

transformation can be high as shown in Huntscha et al. (2013) in a riverbank filtration study where atenolol was almost totally transformed to atenolol acid and further evidenced by studies on the attenuation of selected pharmaceuticals in the hyporheic zone of river beds (Li et al. 2015). Over 80% of acetaminophen was reported to be attenuated in the hyporheic zone of river beds (Li et al. 2016). In some case however, transformation may be partial to zero. For example, partial attenuation of tramadol and sulfamethoxazole (20%) in rivers was reported (Li et al., 2016). In the Li et al. (2016) study, carbamazepine was reported to be persistent (zero transformation), although its biotransformation to carbamazepine-10, 11-epoxide was reported by other workers (Li et al. 2014; Li et al. 2015) and detected in surface water (Huntscha et al. 2013; 2012; Langford & Thomas 2011). Carbamazepine was also reported to be recalcitrant to biodegradation and showed zero elimination during wastewater treatment with activated sludge (Kosjek et al. 2009). This shows the variability in the transformation potential of chemical compounds depending on structural complexity, especially as biological transformation reactions are reported to occur on locations in a compound's structure with the most readily available electrons (Kosjek et al. 2009).

1.2 Research Aim and Objectives

This work aims to investigate removal and degradation of pharmaceuticals in natural (rivers) and artificial (constructed wetland) systems, with a focus on the identification and characterisation of transformation products.

Objectives:

i. Identify compound(s) to study depending on literature evidence on their amenability to biological degradation and degradation half-life.
ii. Decide what systems (effluent receiving rivers or constructed wetland) to undertake transformation studies in, especially considering site accessibility.
iii. Develop analytical methods for the detection of model compounds.

iv. Model pharmaceutical degradation pathways and evaluate possible transformation products from the literature.

v. Undertake degradation studies and identify transformation products.

vi. Assess biological activity of the transformation products.

vii. Study the reproducibility of degradation of model compounds and transformation products formed.

Chapter Two: Literature Review

2.0 Introduction

It is well established that both aerobic and anaerobic processes in wastewater treatment result in breakdown of pharmaceutical compounds, the extent of which is compound specific (Wols et al. 2015; Gros et al. 2014; Salgado et al. 2013; Lee & von Gunten 2010; Khanal et al. 2006). These biodegradation processes are used either alone, or with increasing frequency, alongside advanced processes such as photolysis, adsorption and oxidative technologies in "advanced engineered treatment" which aims to achieve more effective removal of pharmaceutical residues (Chen et al. 2012; Wols et al. 2013). However, it is evident that residues are still discharged to receiving waters, and it is therefore desirable that concentrations of residues are further reduced, along with associated ecotoxicological risks, through natural attenuation to minimize treatment cost and reduce ecological exposure to potent compounds (Long et al. 2013; Cruz-Morató et al. 2013; Sándor et al. 2012; Fayad et al. 2013).

The underlying assumption that drives risk-based approaches to environmental protection and many contaminant fate characterisation models, is that, analytical evidence of the removal of previously identified toxic parent compounds following treatment, translates to removal of its associated adverse biological effects. Thus, after treated effluents are analysed for targeted parent compounds (assuming such molecules degrade), and analytical results shows the none presence of such molecules, elimination of initially observed biological activity is most often inferred without further toxicological investigations. However, evidence emerging from research in the last decade suggests that there are occasions when degradation products formed following treatment may be of some concern (Cwiertny et al., 2014). For example, Diniz et al. (2015) reported that the photolytic degradation of diclofenac following UV irradiation resulted in products that were more toxic to zebrafish (*Danio rerio*) than the parent compound. Degradation products identified by Diniz et al. (2015) were previously reported by Salgado et al. (2013) to be present in drinking water and in treated effluent, thus, showing the potential ubiquity of such bioactive by-products in the water cycle. Similarly, sunlight irradiation of diclofenac contaminated water samples was reported to result in products that exhibited six-

fold phytotoxicity on the unicellular chlorophyte, *Scenedesmus vacuolatus* after two days of direct sunlight exposure (Schmitt-Jansen et al. 2007). A solution resulting from photolytic degradation of 100 mg/L metronidazole was reported to have increased toxicity on *Allium cepa* relative to the parent compound (Dantas et al. 2010).

Worldwide, many degradation products along with their parent compounds have been detected in WwTPs effluent (irrespective of the treatment methods) and in ground and surface waters (Fang et al. 2012; Fatta-Kassinos et al. 2011; Haddad et al. 2015; Heberer, 2002; Lopez et al., 2015; Moreno-González et al., 2014; Pereira et al., 2015; Schlüter-Vorberg et al., 2015; Su et al., 2016). Adverse effects of degradation products on the biolouminscence of *Vibrio fischeri* was reported for the photocatalytic by-products of metoprolol degradation (Veloutsou et al. 2014). Trovó et al. (2009) and Fatta-Kassinos, Vasquez, et al. (2011), reported the formation of 9 degradation products following solar irradiation of distilled water spiked with sulfamethoxazole. After 30 hours of irradiation, sulfamethoxazole was completely degraded. However, the system's toxicity to *Daphnia magna*, measured by the mobility of the test organism, increased from initial 60% prior to irradiation to 100%, indicating that degradation products were more potent than the parent compound. DellaGreca et al., (2003) and Isidori et al. (2005) reported the formation of six (6) by-products following irradiation of water containing naproxen. Toxicity testing of the irradiated solution showed increased acute and chronic toxicity to *Daphnia magna* and *Vibrio fischeri*, relative to naproxen.

Advanced oxidation treatment approaches have been applied in some parts of the world (industry scale) for the treatment of wastewater because of the process reputation for the transformation of a range of organic compounds including pharmaceuticals (Fayad et al. 2013; Wols et al. 2013; Chen et al. 2012; Fatta-Kassinos et al. 2011). For example, Lee & von Gunten (2010) reported the application of oxidation in the treatment of a range of pharmaceuticals including; atenolol, carbamazepine, EE2, ibuprofen and sulfamethoxazole in drinking and wastewater. Chen et al. (2012) reported the oxidative degradation of EE2 with hydrogen peroxide, with the formation of products that retained parent's estrogenic potency. Furthermore, Fayad et al. (2013) reported the oxidative degradation of a range of

progestogens including progesterone spiked at a concentration range of 278-3,070 µg/L, in wastewater using potassium permanganate. Oxidative degradation of clofibric acid by ozonation was reported to result in the formation of seven (7) products (Rosal et al., 2009). Subsequent bioassay using *Daphnia magna* and *Vibrio fischeri also* showed enhanced toxicity of the products relative to the parent compound. Similarly, toxicity of aqueous solution from the oxidative degradation of paracetamol by ozone and hydroxyl radicals on *Vibrio fischeri* was reported to increase, even though paracetamol concentration decreased (Hamdi et al. 2014). Hydroquinone and two other by-products were reported in that study.

The above examples are of chemical pathways; however, there is also evidence that biological transformation can be important. For example, the conversion of the steroid estrone was reported to produce 17*β*-estradiol, a bioactive product much more potent than the parent compound (Khan et al. 2008; Khanal et al., 2006; Yang et al. 2011). Another example, is the use of activated sludge in the treatment of metoprolol, a β-blocker whose ecotoxicological effects on the rainbow trout *Oncorhynchus mykiss* has been reported (Huggett et al. 2002; Triebskorn et al. 2007; van den Brandhof & Montforts, 2010). Metroprolol was shown to form five (5) degradation products including O-desmethylmetoprolol which was reported to moderately inhibit the bioluminescence of the bacterium *Vibrio fischeri*. (Rubirola et al. 2014). In China, the degradation of progesterone by two freshwater algae, *Scenedesmus obliquus* and *Chlorella pyrenoidosa*, was reported to result in the formation of nine (9) products, four (4) of which (Testosterone, 5α-Dihydrotestosterone (5α-DHT), 4-Androstene-3,17-dione (AED) and 17β-Boldenone), are known androgens regularly measured in rivers (Peng et al., 2014; Thomas et al., 2002). Thus, a thorough assessment of the fate of parent pharmaceuticals in the environment will be incomplete without comprehensive information on the formation of their degradation products.

Table 1.2: Examples of Products of Biological Transformation Processes

Parent Compound	Transformation Products	Matrix	Laboratory or Site	Source
Estrone	17*β*-estradiol	Rivers	Site	Khanal et al., 2006
Metroprolol	O-desmethylmetoprolol Metoprolol acid α-hydroxymetoprolol New (unnamed) product	Activated Sludge	Laboratory and Site	Rubirola et al. 2014
Progesterone	Testosterone 5α-Dihydrotestosterone (5α-DHT) 4-Androstene-3,17-dione (AED) 17β-Boldenone Epi-androsterone Androsta-1,4-diene-3,17-dione 1,4-Pregnadiene-3,20-dione 3,20-Allopregnanedione 3β-Hydroxy-5α-pregnan-20-one	Aqueous solution (BG 11 medium)	Laboratory	Peng et al. 2014

2.1 Transformation of Pharmaceuticals

The biotic and abiotic treatment processes that parent pharmaceuticals undergo during engineered treatment and in the environment often do not drastically alter their structure, but transform them into compounds that bear structural resemblance to the parent molecule (Li et al., 2014; Llorca et al., 2015; Rubirola et al., 2014; Schlüter-Vorberg et al., 2015). For example, microbial transformation of clofibric acid in activated sludge produced 4-chlorophenol (Fig. 2.1), a molecule that retained the core parent phenol ring (Kosjek et al. 2009). Wick et al. (2011) reported that TPs resulting from the biological degradation of the opium alkaloid, codeine only had slight molecular modification, such as double bond shift, introduction of hydroxyl functional groups or demethylation of amine, from the parent compound.

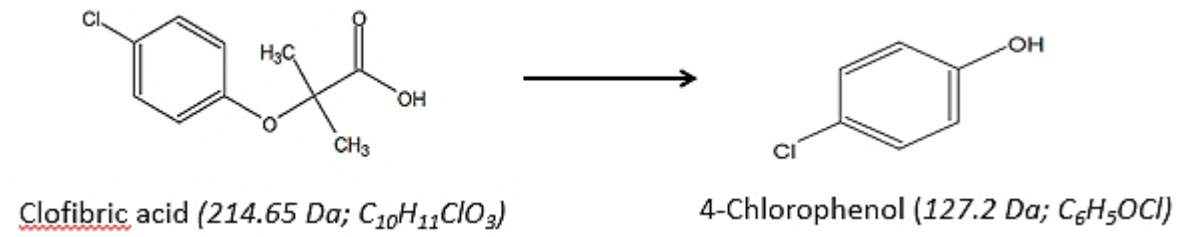


Figure 2.1: Transformation of clofibric acid to 4-Chlorophenol. Product bear structural resemblance to the parent compound keeping the phenol ring structures as the parent (Da: Daltons).

Thus, the term 'transformation' as would be used in this work refers to minor modification in the molecular structure of parent compounds involving the structural reorganisation of composite peripheral elements, functional groups and/or cleavage of side-chain or the substitution of a functional group and/or element with an external one. Pharmaceutical transformation as used in the present study would therefore convey the idea of substantial conservation of core parent structural backbone in products.

Conservation of parent structure in TPs has been widely reported (Diniz et al. 2015; Kumar et al. 2015; Cwiertny et al. 2014; Kolodziej et al. 2013; Wang & Lin 2012; Wick et al. 2011). For example, photolytic degradation of diclofenac and atenolol was reported by Diniz et al. (2015) to result in the formation of five (5) TPs all of which retained the core structural backbone of their respective parent (Fig. 2.2A (diclofenac) and 2.2B (atenolol)). Similarly, algae biotransformation of progesterone resulted in the formation of nine (9) TPs including 3β-Hydroxy-5α-pregnan-20-one and 3, 20-Allopregnanedione (Fig. 2.3A). Reported TPs all had the core parent tetracyclic backbone ring. Furthermore, degradation of 17β-estradiol by activated sludge bacteria *Nitrosomonas europaea* and *Nocardia sp.* was reported to result in the formation of estra-1,3,5(10),16-tetraen-3-oll (Nakai et al., 2011) and estrone (Yu et al. 2013; 2007). Both TPs retained the structural backbone of 17β-estradiol (Fig. 2.3B). Biotransformation of 17α-ethinyl estradiol by nitrifying bacteria was reported by Yi & Harper (2007) to result in the formation of 2-hyroxyl-17α-ethinyl estradiol (Fig. 2.3C). Similarly, biotransformation of 17α-ethinylestradiol by the sphingolipids producing environmental bacteria *Sphingobacterium sp*., was reported by Haiyan et al. (2007) to result in the formation

of estrone (Fig. 2.3C). Direct and indirect photo-transformation of three (3) β-lactam cephalosporin antibiotics, cephradin, cephalexin and cephapirin, resulted in twelve (12) by-products, all of which bore striking structural resemblance to their respective parent molecules (Wang & Lin, 2012).

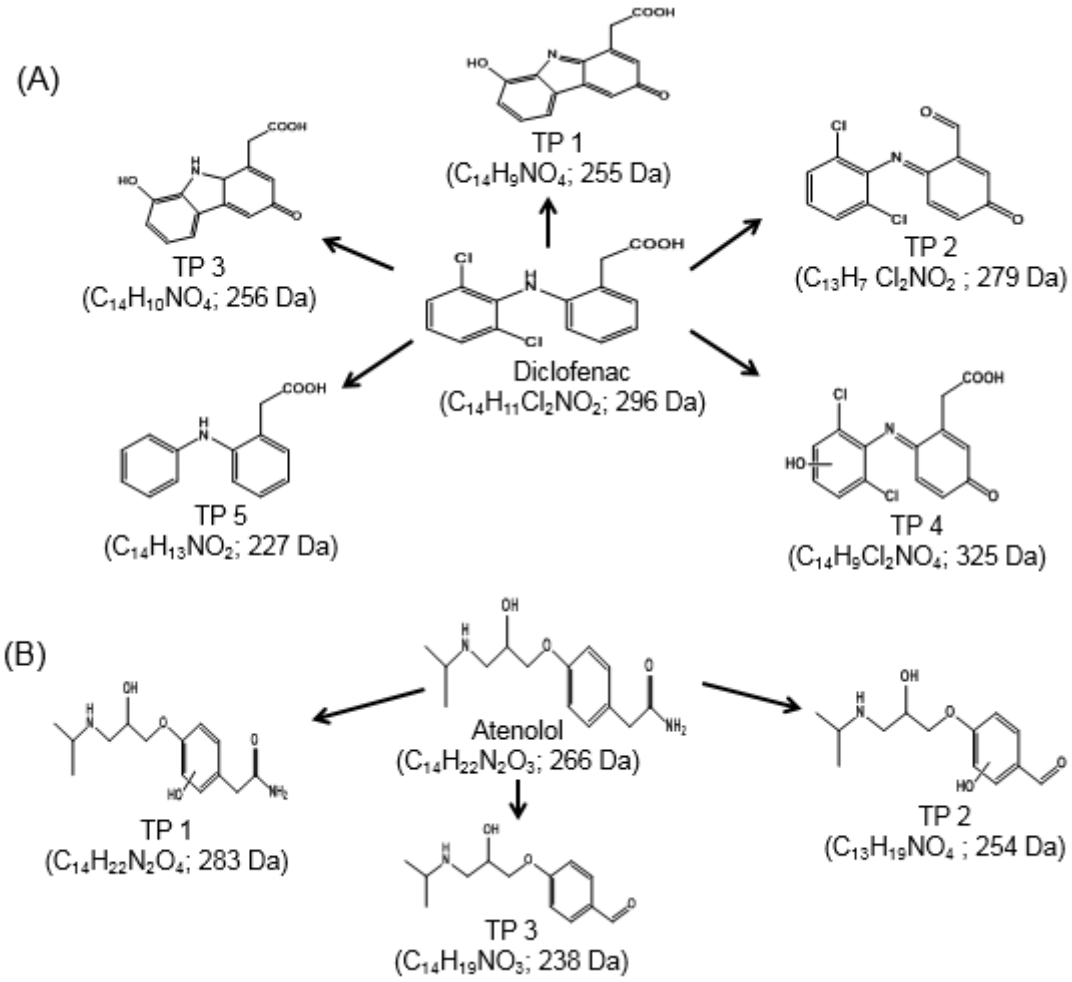


Figure 2.2: Transformation products from photolytic degradation of diclofenac (A) and atenolol (B) showing significant conservation of parent structural backbone in products (Da: Daltons) (Diniz et al. 2015; Kumar et al. 2015)

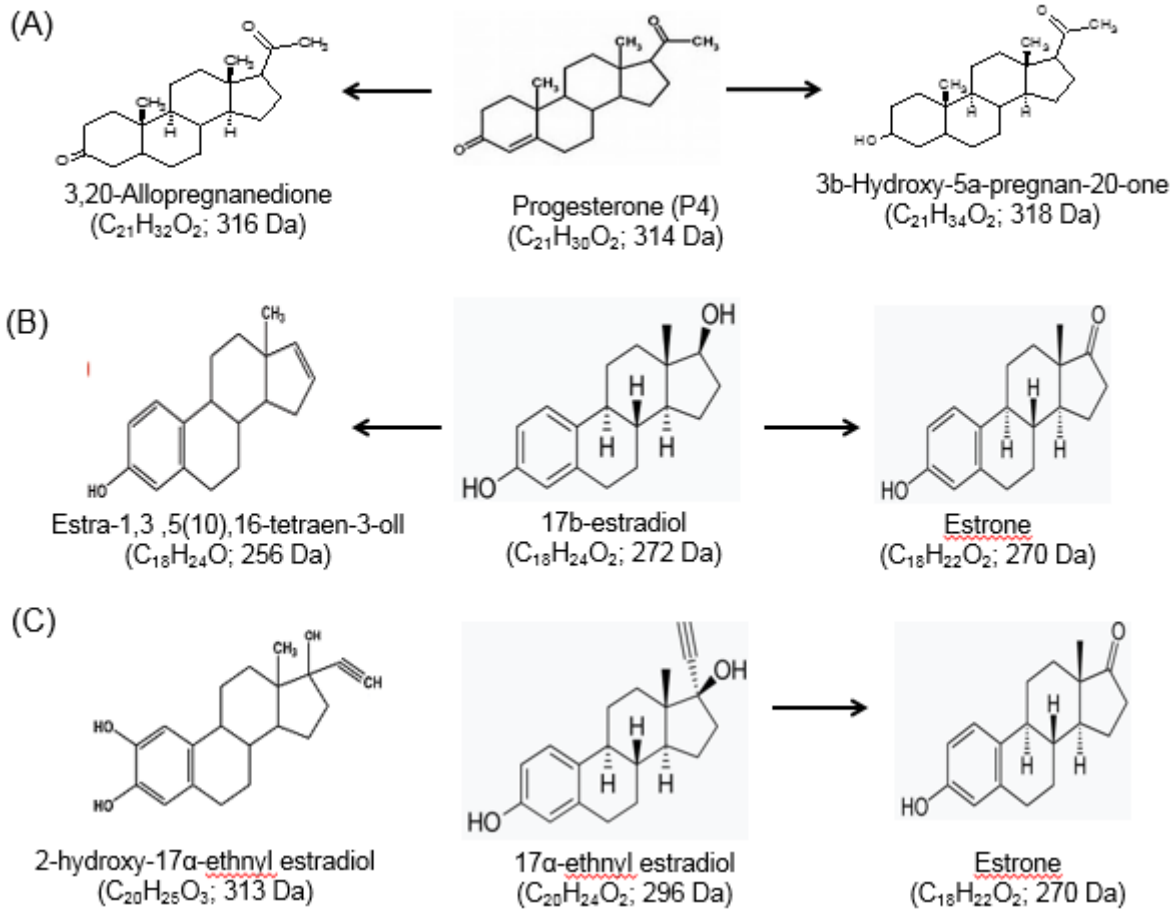


Figure 2.3: Transformation products of progesterone (A), 17β-estradiol (B) and 17α-ethinyl estradiol. All TPs shown have structural resemblance to their respective parent compound. (M: molecular weight) (Peng et al. 2014; Nakai et al., 2011; Yu et al. 2013; 2007)

Structural conservation in transformation products is ecotoxicologically important especially if a parent's potency is structure dependent. The implication is that the given parent's inherent biological acitivity(ies) can be conserved in TPs or new toxicity potency can be created across multiple biological end points (Cwiertny et al. 2014; Forsgren et al. 2014). The latter point is especially relevant with TPs of steroidal hormones. Steroids can exhibit distinct biological activities though differing only by slight structural modification to their side chains. Progestogens, androgens, estrogens and glucocorticoids are examples of natural steroids secreted in the adrenal cortex, testis, ovary and the placenta of humans and animals. Human excretion, animal waste disposal, surface run-off from livestock farms coupled with possibly high, though inadequate, removal in traditional WwTPs results in the regular discharge of

these micropollutants into surface waters (Johnson and Williams, 2004). All four steroid classes (progestogens, estrogens, androgens and glucocorticoids) making up this group of compounds have a common parent tetracyclic backbone. The target nuclear receptors that each steroid – progesterone (P4), estrogen, androgen or glucocorticoid - are designed to interact with, to elicit their intended biological activity, is achieved by slight alteration of their chemical structure (Fig. 2.4). Thus, the slightest modification in the arrangements of the peripheral atoms and/or functional groups of these compounds due to environmental transformation can modify their pharmacophore, the electronic and spatial arrangement of a compound's atoms, which determines its optimal binding with a target biological site to trigger or block biological signalling (Overington et al., 2006). Modification of the pharmacophore due to transformation could make resultant TPs bind to different ligands (nuclear receptors) and eliciting unintended biological responses (e.g. activation or deactivation of certain gene expression) (Cwiertny et al., 2014; Runnalls, et al., 2013; Jenkins, et al., 2004).

To illustrate, structural modification to specific locations in P4, a C-21 progestational steroidal ketone and a progestogen secreted naturally by females, have been shown to not only modify its progestagenic activity, but resultant TPs may also demonstrate activity in other steroid pathways (e.g. androgenicity) (Besse & Garric 2008; 2009). Most transformation reported in the literature, occur in these positions. P4 differs from its androgenic counterpart testosterone, only by having a $–COCH_3$ functional group at carbon 17 (C17). Thus, transformation involving cleavage of the $–COCH_3$ and subsequent hydroxylation (-OH) at the C17, will yield testosterone, an androgen with differing biological activity relative to P4. Another example is the reported bacteria and algae transformation of P4 to known androgens (Carson et al. 2008; Jenkins et al., 2004; Peng et al., 2014). Two freshwater algae, *Scenedesmus obliquus* and *Chlorella pyrenoidosa*, were reported to form four (4) androgens including androsta-1,4-diene-3,17-dione and 4-androstene-3,17-dione (Peng et al. 2014). 4-androstene-3,17-dione was formed by the cleavage of the $–COCH_3$ side chain of P4 at C17. Subsequent dehydrogenation of 4-androstene-3,17-dione at C1 resulted in Androsta-1,4-diene-3,17-dione. Similar transformation and androgenic products (Fig. 2.7) had been previously reported by Carson et

al. (2008) and Jenkins et al. (2004) in a biotransformation reaction mediated by the bacterium *Mycobacterium smegmatis*.

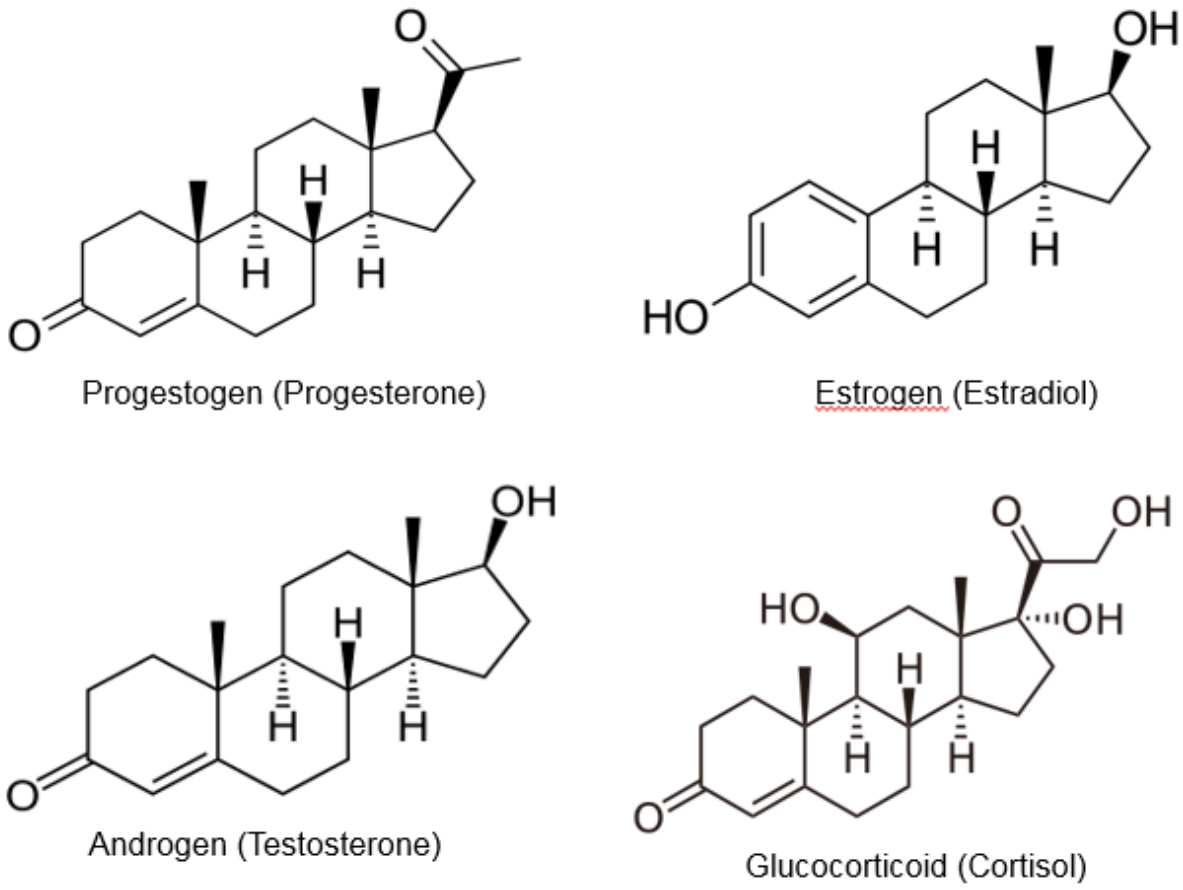


Figure 2.4: Chemical structures of representative members of different classes of steroidal hormones. All steroids shown have a common tetracyclic backbone and differ only by minor modifications molecules (Peng et al. 2014; Cwiertny et al. 2014).

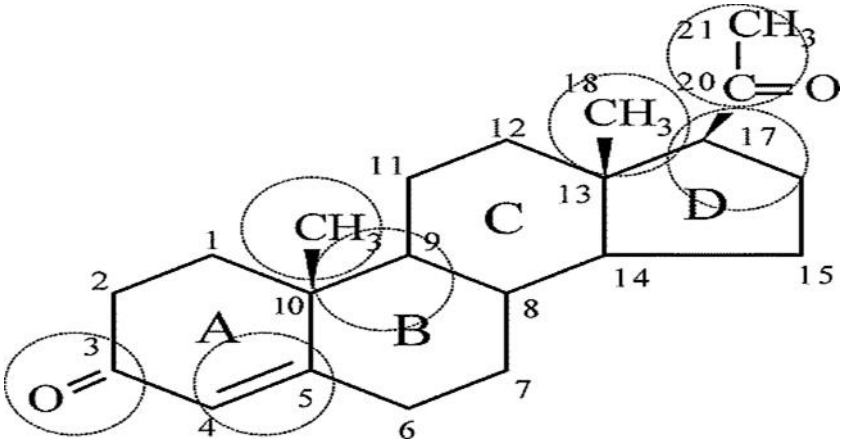


Figure 2.5: The significance of structural transformation on specific points (circled) in the P4 structure and the effect on potency of the steroid. Most transformation reported in the literature, occur in these positions. Changes resulting from substitution or addition of a functional group can alter the affinity for receptors and consequently biological effects (Besse & Garric 2008; 2009)

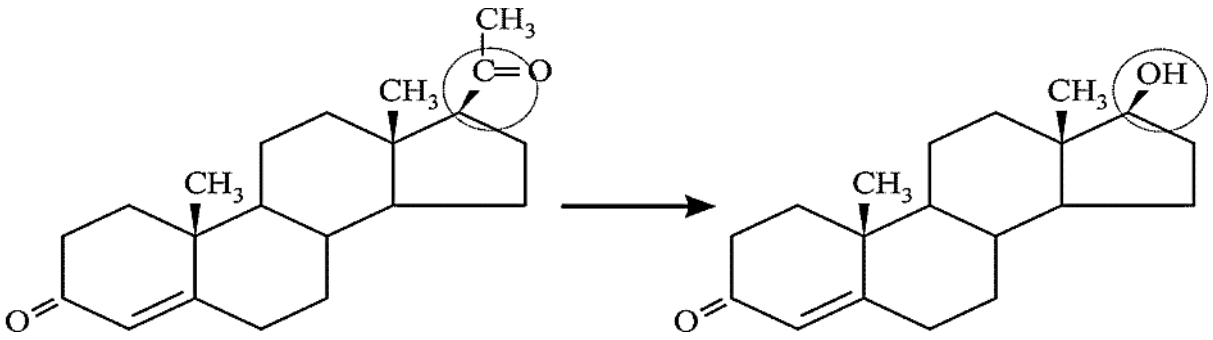


Figure 2.6: The significance of structural transformation on the potency of steroid. Transformation of P4 to testosterone formed by side chain transformation of the parent molecule at C17 (Cwiertny et al. 2014).

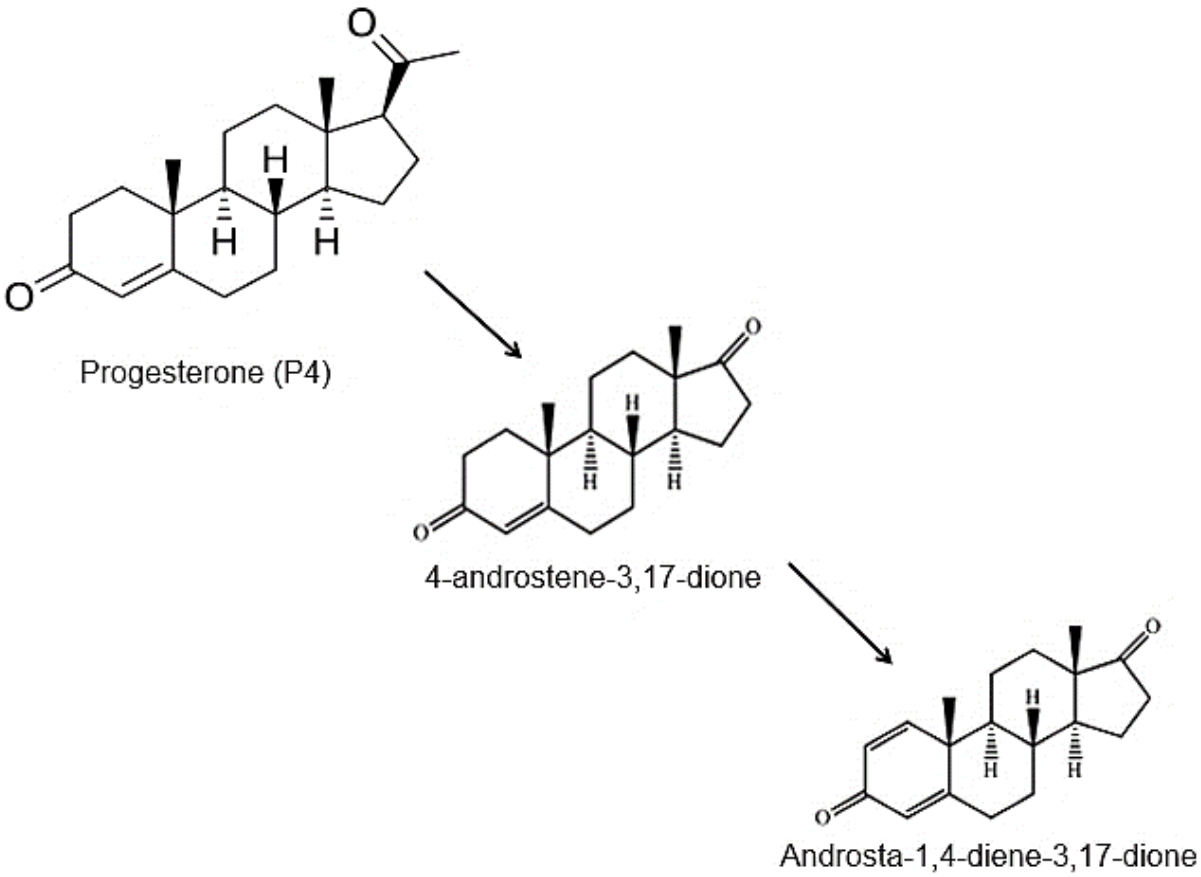


Figure 2.7: The significance of structural transformation on the potency of steroid. Transformation of P4 to 4-androstene-3,17-dione was formed by the cleavage of the –COCH3 side chain of P4 at C17. Subsequent dehydrogenation of 4-androstene-3,17-dione at C1 resulted in Androsta-1,4-diene-3,17-dione (Carson et al. 2008; Jenkins et al. 2004).

Generally, transformation of organic contaminants is often equated with detoxification (Evgenidou et al. 2015; Pico et al. 2015; Cwiertny et al. 2014). Transformation is however, relevant environmentally if it results in products that are recalcitrant to further degradation or products that have similar or enhanced toxicity relative to their parents (Cwiertny et al. 2014; Evgenidou et al. 2015; Pico et al. 2015; Li et al. 2016). To illustrate the latter point, Boxall et al. (2004) showed that pesticide and biocide degradation produced TPs that were three times acutely toxic to daphnids, rainbow trout, earthworms and algae relative to their respective parent molecules. The activated sludge treatment of acyclovir was reported by Schlüter-Vorberg et al. (2015) to result in the formation of carboxy-acyclovir, which was further

transformed into N-(4-carbamoyl-2-imino-5-oxomidazolidin)formamido-n-methoxy-acid by ozonation. Both TPs have been detected in surface water, wastewater treatment influents and effluents and also in drinking water (Prasse et al. 2012; 2011; 2010). Using the Organisation for Economic Co-operation and Development (OECD) 211 guidelines *Daphia magna* 21 days reproduction test, Schlüter-Vorberg et al. showed that 102 mg/L concentration of carboxy-acyclovir reduce reproduction levels of the test organism by 40%. The group also applied the OECD 201 72 hours algal growth inhibition test to the ozonation product and reported that it inhibited the growth of the green alga *Raphidocelis subcapitata* at a concentration of 14.1 mg/L. Acridine and 4-chlorophenol (Fig. 2.1) were reported to be exhibit higher phyotoxicity on germinating seeds of *Lycopersicon esculentum* (tomato) and acute toxicity on the anthropod, *Folsomia candida* than their parent molecules (Chiron et al. 2006; Petrovic & Barceló 2007; Kosjek et al. 2009; Elghniji et al., 2012).The potency of transformation products can differ from that of their parents in the mechanism of their bioconcentration (toxicokinetics) and/or their mode of action (toxicodynamics) (Evgenidou et al. 2015). To illustrate this point, the biological activity of parent pharmaceuticals is generally a function of the interaction of a distinct part (moiety) of the compound with a target receptor in an organism (Boxall et al. 2004). Such interactions are largely a function of the amount of the compounds active moiety reaching the target receptor which is dependent on the compounds octanol-water portioning coefficients or hydrophobicity (Hermens et al. 1984a; 1984b; Deneer et al. 1988; Könemann 1981) and the degree of dissociation (p*K*a) (Boxall et al. 2004). Thus, a transformation products retaining parental active structural moiety and has a higher dissociation constant (less prone to dissociation) or is more hydrophobic (perhaps rare as metabolism often result in more hydrophilic compound) than the parent compound, would be more potent than its parent molecule. The latter point on hydrophobicity could be applied to prodrugs. Prodrugs are compounds administered pharmacologically inactive, and only become active following metabolism (Foe et al. 1998; Wilcox & Avery, 1973). Higher activity following metabolism would also been increased absorption into tissues, which means reasonable hydrophobicity. An example of a prodrug is beclomethasone dipropionate (BDP), a glucocorticoid steroid used mostly by inhalation for treatment of respiratory disorders e.g. asthma. The hydrolytic

breakdown of the compound's ester side chain was reported to yield a much more potent monopropionate derivative, 17- beclomethasone monopropionate (BMP) (Foe et al., 1998). If BDP was to be flushed down the drain and degraded in the environment by a metabolic pathway similar to that of human metabolism, the active molecule, BMP, will be produced and will show higher toxicity relative to BDP in a toxicity test. It is worth mentioning that rarely does transformation results in products with completely new structures (toxicophores) to their parent compounds and that shows higher potency using similar or dissimilar mode of action as their parent molecule (Evgenidou et al. 2015; Farré et al. 2008).

Transformation products exist largely in mixture with their precursor parent compounds (Li et al. 2016; Evgenidou et al. 2015; López-Serna et al. 2012). For example, thirty (30) known TPs of pesticide origin were detected in mixture with their parent compounds in Swiss rivers (Moschet et al. 2014). There may have been many more unknowns present; that the target analytical procedure applied in that study for data acquisition will have missed.

Microbial back-transformation of TPs to parent compounds have been reported in the environment (Qu et al., 2013; Su et al., 2016), a process that potentially increases environmental exposure to parent compounds downstream of effluent discharge points or point of initial transformation. For example, degradation of the antibiotic sulfamethoxazole resulted in transformation products that were reported to be back-transformed by sediment-based bacteria to the parent compound (Su et al., 2016). Similarly, Qu et al. (2013) reported the back-transformation of animal metabolites and TPs of the growth promoter trenbolone acetate at conditions representative of surface waters. Thus, pharmaceutical compounds just do not disappear following treatment or when discharged into the environment, but remain in the environment either as the original parent compounds (in the case of persistent compounds) or as TPs.

2.1.1 The Potency of Natural and Synthetic Steroids and the Effects of Structural Transformation

Synthetic steroids are excellent examples of compounds where minor modifications to the structure of their endogenous analogue(s), a process explored for their synthesis, can result in cross-binding affinity to multiple receptors and triggering biological response(s) (cross-reactivity) that are not immediately obvious from structural inspection (Kumar et al. 2015; Cwiertny et al. 2014; Liu et al. 2011). The relative effect of structural modification on the binding properties of synthetic steroids can be illustrated by the receptor binding affinity of synthetic derivatives of P4 and testosterone (progestins and androgens) relative to their respective natural molecules. Although designed to bind with either the progesterone or androgen receptor and mimic the action of their endogenous compounds, synthetic steroids have been reported to have varying binding affinities to other non-target receptors namely the androgen, estrogen, mineralocorticoid and glucocorticoid receptors (Africander et al. 2011; Kumar et al., 2015; Regine Sitruk-Ware, 2004; Zapater et al. 2013). For example, medroxyP4-acetate is derived from P4 by modification to its side chain. Hydrogen attached to C17 was substituted with a $–OCOCH_3$ functional group (Fig. 2.8A). This structural modification has been reported to result in medroxyP4-acetate possessing 36 and 42% enhanced binding affinity to the androgen and glucocorticoid receptors relative to P4 9 and 5% binding affinity to these receptors (Kumar et al. 2015; Sitruk-Ware 2004; 2008). MedroxyP4-acetate is reported to possess anti-estrogenic and gonadropic properties (Kumar et al. 2015; Schindler et al. 2003) and have been demonstrated to suppress the immune system of stimulated juvenile carp (*Cyprinus carpio* L.) in a 96-hour test. Another example is the synthesis of normegestrol acetate from P4. Normegestrol acetate is synthesized following the same process applied in the synthesis of medroxyP4-acetate but with the substitution of hydrogen at C6 with a methyl group and subsequent dehydrogenation at C7 (Fig. 2.8A). This progestin is also reported bind to have enhance binding affinity for the androgen (42%) and glucocorticoid (6%) receptors (Kuhl, 2005) and possessing anti-estrogenic, androgenic and gonadropic properties (Kumar et al. 2015; Shields-Botella et al. 2003; Schindler et al. 2003). Similarly, levonorgestrel a mirror form of norgestrel is synthesized from testosterone by side chain modification involving

substitution of hydrogen at C17 with alkyne group (CCH) and the addition of a methyl group to C18 (Fig. 2.8B). Gestodene is another progestin derived from testosterone following the same synthetic process as levonorgestel but with the addition of dehydrogenation at C15 and C16 (Fig. 2.8B). Gestodene is reported to have enhanced binding affinity of 71, 38 and 202% to androgen, glucocorticoid and sex hormones binding globulins (present fish gills) (Fuhrmann, Slater, & Fritzemeier, 1995; Schindler et al., 2003). P4 binding affinity to these sex hormones binding globulins is less than 0.5% (Kumar et al. 2015; Kuhl 2005; 1996). Gestodene is reported to show anti-estrogenic, mineralcorticoid and gonadropic activities (Schindler 2014; Africander et al. 2011; Kuhl 2005; Krattenmacher 2000). Furthermore, cleavage of the ester side chain of trenbolone acetate produces trenbolone, the anabolic growth promoter known for its androgenic potency, and exhibiting not only higher (37%) binding affinity to the progesterone receptor than P4, but it also possess anti-glucocorticoids activity (Bauer, Daxenberger, Petri, Sauerwein, & Meyer, 2000). The effect of minor structural modification on higher potency of synthetic steroids can be further illustrated with the toxicity of ethinyl estradiol (EE2). EE2 differs from its natural analogue, 17β-estradiol (E2) by the annealing of an ethyne group to C17. Yet, EE2 is reported to be 27-fold greater estrogenic effects than E2 (Cwiertny et al., 2014; Thorpe et al., 2003).

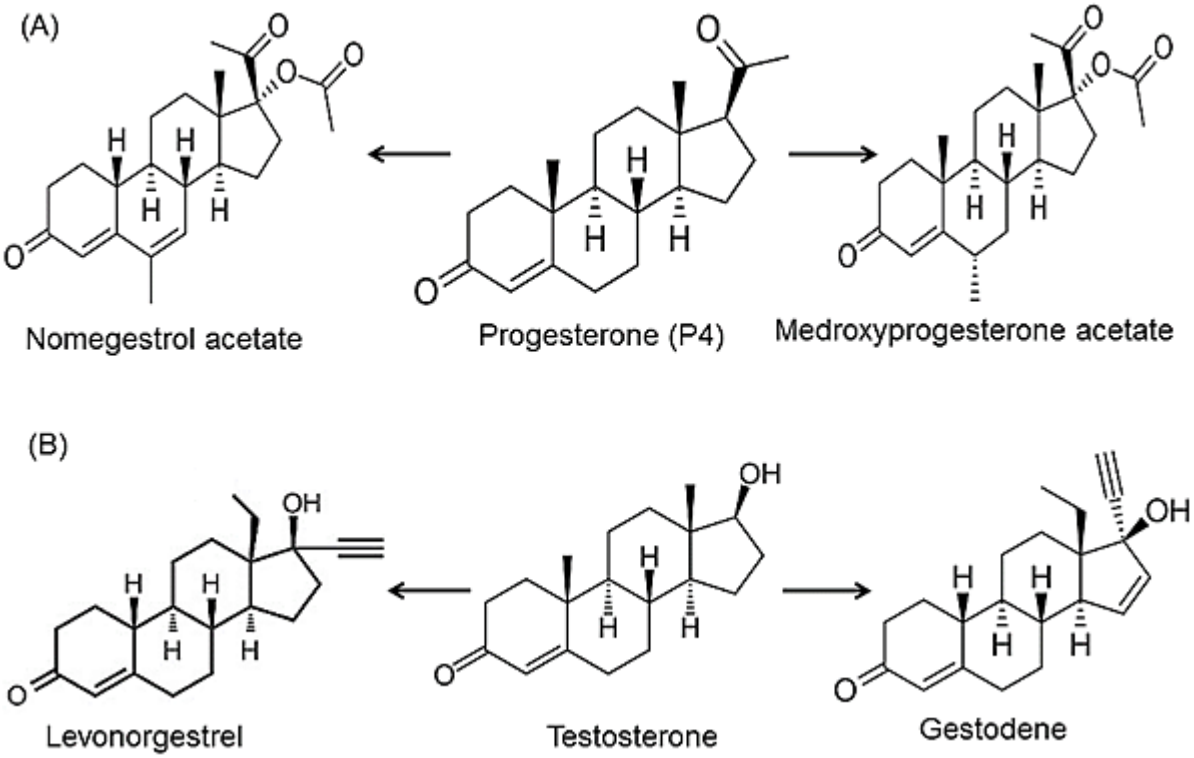


Figure 2.8: Structural modifications explored in the synthesis of progestins from endogenous progesterone and testosterone (Kumar et al. 2015)

2.1.2 Role of TPs in Observed but Unexplained Biological Activity

The general desire for ecological risk mitigation is that the reduction in concentration of a parent bioactive compound is followed by a corresponding reduction in previously observed biological activity. Sometimes, products of transformation are unknown, but when removal of parent compound is marked by significant reduction in system bioactivity, identification of such TPs is often not necessary (Cwiertny et al., 2014; Escher & Fenner, 2011). There are however many instances reported in the literature, where significant activities are detected in environmental systems with results of chemical analysis unable to link such observation to known specific causative chemicals (Cwiertny et al., 2014; Diniz et al., 2015; Evgenidou et al., 2015; Stalter et al. 2010). For example, 27% glucocorticoid and 35% androgen receptor activities were reported by Stavreva et al. (2012) in surface water systems across the United States. However, results of chemical analysis were unable to link such observed potency to

known glucocorticoid and androgenic chemicals present in the samples collected. In another example, screening of 41 known glucocorticoid compounds could not account for the persistent and widespread glucocorticoid activity observed in WwTP effluents and wastewater-impacted rivers in Australia (Escher et al., 2014), even though the level of activity was higher than those observed for estrogen and androgen receptor activities. Still on glucocorticoid activities, Schriks et al. (2013) assessed the presence of glucocorticoids and the extent of their biological activities in Dutch surface waters that serve as sources of water treated for domestic use. Though the assay results of their study were positive for glucocorticoid activities in four out of the eight rivers investigated, the results of chemical analysis were unable to determine which specific glucocorticoid was responsible for the observed effect in the *GR CALUX* bioassay used. *CALUX* bioassays are 96-well plate androgen-and-estrogen-responsive bioassays developed by Sonneveld et al. (2005) through genetic engineering of the *human U2-OS* osteosarcoma cells. In the case of the GR CALUX assay, the human glucocorticoid receptor and the luciferase (bioluminescence) reports gene were transfected into the human osteosarcoma cells. Bioactivity is determined by measuring luciferase bioluminescence activity with a luminometer.

Furthermore, Stalter et al. (2010), reported development retardation in rainbow trout (*Oncorhynchus mykiss*) after exposure to conventional WwTPs effluent, but were unable to attribute the observation to any known chemical agent. Stalter et al. (2010) assessed effluent toxicity using the *fish early life stage toxicity test* and *the yolk-sac larvae test* and reported significant reduction in the fish body weight, length and vitellogenin levels. Likewise, Bellet et al. (2012) investigated the agonist and antagonist activities of steroids in sewage influent from six WwTPs in France. Results of targeted chemical analysis showed that a number of androgens including testosterone, dihydrotestosterone and epiandrosterone were present in tested samples. However, a comparison of the strong androgenic response observed in that study with results of chemical analysis revealed that there may have been other unknown androgens present in samples as combined androgenic effects of measured androgens could only account for part of the observed androgenic response. Van Der Linden et al. (2008) working in The Netherlands, reported a similar finding to Bellet et al. (2012), from their

screening of industrial, hospital and municipal effluent for steroid receptors activity using *human cell derived CALUX reporter gene bioassays* mentioned earlier. Observed receptor activities were generally higher than could be explained with known specific chemicals. Observed glucocorticoid-like receptor activity in surface water was over 2,500 times higher than the conventional response of the natural glucocorticoid, cortisol (about 2900 ng cortisol equivalent/L). Observed receptors activities were generally higher in effluent samples. Van Der Linden et al. (2008) were unable to establish the identity of the compounds responsible for the reported biological activity. Reference to biological activity in WwTPs influent was made in this section because, poor removal during reatment would result in the dischare of potent steroids to receiving surface waters.

2.1.3 Key Transformation Pathways in the Environment

Microbial mediated degradation in the environment and during wastewater treatment is an important micropollutant breakdown process reported to produce TPs, some of which have similar or enhanced potency relative to their parent compounds. This section gives examples of some key biological pathways resulting in the formation of TPs. Discussed pathways have been reported to occur in both biotic and abiotic treatment systems (Li et al. 2014; Kolodziej et al. 2013; Wang & Lin 2012; Fatta-Kassinos, Vasquez, et al. 2011). Thus, reference is made to abiotic treatment processes to highlight similarities in degradation pathways.

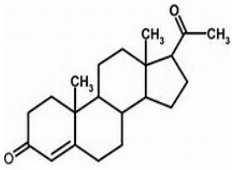
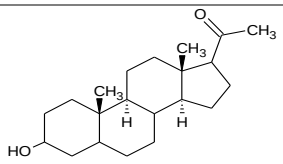
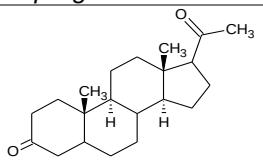
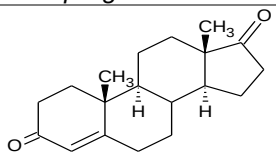
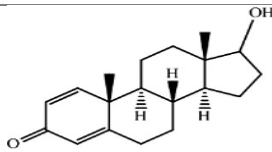
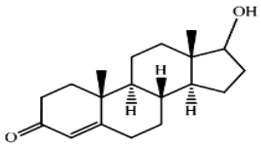
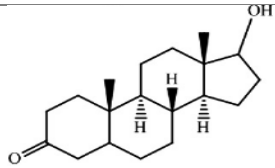
Generally, hydroxylation, oxygenation, hydrogenation, dehydrogenation and sidechain breakdown (Gros et al. 2014; Peng et al. 2014; Peart et al. 2011; Winkler et al. 2001) are the common biological transformation pathways reported in the literature which result in the formation of pharmaceutical TPs. Sometimes, individual pathways proceed solely to form products. But most often, several pathways act together to produce a given TP. Some products of biological degradation have been reported to be highly reactive, serving as precursors for a range of other biotic and abiotic transformation pathways (Wick et al. 2011; Kosjek et al. 2009). For example, activated sludge treatment of the opium alkaloid, codeine

was reported to result in the formation of unsaturated α, β-ketone codeinone, an intermediate product and precursor of a range of abiotic and biotic transformation processes resulting in the formation of nine TPs (Wick et al., 2011).

2.1.3.1 Hydroxylation

Hydroxylation is a dominant pathway in the transformation of pharmaceuticals. For example, the microbial mediated hydroxylation of the ketone group on carbon 14 of 14-Hydroxycodeinone, a metabolite of codeine, to 14-hydroxycodeine was reported by Wick et al. (2011). The hydroxylation of diclofenac and ibuprofen in activated sludge studies was reported by Kosjek et al. (2007). Activated sludge treatment of ibuprofen was reported by Pérez & Barceló (2007) to occur by hydroxylation resulting in the formation of three hydroxyl isomers (1, 2 and 3 hydroxy-ibuprofens) of ibuprofen. Biodegradation of iopromide, the X-ray contrast agent and the antibiotic floroquinolone by the white rot fungus Trametes vesicolor, was reported by Gros et al. (2014) to yield hydroxyl transformation products. Hydroxylation is a key pathway in the degradation of steroid hormones. For example, degradation of 17β-estradiol into 4-hydroxy-17β estradiol by the aerobic bacteria *Sphingomonas sp.* was reported Kurisu et al. (2010) to proceed by the hydroxylation of its C4. Similarly, hydroxylation of C2 in 17α-ethinyl estradiol by nitrifying bacteria was reported to result in the formation of 2-hydroxy-17α-ethinyl estradiol (Yi & Harper, 2007). Similarly, the hydroxylation of C6 of 17α-ethinyl estradiol by the microalgae *Ankistrodesmus braunii* is reported to yield 6-hydroxy-17α-ethinyl estradiol (Della Greca et al., 2008). Hydroxylation is one of the major pathways reported for the microbial degradation of progesterone (Table 2.1). Reference to progesterone stems from its usage patterns globally, regular measurement in the water environment due to its incomplete removal during wastewater treatment, direct human and animal excretion and sometimes direct discharge of untreated wastewater (Liu et al. 2012b) and its reported adverse effects on fish reproduction and chemoreception at environmentally relevant concentrations (Liang et al. 2015a, b; Murack et al. 2011; Havens et al. 2010; Kolodziej et al. 2003; Kobayashi et al. 2002).

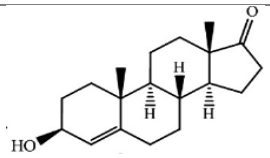
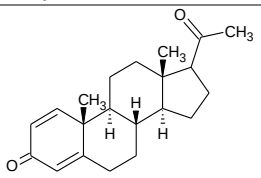
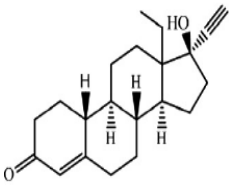
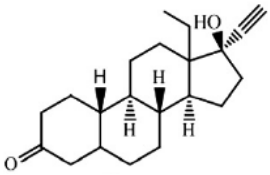
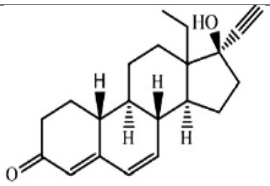
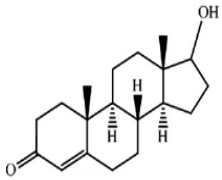
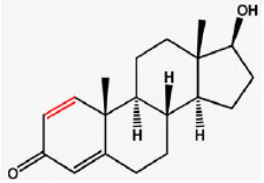
Table 2.1: Pathways of steroids transformation and TPs reported potency

Compound	TP	Reaction	Potency	Treatment
Progesterone (P4)  **314.5 ($C_{21}H_{30}O_2$)**	 *M318 ($C_{21}H_{34}O_2$) 3β-Hydroxy-5α-pregnan-20-one*	Hydroxylation of ketone at C3; Hydrogenation at C4 and C5[1]	Nk	Algae transformation
	 *M316 ($C_{21}H_{32}O_2$) 3,20-Allopregnanedione*	Hydrogenation at C4 and C5[1]	Nk	Algae transformation
	 *M286 ($C_{19}H_{26}O_2$) Androstene*	Cleavage of $–COCH_3$ at C17; Oxygenation at C17 to ketone[1,2]	Yes. Androgenic	
	 *M286* ($C_{19}H_{26}O_2$) Boldenone	Cleavage of $–COCH_3$ at C17; Hydroxylation at C17; Dehydrogenation at C1 and C2[1,3]	Yes. Anabolic agent	Algae transformation
	 *M288 ($C_{19}H_{28}O_2$)* Testosterone	Cleavage of $–COCH_3$ at C17; hydroxylation at C17 [1,2]	Yes	Algae transformation
	 *M290 ($C_{19}H_{30}O_2$) 5α-Dihydrotestosterone*	Cleavage of $–COCH_3$ at C17; Hydroxylation at C17; Hydrogenation at C4 and C5 [1,4]	Yes	Algae transformation

Nk – not known; M – molecular weight

[1] (Peng et al., 2014)
[2] (Thomas et al., 2002)
[3] (Cwiertny et al., 2014)
[4] (Parthasarathi et al. 2004)

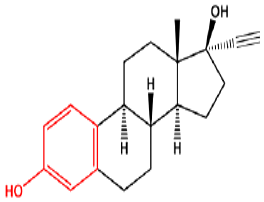
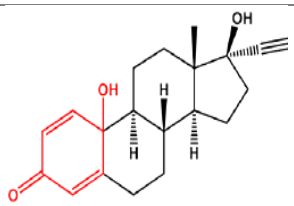
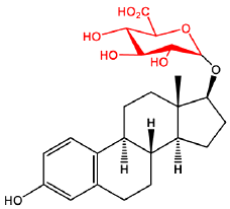
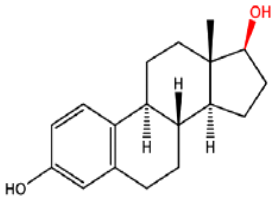
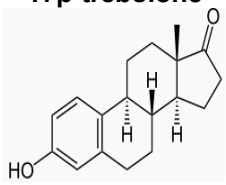
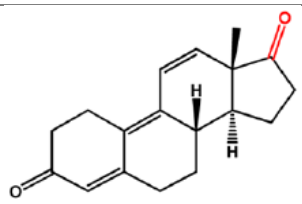
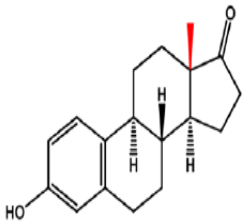
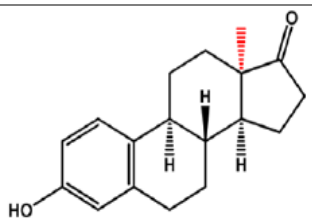
Table 2.1: Pathways of steroids transformation and TPs reported potency (cont'd)

Compound	TP	Reaction	Potency	Treatment
P4	 *M290* ($C_{19}H_{30}O_2$) *Epi-androsterone*	Cleavage of $-COCH_3$ at C17; Oxygenation at C17 to ketone; Hydroxylation of ketone at C3[1,4]	Yes	Algae transformation
	 *M312 ($C_{21}H_{28}O_2$) 1,4-Pregnadiene-3,20-dione*	Dehydrogenation at C1 and C2[1]	Nk	Algae transformation
Norgestrel (NGT)  ***312.5 ($C_{21}H_{28}O_2$)***	 *M314 ($C_{21}H_{30}O_2$) 4,5-Dihydronorgestrel*	Hydrogenation at C4 and C5[1]	Nk	Algae transformation
	 *M310 ($C_{21}H_{26}O_2$) 6,7-Dehydronorgestrel*	Dehydrogenation at C6 and C7[1]	Nk	Algae transformation
Testosterone  ***288.4 ($C_{19}H_{28}O_2$)***	 *M286 ($C_{19}H_{26}O_2$) Boldone or 1-dehydrotestosterone*	Dehydrogenation at C1 and C2[5,6]	Yes	Aerobic bacterial degradation

Nk – not known; M – molecular weight

[5] (Salgado et al., 2013)
[6] (Yang et al., 2011)

Table 2.1: Pathways of steroids transformation and TPs reported potency (cont'd)

Compound	TP	Reaction	Potency	Treatment
Ethinyl estradiol  ***296.4 ($C_{20}H_{24}O_2$)***	 *17α-ethinyl-1-4-estradiene-10,17β-diol-3-one*	Hydroxylation at C19[7,8,9,10]	Yes. More estrogenic than parent	Aerobic bacterial degradation, ozonation and photocatalytic degradation
Estradiol 17β-D-glucuronide  ***470.5 ($C_{24}H_{31}O_8Na$)***	 *M272 ($C_{18}H_{24}O_2$) 17β-estradiol*	Deconjugation at C17; Hydroxylation at the same point[5]	Yes	Hydrolysis
17β-trebolone  ***270.4 ($C_{18}H_{22}O_2$)***	 *Trendione*	Hydroxy-Keto conversion at C3[11,12,13]	Yes	Aerobic degradation
Estrone  ***270.4 ($C_{18}H_{22}O_2$)***	 *Lumiestrone*	Epimerization (methyl group inversion at C13)[14]	Yes. Estrogenic	Photolysis

Nk – not known

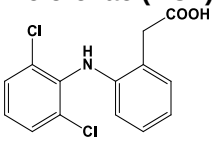
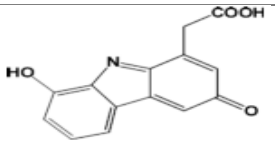
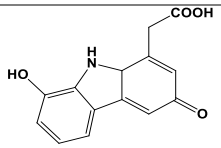
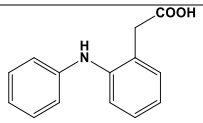
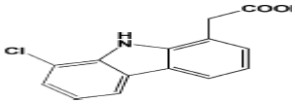
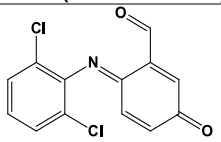
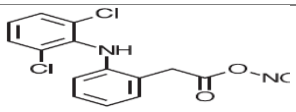
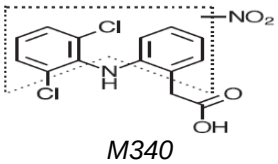
[7] (Chen et al., 2012)
[8] (Frontistis et al., 2012)
[9] (Barr et al. 2012)
[10] (Della Greca et al., 2008)
[11] (Forsgren et al. 2014)
[12] (Kolodziej et al., 2013)
[13] (Khan et al., 2008)
[14] (Whidbey et al., 2012)

The overall observation in Table 2.1 is that microbial breakdown of steroids does not necessarily involve the cleavage of the parental tetracyclic ring, but the transformation of peripheral elements by substitution and/or side chain breakdown. For example, hydroxylation of carbon 17 in P4 following cleavage of its side chain, was a contributory pathway in the formation of boldenone, testosterone and 5α-dihydrotestosterone, all of which are potent TPs (Peng et al., 2014). Biotransformation of a range of other steroids including progesterone and testosterone, was reported by Peart et al. (2011) to occur by hydroxylation of their respective carbon 7. Furthermore, a range of other microbial transformation by hydroxylation at different positions in the P4 structure were reported in the literature. Several species of fungi were noted to introduce a hydroxyl group at the 14α-position of the compound and were further transformed chemically to the 14β-hydroxy substituent (Bhatti and Khera, 2012; Manosroi et al. 2008). For example, P4 was transformed into 14α-hydroxyprogesterone and 9α-hydroxyprogesterone by *Thamnostylum piriforme* (Manosroi et al. 2008). Other reported microbial transformations of progesterone include; 14α-hydroxyprogesterone transformation into 6β, 14α-dihydroxyprogesterone and 7α, 14α-dihydroxyprogesterone (*Mucor griseocyanus*) (Hu et al. 1995), P4 into 11α-hydroxyprogesterone, 15β-hydroxyprogesterone, 7β-hydroxyprogesterone, 7β–15β-dihydroxyprogesterone and 11α–15β-dihydroxyprogesterone (*Aspergillus fumigatous*) (Smith et al. 1994). Bhatti and Khera, (2012) also noted the formation of 4-androstene-3, 17-dione (50), testosterone (2) and testololactone when progesterone was incubated with *Saprolegnia hypogyna*, 15α-hydroxyprogesterone and 12β–15α-dihydroxyprogesterone with *Fusarium culmorum* culture. However, when P4 was incubated with thermophilic bacterium (*Bacillus stearothermophilus*), three monohydroxylated metabolites (20α-hydroxyprogesterone 6β-hydroxyprogesterone and 6α-hydroxyprogesterone) and a new metabolite (9, 10-sco-4-pregnene-3, 9, 20-trione) were reported to be isolated and identified (Bhatti and Khera, 2012).

Hydroxylation reactions are not limited to biological processes alone. Abiotic degradation procedures such as photolysis have been shown to produce hydroxyl TPs. For example, photolytic breakdown of diclofenac was reported to yield hydroxyl TPs (Petrovic & Barceló 2007). Similarly, Salgado et al., (2013) and Diniz et al., (2015) both reported the hydroxylation

as a contributing pathway in the formation of 2-(8-hydroxy-3-oxo-3H-carbazol-1-yl)acetic acid, from the photolysis of diclofenac (Table 2.2).

Table 2.2: Pathways of diclofenac transformation and TPs reported potency

Compound	TP	Reaction	Potency	Treatment
Diclofenac (DCF)  ***296.2 ($C_{14}H_{11}Cl_2NO_2$)***	 *M255 ($C_{14}H_{9N}O_4$) 2-(8-hydroxy-3-oxo-3H-carbazol-1-yl)acetic acid*	Cl_2 loss, hydroxylation, oxidation[5,15]	Yes. Higher toxicity[5,15]	Photolysis
	 *M256 ($C_{14}H_{10}NO_4$)*	Hydrogenation	Yes[5,15]	Photolysis
	 *M227 ($C_{14}H_{13}NO_2$)* *And*  *M260 ($C_{14}H_{10}ClNO_2$)*	loss of Cl or Cl_2[16,17]	M227 (Yes), M260 (Nk)	Photolysis
	 *M279 ($C_{13}H_7Cl_2NO_2$)*	Decarboxylation [14,15]	Yes. Higher than DCF[14,15]	Photolysis
	 *M324* And  *M340*	Nitrification and Nitration[4,18]	Nk	Membrane reactor

[15] (Diniz et al., 2015)
[16] (Jelic et al., 2012)
[17] (Chiron, Minero, & Vione, 2006)
[18] (Tina Kosjek et al., 2009)

Nk – not known

2.1.3.2 Ketone and Hydroxyl Function Groups Interconversions

Interconversions of ketone and hydroxyl functional groups are reported in the literature. For example, androstenedione was formed by conversion of the hydroxyl group at C17 of testosterone to ketone and 17β-estradiol from estrone by the hydroxylation of the ketone group at C17 of parent estrone (Yang et al. 2011; Khanal et al., 2006; 2008). Conversly, backtransformation of 17β-estradiol to estrone by *Sphingomonas sp*. was reported by Kurisu et al. (2010) to occur by the conversion of the hydroxyl group at C17 to ketone, an oxidation pathway. The unicellular algae *Scenedesmus quadricauda* was reported by Della Greca et al. (2008) and Yu et al. (2013) to transform 17α-ethinyl estradiol to 3-keto-17α-ethinyl estradiol by the oxidation of the hydroxyl group at C3 with a ketone group. Similarly, hydroxyl to ketone conversion at C17 of 17α-ethinyl estradiol by *Sphingobacterium sp*. was reported by Haiyan et al. (2007) as a contributory pathway in the formation of five TPs. Hydroxyl to ketone conversion at C3 and subsequent dehydrogenation at C1 and C2 can be observed in table 2.1 to produce trendione from estrone. Biological conversion of hydroxyl groups to ketones is reported to yield tredione from 17β-trenbolone (Fig. 2.9) (Cwiertny et al. 2014; Forsgren et al. 2014; Qu et al. 2013; Khan et al. 2008), thus showing that a given pathway can act with other pathways to produce the same TP from two different parent compounds. Another example of a ketone to hydroxyl interconversion is the contributory pathway in the formation of 3β-Hydroxyl-5α-pregnan-20-one from P4. The hydroxylation of the ketone group in carbon 3 of P4 is followed by hydrogenation at C4 and C5 (Table 2.1). Hydroxylation of Ethinyl estradiol at C19 produced 17α-ethinyl-1-4-estradiene-10,17β-diol-3-one, a TP more estrogenic that the parent compound (Peng et al., 2014) (Table 2.1).

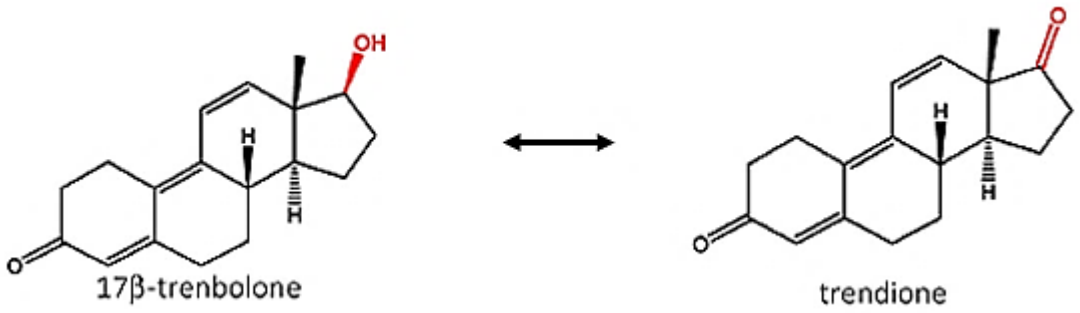


Figure 2.9: Interconversion of one metabolites of trenbolone acetate into the other by the oxidation of a hydroxyl group into a ketone and reduction of ketone to hydroxyl group, an oxidation/reduction pathway (Cwiertny et al. 2014; Forsgren et al. 2014)

2.1.3.3 Hydrogenation

Hydrogenation, the addition of hydrogen atom(s) to a compound is an important biological transformation pathway for pharmaceuticals. For example, Peng et al., (2014) reported algae mediated hydrogenation of carbon 4 and 5 of P4 and norgestrel to produce 3,20-allopregnanedione and 4,5-dihydronorgestrel (Table 2.1). Hydrogenation of carbon 5 of P4 was a contributing pathway in the formation of 3β-Hydroxyl-5α-pregnan-20-one. Similarly, hydrogenation of carbon 5 contributed to the formation of 5α-dihydrotestosterone from progesterone (Table 2.1). Hydrogenation like hydroxylation was reported to account for the formation of photolytic transformation products of diclofenac (Table 2.2), showing the versatility of the pathway to biotic and abiotic treatment processes.

2.1.3.4 Dehydrogenation

Dehydrogenation, the removal of hydrogen atom(s) from a compound, is another major pathway in the breakdown of pharmaceuticals. Boldenone (1-dehydrotestosterone) an anabolic agent exhibiting over 50% greater binding affinity to the androgen receptor relative to testosterone, is formed from a bacteria-mediated dehydrogenation reaction at carbon 1 of the parent molecule, at environmentally relevant conditions (Cwiertny et al., 2014; Yang et al.,

2011). Boldenone thus has a double bond at C1 and C2 relative to testosterone (Fig. 2.10). This is a common transformation reaction occurring in natural and engineered systems. Table 2.1 shows that an algal mediated dehydrogenation reaction has been reported to result in the formation of boldenone from both P4 and testosterone. In the formation of boldenone from P4, dehydrogenation was a contributory reaction pathway occurring before or after cleavage of -$COCH_3$ at C17 and its subsequent hydroxylation. Microbial (*Nocardia sp.*) mediated dehydrogenation of ring D of 17β-estradiol at C17 was a contributory pathway in the formation of estrone (Yu et al. 2013; 2007).

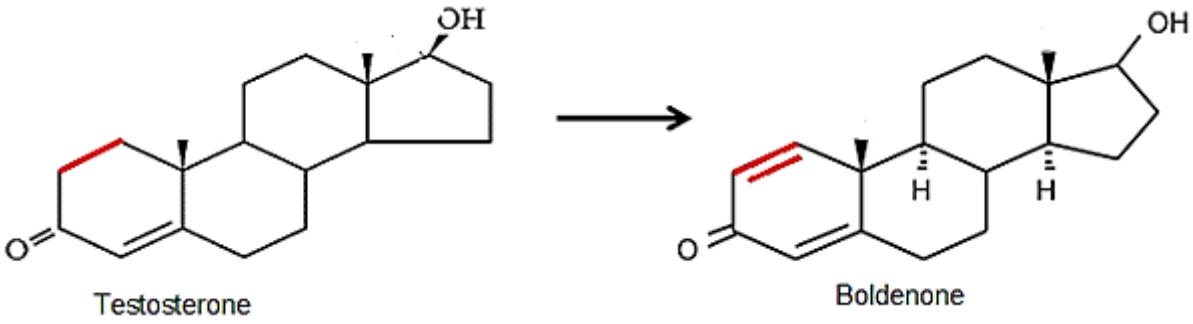


Figure 2.10: Formation of boldenone from testosterone by dehydrogenation (cleavage of the hydrogen atoms) at carbons 1 and 2 (Cwiertny et al., 2014; Peng et al. 2014; Yang et al., 2011).

2.1.3.5 Side Chain Breakdown and other Pathways

Side chain breakdown is one of the major contributing pathways in the formation of algae related TPs from P4 (Table 2.1). Dehydration, the loss of a water molecule is another biologically mediated pathway reported for the transformation of steroids. For example, dehydration of the D-ring in 17β-estradiol was reported by (Nakai et al., 2011) to result in the formation of estratetranol, a TP retaining parent compound's estrogenicity. Nitrification, nitration, carboxylation and decarboxylation, dechlorination, deaklylation and lactam formation (Cwiertny et al., 2014; Kosjek et al. 2007; Kosjek et al., 2009; Qu et al., 2013) are examples of other biological mediated pathways not often reported in the literatures to account for

pharmaceutical transformation, perhaps because of their rare occurrence. Nitrification and nitration (addition of a nitro group into an organic compound) are shown to produce two TPs (M324 and M340) from diclofenac in a membrane reactor treatment (Table 2.2).

Other abiotic treatment processes are also known to produce potent TPs. For example, lumiesterone, a TP formed from epimerization reaction involving the photochemical inversion of the methyl group in C13 of estrone, is reported to be more photopersistent than estrone and retaining 40% estrogenicity of the parent compound (Trudeau et al., 2011; Whidbey et al., 2012). Transformation by oxidative degradation is an important abiotic degredation process which may be chemically or photolytically driven (Chen et al., 2012; Fayad et al., 2013; Frontistis et al., 2012; Lee & von Gunten, 2010; Wols et al., 2015, 2013). Common oxidants that have been used in the breakdown of pharmaceuticals include; chlorine, chlorine dioxide, ozone, hydroxyl radicals, ferrate (IV), potassium permanganate and hydrogen peroxide (Wols et al. 2015; Tisa et al. 2014; Fayad et al. 2013; Wols et al. 2013; Chen et al. 2012; Zhang & Geißen 2010; Lee & von Gunten 2010).

2.2 Approaches to Studying Degradation and Identification of TPs

Two major approaches (laboratory experiments and environmental screening) are generally followed in the study of pharmaceutical degradation and the subsequent identification of their TPs. For laboratory biodegradation experiments, samples can be provided directly from WwTPs or a pilot-scale WwTP or from natural waters (e.g. rivers) (Prasse et al. 2011; Helbling et al. 2010; Kern et al. 2009). The two identified approaches are not mutually exclusive but complimentary of each other. While laboratory studies offer the advantage of simulating degradation in controlled laboratory conditions with appropriate controls that can be compared to treatments, the results of such studies are most relevant if identified TPs are detected in environmental samples (Pico et al., 2015; Jelic et al. 2012; Trautwein and Kümmerer, 2012; Prasse et al. 2011). Bletsou et al. (2015) recommended that following preliminary laboratory studies (e.g. batch experiments), verification of results obtained should be carried out using

real environmental samples. Reason; the result of the latter, will demonstrate the relevance of the former and batch studies results can be reflected on in the design of environmental monitoring studies and in the development of risk mitigation strategies. However, as concentrations of TPs are usually lower in the environmental systems, coupled with the complexity of such samples, analytical methods must be adapted to obtain reliable results (see section 2.2.1 for further details). The advantage of transformation studies performed under well-controlled conditions compared to environmental screening is the relative ease of identification of unknown peaks (TPs). The number of chemically possible molecular formula and structures that could be assigned to an unknown detected peak, is limited to structures showing close relationship to the parent compound (Bletsou et al. 2015; Ruff et al. 2015; Hug et al. 2014; Krauss et al. 2010). Also, it is easy to eliminate non-relevant peaks from identification process, simply my comparison of the peaks detected in control sample with those found in test samples. Nature is known to synthesise a range of products due to its highly diverse microbial communities and the range of physical and chemical process occurring within it (Kind and Fiehn, 2006; 2010). Thus, attempts to identify unknown peaks in environmental samples with uncontrolled conditions is often very challenging involving several work intensive data collections and sophisticated processing steps often involving highly specialised instruments (Pico et al. 2015; Bletsou et al. 2015; Ruff et al. 2015; Hug et al. 2014; Lambropoulou and Nollet, 2014) which may be unavailable locally due to their high cost.

The two identified approaches to studying pharmaceutical transformation, have been applied to studying a range of drugs. For example, Prasse et al. (2011), investigated the degradation of the antiviral drugs acyclovir and penicyclovir and their resultant TPs in a batch activated sludge experiment. Acyclovir was transformed into carboxyl-acyclovir by the oxidation of the hydroxyl group side chain in the compound into a carboxyl functional group. Carboxyl-acyclovir was reported to be detected in drinking water at a concentration of 40 ng/L (Prasse et al., 2011). Penicyclovir was transformed into eight products by a number of pathways occurring alone or as contributory pathway(s). Reported pathways were; oxidation of hydroxyl and aldehyde groups, hydration and dehydration of hydroxyl groups, cleavage of a formaldehyde group and of an acetate group after β-oxidation. Another example is the aerobic degradation

of the opium alkaloid, codeine, a process that yielded ten TPs that were subsequently detected in WwTPs effluents and surface water (Wick et al., 2011). Pathways reported to be responsible for the formation of codeine TPs were hydroxylation, oxidation into ketone, hydrogenation and dehydrogenation. Biotransformation of progesterone was reported to yield amongst others TPs testosterone and Boldenone (Peng et al., 2014), compounds that have been measured in surface waters (Ternes & Joss, 2006; Thomas et al., 2002). Reaction pathways reported by Peng et al. (2014) were side chain breakdown, hydroxylation, hydrogenation and dehydrogenation. These examples show the wider range of pathways which can create subtly different transformation products. Thus, understanding pathways could help more widely in understanding transformation.

2.2.1 Analytical Procedure for the Detection and Identification of TPs

Following degradation studies, a multistep workflow analytical approach is often applied to samples to detect and confirm the identity of TPs. Target analysis, suspect screening and non-target screening are three main methods applied in the analysis of collected samples depending on the availability of reference standards for known TPs or prior knowledge of compounds suspected to be in samples (Fig. 2.11) (Zonja et al. 2014; Schymanski et al. 2014; Kind & Fiehn 2010; Kosjek et al. 2007). Achieving good results (peak detection, identification and confirmation) in the three analytical approaches mentioned above, could depend on the extraction procedures (sample extraction, enrichment/purification, chromatographic separation and ionisation) applied to cleaning-up samples and preconcentrating analytes prior to instrumental analysis (Ibáñez et al. 2012; del Mar Gómez-Ramos et al. 2011; Hernández et al. 2011). These preceding analytical steps are significant in minimizing matrix (background) effects and its associated ion suppression especially in target analysis. For example, sample preparatory step was noted to be crucial in the target analysis of metabolites of the analgesic dipyrone in effluent samples and for the avoidance of assignment of erroneous identifications to detected TPs (Ibáñez et al. 2012). A major drawback however in the application of sample extraction steps to degradation studies involving suspects and non-target screen, is the

potential loss of TPs due to breakthrough. These compounds are mostly unknown and therefore analytical standard are largely unavailable to understand their binding properties to sorbent materials, a factor critical to selecting solid-phase extraction (SPE) procedure and chromatographic column (Pico et al. 2015; Krauss et al. 2010; Kern et al. 2009). Thus, extraction procedures that have been applied by some researchers in transformation studies, are usually not sophisticated, but generic and robust since the physicochemical characteristics of the TPs are sometimes unknown (Pico et al. 2015). Hydrophiliclipophilic balance reversed-phase sorbents (e.g. Oasis HLB) are the most popular owing to their ability to retain several polar compounds. Oasis HLB sorbent is commonly used because of its hydrophilic–lipophilic balance, proven versatility and efficiency in the extraction of analytes of a wide range of polarities and acid/base characteristics at different pH values, including neutral pH (Bletsou et al. 2015; Jelic et al. 2012; Ibáñez et al. 2012; del Mar Gómez-Ramos et al. 2011; Genena et al. 2013; Pérez-Parada et al. 2011; Hernández et al. 2011; Fatta-Kassinos et al. 2011; Kern et al. 2009).

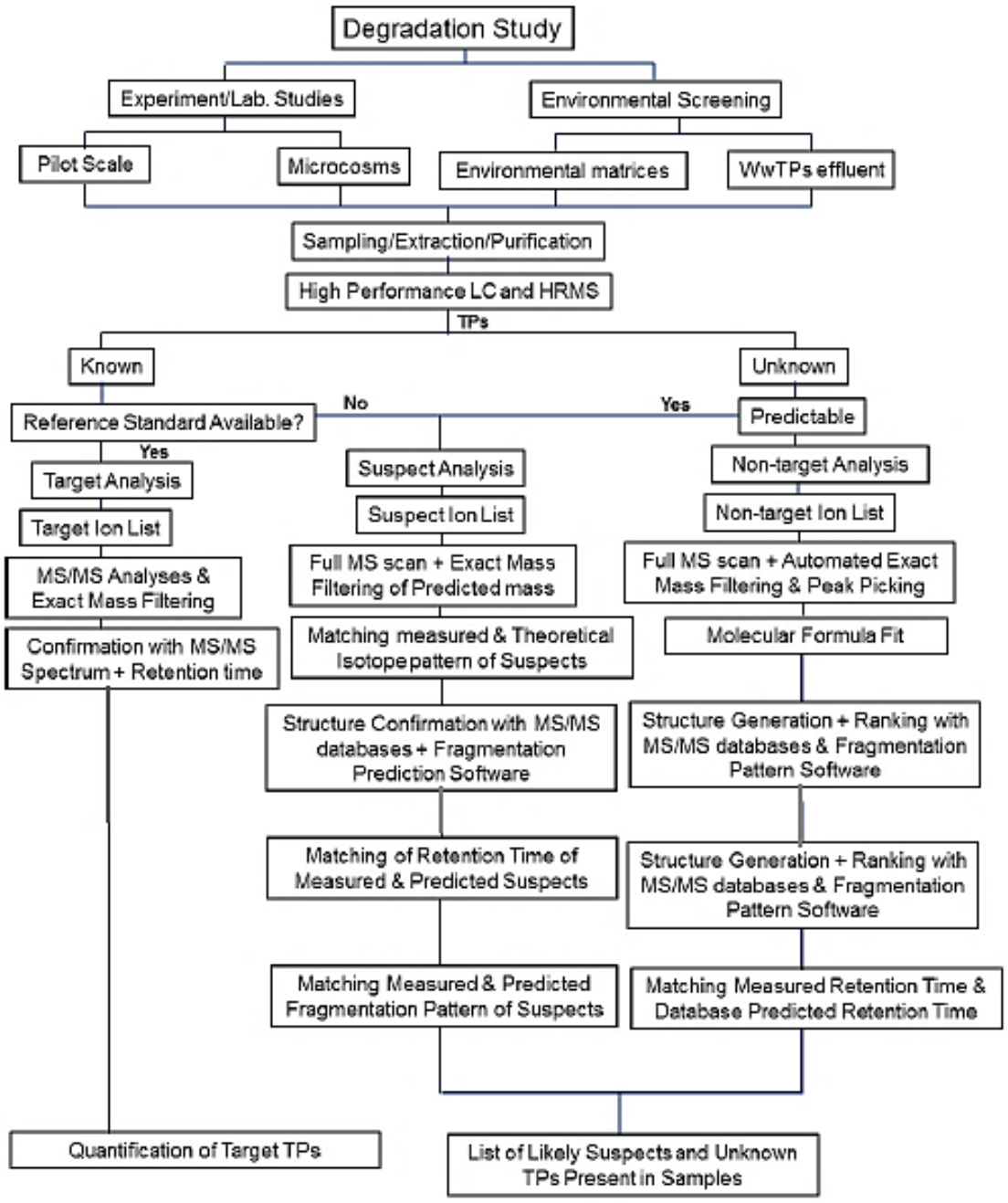


Figure 2.11: Systematic Approaches to Studying Pharmaceutical Degradation and Comparison of Analytical Workflows for Target, Suspect and Non-Target Screening of Transformation Products (Bletsou et al. 2015; Hug et al. 2015; Pico et al. 2015; Krauss et al. 2010)

2.2.1.1 Analytical Method Development and Validation

Analytical approach to studying pharmaceutical compounds in water samples generally involve sample preparatory steps involving the extraction of a suitable volume of water for concentrating-up of desired analytes and the clean-up of matrix component to minimize matrix effect (interferences). This is followed by instrumental analysis for separation and quantification of analytes. The development of analytical methods for the analysis of pharmaceutical contaminants could be very challenging and time-consuming owing to the trace levels of these compounds in environmental samples. Furthermore, these micropollutants often occur as a complex mixture of a range of pollutants from different chemical classes with different chemical and physical properties (Al-Odaini et al. 2010). Extraction is usually performed by SPE utilizing different types of sorbent materials such as silica-based C18 and polymer sorbents (Togola and Budzinski, 2007; Gros et al. 2006). Oasis HLB and MCX are examples of polymer sorbents that have been widely applied in the extraction of pharmaceuticals from environmental samples (Al-Odaini et al. 2010; Kasprzyk-Hordern et al. 2007; 2009a, b). Owing to its hydrophilic–lipophilic balance, Oasis HLB is a multipurpose and efficient sorbent for the extraction of pharmaceuticals with a wide range of polarities and pH values. The drawback however, with using multipurpose sorbents like the HLB is their less selectiveness which often can result either in lower SPE recovery or more likely, higher matrix effect in electrospray ionisation source (Al-Odaini et al. 2010; Kasprzyk-Hordern et al. 2007; 2009a, b).

Extraction recoveries are generally evaluated (validated) with field samples by comparing the concentration of an analyte found in a spiked sample matrix to its corresponding concentration in a reference sample of the same matrix (Pérez-Carrera et al. 2010; Al-Odaini et al. 2010). Replicates samples of a given matrix are spiked prior to extraction with a known volume of an analyte standard or standard mixtures of analytes. This is followed by the addition of an internal standard (I.S.) or a mixture of I.S. (determination of multiple analytes) to the spiked samples, also prior to extraction, to minimize matrix effect (Rodil et al. 2009; Jelic et al. 2009).

2.2.1.2 Target, Suspect and Non-Target Approaches to TPs Identification

The invention of liquid chromatography tandem mass spectrometry technology (LC-MS), particularly the coupling of LC to high resolution mass spectrometers (LC-HRMS), has been very useful in the development of novel analytical methodologies for the detection and identification of TPs in mixture of polar micropollutants (known and unknown), present in very low concentration in complex environmental matrixes (Bletsou et al. 2015; Lambropoulou et al. 2014; Zonja et al. 2014; Schymanski et al. 2014; Krauss et al. 2010). High resolution and accurate hybrid mass spectrometers have been applied in screening environmental samples via a range of analysis (target, suspect and non-target screening) and for the identification and structural elucidation of TPs. Examples of some commercially available mass spectrometers and their characteristic features in decreasing performance, are presented in Table 2.3 for ionic mass (m/z) range 300 - 400.

Table 2.3 Commercially Available Mass Spectrometers and their Characteristic Features (Krauss et al. 2010)

Instrument	Resolution (FWHM)	Mass Prediction Accuracy (ppm)	Dynamic Range (Linear)	Absolute Mass Sensitivity
Fourier Transform Ion Cyclotron Resonance (FT-ICR)	1,000,000	≤1	10^4	Picogram (FS)
Orbitrap	100,000	2	10^3 – 10^4	Femto – picogram (FS)
Time-of-flight (ToF)	20,000	3	10^2 – 10^3	Picogram
Quadrupole Ion Trap (QIT)	10,000	50	10^3	Femto – picogram (SRM; FS)
Triple Quadrupole (QqQ)	Unit resolution	50	10^4	Femto – picogram (SRM)

*FWHM: Peak Full Width at Half Maximum; FS: Full scan mode; SRM: Selected Reaction Monitoring

Target analysis, the quickest and most reliable method of confirming the identity of TPs can be done with low (unit) resolution tandem mass spectrometers or in full-scan in high resolution

mass spectrometers, but is dependent on the availability of reference standard(s) (Evgenidou et al., 2015; Pérez & Barceló, 2007; Petrovic & Barceló, 2007; Pico et al., 2015). Target analysis, is based on the determination of already known TPs, and identification is carried out with standard solutions (Fig. 2.11). In this analytical approach, TPs can be concentrated by solid phase extraction due to the availability of reference standards. In target analysis, known TPs are included within a defined target list in an MS method and can be monitored in routine analysis. LC tandem triple quadrupole (LC-QqQ-MS/MS) is the most commonly used system in target analysis. The QqQ mass analyzer user-friendly interface allows application of MS/MS modes (e.g. selected reaction monitoring (SRM) of parent-precursor ion transitions). Monitoring in two transitions (MS/MS) instead of one is reputed for providing several advantages and interesting characteristics for target analysis, such as reduction in false positives, increased selectivity, reduced interferences and high sensitivity, which allows robust quantification (Bletsou et al. 2015; Ruff et al. 2015; Hug et al. 2015; Krauss et al. 2010). When high resolution mass spectrometer is used in target analysis, limitations of SRM analysis, which allows only the monitoring of selected TPs in target list, is eliminated and almost every compound present in a sample can be determined simultaneously with HRMS instruments operating in full-scan mode (Pico et al. 2015; Bletsou et al. 2015; Fenner et al. 2013; Krauss et al. 2010). This makes it unnecessary to pre-select compounds and associated SRM transitions. Using HRMS, target compounds included in a database are screened based on retention time, isotopic pattern, mass accuracy and MS/MS fragments (Agüera et al. 2013; Nurmi et al. 2012; Díaz et al. 2012). Furthermore, HRMS instruments higher resolution power make them capable of differentiating isobaric compounds with similar nominal mass but different molecular formula (Pico et al. 2015; Bletsou et al. 2015; Nurmi et al. 2012). When hybrid instruments are used, they offer the opportunity of data-dependent MS/MS acquisition, where MS transitions are triggered once a compound from a target-ion list is detected in the full scan (Bletsou et al. 2015; Krauss et al. 2010).

Suspect screening as the name implies, involves screening samples for compounds suspected to be there based on prior knowledge of sample(s) origin and the probable sources of contamination of the sampled matrix. In suspect analysis, a list of possible degradation

product is put together from the literature or from prediction models (Prasse et al. 2011; Helbling et al. 2010). Suspect screening is the technique of choice, when the confirmation of TPs with no reference standard, but whose molecular formula and structure can be predicted (Fig 2.11). Examples of suspect TPs prediction models are EAWAG-BBD (recently replaced with enviPath in 2016) CATABOL, PathPred, Meteor (Wicker et al. 2016; Bletsou et al. 2015; Elis and Wackett, 2012). More details on these prediction tools are presented in section 2.3. The major drawback of this approach is its dependence on databases for information, that are usually very large, for the retention times, accurate mass spectra and isotopic patterns of suspected molecules and their fragments (Evgenidou et al., 2015). Following HRMS analysis, exact mass of each predicted TP is extracted from the chromatogram and compared with control samples. Unclear mass spectra are excluded by applying an intensity-threshold value that stretches mass spectra separating peaks of interest from background noise (Schymanski et al. 2014). Additionally, peaks of interest are further filtered by applying the most plausible retention time, isotopic pattern, ionization efficiency and fragmentation pattern using MS/MS or MS^n (Li et al. 2014). Computer software such as MZmine (peak peaking) and enviMass (chromatograms processing) are used for data processing by noise removal and blank subtraction (Li et al. 2014). The level of confidence of suspect analysis method in the identification of TPs, is dependent on the extent to which the above-mentioned steps were followed. When all the above criteria are fulfilled, a probable structure is proposed based on a library-spectrum match or diagnostic evidence. If some steps are missed, unsure candidates or just clear molecular formulas will be the result of suspect screening (Schymanski et al. 2014; Li et al. 2014). Some workers have applied the characteristic fragmentation pattern of TPs in their identification during suspect analysis. This approached is based on the underlined assumption that TPs bear structural resemblance to parent compounds and will therefore produce similar fragments ions (Bletsou et al. 2015; Kern et al. 2009). Accordingly, searching for specific fragment ions in MS/MS spectra throughout a chromatographic run and applying techniques such as mass-defect filtering run is reported to lead to new TPs (Ruff et al. 2015; Bletsou et al. 2015; Hug et al. 2014; Kern et al. 2009).

Non-target screening, unlike suspect screening involves screening for unknowns without any prior information on possible compounds (Fig. 2.11). Non-target screening and identification of unknown TPs is a challenging and time-consuming venture, but could be facilitated by the availability of information on the molecular formula, MS/MS spectrum, retention time and physicochemical data of potential parent compound (Evgenidou et al., 2015; Pico et al., 2015; Jeon et al. 2013). The confirmation of the identity of detected masses, assignation of molecular formula and reliable interpretation of the MS/MS spectra is highly dependent on instrument performance in producing high resolution, accurate mass and isotopic pattern data of detected TPs (Evgenidou et al., 2015). High resolution mass spectrometers e.g. triple quadrupole time-of-flight mass spectrometers (QToF-MS), linear ion trap (QLIT) and more recently hybrid linear ion trap Orbitrap (LTQ-Orbitrap), a system that combines conventional ion trap system with an exact accurate mass analyser, are examples of high resolution MS instruments (Table 2.3). In the absence of an Orbitrap, QToFs are appraised for their suitability for identification of transformation products of organic compounds (Kosjek et al., 2007; Pérez & Barceló, 2007) due to their sensitivity in full scan mode, high mass resolution and the accuracy of mass data they generate. For example, Kosjek et al. (2009) used QToF to identify the TPs of diclofenac and clofibric acid following activated sludge treatment. ToF instruments measure the time it takes ions of different mass-to-charge ratios (m/z) to travel (accelerate) from the entrance of analyser where they are orthogonally fired in pulsated fashion to the detector. Upgraded versions of ToFs give fragmentation data to a precision in the low parts per million (ppm) range (<5ppm), data that are coupled with products ions accurate mass measurements for TPs identification and confirmation (Hug et al. 2014; Kind & Fiehn, 2010; Llorca et al., 2015).

A major challenge however with the use of HRMS in non-target screen is the generation of huge amounts of data and their subsequent evaluation and results export (Llorca et al., 2015; Pico et al., 2015; Hug et al. 2014). Furthermore, their simultaneous operation in full scan and MS/MS modes produces a lot of data point in a single run. Post-acquisition data-processing tools such as XCMS, MetLin, MZmine, MassBank and enviMass are thus useful in providing rapid, accurate and efficient data mining (Bletsou et al. 2015; Evgenidou et al., 2015; Jeon et al. 2013). Mass Spectrometers vendors often provide associated data processing software,

such as MassHunter by Agilent Technologies, Metabolite Tools and Profile Analysis by Bruker, Applied Biosystems Data Explorer (MDS-Sciex Analyst QS), Waters MassLynx and MetaboLynx, Thermo Scientific Metworks and Sieve. The first and important step in the processing of non-target screen data, is peak picking, involving the comparison of peaks in the sample with control and the exclusion of irrelevant peaks (Bletsou et al. 2015; Krauss et al. 2010). This is followed by the elimination of noise peaks, mass recalibration and breakdown of isotopes and adducts into well-defined and easily identifiable units (Bletsou et al. 2015; Hug et al. 2014). Next, the Kind and Fiehn (2007) seven golden rules for heuristic (empirical) filtering of molecular formulas obtained by accurate mass spectrometry is applied for the assignment of molecular formula to the accurate mass of each peak (Bletsou et al. 2015; Krauss et al. 2010). Plausible structure for determined molecular formula is achieved via search of databases such as PubChem, NIST, ChemSpider and DAIOS database. The latter step often results in several hits which would therefore require ranking with obtained HRMS data (Krauss et al. 2010). To facilitate the structural assignment process, parent compounds' molecular formula and substructures information are useful in restricting the number of hits during databases search and possible structures are likely to be proposed for the unknown TP (Little et al. 2012; Müller et al. 2011; Krauss et al. 2010). It must be noted however that even after following strictly the above described steps in non-target screening, peaks corresponding to possible TPs can still be large, sometimes above a thousand. Thus, prioritisation of the most intense peak is generally recommended (Ruff et al. 2015; Bletsou et al. 2015; Hug et al. 2014; Schymanski et al. 2014). Like in suspect screening, the presence or absence of similar characteristic ions in parent and product fragmentation pattern, showing the stability or reactivity of certain parts of the molecule, often help to reduce the number of peaks (Agüera et al. 2013). Database generated structures are often ranked using a range of databases including MOLGEN-MS, MassFrontier, MetFrag, SIRIUS, MetFusion, ACD/MS Fragmenter including MassBank and MetLin (mentioned earlier) (Ruff et al. 2015; Bletsou et al. 2015; Hug et al. 2014; Schymanski et al. 2014).

There are number of report in the literature on the successful application of the analytical approaches described above in the identification of photolytic and biotransformation products

of a range compounds including pharmaceutical. Hug et al. (2014) reported the application of suspect and non-target screening in the identification of a range of compounds including the herbicide clomazone and UV filter benzophenone-4, and benzothiazole in WwTPs effluent. Huntscha et al. (2014) successfully applied the above mentioned non-target workflow in the identification of biotransformation products of three benzotriazoles (1H-benzotriazole, 4-methyl-1H-benzotriazole and 5-Methyl-1H-benzotriazole). Ruff et al. (2015) applied target screen in the measurement of a range of chemicals (artificial sweeteners, pharmaceuticals, pesticides) in surface water. The authors (Ruff et al. 2015) also applied non-target screen in the identification and confirmation of two substances (the relaxant tizanidine and the solvent 1,3-Dimethyl-2-imidazolidinone) that had never been detected before in their studied river. Kosjek et al. (2007; 2009), reported the application of target and non-target screening in the identification of the biotransformation products of analgesics, antiepileptic and steroidal compounds. Evgenidou et al. (2015), Pérez & Barceló (2008; 2007) and Petrovic & Barceló (2007) all reported the application of the above analytical methods in the identification of TPs of drugs covering all therapeutic classes of pharmaceuticals.

2.3 Biotransformation Prediction Tools

Computer based predictive tools that help understand the fate and possible microbial mediated transformation of organic chemicals especially those of environmental concern, have been developed and applied as a high throughput and cost-effective approach to understanding environmental processes. Examples of such strategic predictive tool of the environmental fate of organic chemicals are; the EAWAG Biocatalysis/Biodegradation Database and Pathway Prediction System (http://eawag-bbd.ethz.ch), CATABOL (http://oasis-lmc.org/products/models/environmental-fate-and-ecotoxicity/catabol-301c.aspx), PathPred (http://www.genome.jp/tools/pathpred/) and very recently enviPath (https://envipath.org/). CATABOL and EAWAG-PPS predict microbial metabolic reactions based on biotransformation rules. The EAWAG BBD associated pathway prediction system's free accessibility, clear assignation of biotransformation rule, relative ease of use and in

obtaining predicted TPs information (structure, molecular formula and mass number), makes it the most common tool used in suspect screening (Helbling et al. 2010; Kern et al. 2009, 2010). PathPred performs a multi-step reaction prediction for the biodegradation pathways of xenobiotic compounds and biosynthesis pathways of secondary metabolites. PathPred is linked to the Kyoto Encyclopedia of Genes and Genomes (KEGG) metabolic pathway maps and thus, capable of linking predicted result to genomic information. Meteor (http://www.lhasalimited.org/products/meteor-nexus.htm), the Human Metabolome Database (HMDB) (http://www.hmdb.ca/) and KEGG (http://www.genome.jp/kegg/) are some examples of other predictive tools, but for the metabolic fate of organic compounds. For example, Meteor was built based on mammalian biotransformation reactions of common functional groups and allows for prediction of metabolic fate in mammals. The above mentioned predictive tools are used together with a range of other data processing software (MassHunter, MassBank, enviMass, MetLin, MZmine, MOLGEN-MS, MassFrontier, MetFrag, SIRIUS, MetFusion, ACD/MS Fragmenter) and molecular structure search databases (e.g. PubChem, NIST, ChemSpider and DAIOS) for the identification and confirmation of suspect and unknown TPs in HRMS (Evgenidou et al. 2015; Llorca et al., 2015; Pico et al., 2015; Bletsou et al. 2015; Hug et al. 2014). MassBank and MetLin are mass spectra library database that help rank candidate structures during suspect and non-target screening by comparing their mass spectra with pattern within database. MOLGEN-MS and MassFrontier helps with TPs identification by matching of a compounds fragmentation pattern with those in the database (Bletsou et al. 2015). MetFusion combines fragmentation pattern and mass spectral search in structural elucidation of TPs.

2.3.1 The EAWAG Biocatalysis/Biodegradation Database and Pathway Prediction System (EAWAG BBD/PPS)

The EAWAG BBD/PPS, originally developed by the University of Minnesota and transferred to EAWAG, has been a unique prediction tool used since the past decade and half for the prediction of microbial transformation of xenobiotic compounds (Wicker et al. 2016; Ellis &

Wackett, 2012). The database was developed by compiling experimental results from published literature and from the Kyoto Encyclopaedia of Genes and Genomes (KEGG) of pathways of microbial (mostly bacterial) breakdown of chemicals of environmental relevance. Soil-based bacterial degradative metabolism dominates the information in the database, as bacteria represent a more diverse community, consisting of rugged strains, able to survive limited resource and constantly changing environmental conditions (Ellis & Wackett, 2012). The integrated Pathway Prediction System (PPS) predicts possible bacterial catabolic reactions using atom-to-atom mapping, substructure searching and, most especially, a biotransformation (bt) rule-based approach. Biotransformation rules which are updated periodically, are ranked “very likely”, “likely” and “unlikely” according to the likelihood of their occurrence during aerobic biodegradation. However, some anaerobes can tolerate oxygen to certain degree and thus survive in aerobic systems (Kato et al., 1997). A compound whose transformation pathway is to be predicted, is submitted to the pathway prediction system using its *simplified molecular-input line-entry system* (SMILES). For example, the SMILES for progesterone is CC(=O)[C@H]1CC[C@@H]2[C@@]1(CC[C@H]3[C@H]2CCC4=CC(=O)CC[C@]34C)C. Following the submission of a compound during each prediction cycle, the PPS examines and detects organic functional groups present in compound(s) submitted, and matches such functional groups to appropriate bt rules incorporated into the database from literature reports and/or from KEGG, and thus predicts possible degradation pathways based on built-in biotransformation rules. The prediction pathway terminates only when it reaches a compound listed as a termination compound by the PPS or when it reaches a compound that cannot be further degraded using existing bt rules. The EAWAG BBD/PPS prediction output for the pathway for progesterone transformation in the environment is illustrated in Fig 2.12 (only aerobically likely pathways) and Fig 2.13 (all available pathways). The aerobic likelihood of the formation of the predicted intermediate product was *neutral* indicating that there was a 50% chance of its formation in aerobic and anaerobic systems. The implication is that, where the odds are against its formation in a given system (aerobic or anaerobic), subsequent TPs resulting from its breakdown, would not be produced. The same is true of the formation of

products 5 and 6. However, the aerobic likelihood of the formation of products 3 and 4 is 'very likely'. The pathway of formation of product 3, a product that does not involve cleavage of the B ring (Fig 2.13) is aerobically unlikely thus showing it is a product from anaerobic degradation pathways. See Table 4.4 in the result section of this thesis for the name of products 2-6.

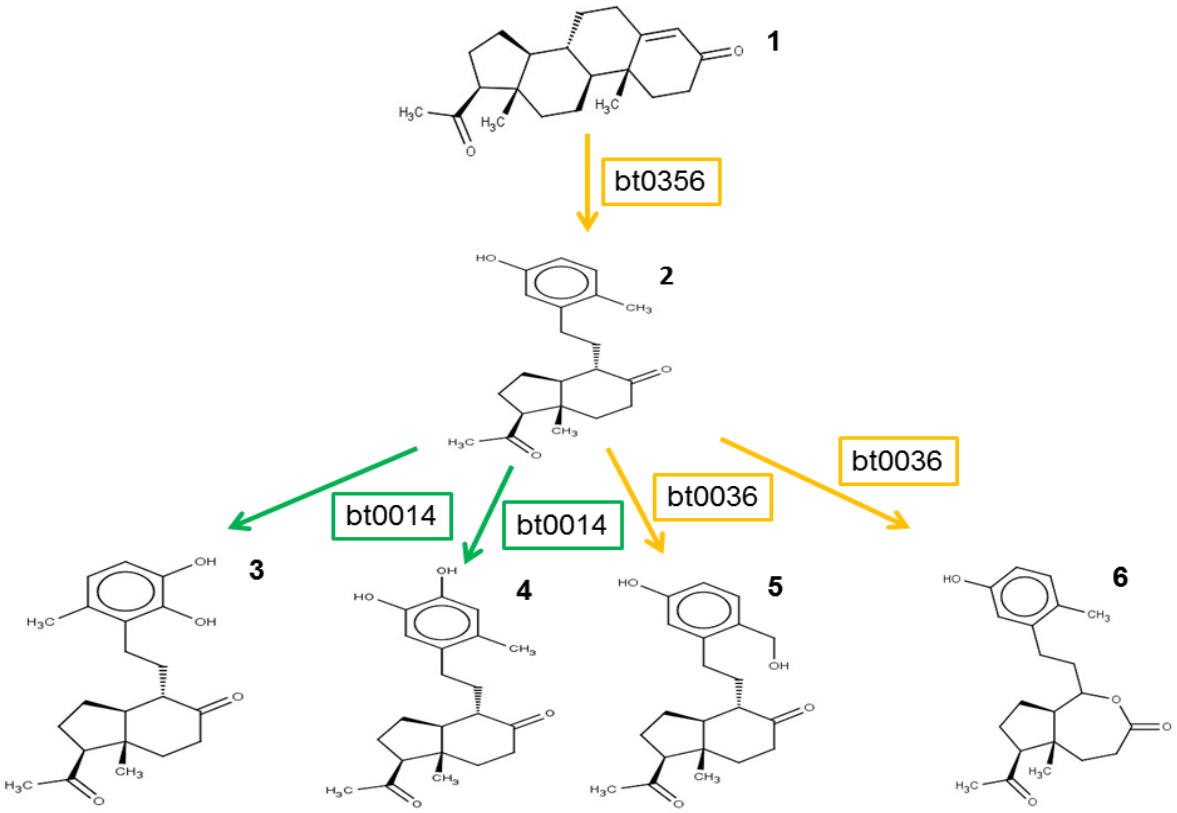


Figure 2.12: Progesterone (1) microbial biotransformation pathway prediction results from EAWAG-PPS, http://umbbd.ethz.ch/predict/ (JobID 2017.04.08-04.48.16-24). Green arrows mean that associated product have a 100% likelihood of being formed in aerobic conditions. bt: biotransformation rules that resulting the formation of associated product(s). (bt0356: oxidation, aromatization and spontaneous ring cleavage; bt0036: aromatic Methyl to primary Alcohol; bt0014: Hydroxylation).

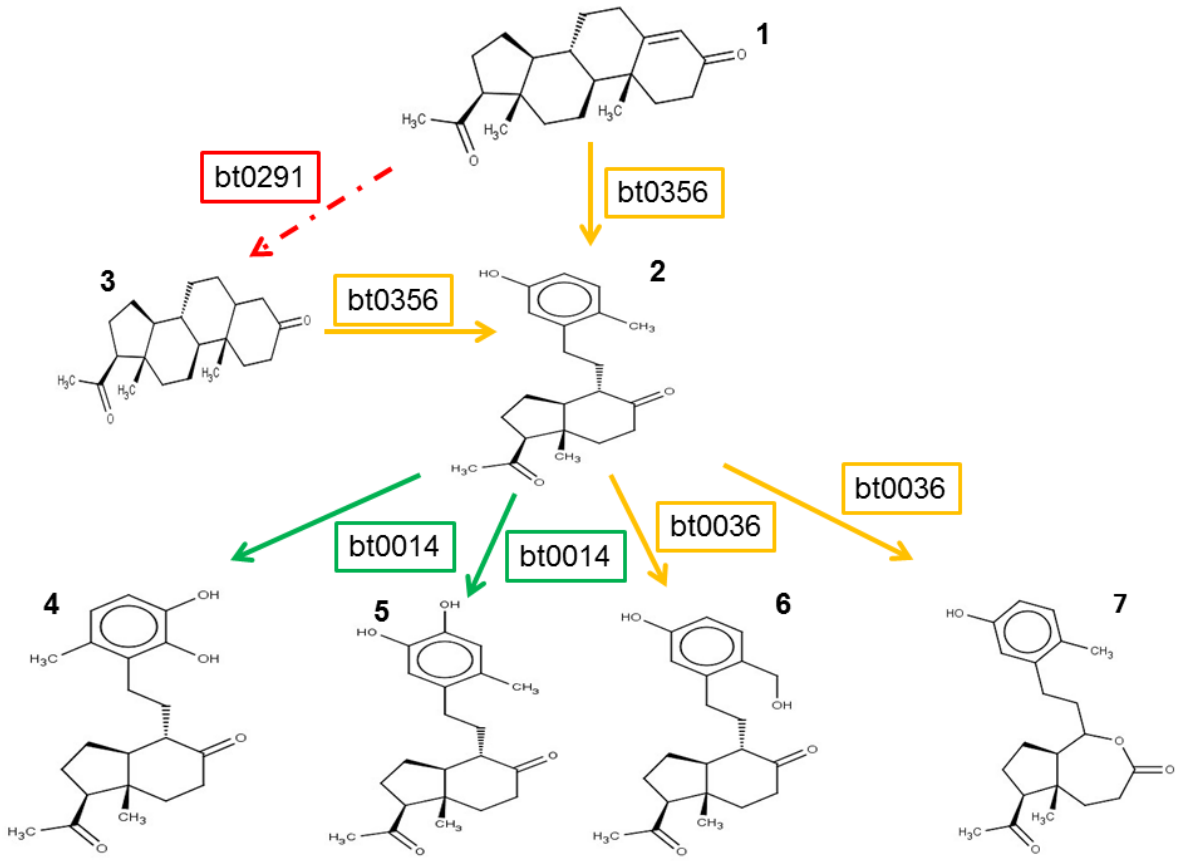


Figure 2.13: Progesterone (1) degradation pathway prediction results from EAWAG-PPS, http://umbbd.ethz.ch/predict/ (JobID 2017.04.12-12.26.26-50). Green arrows mean that associated product have a 100% likelihood of being formed in aerobic conditions. Yellow arrows indicate 50% likelihood of products formation under aerobic conditions. Red arrow indicates an anaerobic pathway. bt: biotransformation rules that resulting the formation of associated product(s). bt0291: Alkene to Alkane

The EAWAG BBD/PPS thus provides useful information on genes, enzymes, and pathways involved in biological transformation of contaminants, enabling us to appreciate environmental transformation processes, identify potential TPs and predict chemical fate in the environment. In the environment, biodegradation reactions occur alongside a number of physical breakdown processes, such as photolysis and hydrolysis. These physical processes influence the extent to which transformation processes occur in the environment, and are also very relevant in understanding the fate of certain compounds, especially those originally designed to resist microbial degradation (Fatta-Kassinos et al., 2011). The bio-persistence of many organic

compounds, limits the extent of biological transformation processes in the environment. While biochemical reactions are governed by specific pathways and enzymes, non-biotic degradation processes are less guided. This is especially true if radicals are involved, resulting in many TPs due to the non-specificity of radicals mediated abiotic reactions (Fatta-Kassinos et al. 2011). Microbes in the environment are diverse and constantly evolving in response to the influx of new chemicals and changing environmental conditions. Thus, metabolic pathways built into prediction tools such as the BBD, based on available knowledge at the time of their design, may minimise or overstate the reactions that occur in the field. To illustrate, certain microbial genes that encode enzymes for metabolic breakdown of some compounds can evolve a range of new metabolic pathways, due to current environmental pressures, that have not been previously studied, but that allow for the metabolism of new chemicals or mixtures of chemicals (Ellis & Wackett, 2012; Copley, 2009; Seffernick & Wacket, 2001).

Despite the aforementioned limitations, recent studies evaluating the performance of the EAWAG BBD in predicting real time metabolism of pharmaceuticals and other contaminants (including pesticides), have established its efficacy (Prasse et al. 2011; Helbling et al. 2010; Kern et al., 2009). For example, the EAWAG BBD was reported to have successfully predicted 21 TPs out of the 26 TPs detected in a batch reactor experiment seeded with activated sludge (Helbling et al., 2010). Database predicted TPs which were the result of a two-step (intermediate and final products) aerobic prediction system were compared to non-target screening full scan mass spectrometry data. Batch reactors were individually spiked with six pharmaceuticals and six pesticides.

2.3.2 The Environmental Contaminant Biotransformation Pathway (enviPath)

The environmental contaminant biotransformation pathway (enviPath) is also a database and prediction system designed for the prediction of microbial biotransformation pathways of organic contaminants in the environment. enviPath, also a property of EAWAG, was developed as an improvement on the low selectivity of the existing EAWAG BBD/PPS

prediction pathways and a planned future replacement for the latter (Fenner et al. 2008; Wicker et al., 2016). Relative to its predecessor reported to predict a number of irrelevant transformation products that are unlikely to occur in the environment (Fenner et al., 2008), enviPath is believed to focus on key enzyme catalysed biotransformation reactions that organic contaminants undergo in the environment. enviPath uses the EAWAG BBD/PPS database of experimental results of organic chemical biodegradation as a basis, but improves on this by allowing individual users to populate the database with their own data so as to increase the versatility of its use. The goal is that enviPath would provide predictions that could inform bioremediation technologies and that can be applied in understanding the fate and risk of organic contaminants in the environment. The full detail of the design of enviPath is available in the supplementary information of Wicker et al. (2016). enviPath was not used in this work because it was launched at a late stage of the present study. However, it was used to model P4 degradation at a later stage in this work and associated results are presented and compared to those of the BBD in chapter five of the present work. A review of literature on evidence of enviPath application was not successful. Publications available either referred to the Eawag-Soil public database developed in enviPath, and containing all freely accessible regulatory data on pesticide degradation in laboratory soil simulation studies for pesticides registered in the EU (Latino et al. 2017), or applied EAWAG BBD and only passively mentioned enviPath as a new tool (Tratnyek et al. 2017; Çeçen, 2017; Letzel et al. 2016).

2.4 Model Compounds

2.4.1 Diclofenac

Diclofenac (2-(2-(2,6-dichlorophenylamino)phenyl)acetic acid) (DCF) is a common over-the-counter non-steroidal anti-inflammatory drug (NSAID) sold under various trade names such as Acoflam, Arthrex, Diclac, Dicloabac, and Voltarol (Vieno and Sillanpää, 2014). Diclofenac is used orally as tablets or applied topically as a gel. Table 2.4 presents some relevant physicochemical properties of the compound. Dissociation constant (pKa) and partitioning coefficients Kow (octanol-water coefficient) and Kd (solid-water distribution coefficient) of DCF

in different sludge types have been included in the table because they influence the compound's removal during wastewater treatment, and in the environment. Bioconcentration factor (BCF, µg/g wet weight)/(µg/mL), a parameter that expresses a chemical's concentration in an organism relative to its surrounding environment, has not been included in Table 1. This is because reported BCF for DCF vary depending on the test organism and the prevailing pH of the test medium. Bioconcentration factor is often used to represent regulatory classification of a compound's potential for chronic toxic effects and/or biomagnification across a food chain especially in higher trophic levels (Gomez et al. 2010). This concept took on greater importance with the implementation of the European Union regulation on the Registration, Evaluation and Authorisation of Chemicals (REACH) and the Canadian assessment of chemicals on the Domestic Substances List (DSL) (Gomez et al. 2010). Bioconcentration factor is reported in varying units such as µg/mL, g/mL, g/L or kg/L (Ding et al. 2016; 2015; Brozinski et al. 2012; Kallio et al. 2010; Ericson et al. 2010). Some reports on diclofenac BCF are discussed in the fourth paragraph of this section of the thesis.

Table 2.4: Physicochemical Properties of Diclofenac

Parameter	Value	Reference
Chemical formula	$C_{14}H_{11}Cl_2NO_2$	Pubchem
CAS number	15307-86-5	Vieno and Sillanpää, 2014
Molecular weight	296.15 g/mol	Pubchem
Solubility in water	2.37 mg/L @ 25^0C	DrugBank
Dissociation constant (pK_a)	4.15	DrugBank
Octanol-water coefficient (Log K_{ow})	4.51	DrugBank
Log $K_{d,\ primary\ sludge}$	2.7; 2.3	Ternes et al. 2004; Radjenovic et al. 2009
Log $K_{d,\ secondary\ sludge}$	1.2; 2.1	Ternes et al. 2004; Radjenovic et al. 2009
Log $K_{d,\ membrane\ bioreactor}$	2.3-2.5	Vieno and Silapanää, 2014; Radjenovic et al., 2009
Log $K_{d,\ digested\ sludge}$	1.3-2.2	Vieno and Silapanää, 2014; Carballa et al. 2008

Applying Rogers (1996) general rule of thumb in using K_{OW} as an estimate of sorption (log K_{OW} < 2.5: low sorption potential, 2.5 < log K_{OW} < 4: medium sorption potential, and log K_{OW} > 4:

high sorption potential), DCF will have a high potential for adsorption to the lipophilic cell membrane of microorganisms and to the organic fractions of sludge/sediment. However, the reported effects of environmental pH on pharmaceuticals physicochemical properties (e.g. log KOW, alteration in sorption behaviours) (Luo et al. 2014; Schäfer et al. 2011), could be applied to DCF. For example, a pH greater than the dissociation constant (pKa) of chemicals was reported to potentially alter their sorption behaviour (Luo et al. 2014; Schäfer et al. 2011). Luo et al. (2014) noted that a compound's acidity (pH) (determined by its functional group) can modify its sorption or/and electrostatic interactions. Schäfer et al. (2011) indicated that at pH above the acid dissociation constant (pKa), pharmaceuticals and hormones hydroxyl functional group dissociates, making them negatively charged and facilitating charge repulsion with the negatively charged adsorbing surfaces. Thus, at pH > 4, charge repulsion can be expected to occur between DCF and the negatively charged membrane surfaces and limiting adsorption. Nevertheless, it is worth noting that adsorption of pharmaceuticals onto sludge can also be influenced by weak intermolecular forces such as Van der Waals forces (Hyland et al. 2012; Radjenović et al. 2009; Kulshrestha et al. 2004). For example, Kulshrestha et al. (2004) noted that oxytetracycline could sorb onto sludge even in the form of zwitterion (a neutral molecule with both positive and negative electrical charges), which implies that hydrophobic interactions with sludge matrix could occur despite the presence of ionic charges and/or low octanol–water partition coefficient (log K_{OW}). Solid-water distribution coefficient (Kd) is another parameter used in the determination of pharmaceuticals partitioning between sludge and the water phase. For compounds with K_d values below 300 L/kg (log Kd < 2.48), their sorption onto sludge is considered insignificant while those with log K_d > 4 are considered to have high sorption potential (Luo et al. 2014; Tadkaew et al. 2011). Thus, the low log K_d for diclofenac indicates that it is not likely to adsorb to solids and be removed by primary treatment (sedimentation).

Diclofenac is an environmentally relevant compound due to reports of its regular detection in the water environment and its adverse effects on aquatic species at environmentally relevant concentration ≤1.0 µg/L (Acuña et al. 2015; Diniz et al. 2015; Vieno and Silapanää, 2014). In the effluents of municipal wastewater treatment plants, DCF is among the most frequently

detected pharmaceuticals (Verlicchi et al., 2012). Verlicchi et al., 2012 reported a daily average mass load of 240 mg/L. Some reported lowest observed effect concentrations of diclofenac are presented in Table 2.5. During a review of the Water Framework Directive (200/60/EC), the European Commission panel included diclofenac and two estrogenic hormones in a watchlist of compounds that could be classified as hazardous environmental compounds, depending on results of a four-year EU-wide environmental risk monitoring campaign (EU, 2013).

In addition to its toxic effects, DCF apparent high octanol-water partition coefficient (log K_{OW} >4 (Table 2.4)) has raised concerns about its potential for bioconcentration in fish and the subsequent possibility for secondary poisoning within the aquatic food chain and arboreal food chain (through fish-eating birds) (Schwaiger et al. 2004). Generally, the BCFs of hydrophobic compounds are closely correlated with their octanol-water partition coefficient (log Kow) (Dai et al., 2013). Bioconcentration of DCF in some aquatic organisms including fish has been reported. For example, a higher concentration of diclofenac relative to measured water concentration was reported in mussels (Ericson et al. 2010). At a water concentration of 1 µg/L in an 8-day exposure study, the authors reported a bioconcentration factor of 175. Similarly, at a 1 µg/L aqueous DCF concentration in a 28 days exposure study, bioconcentration factors of 2732, 971, 763 and 69, were respectively reported for the liver, kidney, gills and muscle tissues of the rainbow trout (Brozinski et al. 2012; Kallio et al. 2010; Schwaiger et al. 2004). Following exposure to aqueous DCF concentration of 1.7 µg/L in a 10-day exposure study, Kallio et al. (2010) reported bioconcentration factors in the bile of the rainbow trout in the range of 320–950. In another study however, the BCF of DCF in rainbow trout was reported to be below 10, and the authors noted that this represented no relevant bioconcentration in the trout (Memmert et al. 2013). The variation in reports on potential bioconcentration of DCF in fish tissues may be related to the effect of pH on DCF solubility and octanol-water distribution coefficient (the common method of BCF determination). For example, Avdeef et al. (1998) reported a log K_{OW} (octanol-water partition coefficient) of 4.51 and 1.3 for nonionised DCF at pH 3 and 7 respectively. Scheytt, et al. (2005) reported a log K_{OW} of 1.9 at pH 7.0. Thus, as suggested by Memmert et al. (2013,) a more objective estimate

of fish DCF bioconcentration by the octanol-water distribution coefficient, should consider environmentally relevant pH range of 6 to 9.

Table 2.5: Lowest Observed Effect Concentration of Diclofenac in Environmental Species

Species	Exposure time	Endpoint	Conc. (µg/L)	Reference
Rainbow Trout (*Oncorhynchus mykiss)*	28 days	Changes in liver ultrastructures, liver glycogen	1	Triebskorn et al. 2004
Mollusca (*Dreissena polymorpha*)	96 hr	Reduction of haemocyte (blood cell) viability; Oxidative stress with high lipid peroxidation*	1	Acuña et al. 2015; Parolini et al. 2009; Schmidt et al. 2011
Green shore crab (*Carcinus maenas*)	Not reported	Increased haemolymph osmolarity	0.01	Eades and Waring, 2010
Fish (*Cirrhinus mrigala*)	96 hr	Changes in enzymatic activity	1	Acuña et al. 2015; Saravanan and Ramesh 2013
Zebra mussels (*Dreissena polymorpha)*	96 hr	Increases lipid peroxidation	1	Quinn et al., 2011
Zebra fish (*Danio rerio*) embryos	90 mins; 96 hr	Reduction in lipid peroxidation	0.03	Feito et al. 2012
Fern (*Polystichum setiferum)*	48 hrs	Increased mitochondria activity	0.3	Acuña et al. 2015; Vieno and Silanpanää, 2014; Feito et al. 2012
Baltic sea blue mussel (*Mytilus edulis trossulus)*	8 days	Adverse effects on byssus strength	1	Ericson et al. 2010

* Lipid peroxidation refers to the process in which free radicals "steal" electrons from the lipids in cell membranes, resulting in oxidative cell damage. Conc.: concentration

2.4.1.1 Environmental Occurrence of Diclofenac

Measured concentrations of DCF in the water environment vary depending on the source and type of water environment; surface, groundwater or wastewater, with the highest recorded concentrations being in hospital and pharmaceutical manufacturers wastewater (Vieno and Silanpää, 2014; Sim et al. 2011; Zhang et al. 2008). Some reported concentrations of DFC in the water environmental are presented in Table 2.6. Generally, reported surface water concentrations are <0.1-0.5 µg/L (Hernando et al., 2006; Hilton and Thomas, 2003; Kim et al., 2007; Lin et al., 2005; Rabiet et al., 2006; Vieno, 2007). However, there are cases were higher concentrations have been measured (Table 3). Groundwater concentrations are often below instrument detection limits, although there are also cases where high values have also been measure (Table 3) (Lin et al., 2005; Loos et al., 2010; López-Serna et al., 2013; Rabiet et al., 2006). Comparing the measured environmental concentration of DCF (Table 2.6) and the reported low effect concentration (Table 2.5), it is apparent that concentrations often detected in surface and groundwater may represent a problem for aquatic biota, thus, lending support to the EU's decision to include the compound in a watchlist of potentially hazardous chemicals.

Table 2.6: Measured Environmental Concentrations of Diclofenac and its Metabolites

Compound	Type of water	Source	Conc. (µg/L)	Reference
DCF	Wastewater	Hospital	6.9	Sim et al., 2011
DCF	Wastewater	Pharmaceutical manufacturers	203.0	Sim et al. 2011
DCF	Wastewater	Hospital/Municipal	0.2	Zorita et al., 2009
5-OH-DCF	Wastewater	Municipal	3.7	Langford and Thomas, 2011
DCF	Surface water	Rivers	1.2	Ternes, 1998
DCF	Surface water		8.5	Scheurell et al., 2009
3'-OH-DCF	Surface water		0.3	Scheurell et al., 2009
4'-OH-DCF	Surface water		1.8	Scheurell et al., 2009
DCF	Groundwater		0.4	Lopez-Serna et al. 2013, Heberer, 2002
4-OH-DCF	Groundwater		0.2	Lopez-Serna et al. 2013
DCF	Drinking water		<0.01	Vulliet et al. 2011

2.4.1.2 Removal of Diclofenac During Biological Wastewater Treatment

Diclofenac is a poorly biodegradable compound resulting in its low removal during biological treatment process (Vieno and Silanpaää, 2014; Lee et al. 2012). A pseudo-first order rate constant of 0.018d^{-1} (approximately a 40 days' half-life) was reported for DCF under biotic conditions (Vieno and Silanpaää, 2014; Lee et al. 2012). A biological degradation constant of <0.1 L g^{-1}ss d^{-1} has been generally assigned to DCF (Vieno and Silanpaää, 2014; Suárez et al. 2012; 2010; Zhang et al. 2008), indicating very slow to no biodegradation potential. The biodegradation constant (k_{biol}) is an estimate of a compound's biodegradation potential considering a first order degradation reaction (Joss et al. 2006). This classification system which was first suggested by Joss et al. (2006), revised by Suárez et al. (2010) for measuring micropollutants biological degradation potential and applied to DCF, is presented below;

k_{biol} <0.5 – hardly biodegradable

0.5 < k_{biol} < 1 – moderately biodegradable

1 < k_{biol} < 5 – highly biodegradable

k_{biol} > 5 – very highly biodegradable

Diclofenac biodegradation constants (k_{biol} value) for different treatment configurations are presented in Table 2.7. Where removal and length of study information are available for the treatment method reported, these have been included in the table. Examples of process configurations reported to influence DCF removal are; conventional activated sludge (CAS), biological nutrient removal (BNR), membrane bioreactor (MBR) and a moving bed biofilm reactor (MBBR) (Vieno and Silanpää, 2014). Operational and environmental conditions such as sludge retention time, hydraulic retention time, pH, temperature and presence or absence of oxygen, are other factors reported to influence DCF removal during biotic treatment (Vieno and Silanpää, 2014; Zhang et al. 2008). Generally, k_{biol} for DCF in different treatment systems (including anoxic conditions) is <0.5 indicating poor removal via biodegradation. However,

nitrifying conditions were reported by Suarez et al. (2010), to account for moderate biodegradation of DCF, perhaps by co-metabolism by ammonium monooxygenase enzyme. Nevertheless, k_{biol} value of 1.2 l g^{-1} $_{SS}$ d^{-1} was reported for DCF under aerobic nitrifying conditions, thus, suggesting a moderate to high biodegradation potential (Vieno and Silanpää, 2014). Similarly, Tran et al. (2009) noted that DCF was moderately biodegradable when enriched nitrifying activated sludge was used (k_{biol} of 0.31–0.52 l $g^{-1}{}_{SS}$ d^{-1}). These reports highlight that biological elimination of DCF can be improved depending on the treatment process configuration and operational conditions. Extended sludge retention time (SRT), which facilitates the build-up of the slowly growing nitrifying bacteria population (reputed for removal of a range of micropollutants) will be an important operational factor in the biological elimination of DCF during wastewater treatment.

Diclofenac however, has been shown to rapidly phototransform in environmental waters, although with concerns over the increased toxicity of its phototransformation products relative to the parent compound (Avetta et al. 2016; Dinz et al. 2015; Salado et al., 2013: Svanfelt, 2013; Zhang et al. 2008). For example, Zhang et al. (2008) reported the rapid photodegradation of DCF, with a half-life of 1 hour when an aqueous solution was exposed to sunlight. Incubation of lake water, spiked with DCF, showed no degradation in the dark, but rapidly phototransformed (half-life: 1 hr) when exposed to sunlight (Buser et al. 1998).

Table 2.7: DCF Biodegradation Constant and Removal in Different System Configuration

Treatment System	k_{biol}	Study Length	% Removal	References
CAS	<0.1	> 28 days	1 - 4	Vieno and Silapanää, 2014
MBR	<0.1	-	-	Zhang et al. 2008
Anoxic+Aerobic	<0.1	-	-	Joss et al. 2006
Anoxic	0.4	-	-	Suarez et al. 2012
Nitrifying conditions	1.2	-	-	Suarez et al. 2010
Nitrification culture	0.31-0.52	-	-	Tran et al. 2009
Aerobic	<0.1	55 hours	1-4	Vieno and Silapanää, 2014
Oxic MBBR	-	48 hours	34-38	Vieno and Silapanää, 2014

2.4.1.3 Human Metabolism and Metabolites of Diclofenac

Globally, the annual consumption of diclofenac varies from country to country, and from individual to individual (Zhang et al., 2008). For clarity and where data are available, the World Health Organization (WHO) statistically standardized measure of drug consumption, *defined daily dose*, has been used in this work. The WHO defined daily dose for diclofenac is 100 mg (Vieno and Silanpää, 2014). Oral and dermal (topical) administration are the two forms of use of DCF with oral application being the dominant mode of use. Topically applied diclofenac gel is absorbed up to 6-7% by the skin and the remainder is washed off during bathing or adheres to clothing and is subsequently washed off during laundry (Davies and Anderson, 1997). Dermal use is reported to limit the bioavailability of the compound by 50% by by-passing metabolism in the liver (Zhang et al., 2008) and could therefore result in the greatest amount of parent DCF discharged into rivers (Heberer and Feldmann, 2005). Orally administered DCF is almost completely metabolised, with a reported half-life of 2 hours (Wishart et al., 2006), with <1% excreted as unmetabolized DCF (Vieno and Silanpää, 2014). Due to Phase I hepatic reaction (oxidation, reduction and hydrolytic cleavage) and Phase II metabolism involving glucuronic acid and taurine, metabolites, glucuronide and sulfate conjugates are formed (Davies and Anderson, 1997). About 11% of the daily administered DCF is eliminated from the body as conjugates and the remaining 88% as metabolites (Stierlin and Faigle, 1979). Generally, there is an increasing number of -OH groups (representing increased polarity and facilitating quick elimination from the body), but no apparent ring cleavage. Between 65-70% of DCF is excreted in urine and 20-30% in faeces as a mixture of parent DCF, conjugates and mostly metabolites. The metabolic pathway of diclofenac in the human body and resultant conjugates and metabolites are presented in Figure 2.14.

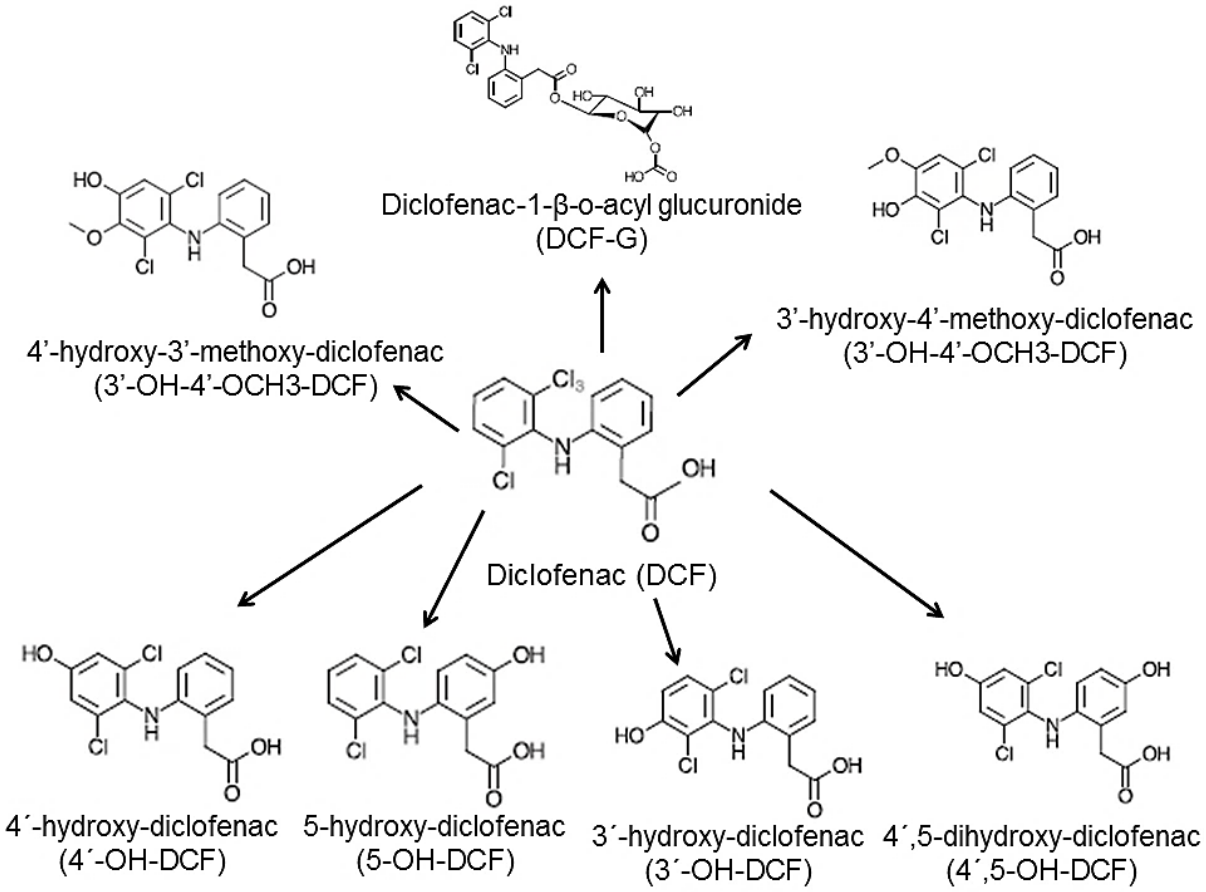


Figure 2.14: Metabolic pathway and metabolites/conjugates of diclofenac (Adapted from Davies and Anderson, 1997; Blum et al. 1996; Fiagle et al. 1988; Sterlin and Faigle, 1979; Steirlin et al. 1979)

2.4.2 Propranolol

Propranolol (hydrochloride, 1-(isopropylamino)-3-(1-napthyloxy)-2-propanol hydrochloride), is a beta-blocker and one of the cardiovascular pharmaceuticals used to regulate blood flow through arteries and veins in the heart (Dantas et al., 2010). It is widely used for the treatment of tremors, angina (chest pain), hypertension (high blood pressure), heart rhythm disorders, and other heart or circulatory conditions (Uwai et al. 2005). It is also used to treat or prevent heart attack, and to reduce the severity and frequency of migraine headaches (Dantas et al., 2010; Uwai et al. 2005). The physicochemical properties of propranolol are presented in Table 2.8. Environmental fate parameters (Log K_{OW} and Log K_d) have been included.

Bioconcentration factor (BCF) discussed in the work, is not shown in the table for similar reason presented for diclofenac.

Table 2.8: Physicochemical Properties of Propranolol

Parameter	Value	Reference
Chemical formula	$C_{16}H_{21}NO_2$	Pubchem
CAS number	525-66-6	Lin et al., 2010
Molecular weight	259.35 g/mol	Pubchem
Solubility in water	61.7 mg/L @ 25^0C	DrugBank
Dissociation constant (pK_a)	9.42; 9.14	DrugBank; Balon et al. 1999
Octanol-water coefficient (Log K_{OW})	2.60-3.48	ChemSpider
Log $K_{d,\ primary\ sludge}$	641 ± 478 L/kg	Radjenović et al. 2009
Log $K_{d,\ secondary\ sludge}$	320 ± 58 L/kg	Maurer et al. 2007; Lin et al. 2010; Alder et al. 2010

Going by the earlier mentioned classification of chemical sorption potential based on the octanol-water coefficient (Rogers, 1996), the Log K_{OW} of 2.60–3.48, indicates a medium potential for bioconcentration in aquatic biota and a moderate potential for sorption into sludge. Furthermore, the compound's high log K_d values (>300 L/kg) indicates that sorption to sludge is an important removal mechanism during wastewater treatment. Due to ionization of the PRPL amino group, the compound will have positive charges when the pH of the solution it is found is below its pKa and then carries negative charge as the solution's pH rises above its pKa (Deng et al. 2011; Hansen et al. 2006). Maurer et al. (2007) noted that PRPL is a weak base that becomes protonated and positively charged at environmental pH values of around 7.

Sorption of propranolol onto solids was reported to be influenced by pH and ionic strength. For example, the sorption capacity of PRPL onto acid-activated attapulgite clay was reported to be higher than the natural attapulgite (Deng et al. 2011). The authors attributed their observation to the non-electrostatic surface complexation, electrostatic interactions and cation

exchange properties of the acid-activated attapulgite. Attapulgite natural sorbent (crystalline hydrated magnesium silicate with a fibrous morphology) is reported to be a low-cost and environmentally more relevant sorbent relative to activated carbon (Wang et al. 2011; Liu et al. 2011). Significant removal (83%) of PRPL concentration by sorption into sediment was reported following a 14-day study with a half-life of 2.2 days (Lin et al. 2010). Similarly, sorption potential was reported for low- and high-organic-content soils as well as in acclimatized and non-acclimatized activated sludge (Drillia et al. 2005; Daniel et al. 2005). These reports suggest that sorption will be an Important dominant removal mechanism for PRPL during wastewater treatment.

Following a 24-hour *Daphnia* exposure study at aqueous PRPL concentration of 5 µg/L, a bioconcentration factor of 83 kg/L was reported (Ding et al. 2016). The importance for accounting for pH influences when assessing the bioaccumulation and ecological risks of PRPL, being an ionizable organic compound, was also highlighted in the Ding et al. 2016 study. Propranolol BCF was reported by the authors to increase in *Daphnia* with increasing pH levels, ranging from 7 to 9. The authors also noted the influence of PRPL hydrophobicity (log K_{ow} 3.48) on its bioconcentration potential. At a nominal PRPL concentration of 1 µg/L, Ericson et al. (2010) reported a BCF of 160 in the Baltic Sea blue mussels, *Mytilus edulis trossulus* following an 8 days exposure study. Following a 21 days exposure study, Giltrow et al. (2009) reported an increased plasma concentrations (340 µg/L) of PRPL in male fathead minnows exposed to nominal PRPL concentrations of 100 µg/L. At an aqueous PRPL concentration of 10 µg/L, a BCF of 103.4 was reported for algae following a 48-hour exposure study (Ding et al. 2015).

Propranolol (PRPL) is relevant environmentally because it is regularly detected in the water environment, especially in wastewater (Godoy et al. 2015; Gros et al. 2010; Alder et al. 2010; Robert and Thomas, 2006; Thomas and Hilton, 2004) and reported to have toxic effects on aquatic organisms (Ribeiro et al. 2014; Huggett et al., 2002; Ferrari et al., 2004; Escher et al. 2005; 2006). The reported daily loading rate of PRPL in WwTPs effluent was 0.39 ± 0.07 L d^{-1} g^{-1} (Alder et al. 2010; Maurer et al. 2007). Alder et al. (2010) reported that PRPL was detected

in 40 German rivers studied, at median and maximum concentration of 0.012 µg/L and 0.59 µg/L respectively. Similarly, Thomas and Hilton, (2004) reported a widespread detection of PRPL in UK rivers being present in approximately 90% of the rivers sampled (*n=45*) at a median concentration of 0.029 µg/L. Following a 3-year monitoring study, Gros et al. (2010) reported a 100 and 79% detection rate for PRPL in effluent and river waters respectively. In terms of ecotoxicological effects, Escher et al. (2005; 2006) demonstrated that PRPL inhibited the photosynthetic efficiency of the green algae *Desmodesmus subspicatus* at environmentally relevant concentrations. Ribeiro et al. (2014) reported the death of Zebra fish embryos and a decrease in sea urchin larva exposed to 125 µg/L of propranolol. Huggett et al. (2002) reported a decrease in the reproduction of *Hyalella azteca* (amphipod crustacean) at a PRPL concentration of 100 µg/L. A 4-week propranolol exposure study, at concentrations as low as 0.5 µg/L, resulted in a decrease in the total number of eggs produced by Medaka, Japanese rice fish (*Oryzias latipes*) and the number of viable eggs that were hatched (Huggett et al. 2002). Table 2.9 presents a summary of the reported lowest observed effect concentrations (LOEC) of propranolol.

Table 2.9: Lowest Observed Effect Concentration of Propranolol in Environmental Species

Species	Exposure time	Endpoint	Conc. (µg/L)	Reference
Daphnia magna	9 days	Reduced growth	440	Dzialowski et al. 2006
Daphnia magna	9 days	Reduced reproduction	110	Dzialowski et al. 2006
Daphnia magna	9 days	Changes in heart rate	55	Dzialowski et al. 2006
Adult *Mytilus galloprovincialis*	10 days	Changes in feeding rate	147	Solé et al. 2010
Adult *Mytilus galloprovincialis*	10 days	Enhanced gill GST (glutathione *S*-transferase) activity	11	Solé et al. 2010
Daphnia magna	21 days	Increased reproduction	50	Stanley et al. 2006
Pimephales promelas	48 hrs	Decreased growth	1210	Stanley et al. 2006
Hyalella azteca	48 hrs	Decreased reproduction	100	Huggets et al., 2002
Ceriodaphnia dubia	48 hrs	Decreased reproduction	250	Huggets et al., 2002
Medaka (*Oryzias latipes*)	4 weeks	Decreased reproduction	0.5	Huggets et al., 2002

2.4.2.1 Environmental Occurrence of Propranolol

Like most pharmaceutical compounds, measured concentrations of PRPL vary depending on water type with the highest concentrations being in wastewater influent and effluents. Some measured concentrations of PRPL in the environment are presented in Table 2.10. Concentrations in drinking water were generally below instrument detection limits and environmental concentrations of its metabolites are scarce in the literature. Overall, measured environmental concentrations of PRPL are below the reported LOEC values. However, constant input of the compound with effluent into the environment and PRPL potential for bioconcentration and subsequent bioaccumulation makes it a chemical of concern.

Table 2.10: Measured Environmental Concentrations of Propranolol

Type of water	Source	Concentration (µg/L)	Reference
Effluent	Municipal	0.3	Ternes, 1998
Surface water	WwTPs effluent	0.6	Ternes, 1998
Effluent	Municipal	0.3	Ashton et al., 2004
Surface water	WwTPs effluent	0.2	Ashton et al. 2004
Effluent	WwTPs effluent	0.4	Roberts and Thomas, 2006
Surface water	WwTPs effleunt	0.1	Roberts and Thomas, 2006
Effluent	Municipal	0.2	Fono and Sedlak, 2005
Surface water		<0.1	Fono and Sedlak, 2005
Effluent	Municipal	0.2	Morasch et al. 2010
Drinking water		<0.01	Morasch et al, 2010

2.4.2.2 Removal of Propranolol During Biological Wastewater Treatment

Poor biodegradability and incomplete removal during wastewater treatment is an important pathway of propranolol contamination of surface water (Godoy et al. 2015; Maurer et al. 2007; Bendz et al., 2005). For example, a Swedish report noted poor removal (32%) of PRPL following activated sludge treatment (Bendz et al., 2005). Gros et al. (2010) reported 0-20% removal for PRPL in various activated sludge treatment plants operated at hydraulic retention times ranging from 6-33 hrs. However, Radjenović et al. (2009) reported a 60% (conventional activated sludge) and 66-78% (membrane bioreactors) removal rate for propranolol, perhaps due to sorption into sludge and not microbial degradation. The authors however noted that operation of one of their membrane bioreactors at prolonged sludge retention time, was unfavourable for the elimination of PRPL. Kasprzyk-Hordern et al. (2009) reported a 35% removal rate for PRPL following activated sludge treatment. Furthermore, PRPL is reported to exhibit a high degree of persistence in surface waters (Maszkowska et al. 2014; Bendz et al., 2005), a factor that may contribute to its frequent detection in surface waters. Maszkowska et al. (2014) reported that the half-live of propranolol in surface water at 25 °C could be >1 year

and that in addition to the compound's high hydrolytic stability, relatively limited immobilization in natural soils and/or sediments may cause PRPL to accumulate in the aquatic environment.

2.4.2.3 Human Metabolism and Metabolites of Propranolol

In Europe, the use of beta-blockers varies across member states, with the UK reported to have the highest consumption rate (approx. 3.2 g cap^{-1} a^{-1}) in 2001 (Alder et al., 2010). In 2004, Robinson et al. (2007) reported an estimated UK use of PRPL as 10,000 $kg^{-1}a^{-1}$. The WHO defined daily dose for propranolol is 160 mg (WHOCC, 2016). Propranolol is administered orally or intravenously. Following use, PRPL is completely absorbed and extensively metabolised with a half-life of approximately 4 hours, with only 1–4% excreted unchanged with faeces (Alder et al. 2010). Because of Phase I and II metabolisms in the liver, the remainder of the administered dose is extensively metabolized and metabolites including conjugates are excreted via urine (Masubuchi et al. 1994; Walle et al., 1985). Walle et al. (1985) noted that the primary metabolic pathways and their relative contributions were; glucuronidation (17%), side-chain oxidation (41%) and ring oxidation (42%). Some reported metabolites of propranolol are; propranolol-glucoronide; 4-hydroxy-, 5-hydroxy-, 7-hydroxy-, N-desisopropylpropranolol, propranolol glycol and naphthoxylactic acid (Figure 2.15) (Celiz et al. 2009; Masubuchi et al. 1994; Walle et al., 1985; Paterson et al. 1970).

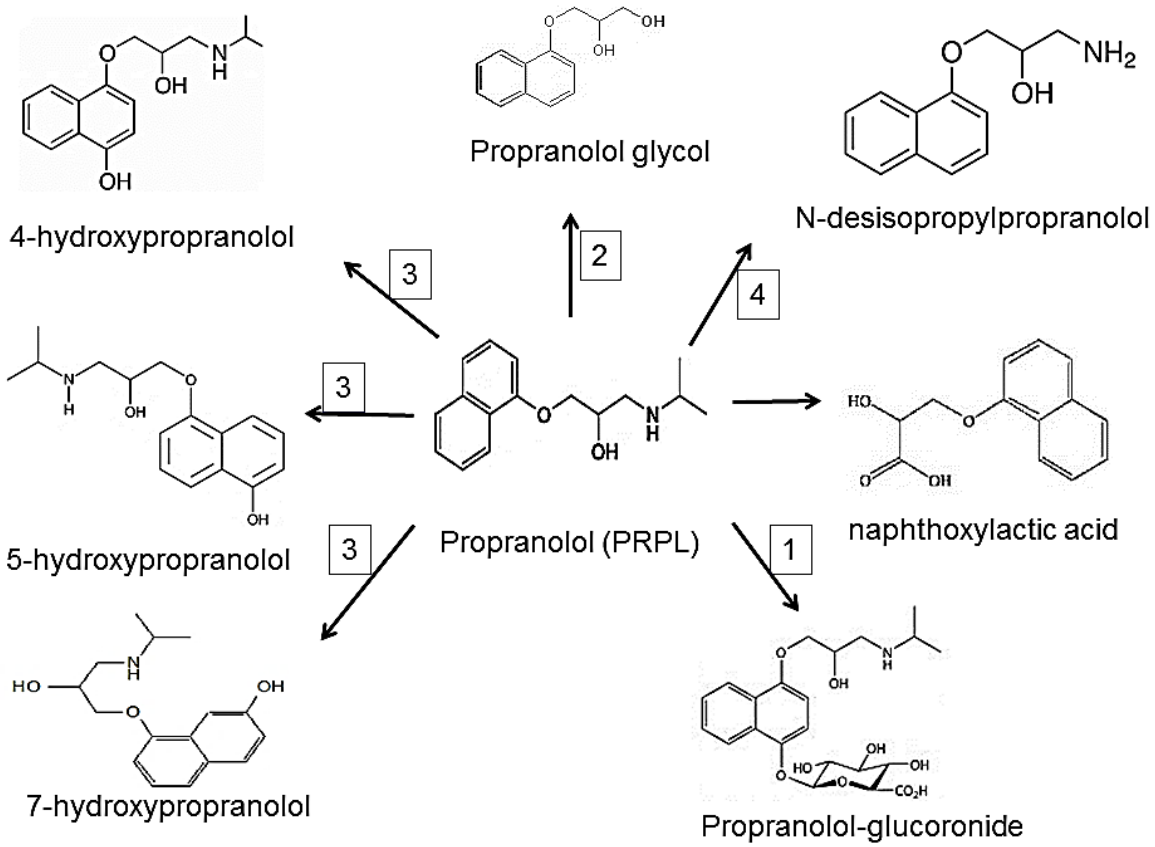


Figure 2.15: The metabolic pathways for propranolol reported in human, showing glucuronidation (1); side-chain oxidation (2); ring oxidation (3) and N-desisopropylation (4) (Celiz et al. 2009; Masubuchi et al. 1994; Walle et al., 1985; Paterson et al.1970)

2.4.3 Fluoxetine

Fluoxetine ((±)-N-methyl-3-phenyl-3-[(α,α,α-trifluoro-p-tolyl)oxy]propylamine) (FLX) is a prescription only antidepressant sold popularly as Prozac, and a member of the selective serotonin reuptake inhibitor (SSRI) drugs class (Hazelton et al. 2014; Morando et al. 2009). Fluoxetine is administered orally for the treatment of major depression along with panic, bulimia nervosa, obsessive–compulsive behaviours and premenstrual dysphoric disorders (Hazelton et al. 2014; Morando et al. 2009). Fluoxetine acts by strongly and selectively inhibiting a transporter enzyme for serotonin reuptake at the presynaptic membrane, causing an increase in serotonin (5-hydroxytryptamine) concentrations at postsynaptic receptor sites

(Vaswani et al. 2003; Wong et al. 1995). It is mainly metabolized by the cytochrome P450 enzyme system in the liver to yield several metabolites including norfluoxetine (Preskorn et al. 1997; DeVane, 1999; Benfield et al. 1986). Norfluoxetine (N-FLX) is a pharmacologically active metabolite known to inhibit serotonin uptake (Lister et al. 2009; Fong et al. 2008) and which compared to the parent compound, has a longer elimination half-life in both rats (7-15 days) and humans (1-3 days) (Kwon and Armbrust, 2006; Brooks et al. 2003). The physicochemical properties of FLX are presented in Table 2.11. Relevant treatment and environmental fate parameters such as Log K_{OW}, Log K_{OC} and Log K_d have been included.

Table 2.11: Physicochemical Properties of Fluoxetine

Parameter	Value	Reference
Chemical formula	$C_{17}H_{18}F_3NO$	Pubchem
CAS number	54910-89-3	IPCS INCHEM
Molecular weight	309.33 g/mol	Pubchem
Solubility in water	50 mg/mL at 25 °C	DrugBank
Dissociation constant (pKa)	10.06 ± 0.10	Brooks et al. 2003
Octanol-water coefficient (Log K_{OW})	4 - 4.35	Radjenović et al. 2009; Brooks et al. 2003
Octanol-carbon coefficient (Log K_{OC})	3 – 4.50	Hyland et al. 2012; Brooks et al. 2003
Sediment-water coefficient (K_d)	368 L/Kg	Bringolf et al. 2010

Fluoxetine is environmentally relevant because research has shown that the compound is regularly detected in the water environment (Fernández et al. 2010; Neuwoehner et al. 2009; Radjenović et al. 2009; Kim et al. 2007). For example, Radjenović et al (2009) reported that on an average, 13 g/day of FLX is discharge with effluent into surface waters. Fluoxetine is reported to be relatively stable to hydrolysis, photolysis, and microbial degradation during engineered treatment and in the environment (Radjenović et al. 2009; Redshaw et al. 2008; Kwon and Armbrust, 2006). The compounds relatively high Log K_{OW}, K_{OC} and K_d indicates that adsorption to sludge and sediment will be an important removal mechanism during wastewater treatment and from surface waters. Radjenović et al. (2009) and Redshaw et al. (2008)

independently reported that FLX partitioned extensively to sediment. Kwon and Armbrust, (2006) reported higher concentrations of FLX in sediment relative to the aqueous concentration, indicating persistence and potential for bioaccumulation across the aquatic food chain (through benthos feeders). Fluoxetine was detected in sediment at 968-fold higher concentrations relative to aqueous levels (Furlong et al. 2004). Following their 44-days laboratory based FLX fate and exposure study, Sánchez-Argüello et al. (2009) reported a 10-fold higher concentration of FLX in sediment relative to overlaying water concentration. The authors noted that at a water concentration of 125 µg/L, approximately 900 µg/kg of FLX was measured in sediment. Partitioning of FLX is potentially dependent on the content of organic material in water and sediment. The presence of suspended organic matter (OM) enhances removal via adsorption, but on the other hand, high OM content in sediment can increase adsorption to sediment through covalently bound residues (Kwon and Armbrust, 2006) and results in persistence. Sorption of FLX to aerobic granular sludge was observed by Moreira et al. (2015) in a sequential batch reactor study. Like diclofenac and propranolol, pH was reported to alter the sorption properties of FLX. For example, Brooks et al. (2003a, b) predicted FLX log K_{OW} at pH 2.0 and 11.0 to be 1.25 and 4.30 respectively. Also, at pH 7.0, log K_{OW} for FLX have been variously reported as 1.57 (Brooks et al. 2003a), 1.8 (Huggett et al. 2004), 4.05 (Christensen et al. 2007), and 4.51 (Lienert et al. 2007).

Fluoxetine has been reported to potentially accumulate in organisms (Bringolf et al. 2010; Schultz et al. 2010; Chu and Metcalfe, 2007; Brooks et al. 2005; Furlong et al. 2004). For example, BCF values of 74 and 80 were measured for FLX and N-FLX after a 5-day exposure of the Japanese Medaka to FLX concentration of 0.64 µg/L (Paterson and Metcalfe, 2008). Meredith-Williams et al. (2012) reported a BCF of 185,900 for the freshwater shrimp (*Gammarus pulex*) at aqueous FLX concentration of 100 µg/L. Bringolf et al. (2010) and Schultz et al. (2010) independently reported the accumulation of FLX and N-FLX in freshwater fishes. Like diclofenac and propranolol, reported BCF values for FLX vary with pH. For example, Nakamura et al. (2008) reported BCF values of 330, 580 and 310 µg/L at pH 7.0, 8.0 and 9.0 respectively for the Japanese Medaka exposed to 15 µg/L FLX concentration.

Brooks et al. (2003a, b) predicted BCF values for Medaka at pH 2.0, 7.0 and 11.0 following a four-week FLX exposure at 1 µg/L were 1, 2 and 1071.5.

Fluoxetine and Norfluoxetine have been demonstrated to be toxic to aquatic biota (Hazelton et al. 2014; Mennigen et al. 2010; Fong et al. 2008; Brooks et al. 2003). For example, FLX was reported to induce disruption in movement and burrowing of freshwater mussels at concentrations of 2.5 and 23.3 µg/L (Hazelton et al. 2014). Some reported LOEC for FLX and N-FLX and their corresponding biological endpoints are presented in Table 2.12.

Table 2.12: Lowest Observed Effect Concentration of Fluoxetine in Environmental Species

Species	Compound	Endpoint	Conc. (ng/L)	Reference
Potamopyrgus antipodarum	FLX	Embryos without shell	0.81	Santos et al. 2010
Chironomus riparius	FLX	Reduced emergence	1120	Nentwig, 2007
Danio rerio	FLX	Reduced egg production and decreased 17β-estradiol levels	0.32	Lister et al. 2009
Ceriodaphnia dubia	FLX	Increased fecundity	56	Santos et al. 2010
Daphnia mana	FLX	Increased fecundity	36	Flaherty and Dodson 2010
Daphnia magna	FLX	Deformity and Mortality	36	Flaherty and Dodson 2010
Oryzias latipes	FLX	Increased 17β-estradiol levels	0.1	Foran et al. 2010
Raphidocelis subcapitata	FLX	Growth inhibition	13.6	Santos et al. 2010
Dreissena polymorpha	N-FLX	Increased reproduction	1	Fong and Molnar 2008
Mytilopsis leucophaeata	N-FLX	Increased reproduction	0.5	Fong and Molnar 2008
Sphaerium striatinum	N-FLX	Increased reproduction	10	Fong and Molnar, 2008

2.4.3.1 Environmental Concentrations of Fluoxetine

Measured environmental concentrations of FLX and its active metabolite N-FLX are mostly in the low nanogram per litre levels. Thus, concentrations of these compounds are presented in Table 2.13 in nanogram per litre (ng/L) concentrations compared to the units used for diclofenac and propranolol (µg/L). Measured concentrations in water are in the region of 50% of cases where effects have been observed. However, with the reported partitioning and persistence of FLX in sediment, targeted risk assessment of fluoxetine must consider water and sediment exposures for epibenthic and benthic (sediment dwelling organisms).

Table 2.13: Measured Environmental concentrations of Fluoxetine and Norfluoxetine

Compound	Type of water	Conc. (ng/L)	Reference
FLX	Effluent	0.6-8.4	Vasskog et al. 2006
FLX	Surface water	1.7	Santos et al. 2010
FLX	Effluent	12	Fent et al. 2006
FLX	Surface water	0.42-1.3	Santos et al. 2006
FLX	Surface water	21.4	Bedner and MacCrehan, 2006
FLX	Surface water	12	Santos et al. 2010
FLX	Ground water	56	Fent et al. 2006
FLX	Drinking water	0.64	Santos et al. 2010
N-FLX	Effluent	<0.54-2.4	Vasskog et al. 2008
N-FLX	Effluent	1.7-1.8	Santos et al. 2010
N-FLX	Surface water	1.2-1.3	Santos et al. 2010
N-FLX	Drinking water	0.77	Santos et al. 2010

2.4.3.2 Removal of Fluoxetine During Biological Wastewater Treatment

Kwon and Armbrust, (2006) reported that FLX was recalcitrant to microbial degradation. In a 28-day biodegradation study, seeded with varied microbial concentrations, the fluoxetine concentration remained constant, and no metabolites were observed (Kwon and Armbrust, 2006). Borges et al. (2009) reported a zero biotransformation of FLX by endophytic fungi. Similarly, Redshaw et al. (2008) reported that FLX and NFLX were not degraded in sewage sludge-amended soils or in liquid cultures supplied with sewage sludge-amended soils as

inoculum. Moreira et al. 2014 reported a biodegradation half-life of 18 days and a complete removal of FLX in 30 days when the compound was degraded with *Labrys portucalensis* F11, a microbial strain with the capacity to degrade a range of fluorinated aromatic compounds. There are, however, reports were modification of operation procedures and/or the use of supplement or consortia of microbes, has resulted in significant (>50%) and up to 100% biodegradation of FLX (Rodarte-Morales et al. 2011; Suarez et al. 2010; Vasskog et al. 2009).

2.4.3.3 Human Metabolism of Fluoxetine

Following oral administration, fluoxetine is almost completely absorbed into blood through the gut. Due to hepatic metabolism, the concentration of FLX is significantly reduced before it reaches the systemic circulation. (Hiemke and Härtter, 2000; Catterson and Preskorn, 1996). After consumption, FLX undergoes extensive metabolic conversion, leading to the active metabolite norfluoxetine, which can act as an SSRI with similar potency to that of fluoxetine, and the formation of multiple other metabolites (Fuller et al. 1992; Hiemke and Härtter, 2000). Fluoxetine and norfluoxetine are then excreted from the human body primarily through urine, with up to 11% of the administered fluoxetine dose being excreted as the unchanged parent compound, 7% as norfluoxetine (N-FLX) and 73% as a combination of other metabolites and conjugates (Zhou et al. 2009; Hiemke and Härtter, 2000; Preskorn, 1997; Altamura et al., 1994; Wong et al. 1974) (Figure 2.16).

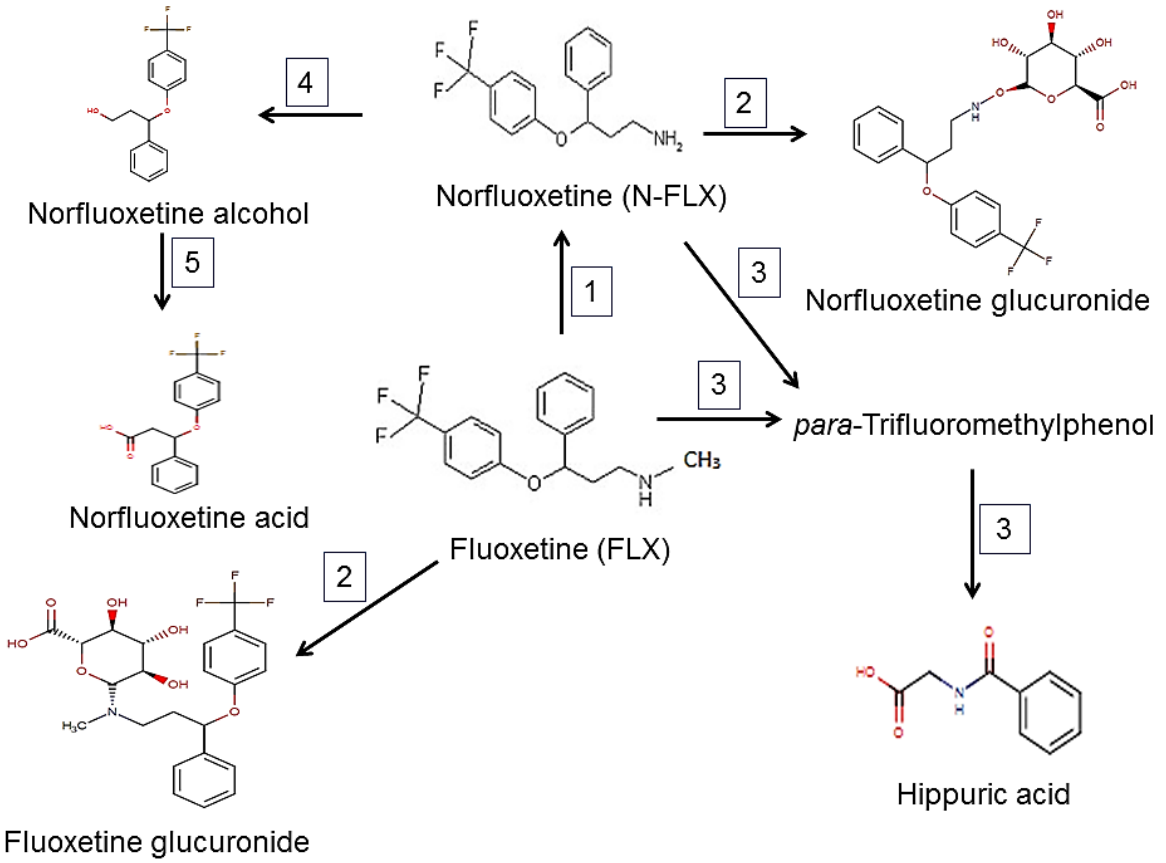


Figure 2.16: Fluoxetine metabolic pathway showing N-demethylation (1); glucuronidation (2); O-dealkylation (3); Oxidative deamination (4) and oxidation (5). FLX and N-FLX are believed to undergo O-dealkylation to form para-trifluoromethylphenol, which is then subsequently metabolized to hippuric acid (Zhou et al. 2009; Hiemke and Härtter, 2000; Preskorn, 1997; Altamura et al., 1994; Wong et al. 1974)

2.4.4 Progesterone

Progesterone (P4), a C-21 progestational steroidal ketone (aka Pregn-4-en-3,20-dione), is secreted naturally by females (Besse and Garric, 2009). The production rate and blood plasma levels are highest in females of reproductive age and during pregnancy (Quinkler et al. 2002). The production rate varies from 92 to 563 mg/day and blood plasma levels during pregnancy vary from 21 to 200 ng/mL in humans and 7 to 25 ng/mL in animals (EMEA, 2004). During a woman's monthly menstrual cycle, an egg matures and is released from the ovaries (ovulation). The ovary then produces P4 that prevent further release of eggs and prepare the lining of the womb for a possible conception (Schumacher et al. 2004). If pregnancy occurs,

natural P4 levels remain high in the body, maintaining the womb lining. If pregnancy does not occur, progesterone levels in the body fall, resulting in a menstrual period. Progesterone is rapidly conjugated and excreted by the body (Besse and Garric, 2009). Since natural P4 is inactivated very rapidly in the body, several synthetic forms of P4 known collectively as progestins have been developed and are now widely produced and regularly used in oral contraceptive formulations and as part of hormone replacement therapy (Fayad et al., 2013). Worldwide, progestins are probably the most widely used of all steroids (Zhang et al., 2014; Dinger et al. 2007), as contraception methods that use them have been reported to be the most desired by patients (Fayad et al., 2013). Reported yearly use of P4 in France (2004) and Switzerland (2010) were 9864.25 and 495 kg respectively (Fent, 2015; Besse and Garric, 2009). The increased use of synthetic progesterone (progestins) may partly be due to their superior effectiveness in the inhibition of pituitary gonadotropins relative to the natural P4 compound (Dukes, 2003; Sitruk-Ware and Nath, 2010; Liu et al. 2011). Furthermore, their combination with certain estrogens in oral contraception formulations means consumers can have an all-in-one combination therapy. Literature available physicochemical properties of P4 are presented in Table 2.14.

Table 2.14: Physicochemical Properties of Progesterone

Parameter	Value	Reference
Chemical formula	$C_{21}H_{30}O_2$	Pubchem
CAS number	57-83-0	Pubchem
Molecular weight	314.46 g/mol	Pubchem
Solubility in water	8.81 mg/L at 25 °C	DrugBank
Octanol-water coefficient (Log K_{OW})	3.87	HSDB; Lui et al. 2009

* HSDB – Hazardous Substances Database

Progesterone is relevant environmentally because it has been measured in the water environment due to its incomplete removal during wastewater treatment, direct human and animal excretion and sometimes direct discharge of untreated wastewater (Liu et al. 2012b). Surface water runoff of farms with concentrated animal feeding operations was reported to result in P4 concentrations as high as 375 ng/L in receiving surface waters (Havens et al.

2010). Furthermore, P4 has been reported to have adverse effects on fish reproduction and chemoreception at environmentally relevant concentrations (Liang et al. 2015a, b; Murack et al. 2011; Havens et al. 2010; Kolodziej et al. 2003; Kobayashi et al. 2002). For example, long-term exposure to environmentally relevant concentration (63 ng/L) of progesterone was reported by Liang et al. (2015) to affect sex differentiation (higher female population relative to male) in zebrafish (*Danio rerio*). A reduction in sperm motility was reported following a one-week exposure of the male fathead minnows to a P4 nominal concentration of 300 ng/L (Murack et al. 2011). Furthermore, P4 was reported to cause substantial decrease in fertilization of the male fat head minnows following a 21 days exposure at concentration of 1,000 ng/L (Havens et al. 2010). Following a 21-day exposure study, DeQuattro et al. (2012) reported a P4 dose-dependent (10, 100, and1,000 ng/L) decrease in fecundity and fertility in fathead minnow and significant reduction in gonadosomatic index and vitellogenin gene expression in females. Table 2.15 presents reported LOEC of P4. Surface water concentrations are around the LOEC values. Table 2.16 presents some measured concentrations of P4 in different water systems with the highest concentrations being in untreated effluent from animal farms and hospitals.

Table 2.15: Lowest Observed Effect Concentration of Progesterone in Aquatic Species

Species	Endpoint	Conc. (ng/L)	Duration	Reference
Pimephales promelas	Fecundity, reproduction	100	21 days	Fent, 2015; DeQuattro et al. (2012)
	Vitellogenin mRNA decrease	10	21 days	DeQuattro et al. (2012)
	Transcripts increase/decrease	500	4 hours	Garcia-Reyero et al. (2013)
	Reduced sperm motility	300	7 days	Murack et al. (2011)
Danio rerio	Transcripts activity	2	48 -144 hours	Zucchi et al. 2012
	Sex ratio and transcript activity	63	40 days	Liang et al. 2015
	Transcripts, gonado-somatic index	25	21 days	Blüthgen et al. 2013
	Transcripts activity	254	120 hours	Blüthgen et al. 2013
	Transcripts activity	3.5	14 days	Zucchi et al. 2013
	Vitellogenin protein	304	14 days	Zucchi et al. 2013
Gambusia affinis	Transcripts activity	1000	8 days	Huang et al. 2013

Table 2.16: Measured Environmental concentrations of Progesterone

Type of water	Conc. (ng/L)	Reference
Surface water	5 - 199	Koplin et al. 2002
Surface water	30.5	Fent, 2015; Liu et al. 2012a
Surface water	1.7 - 3.5	Vulliet et al. 2008
Surface water	5 - 27	Kolodziej and Sedlak, 2007
River & Drinking water	<0.24 –1.39	Kuster et al. 2008
Sediment	6.82	López de Alda et al. 2002
Snowmelt runoff	375	DeQuattro et al 2012
Groundwater	2.1 – 4.8	Vulliet et al. 2008; Besse and Garric, 2009
Effluent	1	Liu et al. 2012b
Wastewater (farm)	3470 - 11900	Liu et al. 2012a
Effluent	33	Pauwels et al. 2008
Effluent	3.2	Pauwels et al. 2008

2.2.4.1 Removal of Progesterone During Biological Wastewater Treatment

Progesterone is reported to be rapidly biodegradable in surface water and as having a high removal rate during engineered treatment (Peng et al. 2014; Liu et al. 2013; Chang et al. 2008; Esperanza et al. 2007; Labadie and Budzinski, 2005; Jenkins et al. 2004). More than 95% of progesterone was reported to be transformed by the two freshwater microalgae (*Scenedesmus obliquus* and *Chlorella pyrenoidosa*) following a 5 days study (half-life: 16 and 39 hours respectively; rate constants: 0.044 and 0.018) (Peng et al. 2014). The authors noted rapid transformation of progesterone within the first 48 h, followed by a slow transformation. Progesterone was reported to be completely biodegraded in an aerobic system seeded with activated sludge (half-life: 4.3 hours) (Liu et al. 2013). Chang et al. (2008) reported a 90 – 96% removal of P4 following aerobic biological treatment. Similarly, Carson et al. (2008) and Jenkins et al. (2004) observed a complete microbial breakdown of P4 resulting in the formation of androgens that may retain biological activity.

2.4.4.2 Human Metabolism of Progesterone

Progesterone is metabolized extensively in the liver mainly by reduction and hydroxylation (Stanczyk, 2003; Grady et al. 1952). Metabolism by oxidation of the P4 side chain has been reported (Grady et al. 1952). Reduction occurs primarily on a double-bond of ring A and then on the ketone function of carbon 3 (Besse and Garric, 2009; Wasser et al. 1994). Natural or oral or intravenously administered progesterone (P4) (Kuhl, 2005) is transformed into a range of metabolites, excreted mainly free (rather than conjugated), with the conjugated metabolite, pregnanediol-glucuronide, reported in some studies (Stanczyk, 2003; Wasser et al. 1994; 1996; Adlercreutz and Martin, 1980). Progesterone is reported to be transformed to two isomers of dihydroprogesterone, four pregnanolone isomers, and eight isomers of pregnanediol (Stanczyk, 2003). Stanczyk, 2003 further noted that about 35 identified and theoretical unconjugated metabolites of P4 that may be found in blood, urine, and/or faeces. Figure 2.17 present some examples of these metabolites including the reported conjugate. Progesterone metabolites are excreted mainly through faeces followed by urine. (Stanczyk, 2003; Wasser et al. 1994; 1996; Adlercreutz and Martin, 1980). Pregnanediol is the main

metabolite of P4 (Besse and Garric, 2009; Stanczyk, 1996) with a daily excretion rates ranging from 1 mg for men to 70 mg for pregnant women (Hardman et al.,1996). Pregnanediol is not reported to be pharmacologically active. However, some metabolites such as 17-hydroxyprogesterone and 20α-dihydroprogesterone are noted to still display parental progestagenic activity (Besse and Garric, 2009).

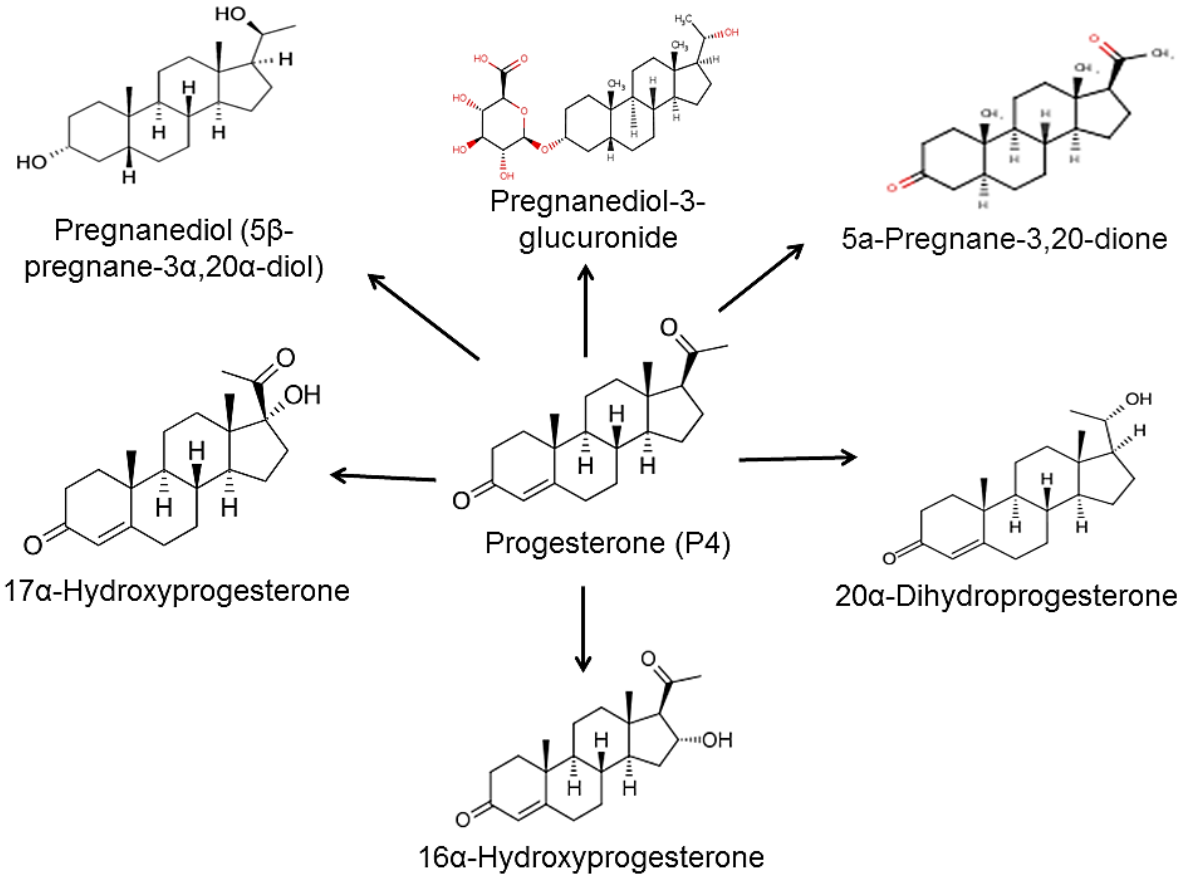


Figure 2.17: Progesterone metabolic pathway (Besse and Garric, 2009; Stanczyk, 2003; Wasser et al., 1994; 1996; Adlercreutz and Martin, 1980)

2.5 Selection of a Model compound for Degradation Studies – Progesterone

Progesterone was selected based on its rapid biodegradability relative to the other model compounds (compare sections 2.4.1.2, 2.4.2.2, 2.4.3.2 and 2.4.4.1) and its usage patterns globally. In the United Kingdom, it is used as part of, hormone replacement therapy (Lobo,

1992; Rosano et al., 2001), preventive measure of endometrial cancer (Gambrell et al. 1983), as palliative appetite stimulant for cancer patients (Kornek et al., 1996) and for the treatment of dysfunctional uterine bleeding (Kumar et al., 2015; Wilkinson & Kadir, 2010). Furthermore, progesterone has been measured in wastewater/surface waters and reported to have effects on aquatic organisms (see Table 2.15). Previous studies at Brunel by Janna (2011) had identified that P4 degrades to below detectable concentrations over 72 hours, a timescale that allows for laboratory scale microcosm studies. The review of literature highlighted some likely transformation products of P4, but no studies were identified where progesterone degradation studies had been undertaken in environmentally relevant samples, such as surface waters.

2.6 Techniques for Screening for Biological Activity

The assays used in the present study for screening for progestogenic and androgenic activity were based on well characterised methods using genetically modified yeast (*Saccharomyces cerevisiae).* These assays have been used to evaluate the activities of steroids and surfactants in environmental samples (Gaido et al., 1997; Lorenzen et al., 2004; Purvis et al., 1991; Routledge & Sumpter, 1996; Runnalls, Beresford, Losty, Scott, & Sumpter, 2013; Sohoni & Sumpter, 1998).

2.6.1 Recombinant Yeast Development

Two genetically modified strains of the yeast *Saccharomyces cerevisiae* are generally used for screening for progestogenic and androgenic activities. These recombinant yeast strains are engineered respectively by integrating the DNA sequence of the human progesterone receptor (hPR) (Gaido et al., 1997) and human androgen receptor (hAR) (Routledge & Sumpter, 1996) into the yeast genome. This genetic engineering process is necessary as yeast cells do not inherently have hPR and hAR, genetic receptors that provide binding sites for the compounds of interest and thus help identify biologically active compounds. The suitability of *Saccharomyces cerevisiae* stems from its possession of certain expression plasmids that carry progesterone or androgen-responsive sequences. These responsive sequences control the expression of the reporter gene *lac-Z*, a gene responsible for producing

the enzyme β-galactosidase, used for measuring the activities of hPR and hAR. Upon binding the test compound otherwise referred to as 'active ligand', the progesterone receptor or androgen receptor interacts with transcriptional factors in the cell resulting in the expression of *lac-Z* and the subsequent secretion of β-galactosidase into the test medium.

Testing for progestogenic or androgenic activities is usually performed by incubating the hPR and hAR cells in a test medium containing the test compound. Incubation is performed so that on interaction of the test chemical with the relevant receptors, β-galactosidase is synthesized and secreted into the medium, where it breaks down the chromogenic substrate in such medium. Breakdown of the chromogenic substrate cause a measurable colour change from yellow to red. In the progesterone screen, the yellow colour comes from the production of orthonitrophenol, a yellow product resulting from the cleavage of 2-Nitrophenyl β-D-galactopyranoside (ONPG) by β-galactosidase enzyme. The chromogenic material in the androgen screen is *Chlorophenol red-β-D-galactopyranoside (CRPG).* The intensity of the colour change, is measured at absorbance of 420nm (P4) and 540nm (androgens) using Molecular Devices plate reader (kinetic microtiter plate reader). Progesterone or dihydrotestosterone is appropriately used as positive controls in each screen. The assays have been reported to show no activity at P4 concentrations below 10^{-8} M, approximately 3.15 µg/L (Gaido et al. 1997; Sohoni & Sumpter, 1998) and androgen concentrations below 10^{-10} M (Runnalls et al. 2013; Sohoni & Sumpter 1998).

2.6.2 Standard Procedure for Assay Reagents Preparation

2.6.2.1 Progesterone Screen (Lorenzen et al. 2004; Gaido et al. 1997)

Selection Medium: 6.7 g of yeast nitrogen base with ammonium sulphate, 0.15 g of adenine, 0.15 g of lysine, 0.08 g of tryptophan is dissolved in 900 ml double distilled water (ddH_2O) and stirred. 45 ml of the medium is dispensed in solvent (ethanol) rinsed 100 mL bottle and autoclaved at 121^0C for 15 minutes.

20% D-(+)-Glucose: 20 g of glucose is weighed and a final volume of 100 mL is made by adding ddH_2O. The content is stirred well and autoclaved 121^0C for 15 minutes.

20mM Copper (II) Sulphate: 0.1596 g of $CuSO_4$ is weighed and a final volume of 50 mL is made by adding ddH_2O. The solution is then sterilised by filtering 5 mL aliquots into sterile glass bottles through a 0.2 µm pore size disposable syringe filter. Sterilisation is done inside a laminar air flow cabinet.

10% SDS (Soduim dodecyl Sulphate): 10 g of lauryl sulphate is weighed and made up to 100 mL final volume by the addition of ddH_2O. The mixture is stirred and transferred into a 100 mL sterile bottle.

Z-Buffer: 8.05 g $Na_2HPO_4.7H_2O$, 2.75 g $Na_2HPO_4.7H_2O$, 0.375 g KCl and 0.123 g $Mg_2SO_4.7H_2O$ are added to 400 mL ddH_2O and stirred. The pH of the solution is adjusted to 7.0. The volume is subsequently adjusted to 500 mL and autoclaved at 121^0C for 15 minutes.

Assay Buffer: Depending on the number of plates to be screened a given volume of 2 mg/mL 2-Nitrophenyl β-D-galactopyranoside (ONPG, 98% purity) into a given volume of z-buffer. To illustrate, assume two plates are being screened, 48 mg of ONPG is added to 23.8 mL z-buffer. In a similar vein, if a plate is being screened, 24 mg ONPG is added to 11.9 mL z-buffer. To dissolve the ONPG, the mixture is vortexed and put in 28^0C shaking incubator for approximately 30 minutes. It is important to ensure that the ONPG is in solution before adding any other components. To complete the preparation of the assay buffer, 65 µL (2 plates screening) of 50mM 2-ME (2-Mercaptoethanol) and 240 µL 10% SDS are added and inverted to mix. The assay buffer is prepared just before use as it is stable only for an hour.

Preparation and Storage of 10X concentrated yeast stocks

Day 1: Growth medium is prepared by the addition of 5 mL glucose solution to 45 mL selection medium (SM) in a sterile conical flask (final volume was approximately 50 mL). 125 µL of 10X concentrated yeast stock from a cryogenic vial stored at -20^0C is added to the growth medium.

The medium is incubated at 28^0C for approximately 24 hours on an orbital shaker until it is turbid.

Day 2: Freshly prepared growth media are added 50 mL each to two conical flasks. 1 mL of yeast from the 24 hours culture is added to each flask. These new cultures are incubated at 28^0C for approximately 24 hours on an orbital shaker until culture is turbid.

Day 3: The culture from the previous day is transferred to a sterile 50 mL centrifuge tube and centrifuged at 4^0C for 10 minutes at 2,000 g. The supernatant is decanted, and the culture resuspended in 5 mL selection medium with 15% glycerol (8 mL sterile glycerol/45 mL SM). 0.5 mL aliquots of the 10X concentrated stock solution is then transferred to 1.2 mL sterile cryovials and stored at -20^0C. Short term storage should be at -70^0C/-80^0C for a shelf-life of 3-4 months.

2.6.2.2 Androgen Screen (Beresford et al. 2000; Sohoni and Sumpter et al. 1998)

Selection Medium (pH 7.1): To 1 L of ddH_2O, 13.61 kg KH_2PO_4, 1.98 g $(NH_4)_2SO_4$, 4.2 g KOH pellets, 0.2 g $MgSO_4$, 1 mL $Fe_2(SO_4)_3$ solution (40 mg/50 mL H_2O), 50 mg L-leucine, 50 mg L-histidine, 50 mg adenine, 20 mg L-arginine-HCl, 20 mg L-methionine, 30 mg L-tyrosine, 30 mg L-isoleucine, 30 mg L-lysine-HCl, 25 mg L-phenylalanine, 100 mg L-glutamic acid, 150 mg L-valine and 375 mg L-serine, are added and place on heated stirrer to dissolve. 45 mL aliquots are dispensed into glass bottles and sterilised at 121^0C for 15 minutes, and store at room temperature.

D-(+)-Glucose: 20 g of glucose is weighed and a final volume of 100 mL is made by adding ddH_2O (20% w/v). The solution is sterilised in 20 mL aliquots at 121^0C for 10 minutes and stored at room temperature.

L-Aspartic Acid: A stock solution of 4 mg/mL is made and sterilised in 20 mL aliquots at 121^0C for 10 minutes and stored thereafter in room temperature.

Vitamin Solution: To 180 mL ddH_2O, 8 mg thiamine, 8 mg pyridoxine, 8 mg pantothenic acid, 40 mg inositol and 20 mL biotin solution (2 mg/100 mL H_2O). Solution is sterilised by filtering through a 0.2 µm pore size disposable syringe filter in a laminar air flow cabinet. 10 mL aliquots of the solution were filtered into sterile glass bottles and stored at 4^0C.

L-Threonine: A stock solution of 24 mg/mL is prepared. The solution is sterilised in 10 mL aliquots and stored at 4^0C.

Copper (II) Sulphate: A 20mM solution is prepared and sterilised by filtering through a 0.2 µm pore size disposable filter in a laminar air flow cabinet. 5 mL of the solution is filtered into sterile glass bottles and stored at room temperature.

Chlorophenol red-β-D-galactopyranoside (CPRG): A stock solution of 10 mg/mL is made and sterilised by filtering through a 0.2 µm pore size disposable syringe filter into sterile glass bottles, in a laminar air flow cabinet and stored at 4^0C.

Preparation and Storage of 10X concentrated yeast stocks

Day 1: Growth medium is prepared by the addition of 5 mL glucose solution, 1.25 mL L-aspartic acid solution, 0.5 mL vitamin solution, 0.4 mL L-threonine solution and 125 µL copper (II) sulphate to 45 mL minimal medium in a sterile conical flask (final volume is approximately 50 mL). 125 µL of 10X concentrated yeast stock from cryogenic vial stored at -20^0C is added to growth medium. The medium is incubated at 28^0C for approximately 24 hours on an orbital shaker until it is turbid.

Day 2: Freshly prepared growth media are added 50 mL each to two conical flasks. 1 mL of yeast from the 24 hours culture is added to each flask. These new cultures are incubated at 28^0C for approximately 24 hours on an orbital shaker until culture is turbid.

Day 3: The culture from the previous day is transferred to a sterile 50 mL centrifuge tube and centrifuged at 4^0C for 10 minutes at 2,000 g. The supernatant is decanted, and the culture resuspended in 5 mL minimal medium (MM) with 15% glycerol (8 mL sterile glycerol/45 mL

MM). 0.5 mL aliquots of the 10X concentrated stock solution is transferred to 1.2 mL sterile cryovials and stored at -20^{0}C. Short term storage was done at -70^{0}C/-80^{0}C for a shelf-life of 3-4 months.

Chapter Three: Methodology

Overview

Analytical method development is a critical component in analytical chemistry. The effectiveness of the process determines the reliability of data generated from the final analytical procedure and the quality of conclusions reached based on such data. This chapter presents the detailed analytical methods/procedures that were developed and applied for data acquisition in this research. Some of the methods described in this chapter have been published (see appendix). The standards and reagents used and their purity are presented. Sample preparation procedures and analytical instrumental methods used are also described. Field work and details of laboratory degradation study performed are presented.

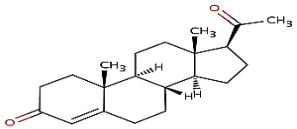
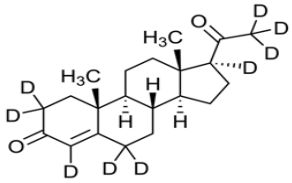
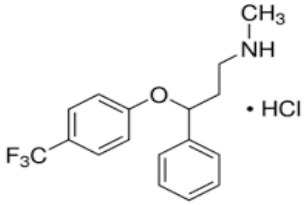
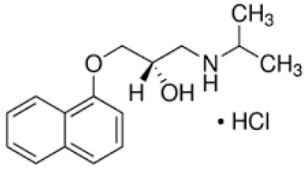
3.1 Reagents

All analytical standards including deuterated internal standards used were greater than 98% in purity. Progesterone (P4) was purchased from QMx, (Essex, UK). Diclofenac sodium salt, Fluoxetine hydrochloride and Propranolol hydrochloride were purchased from Sigma Aldrich (Gillingham, UK). Table 3.1 provides summary information on the standards used. Isotopically labelled progesterone ($P4_{d9}$) was used during the analytical method development stage of this study as the sole internal standard (IS) for the quantification of all compounds of interest. $P4_{d9}$ was purchased from Sigma Aldrich (Gillingham, UK). Summarised information on $P4_{d9}$ is also presented in table 3.1. All reagents (Methanol and Acetonitrile) used were HPLC grade. Reference standards were made in methanol purchased from Rathburn Chemicals (Walkerburn, UK). Reagent grade MilliQ water (18.2MΩ) (Millipore, Watford, UK) was spiked, used as blank and for the preparation of standards. Ammonium hydroxide (NH_4OH) and formic acid used for aqueous phase preparation were also purchased from Sigma Aldrich (Gillingham, UK). The ammonium hydroxide and formic acid used were TraceSELECT ultra and reagent grades respectively. Acetonitrile (Rathburn Chemicals (Walkerburn, UK)) and methanol were used for elution of target analytes during solid phase extraction.

Individually, standard stock solutions of model compounds and the isotopically labelled internal standard ($P4_{d9}$) were prepared on weight/volume (w/v) basis in methanol. A 1000 mg/L individual stock of each compound was prepared by dissolving 10 mg of the standard in 10 mL methanol respectively. A 10 µg/mL (10,000 ng/mL) sub-standard of each compound was prepared by 1-100 dilutions. Four sub-standards namely; 1000 ng/mL; 100 ng/mL; 10 ng/mL and 1 ng/mL were respectively prepared starting with the 10 mg/L sub-standard for each compound through serial dilution (precisely 1-10 dilutions with each new standard serving as the stock for the next). Similarly, an 800 ng/mL (0.8 µg/L) sub-standard of $P4_{d9}$ was prepared by appropriate dilution (10 µL in 12.5 mL) of stock solution in methanol. Prepared standards were stored at -20^0C for preservation. Prepared standards were used within two weeks and new ones prepared following every field work and laboratory degradation study.

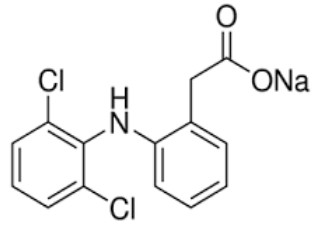
For the analysis of field samples, a series of five-point working (calibration) standards ranging from 1 - 100 ng/mL and containing all four analytes and 200 ng/mL (0.2 µg/L) IS respectively were prepared in chromatographic vials by appropriate dilution of sub-standards with methanol/water (50/50, v/v). For the analysis of samples from laboratory based degradation study, another five-point calibration standard ranging from 0.25-2500 ng/mL, each containing 200 ng/mL of the IS was also prepared. Working standards were freshly prepared for each run by the mixture of appropriate amount of the intermediate sub-standards.

Table 3.1: Summarized information of Analytical Standards

Compound	Abbr.	CAS [a,d]	Mol. Wt. [a,b,d]	Formula[a,d]	pKa[a,c,e]	IUPAC Name [a,d]	Structure [a,d]
Progesterone	P4	57-83-0	314.46	$C_{21}H_{30}O_2$	-	Pregn-4-ene-3,20-dione	
Progesterone d9	$P4_{d9}$	15775-74-3	323.52	$C_{21}D_9H_{21}O_2$	-	Progesterone-2,2,4,6,6,17α,21,21,21-d9	
Fluoxetine Hydrochloride	FLX	56296-78-7	345.79	$C_{17}H_{18}F_3NO \cdot HCl$	10.06 ± 0.10	(±)-N-Methyl-γ-[4-(trifluoromethyl)phenoxy]benzenepropanamine hydrochloride	
Propranolol Hydrochloride	PRPL	318-98-9	295.8	$C_{16}H_{21}NO_2 \cdot HCl$	9.42; 9.14	[2-Hydroxy-3-(naphthyloxy)propyl]isopropylammonium chloride	

a: DrugBank; b: Chemspider; c: TOXNET; d: Sigma-Aldrich MSDS; e: Brooks et al. 2003

Table 3.1: Summarized information of Analytical Standards (continued)

Compound	Abbr.	CAS [a,d]	Mol. Wt. [a,b,d]	Formula [a,d]	pKa [a,c]	IUPAC Name [a,d]	Structure [a,d]
Diclofenac Sodium Salt	DCF	15307-79-6	318.13	$C_{14}H_{10}Cl_2NNaO_2$	4.15	2-{2-[(2,6-dichlorophenyl)amino] phenyl}acetic acid	

a: DrugBank; b: Chemspider; c: TOXNET; d: Sigma-Aldrich MSDS

3.2 Method Development

3.2.1 Solid Phase Extraction (SPE)

Solid phase extraction (SPE) was performed using Oasis HLB (500mg, 6cm^3) cartridges purchased from Waters Ltd. (Watford, UK). In this work, only Oasis HLB cartridges were used for extraction of target analytes although a common procedure is to examine different cartridges for best recoveries. Oasis HLB was the sorbent selected because of its hydrophilic–lipophilic balance, proved versatility and efficiency in the extraction of analytes of a wide range of polarities and acid/base character at different pH values, including neutral pH (see section 2.2.1) The SPE protocol was optimized through several preliminary experiments involving the following;

- Eluting with different types of solvents (Methanol and Acetonitrile) to determine which gave better recovery. Methanol and Acetonitrile are the most widely reported solvents for the elution of model compounds.
- Eluting with a different volume (4 mL and 8 mL (2 x 4mL)) of selected eluent to determine which volume gave better recovery. The two solvent volumes used are those commonly reported in the literature.
- Spiking different volumes (0.5 and 1 Litres) of MilliQ water respectively with 0.1mL of 10 µg/mL mixture of all four pharmaceuticals. This was performed to determine if loading a high volume of sample into Oasis HLB resulted in loss of any of the analytes of interest due to breakthrough. This is with a view to determining what volume of field sample to extract assuming that distribution of a given analyte in a sample, may not be even.
- Sample loading rate was adjusted manually (timed) to have a fast (5 mL/min) and slow flow (2 mL/min) rates to determine which gave a better recovery.
- The comparable effects of drying samples down and re-concentration with a given volume of solvent sample vis-à-vis samples used wet (not dried down), on the percentage of any given analyte recovered following extraction was tested. Sample

drying is a recommended step for concentrating up analytes for target analysis but uncertain for suspect and non-target screening.

The investigation of the first four SPE condition above, worked with high concentrations of each compound (100 µL of 10 mg/L standard mixture of all model compounds in 500 mL milliQ water), such that sample drying and concentrating up was not necessary. Spiking with high concentration of analytes was performed to determine if locally available mass spectrometer (unit resolution) was sensitivity enough to detect and quantify analytes of interest when sample extraction step is not done prior to instrumental analysis. This investigation was important as the plan was not to extract degradation samples prior to instrumental analysis for suspect and non-target TPs based on the drawback highlighted in section 2.2.1, the potential loss of TPs due to breakthrough as the binding properties of these TPs to sorbent materials are largely unknown, a factor critical to selecting solid-phase extraction (SPE) procedure. Furthermore, there will be uncertainty in results obtained for presence or absence of TPs when SPE is applied. If not detected, it would be thought that they were present but lost during extraction process. Prior to sample loading, each cartridge was conditioned with 5 mL of MilliQ water and the respective eluent to be used. This was done to activate by protonation the strong cation-exchange sulfonic acid groups in each sorbent surface.

For the investigation of the best type of eluent to use, 4 mL of methanol and acetonitrile were used respectively for elution of cartridges. Thus, the representative concentration of individual compounds in the 4 mL extract (eluate) was 0.25 µg/mL. Concentration of analytes in chromatographic vial was 0.125 µg/mL, as this represented a 50:50 dilution of the eluate sample with double deionised water. Following the determination of what eluent (methanol) to use, the best volume of MeOH to use for elution was investigated by eluting cartridges with 4 mL and 8 mL (i.e. 2 x 4 mL) of methanol respectively. Subsequently, different volumes (0.5 and 1 L) of MilliQ were respectively spiked with 100 µL of the analytes mixture to investigate the relative effects that loading varying volumes of a sample will have on analytes recovery. Following this, loading rates were adjusted for the optimal sample volume determined to see what effects this will have on recovery.

For the investigation of the last SPE condition, 0.5 L of MilliQ water was spiked with 10 µL of the standard mixture of all model compounds, a much lower concentration relative to the initial studies, since model compounds are often present at very low concentrations in the environment and sample extraction was to be performed. This last SPE optimization protocol was performed because of the planned measurement of model parent pharmaceuticals in reed beds. Accordingly, 0.5 L of MilliQ water was spiked with 10 µL of the 10 mg/L standard mixture of all model compounds. Cartridges were eluted with 8 mL (2 x 4mL) of MeOH, such that concentration of analytes in eluent was 0.125 µg/mL. Extracts were then dried under a gentle stream of nitrogen gas and reconstituted to 1.2 mL with a mixture of methanol and water (50/50 v/v). A representative concentration of 0.08 µg/mL was thus expected for individual analytes in the chromatographic vial. Analytical results for concentrated samples were compared to the non-concentrated samples where 0.5 L of MilliQ water was spiked with 100 µL of 10 mg/L standard mixture of all model compounds.

3.2.2 SPE Method Validation

The SPE conditions developed were validated using wastewater influent and effluent sampled from pilot scale reed beds at a sewage treatment works (see section 3.2.2.1 for description of sample pilot reed bed). An additional aim of this validation exercise, was to determine the number of TPs that will be detected in samples, seeing that SPE extraction step is applied to samples processing and considering that the concentrations and degradability of some of the model compounds. Seeing that P4 degrades rapidly relative to the other model compounds (see section 2.4), attention was focussed on the number of detected P4 suspected TPs in the reed beds' samples. Wastewater samples were collected in amber coloured bottles pre-rinsed with MilliQ water and immediately transported to the laboratory for analysis. Samples were manually extracted using vacuum manifold and with Oasis HLB cartridges (500 mg/6cm^3) after the addition of the internal standard. Pilot scale reed beds sampling was undertaken on 30th March 2015 at Severn Trent sewage treatment works (STW) located in Barston. The primary

aim of this sampling session was the validation of the developed SPE method, establishing the concentrations of target parent compounds in influent and final effluent of reed beds. The secondary aim was to determine reed bed performance in removing model compounds. However, because spot samples (zero hydraulic retention time) were taken at the in/out of reed beds and further access to site was not possible, the results presented for performance of reed beds will require further study to verify their reliability. Two litres (2L) samples were respectively taken in triplicate. Samples were divided into two groups. One group was spiked with a 100 µL of a 10 µg/L concentration of the target pharmaceutical mix and the other group were not. Before field samples were extracted, they were respectively spiked with 0.1 mL of 0.8 µg/L of the IS ($P4_{d9}$) as the quantitation method used in this study was based on internal standard calibration. Addition of the IS was done to correct for any losses in extraction and matrix effects that could result in suppression or enhancement of ion signal (Note: SPE method development was done with distilled water and no IS was used). Initial work in reed beds used $P4d_9$ as a sole IS, with the intention that further work will use compound specific IS, which were not available at the time the first sampling was carried out. The sole use of one IS was thought to lead to some bias, in that we were correcting for recovery using a compound with different characteristics, which may respond differently in different matrices. Extracts were dried under a nitrogen stream and reconstituted to 1.2 mL with a mixture of methanol and water. Percentage recovery and reed bed performance for removal of target compounds were calculated using the mathematical relations below;

Equation 1: Analyte Recovery (%)

$$\text{Recovery (\%)} = \frac{\text{Measured Concentration}}{\text{Expected Concentration}} \text{ X } 100$$

Equation 2: Model Compounds Removal Efficiency (%)

$$\text{Removal Efficiency (\%)} = \frac{\text{Mean (Influent) - Mean (Effluent)}}{\text{Mean (Influent)}} \text{ X } 100$$

3.2.2.1 General Process Description of the Barston STW

The STW used biological trickling filter technology for the treatment of sewage. The operating procedure involved continuous spraying (trickling) of post primary sedimentation sewage from a settlement tank through the surface of a fixed filter bed filled with gravel to which approximately 0.1 - 0.2 mm thick biofilm layer had grown over time. With the help of the good oxygenation provided by the rotating sprinkler, favourable aerobic conditions were created in the filter, which allows the aerobes in the thin layer of biofilm to oxidize organic compounds present in sprinkled wastewater to water and carbon dioxide, raw materials that support further photoautotrophic production of new biomass.

Pilot scale vertical subsurface flow reed beds used as a tertiary treatment step for polishing effluents prior to discharge were sampled. They were 2.5 m x 2.5 m x 1m high (area: 6.25 m^2) in size. The reed bed composition from bottom up were; drainage, 0.15 m of large gravel (diameter: 16 to 32 mm), transition, 0.10 m of pea gravel (diameter 4 to 8 mm), main layer, 0.5 m of concrete sand (<4 mm) and the remaining 0.25 m was freeboard. Influent loading rate was 0.4 m^3/m^2/day.

3.2.3 Chromatography and Mass Spectrometry Analysis

Samples were analysed using high performance liquid chromatography (HPLC) coupled to a triple quadrupole mass spectrometer using an electrospray ionisation source. A high-resolution Agilent 6540 accurate-mass quadrupole time-of-flight mass spectrometer was used for accurate mass analysis. The two mass spectrometers used are described in detail in sections 3.2.3.2 and 3.2.3.3. Agilent MassHunter software and Analyst software 1.4.2 (Applied Biosystems, Warrington, UK) were used for data acquisition and evaluation as well as instrument control. HPLC analysis was performed using an ACE C18 (10 cm x 2.1 mm) 3 µm particle size column (Hichrom, UK) and protected by a C18 guard column.

3.2.3.1 High Performance Liquid Chromatography Method

Column separation was realised by pumping two eluents (Mobile phase A and B) at a flow rate of 210 µL/min. Mobile phase A (aqueous phase) varied depending on whether analyte of interest was positively (P4, FLX and PRPL) or negatively (DCF) ionised. Variation in pH of the aqueous phase was observed to cause significant changes in ion selectivity, peak shape and retention time. Peak shape and retention time was observed to improve at low aqueous phase pH (lower than pKa of the positively ionized analytes) and at higher pH (higher than pKa for DFC). This because when acidic analytes (positively ionized analytes) are present in acidic solution (at pH at least two 2 units below their pKa), there is an abundance of protons (H^+) in such a solution that these analytes (acidic) retain their protons and thus become ion-suppressed, a condition that improves their retention on HPLC column. On the other hand, when basic analytes (negatively ionized analytes) are present in a basic solution (at a pH at least two units above their pKa), there are too few protons (H^+) in solution for the basic analyte to become protonated, and so they remain in their ion-suppressed, neutral form, improving their retention on HPLC column. See section 2.4 on the effect of pKa on the sorption properties of model compounds.

Thus, in this work, the final method aqueous phase was MilliQ water (+ formic acid (999:1 v/v)) (pH: 2.6) for the positively ionised analytes and MilliQ water (+ ammonium formate (97:3 v/v) (pH; 8.2) for negatively ionised analytes. Mobile phase B was acetonitrile. Total run time was 43 minutes (Fig. 3.1) with data acquisition over gradient program of;

1. 5% acetonitrile for 11 minutes (Isocratic period to allow separation. Methods with gradient starting earlier were tried but did not allow for separation, and gave broader peaks),
2. linear gradient to 75% acetonitrile over 19 minutes and
3. held at 75% for 1 minute. Column wash (short column wash, no evidence of peaks in blanks) followed for 1 minute and thereafter
4. equilibration to beginning conditions (5%) for 11 minutes for a 43-minute cycle.

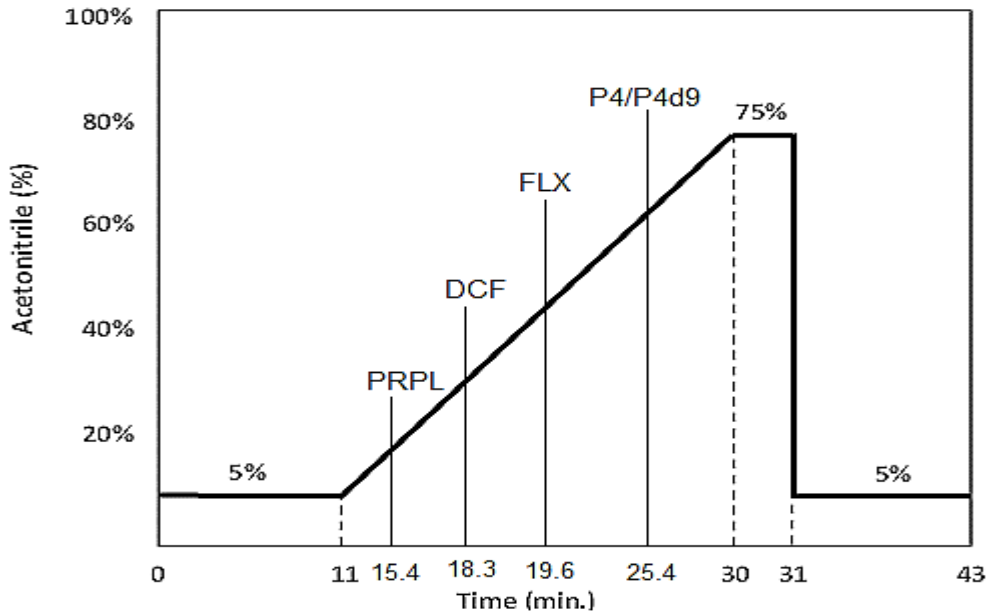


Figure 3.1: Acetonitrile (Eluent B) gradient profile for model compounds HPLC analysis showing retention times. Aqueous phase was MilliQ water (+ formic acid (999:1 v/v)). (P4: Progesterone; FLX: Fluoxetine; DCF: Diclofenac; PRPL: propranolol)

3.2.3.2 Unit Resolution LC-MS/MS

The unit resolution analytical system consisted of a Perkin Elmer Series 200 pump equipped with autosampler and mobile phase mixer connected in series to a PE SCIEX API365 triple quadrupole mass spectrometer with turbo ion spray source in positive and negative ionisation modes (LC/ESI$^+$/MS/MS). System parameters (e.g. Declustering, Focusing and Entrance Potentials (Fig. 3.2)) were optimized by varying values for each parameter to obtain higher signal strength (sensitivity). Instrument calibration for model compounds (P4, FLX, PRPL and DCF) masses and the optimization of source-dependent parameters was achieved by manual tuning. Prior to sample analysis and as part of method development, the mass spectrometer was manually tuned to determine the best possible ion source of two available ion sources (electrospray ionisation (ESI$^+$) and atmospheric pressure chemical ionisation (APCI$^+$)). Optimization of source parameters also involved the determination of the best mode (+) or (−) suitable for each analyte. Using positive and negative modes respectively, the instrument was

manually tuned to determine which source gave the best ionisation judged by signal to background noise (sensitivity), signal strength (intensity) and nature of peak produced. Using a 250 µl syringe installed on syringe pump, each analyte standard (standard diluted (50:50) with corresponding aqueous mobile phase), was infused at a rate of 50 µl/min. For the acidic analytes, the positive mode on the ESI produced more parent ions relative to the negative mode on either ion sources. Running on the negative mode, was good for DCF. Consequently, other system parameters were optimized to get higher signal strength. Choice of a unique product ion that can be monitored and quantified for each compound was also determined by manual tuning using the parent to product ion transition scan mode. The most intense product ion was then selected for multiple reaction monitoring mode (MRM) analysis. Parent and product ions monitored are presented in Table 3.2. Other instrument parameters were optimized as presented in Table 3.3 below to get better signal strength.

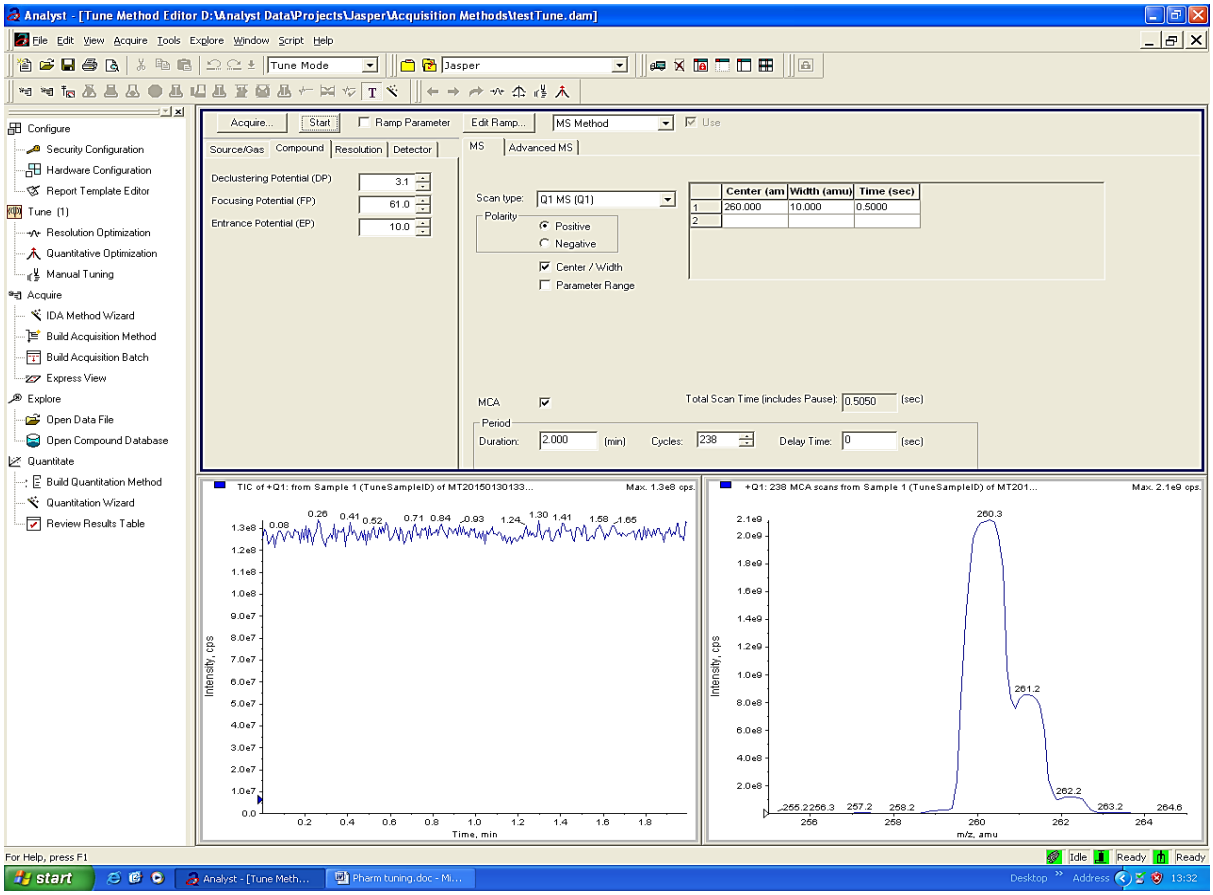


Figure 3.2: Screenshot of system parameters optimization output for propranolol

Table 3.2: Parent and Fragment Ions of Model Compounds

Compounds	Retention Time (Min)	Ions (m/z)	
		Parent (M1)	Product (M2)
P4	25.50	315.3	97.1
P4d9	25.30	324.3	100.2
PRPL	15.19	260.0	116.2
FLX	20.07	310.0	44.0
DCF	15.16	294.0	250.0

Table 3.3: Optimized Instrument Parameters for Enhanced Signal Strength

Mass Spec Parameter	Compound			
	P4	**PRPL**	**FLX**	**DCF**
Nebulizer Gas (NEB)	9	9	9	8
Curtain Gas (CE)	8	8	8	8
IonSpray Voltage (IV)	4500	4500	4500	-4300
Collision Gas (CAD)	10	2	2	11
Temperature (TEM)(^{0}C)	350	350	350	350
Declustering Potential (DP) (V)	35	3.1	11	-55
Focusing Potential (FP) (V)	92	61	69	-55
Entrance Potential (EP) (V)	4.5	10	2.5	-2.5
Collision Energy (CE) (eV)	36	25	30	-20
Collision Cell Exit Potential (CXP)	8	4.5	3.3	-15

3.2.3.3 Accurate Mass Measurements and Collision Induced Dissociation

Separation was conducted on a 1290 Series LC from Agilent (Stockport, UK) equipped with the same ACE column. The injection volume was 20 μL. The mobile phase consisted of water (A) and acetonitrile (B), with both eluents containing 0.1% formic acid. An elution gradient (0.3 mL/min) was applied with the initial concentration (5% B) held for 1 min, after which it was linearly increased to 75% B for 9 min and then held for 5 min. A 3-min post run was required after analysis. The LC was connected to an Agilent 6540 accurate-mass quadrupole time-of-flight mass spectrometer. Sample ionization was achieved with jet stream electrospray operated in positive ion mode under the following conditions: sheath gas temperature 250 °C; nebulizer 45 psi, gas flow 8 L/min; gas temperature 250 °C; skimmer 65 V, fragmentor 175 V, nozzle 0 V; octopole RF 750 V; and capillary 3500 V. Accurate mass spectra were acquired in scan mode (50 - 1200 *m/z*). Reference masses were 121.0509 and 922.0098 *m/z* with a resolution of 19,546 at 922.0106 *m/z*. Data were acquired with Agilent MassHunter software. Considering P4 as the parent compound, a mass defect filter (0.2246 ± 0.05 Da) was applied to identify TPs.

Ion fragmentation was performed by collision-induced dissociation (CID) mass spectrometry in the Agilent 6540 time-of-flight instrument equipped with the column used previously. Collision-induced dissociation and source-induced dissociation (SID) are two common methods applied in mass spectrometry for ion fragmentation. CID was used in this work because of its reputation for being a simpler analytical method and for producing effectively controlled fragmentation data that are reproducible and easy to interpret. Acquisition parameters remained the same, with fragmentation carried out at collision energy of 15 V. Ion fragmentation was monitored over an *m/z* range of 40 – 400.

3.3 Progesterone Degradation Study

3.3.1 River Water Sampling

A total of three river sampling campaign on 7th December 2014; 5th November 2015 and 23rd November 2016, were carried out for this study. Sampling events were carried out around the same season (winter) for easy comparison of results. River water (20 litres) for the first two degradation studies were collected from the Grand Union Canal in Maple Cross, Hertfordshire (Figure 3.3) at approximately 1 km downstream of the effluent discharge point of the Maple Lodge Sewage Treatment Works (National Grid Reference (NGR): (TQ0406491270). Sampling was carried out at 1 km downstream and from sewage works effluent discharge points, to allow for mixing the of effluent (and associated microbial community) with the river, to be as representative as possible of the microbial community downstream of sewage works. River waters for the last degradation study were collected from approximately the same distance downstream of the effluent discharge point of selected STW. Sample locations according to their related STW were; Blackbirds ((NGR): TL1371001060), Chesham (NGR: SU9810099601) and High Wycombe ((NGR): SU8825092210) (Figure 3.3). Water samples were collected with the aid of a sampling bucket (1 L) with an attached adjustable 2-3 metres handle. Prior to sample collection, sampling bucket and sample container where rinsed twice with river water. Afterward, sampling bucket handle was adjusted such that a representative sample was picked from a point 2 metres from the bank of the canal. Samples were taken 2 metres from the river bank to get close to mid-stream flow. Sample was immediately transported to the laboratory and processed within 48 hours.

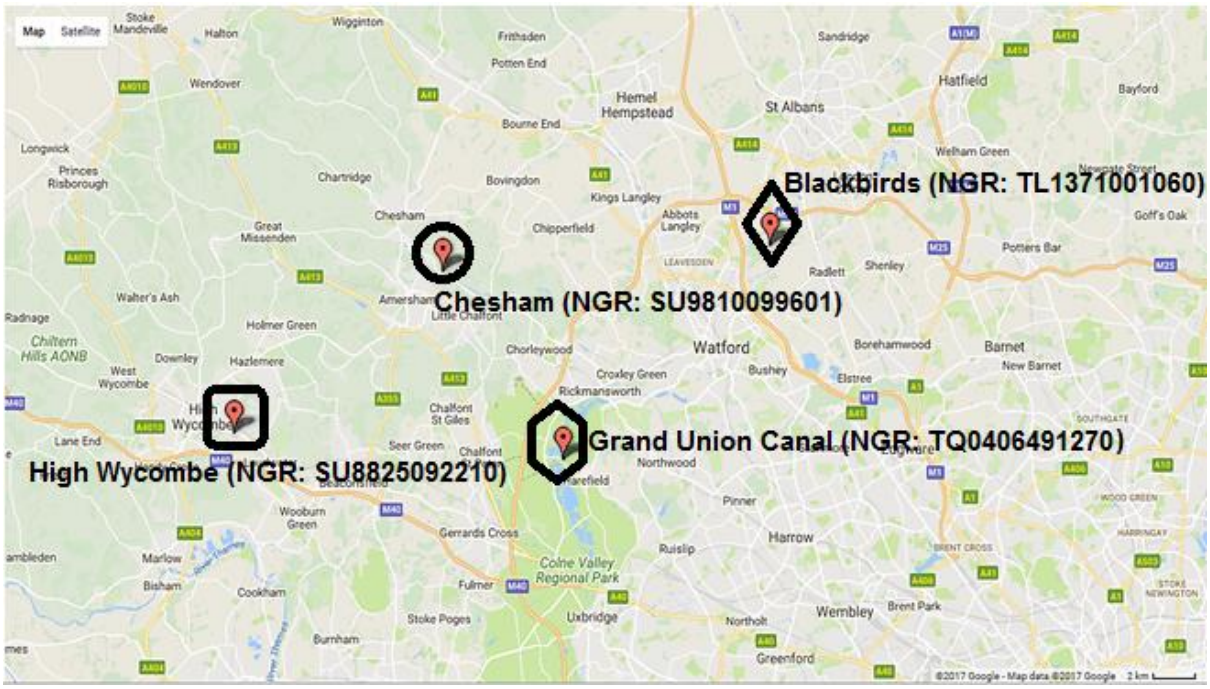


Figure 3.3: Sampling Locations and their corresponding National Grid Reference (NGR)

3.3.2 Laboratory Progesterone Degradation Study

Three degradation studies using river water from four different locations, were conducted. The first set-up on 7th December 2014 used river water from the Grand Union Canal. This initial study was designed to test the overarching hypothesis that P4 would degrade in rivers. The second study set up on the 5th of November 2015 and used sample from the same location as the first study, was aimed at confirming the first study results with the addition of biological assays to test for progestogenic and androgenic activities. The third study set up on the 23rd of November 2016 was designed to test the hypothesis in three other locations since the first two studies were based on samples collected from the same location.

The initial study used 9 L degradation tanks. However, the subsequent unavailability of the tanks used in the first study led to the use of 4 L tanks for subsequent degradation studies (Figure 3.4). Tanks were grouped into two categories; Spiked and Control Tanks. Prior to the start of study, the tanks were respectively rinsed twice with the river water sample after which 4 litres of associated river water was measured into each tank. Aerators with fitted air stone

endings and connected to air pumps, along with agitators (magnetic stirrers) were installed in each tank to ensure even aeration and good mixing of tank content. The tanks containing river water were left to stabilise (equilibrate) and assume stable laboratory conditions for 24 hours to get dissolved oxygen (DO) and temperature stable. Prior to the installation of air pumps and stirrer, the initial DO levels, pH and temperature of samples was measured. These parameters were regularly measured in each sampling regime for the spiked and control tanks. Following the 24hour equilibration period, the 'Spiked' tanks were spiked with 4 mL of 1,000 mg/L P4 stock solution and the 'control' tanks were not. Sampling regimes in hours were 0 (20 minutes following spiking), 4, 8, 12, 24, 48 and 72. For each sampling period, 0.6 mL of water was sampled respectively for the spiked and control set-up into a chromatographic vial followed by the addition of 0.3 mL each of methanol and the IS ($P4d_9$). Samples were taken in triplicate for each sampling time. Overall, three batch of samples were taken altogether and analysed. Tanks were spiked at 1,000 mg/L so that any major degradation products were expected to be detected without pre-concentration using SPE. This was also appropriate as recovery by SPE could not be tested for unknown compounds (see section 2.2.1).

Progesterone degradation kinetic rate constant was calculated using the first-order reaction kinetic model (equation 3). Degradation half-life was calculated using the relationship shown in equation 4.

Equation 3: Kinetic rate constant (h)

$$\ln \frac{C_t}{C_0} = -kt$$

Equation 4: Degradation half-life (h)

$$\frac{0.693}{k} = t_{1/2}$$

*where C_t: concentration at a given time; C_0: starting concentration; k: kinetic rate constant; t½: half-life of degradation; In: natural log; 0.693: In (0.5)

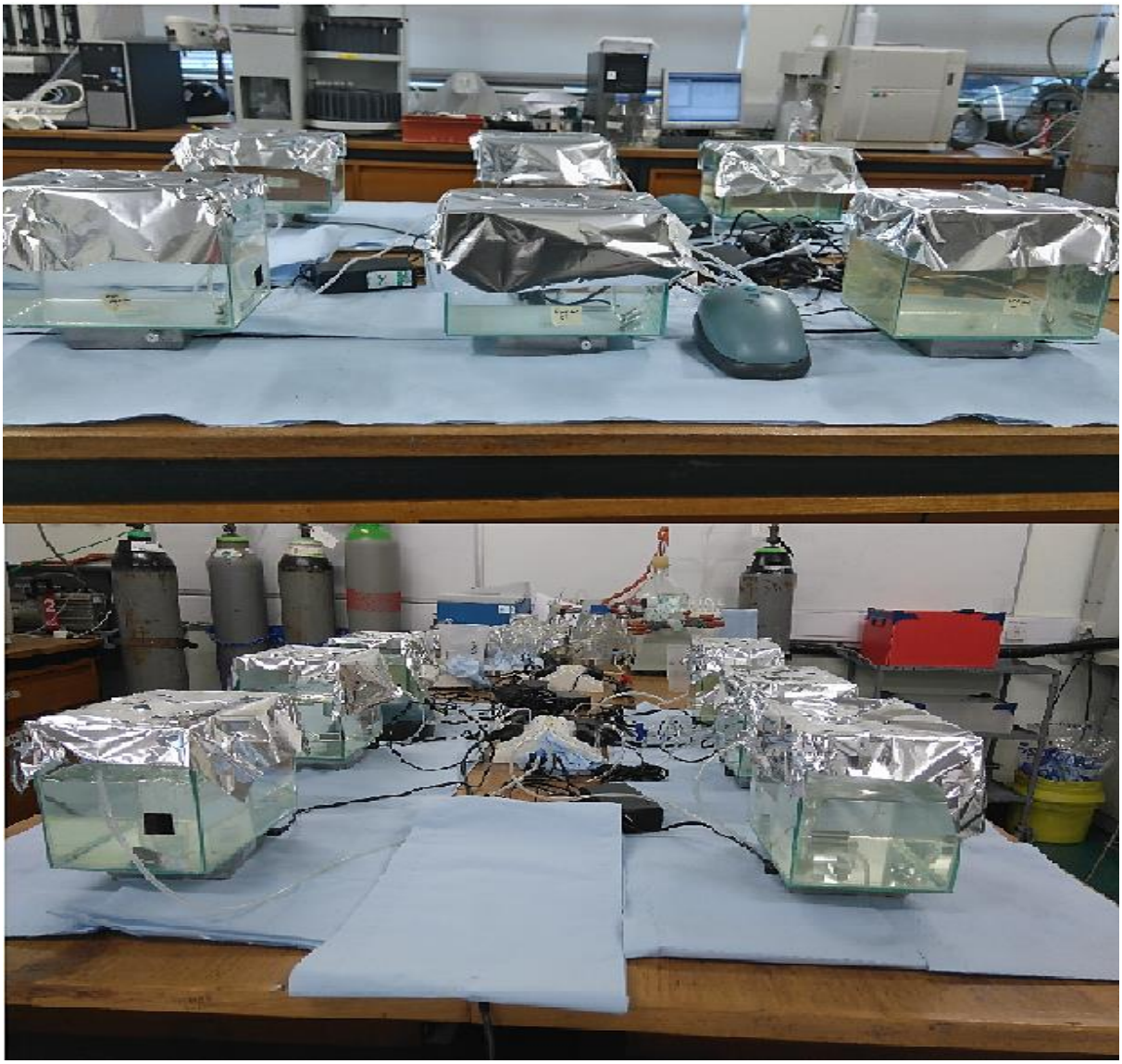


Figure 3.4: Laboratory Degradation study. Tinfoil was used to minimise evaporation and to allow air flow.

3.4 Selection of Suspect TPs to be monitored

The choice of target progesterone TPs to be monitored under low resolution was guided by the outputs from the EAWAG BBD predictions alongside literature reports of products resulting from the biodegradation of P4 (Peng et al. 2014; Ellis & Wackett, 2012). Progesterone was submitted to the EAWAG BBD associated pathway prediction system (PPS) using its simplified molecular-input line-entry system (SMILES). Submitted P4 SMILES was

CC(=O)[C@H]1CC[C@@H]2[C@@]1(CC[C@H]3[C@H]2CCC4=CC(=O)CC[C@]34C)C). Later, in the present study, the improved version of the EAWAG BBD (enviPath), was used to model P4 degradation and results were compared. Putative products detected under low resolution and those confirmed by high resolution mass spectrometry (HMRS) are presented in the Results section. Comparison of P4 predicted TPs and associated pathways by the two databases is presented in the discussion chapter (Chapter 5) of this thesis.

3.5 Methodology for Screening for Biological Activity

3.5.1 Progesterone Screen Procedure:

Planning was important and timings critical to get the assays to work. Progesterone screen procedure was based on the protocol described by Gaido et al., 1997 and modified for use with 96-well assay plates by Lorenzen et al., 2004 (see section 2.6.2.1).

Day 0: The inner chamber of a type II laminar flow cabinet where yeast work was to be performed was wiped with 70% ethanol. Thereafter, 5 mL of 20% glucose was added to 45 mL selection medium. At 10am, a yeast culture was started by adding 125 µL human progesterone (hPR) yeast from cryovial stored at -20^{0}C into the 50 mL growth medium. The yeast culture was placed on a PSU-10i orbital shaker set at 200 rpm and incubated at 28^{0}C for 24 hours.

Day 1: At 10am, the incubated yeast culture from day 0 was diluted 50% by the addition of 25 mL of the culture to 25 mL minimal growth medium in a fresh flask. The old culture was disposed of for autoclaving and the new one shaken at 28^{0}C and incubated for 4 hours. At 1 pm, 3 hours following incubation of the new yeast culture, two 96-well assay plates were respectively designated as ‘dilution plate’ and ‘progesterone screen plate’. 100 µL ethanol was pipetted respectively into wells A2 - A12. 100 µL P4 stock (5 x 10^{-6}M) (positive control) was pipetted into well A1 and also well A2. The content of well A2 was mixed by simultaneous

pipetting and expelling for 5 times. Thereafter, by serial dilution, 100 µL of the diluted content in one well was used to dilute the well after it.

Using an automated 120 µL 12 point multichannel pipette, 10 µL dilutions of progesterone in ethanol, were transferred to designated wells in the 'progesterone screen plate' in duplicate and allowed to evaporate to dryness for approximately 30 minutes. Similarly, 10 µL ethanol was also transferred to control wells in 'progesterone screen plate' and left to dry. At 2pm, 4 hours after incubation, the turbidity of the new yeast culture was checked using a SpectraMax 340pc Molecular Devices plate reader to ascertain if the turbidity was within the acceptable range of 1-2 x 10^7 cells/mL. The absorbance for the device was set at 600 nm for a 100 µL culture volume. If the culture turbidity was high, it was diluted appropriately to the acceptable range. Thereafter, 250 µL (50mL culture; 500 µL/100 mL) 20mM $CuSO_4$ and 200 µL aliquots were transferred to the 96-well assay plate. Plate was sealed and then shaken for 2 minutes after which it was incubated at 32^0C for 19–20 hours.

Day 2: At 10am, yeast cells in previous day incubated plate were resuspended by shaking for 5 minutes. The cells were pipetted to mix further. Thereafter, 50 µL of the suspension was transferred to corresponding wells of a new 96-well plate to which 50 µL minimal medium had been previously added. The new assay plate was shaken for 2 minutes and read at 600nm to determine where cell lysis had occurred.

Thereafter, cells were lysed by freeze thawing in 3 cycles of 4 mins on dry ice, followed by 4 minutes at 42^0C. Assay plate was thereafter shaken for 5 mins. Using a reverse pipetting method, 100 µL of assay buffer was added to all wells in the multiwall plate. The assay plate was shaken for 2 minutes at a low speed of 2 m/s to minimize foaming of the SDS and then incubated at 37^0C. Plate was read every 30 minutes of incubation at 420 nm and 600 nm until colour development stopped. To correct for turbidity, the following equation was applied;

Corrected value = chem. abs. (420 nm) – [chem. abs. (600 nm) – blank abs. (600 nm)]

3.5.2 Androgen Screen Procedure

Like the progestogenic screen, planning and timing were critical to get the assays to work. The androgenic screen procedure was based on the protocol described by Sohoni & Sumpter, 1998 (see section 2.6.2.2).

Day 0: Growth medium was prepared by the addition of 5 mL glucose solution, 1.25 mL L-aspartic acid solution, 0.5 mL vitamin solution, 0.4 mL L-threonine solution and 125 µL copper (II) sulphate to 45 mL minimal medium in a sterile conical flask (final volume was approximately 50 mL). 125 µL of 10X concentrated yeast stock from cryogenic vial stored at -20^{0}C (see day 3 of '*preparation and storage of 10X yeast stock*' above) was added to growth medium. The medium was incubated at 28^{0}C for approximately 24 hours on an orbital shaker until it was turbid.

Day 1: Similar to the progesterone screen, test chemical 10^{-6} M (290.4 µg/L ethanol) of dihydrotestosterone (DHT) (positive control) was serially diluted and 10 µL aliquots were transferred to 96-well optically flat bottom assay plate and left to evaporate to dryness. 10 µL absolute ethanol was also transferred to blank wells. Thereafter, assay medium was prepared by the addition of 0.5 ml CPRG to 50 mL fresh growth medium. The assay medium was seeded with $4x10^7$ yeast cells from the previous day (24 hours) culture (about 0.5 mL to 2 mL, determined after absorbance readings). 50 mL assay medium was prepared (enough for 2.5 plates). 200 µL of the seeded assay medium was pipetted with a multichannel pipette into wells in assay plate. This gave a 1/20 dilution in each well (10 µL/200 µL). Plates were sealed with autoclave tape and shaken vigorously for 2 minutes on a titre plate shaker. Thereafter, assay pales were incubated at 32^{0}C in a naturally ventilated heating cabinet.

Day 2: Assay plates were again shaken vigorously for 2 minutes to mix and disperse the growing cells. Plates were returned to 32^{0}c incubator after shaking.

Day 3: After 3 days of incubation, assay plates were again shaken for 2 minutes and left for an hour to allow yeast cells to settle. Plates were read with plate reader at 540 nm (optimum absorbance for CPRG) and 620 nm (for turbidity). To correct for turbidity, the following equation was used;

Corrected value = chem. abs. (540 nm) – [chem. abs. (620 nm) –blank abs. (620 nm)]

3.5.3 Calculation of Assay Results

The absorbance readings for the individual wells in assay plate were plotted against the log of the molar concentration of the test compound in the corresponding wells. The position of the activity curve produced for the test compound relative to that of the positive control is used to determine and measure the presence and extent of biological activity. The activity expressed in these assays was based on effective dose 50 (ED_{50}) as described by Sohoni & Sumpter 1998.

Chapter Four: Results

4.1 Analytical Methods Development and Validation

4.1.1 Method Development

The first step in the development of analytical method for the present study was the optimization of system parameters in the locally available PE SCIEX API365 triple quadrupole mass spectrometer (MS) to obtain higher signal strength (sensitivity). The data for MS parameters optimization had been presented in section 3.2.3.2 of this thesis, but repeated here for easy reference. Using the electrospray source and in the manual tuning mode, parameters were optimized in the positive mode for positively ionised analytes and in the negative mode for the negatively ionised analytes. The choice of product ion to monitor for each compound was also determined by manual tuning using the parent to product ion transition scan mode. Previously reported most intense product ion selected for multiple reaction monitoring mode (MRM) analysis and final values of other optimized instrument parameters are also presented in Table 4.1 and 4.2. An example of the most intense product ion peak selected for MRM is illustrated in Fig 4.1 for propranolol. Following set up of parameters for the electrospray source and mass spectrometry, chromatographic separation was then developed.

Table 4.1: Monitored Parent and Fragment Ions of Model Compounds

Compounds	Retention Time (Min)	Ions (m/z)	
		Parent (M1)	Product (M2)
P4	25.50	315.3	97.1
P4d9	25.30	324.3	100.2
PRPL	15.19	260.0	116.2
FLX	20.07	310.0	44.0
DCF	15.16	294.0	250.0

Table 4.2: Final Optimized Values of MS Parameters for Higher Sensitivity

Mass Spec Parameter	Compound			
	P4	**PRPL**	**FLX**	**DCF**
Nebulizer Gas (NEB)	9	9	9	8
Curtain Gas (CE)	8	8	8	8
IonSpray Voltage (IS)	4500	4500	4500	-4300
Collision Gas (CAD)	10	2	2	11
Temperature (TEM)(^{0}C)	350	350	350	350
Declustering Potential (DP) (V)	35	3.1	11	-55
Focusing Potential (FP) (V)	92	61	69	-55
Entrance Potential (EP) (V)	4.5	10	2.5	-2.5
Collision Energy (CE)	36	25	30	-20
Collision Cell Exit Potential (CXP)	8	4.5	3.3	-15

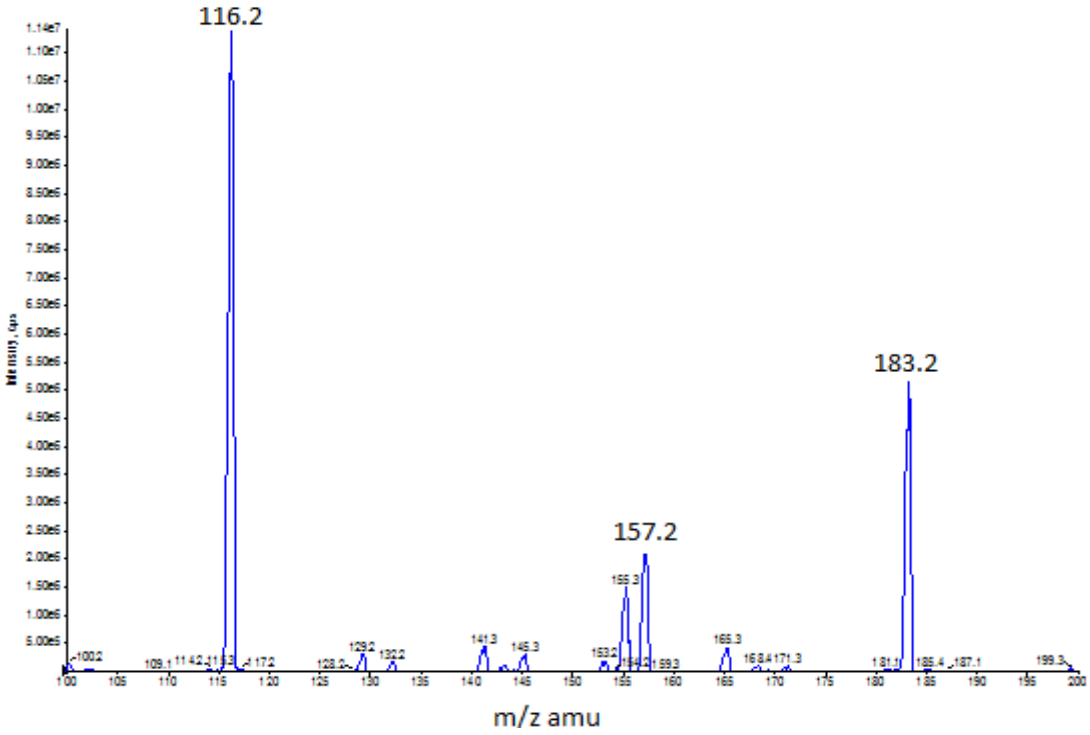


Figure 4.1: Selected PRPL product ion for MRM obtained from parent to product ion transition scan mode. Peak116.2 was more intense relative to other product ion peaks and was therefore selected for MRM.

4.1.1.1 Liquid Chromatography

For the development of chromatographic methods, a series of five-point working (calibration) standards ranging from 1 - 100 ng/mL prepared in chromatographic vials by appropriate dilution of sub-standards with methanol/water (50/50, v/v) were used. Analytical standards contained all four analytes and 200 ng/mL of the internal standard ($P4_{d9}$) respectively. Calibration curves for the four model compounds and their corresponding correlation coefficient are presented in Figures 4.2 – 4.5. Three of the target compounds, P4, FLX, and PRPL separated well producing distinct peaks with good resolution, peak shapes and response. Diclofenac also produced a distinct peak. However, the instrument sensitivity was lower in the negative mode. The total ion chromatograms of the target compounds for a 100 ng/mL standard are presented in Figures 4.6 and 4.7. The intensity of DCF peak ($3x10^3$ cps) was low relative to propranolol response ($7x10^4$ cps), which was the lowest response of the positively ionised analytes.

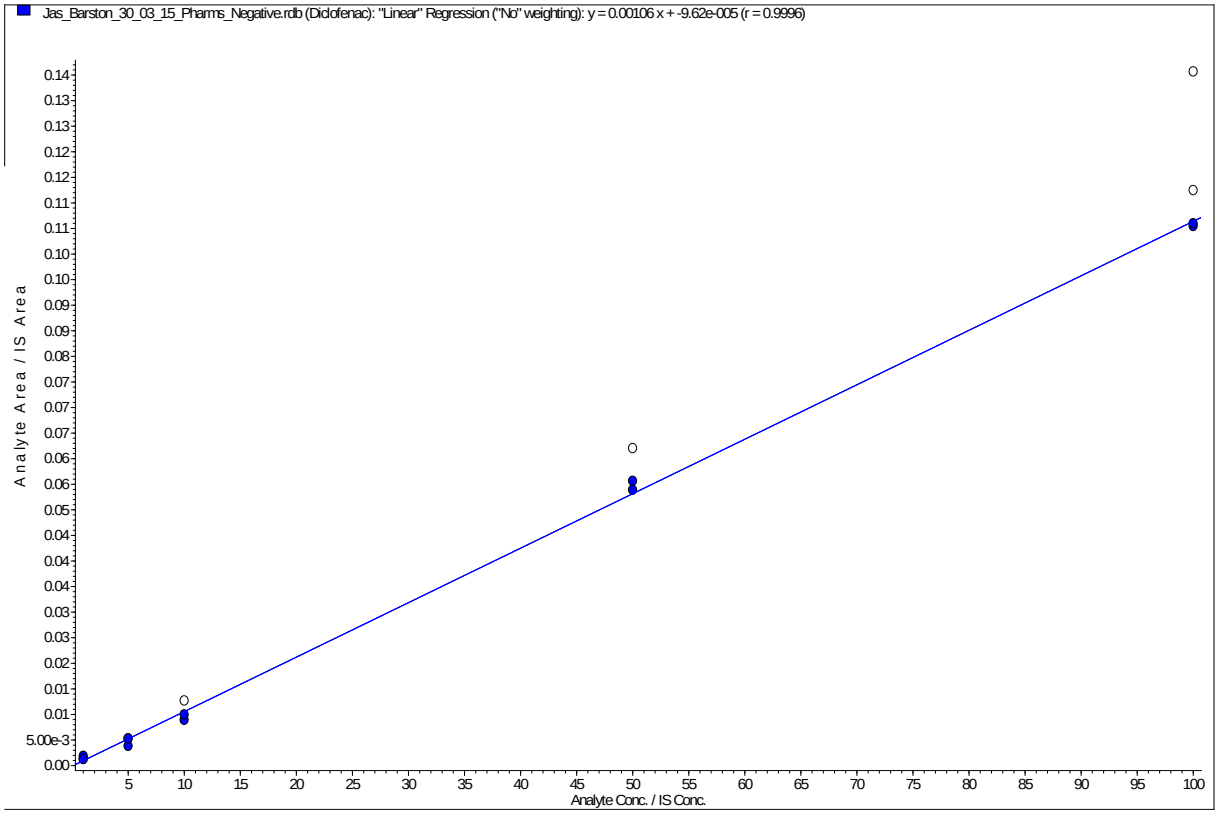


Figure 4.2: Calibration curve for diclofenac (standard concentration: 1 – 100 ng/mL (R2=0.99))

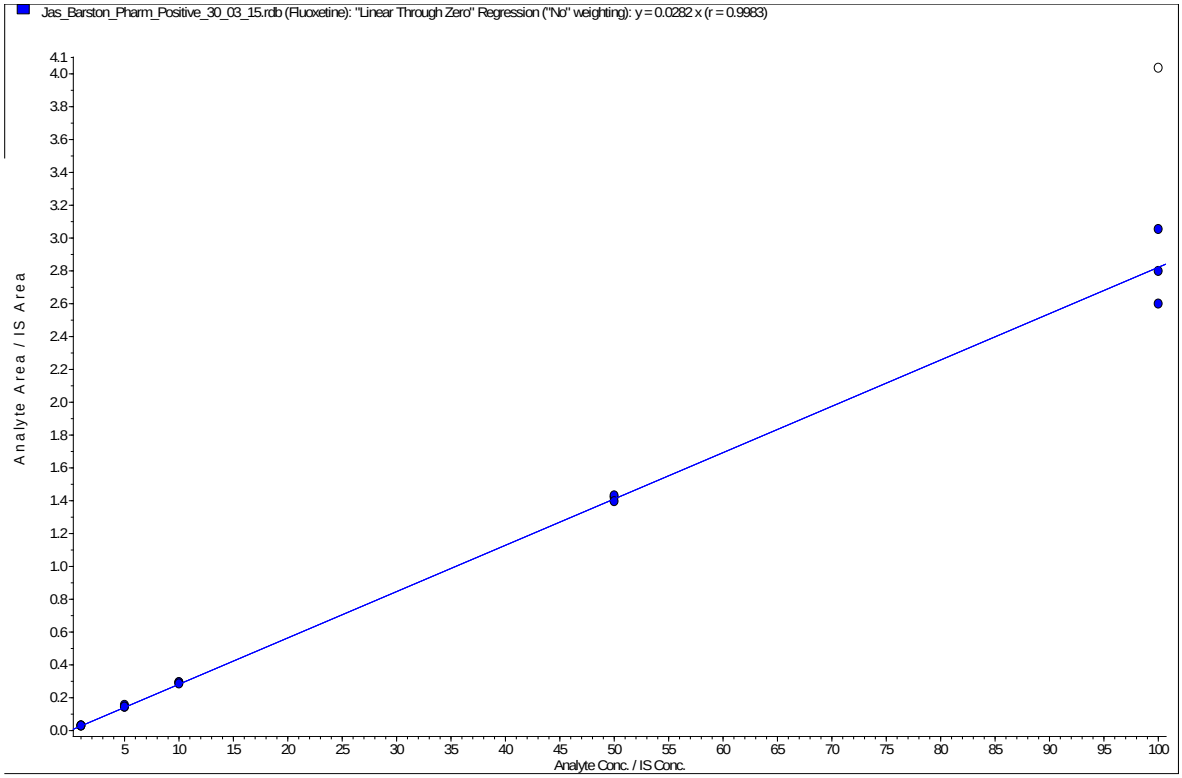


Figure 4.3: Calibration curve for fluoxetine (standard concentration: 1–100 ng/mL (R2=0.99))

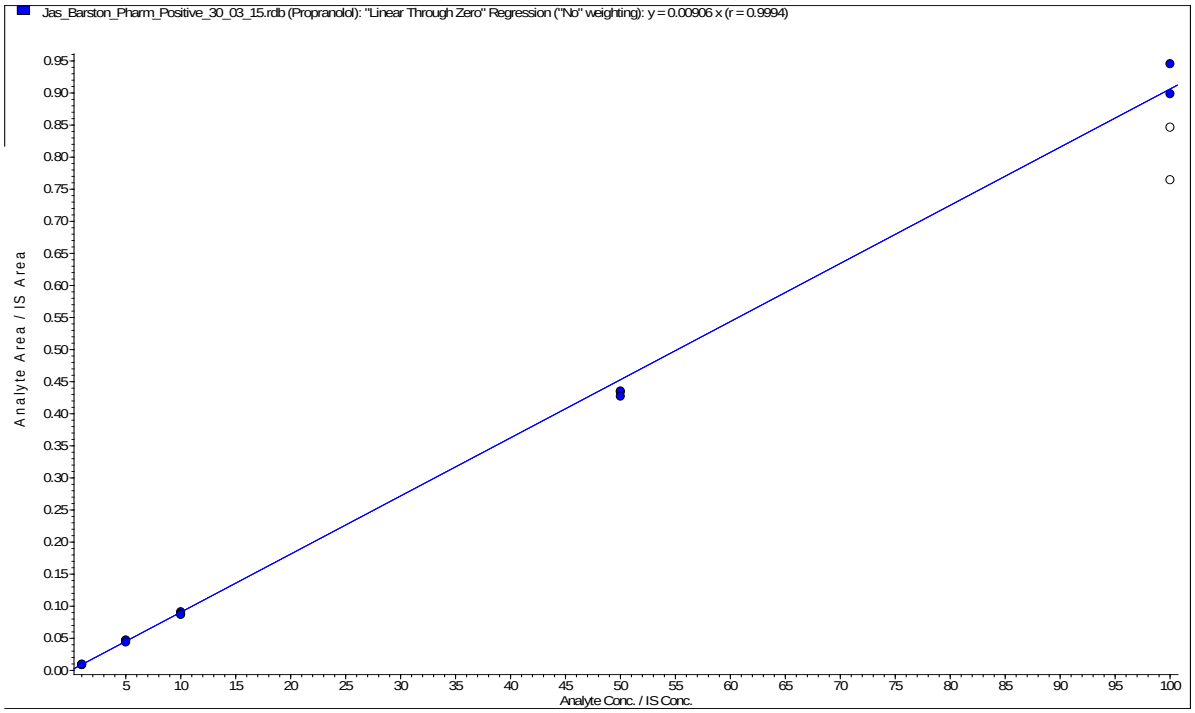


Figure 4.4: Calibration curve for propranolol (standards concentration: 1–100 ng/mL (R2=0.99))

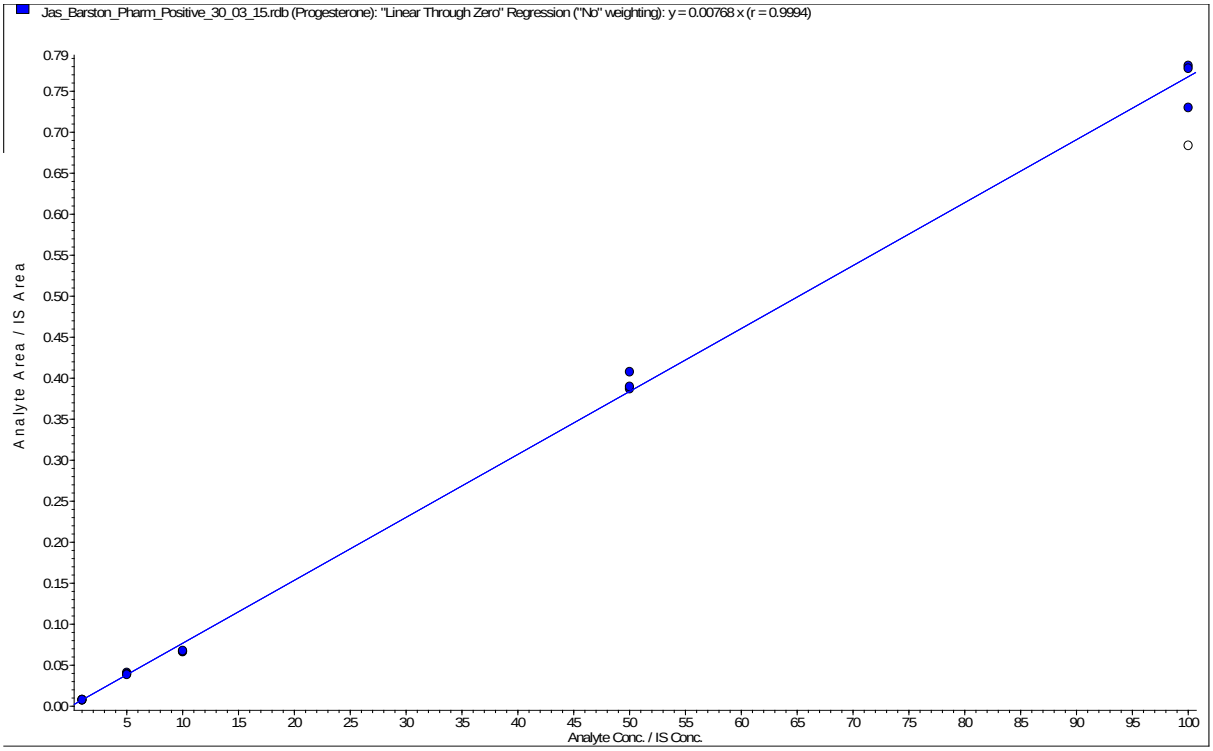


Figure 4.5: Calibration curve for progesterone (standard concentration: 1–100 ng/mL (R2=0.99))

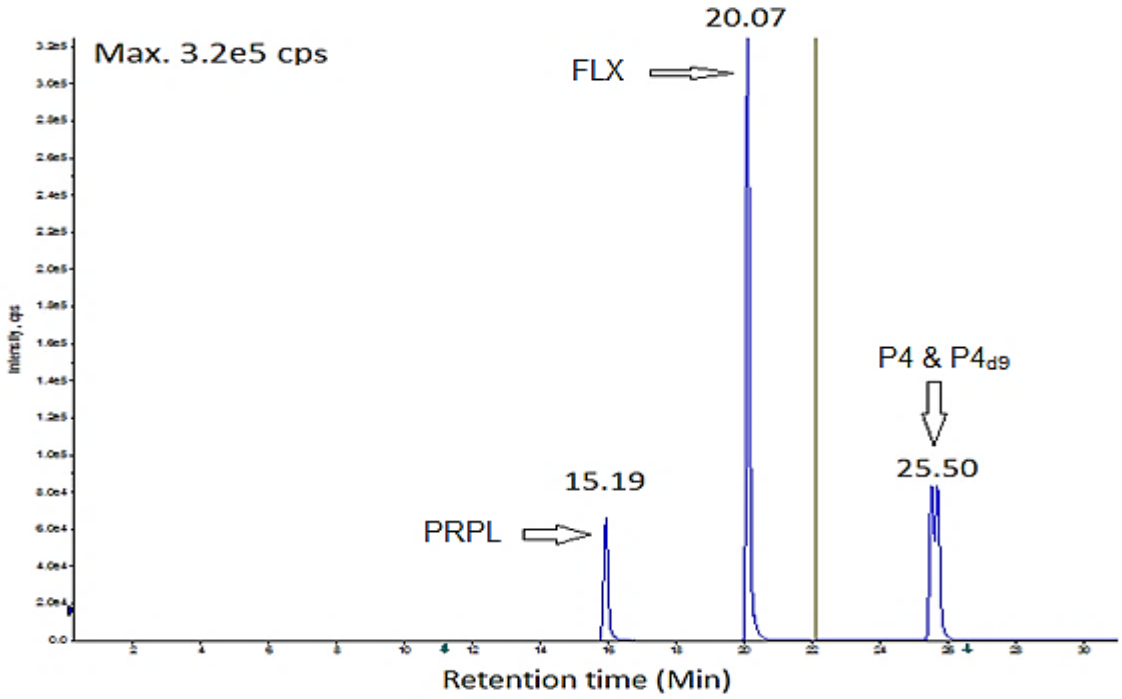


Figure 4.6: Total ion chromatogram of the positively ionised target compounds in a 100 ng/mL standard. Propranolol response of 7x104 was the least of the positively ionised analytes. Progesterone (P4) peak and that of the IS (P4d9) are combined as shown in the figure.

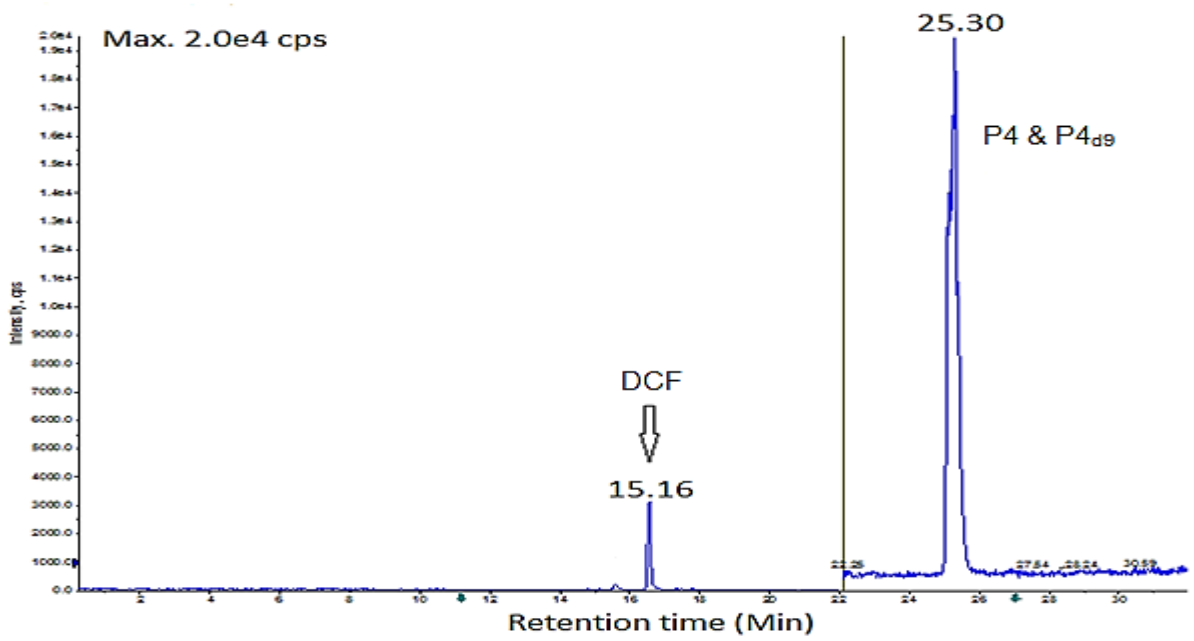


Figure 4.7: Total ion chromatogram for diclofenac for 100 ng/mL standard. Period 1 was in the negative ionisation mode Period 2 in the positive mode.

4.1.1.2 Recoveries from SPE

This section of the present work presents result of experiments on SPE protocol optimization. Some brief of the methods described in chapter 3 are mentioned here to provide context for the results presented. Initial studies focussed on the relative effect that the following parameters, type of elution solvent, volume of elution solvent used for cartridge washing, volume of sample loaded on the cartridges and the rate of loading would have on analyte recovery. These studies worked with high concentrations of each compound (100 µL of 10 mg/L standard mixture of all model compounds in 500 mL milliQ water), such that sample drying and concentrating up was not necessary. For the investigation of the best type of elution solvent to use, 4 mL of methanol and acetonitrile were used respectively for elution of cartridges. Thus, the expected concentration of individual compounds in the eluent was 0.25 µg/mL. Eluent sample for instrumental analysis were diluted 50:50 with MilliQ water, making the concentration of analytes in chromatographic vial for LC-MS/MS analysis to be 0.125 µg/mL. The result demonstrated that methanol was a better elution solvent for the recovery of model compounds relative to acetonitrile (Fig. 4.8A). Overall, recovery for all compounds

was above 80% when cartridges were eluted with methanol, whereas with acetonitrile they were below 50%.

Following the determination of what elution solvent (methanol) to use, the best volume of methanol to use for elution was investigated by eluting cartridges with 4 mL and 8 mL (2 x 4 mL) of methanol respectively. The recovery obtained for all four model compounds were 20% higher when cartridges were washed with 2 x 4 mL (8 mL) of methanol relative to washing with 4 mL methanol (Fig. 4.8B). The higher recovery observed when eluting with increased volume of methanol, is probably because, eluting with a second 4 mL solvent volume helped free-up any residue of target analytes still bonded to the cartridge adsorbent material after the initial wash with 4 mL MeOH. Irrespective of the volume of elution solvent used, P4 had 20% more recovery when compared to the other compounds.

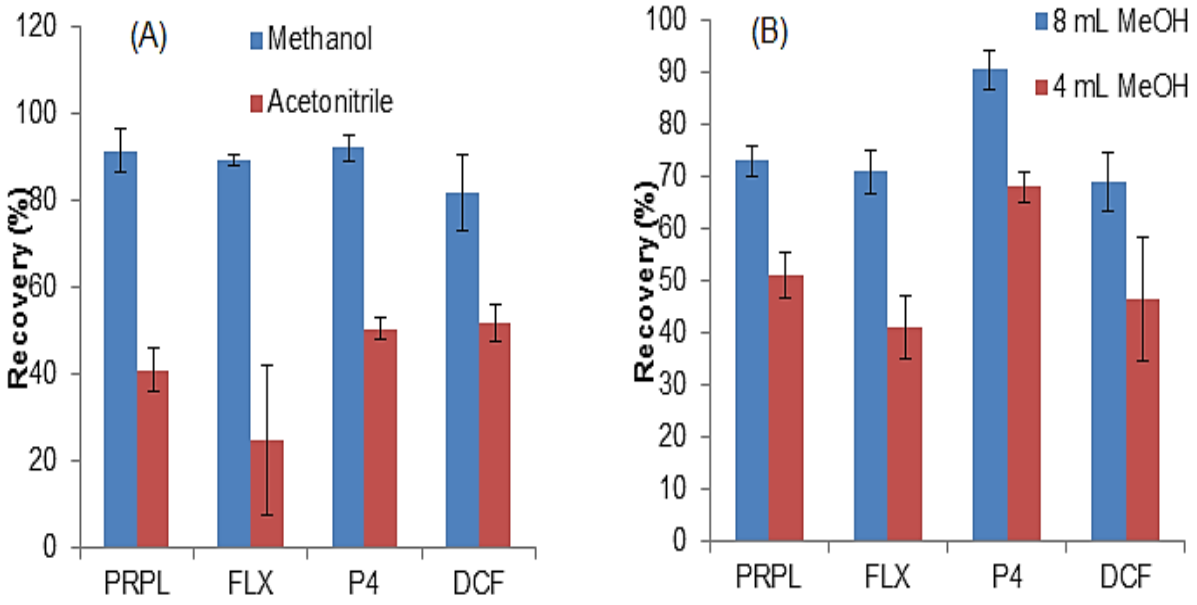


Figure 4.8: Relative effects of type of elution solvent (A) and volume used (B) in washing cartridges on model compounds recovery (Error bars = SD; n=3; Flow rate: 2 mL/min)

Additionally, different volumes (0.5 and 1 L) of MilliQ water were respectively spiked with 100 µL of 10 mg/L mixture of all five pharmaceuticals. This was done to determine if loading a

higher volume of water resulted in loss of analytes due to breakthrough. The extraction of 0.5 L of MilliQ water spiked with mixture of model compounds in this study, produced a recovery that was 50% higher relative to extracting a 1 L (Fig. 4.9A), the common approach reported in literature. The results thus, showed that extracting a lower volume of samples would generally result in improved recovery of compounds of interest. An advantage of extracting lower sample volume, is high throughput, and results obtained can be used to estimate analytes concentration in higher sample volume. The lower recovery observed when 1 L of water was extracted may be due to breakthrough of previously bonded compounds to cartridge adsorbent material. Furthermore, 0.5 L of water was loaded at different rates corresponding to fast (5 mL/min) and slow flow (2 mL/min) to determine which loading rate gave better recovery. The two loading rates were randomly chosen as variable loading rates (1–20 mL/min) are reported in the literature for both manual and automated SPE operations. Furthermore, there was also the desire for a quicker procedure, hence the 5 mL/min flow (loading) rate was tested against the 2 mL/min used in the optimization of the previous SPE conditions. Overall, analyte recovery was 20% better when cartridges were loaded at a slow rate of 2 mL/min relative to loading at a higher rate of 5 mL/min (Fig. 4.9B).

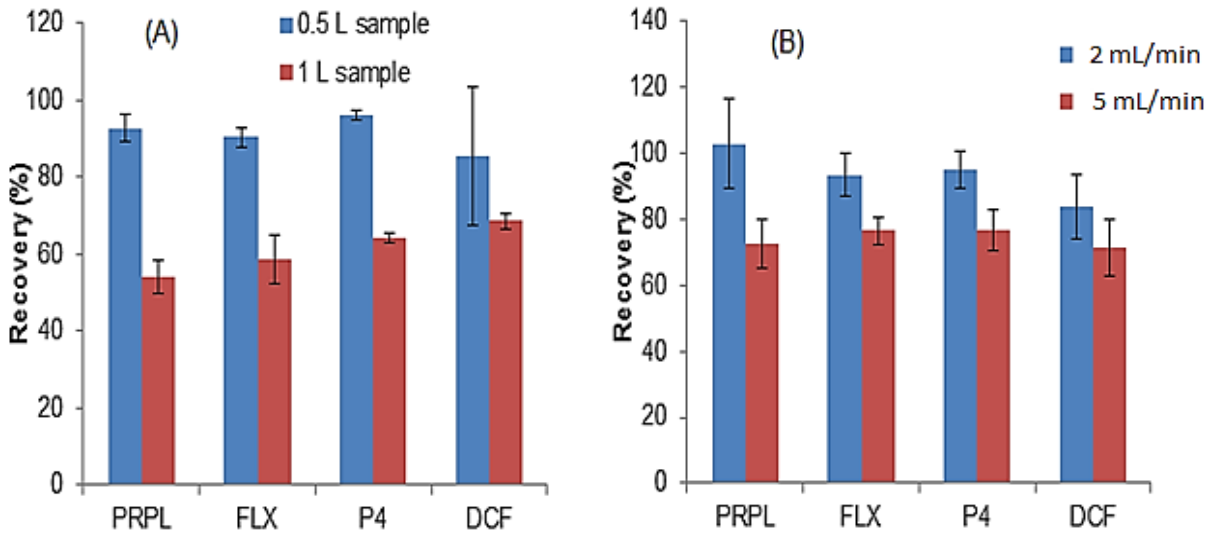


Figure 4.9: Relative effects of extracting varying volume of samples (A: 4 mL volume used; flow rate: 2 mL/min) and loading rates (B) on recovery of target compounds (Error bars = SD; n=3)

Next, the relative effect of drying SPE extracts down relative to when they were not, on target compounds recovery was investigated, especially since model compounds are often present at very low concentrations in the environment. Thus, 0.5 L of MilliQ water was spiked with 10 µL of the 10 mg/L standard mixture of all model compounds, a much lower volume of pharmaceuticals standard mixture relative to the initial studies (100 µl of 10 mg/L). Cartridges were eluted with 2 x 4 mL of methanol, such that concentration of analytes in extracts not dried down (referred to as 'non-concentrated' in Fig 4.10) was 0.0125 µg/mL, a lower concentration compared to the initial study of 0.125 µg/mL. Extracts for samples designated for drying ('concentrated' in Fig 4.10), were then dried under a nitrogen stream and reconstituted to 1.2 mL with a mixture of methanol and water (50/50 v/v). Dried extracts were reconstituted to 1.2 mL to have enough sample volume for multiple instrumental run. A representative concentration of 0.08 µg/mL was thus expected for individual analytes in the chromatographic vial. Analytical results for concentrated samples were compared to the non-concentrated samples. The study result demonstrated that samples that were concentrated up, had better recovery for target analytes relative to the non-concentrated samples (Fig 4.10). Overall, locally available unit resolution mass spectrometer could hardly detect and quantify the low concentrations of model compounds in non-concentrated samples.

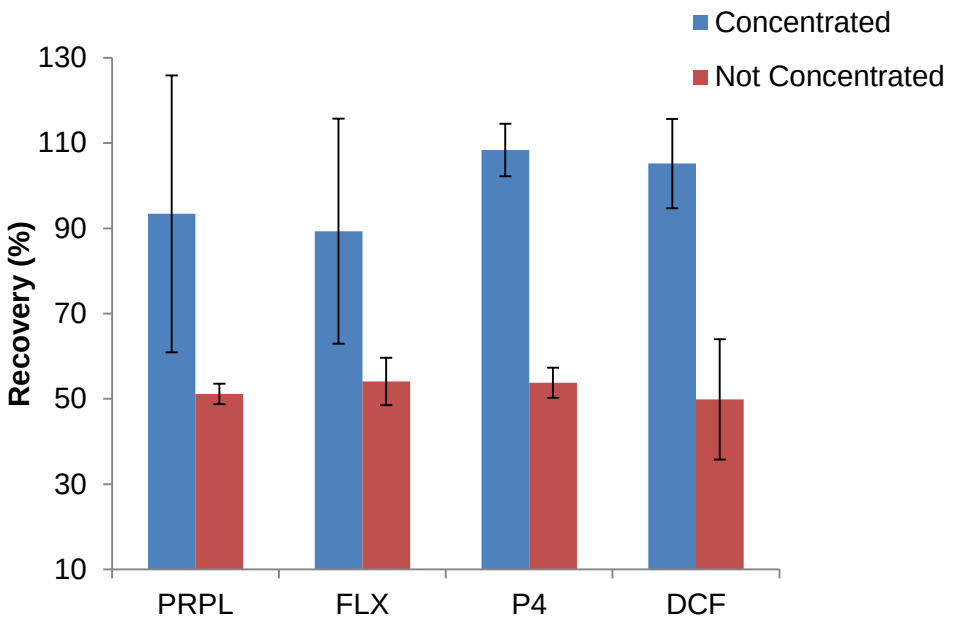


Figure 4.10: Effect of sample dry down on recovery of target compounds (Error Bars = SD; n=3). Concentrated: extracts dried down and reconstituted; Not Concentrated: extracts not dried down.

4.1.1.2.1 Validation of SPE Method

The SPE method developed was validated using wastewater influent and effluent sampled from pilot scale reed beds at a Severn Trent Water STW located in Barston. Two litres (2L) spot samples (HRT <1 min) were respectively taken in triplicate. In the laboratory, samples were subsequently then divided into two groups of 0.5 L each. One group was spiked with a 100 µL of a 10 mg/L concentration of the target pharmaceutical mix and the isotopically labelled $P4_{d9}$ and the other group was used as a control. The internal standard was added prior to sample extraction, to minimize matrix effect. However, as noted in section 3.2.2, Initial work in reed beds used $P4d_9$ as a sole IS, the use of $P4d_9$ as a sole IS in the initial study with the reed beds, was thought to lead to some bias, in that we were correcting for recovery using a compound with different characteristics, which may respond differently in different matrices. Overall, the optimized SPE protocol was adequate for recovery of all model compounds. Recovery was however higher in influent samples when compared to effluent. Also, the influent data were more varied relative to the effluent as shown by the percentage relative

standard deviation (%RSD aka Coefficient of Variation (CV)) (Fig. 4.11). Variation in influent data can be attributed to the more complex nature and possibly presence of more interference in these samples relative to the effluent. The latter point must have also accounted for the enhanced recovery recorded for the influent samples (slower flow rate through cartridge and therefore higher contact time with cartridge sorbent material). Interferences in these samples may have slowed down the rate of flow of target analytes through cartridge adsorbent materials allowing for higher contact time, which makes for increased bonding. DCF recovery was relatively high when compared to other target compounds in both influent and effluent samples. DCF recovery was >50% in influent relative to other compounds and about 80% in effluent. Perhaps, the use of P4d_9 as a sole IS in this study, coupled with the switching of ionisation modes from negative for DCF in period 1 to positive for the IS in period 2 (Fig. 4.7) may have resulted in false positives. See Figure 4.2 for the DCF calibration curve showing that standards selected for quantitation covered a broad range of concentration points, like the other model compounds. Recovery data were used to correct for concentration of model compounds in reed beds (Table 4.1). However, because of the uncertainty in recovery of model compounds, spot sampling (zero hydraulic retention time) of the in/out of reed beds and lack of further access to site, the results presented for performance of reed beds will require further study to verify their reliability.

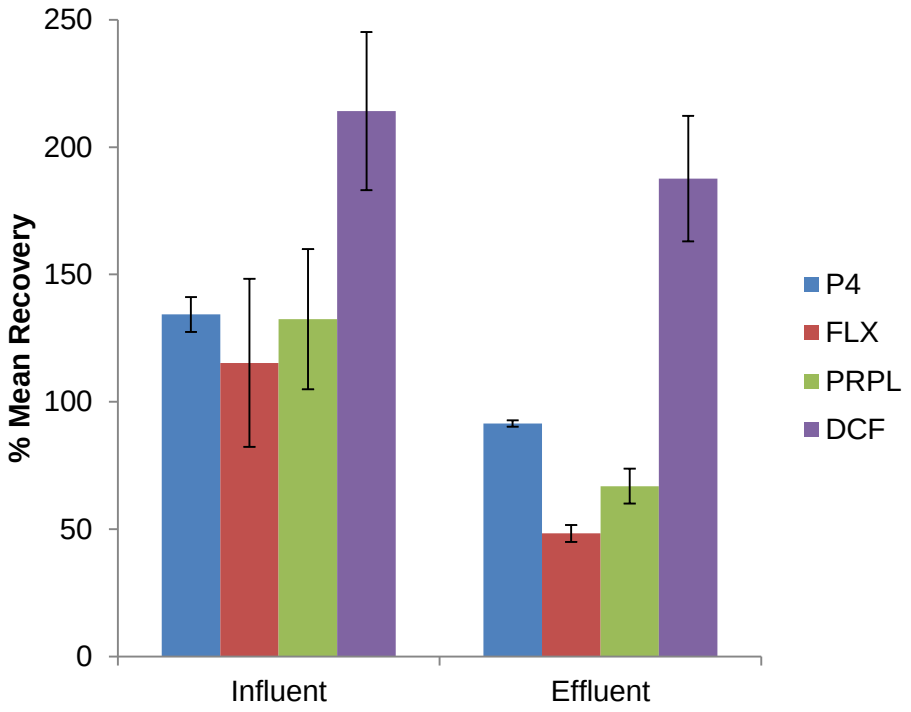


Figure 4.11: Model compounds recovery in influent and effluent of pilot reed beds (Error Bars = %RSD; n=3)

4.2 Application of Analytical Methods in the Measurement of Target Compounds in Reed Beds

4.2.1 Concentration and Removal of Target Analytes in Reed Beds

A further aim to the sampling of reed beds was to establish the concentrations of target compounds in influent and final effluent. Percentage recovery values were used to correct for analytes recovery in influent and effluent. Target compounds were present in both influent and effluent of reed beds (Table 4.3 and Fig. 4.12). Similar to data for SPE validation, influent data were more varied relative to the effluent. Progesterone concentration in the samples was the lowest of all four compounds. Influent B data point for P4 appears to be an outlier. A review of the raw datasets reveals this was not a typo. Data reported for diclofenac in Table 4.3 are uncertain due to the probable false positive mentioned in 4.1.1.2.1.

Also, reed bed performance in removing model compounds was investigated by comparing target compounds concentration in influent with their corresponding concentrations in effluent following influent polishing by the reed bed. Generally, all analytes were removed to varying degrees by the reed beds. Removal of target compounds was generally above 65% for all model compounds except DCF (Fig 4.13). As noted in section 4.1.1.2.1, further work will be needed to validate reed bed performance results.

Table 4.3: Concentration of target compounds in reed beds (±SD)

	Concentration (ng/L)			
Sample Name	P4	FLX	PRPL	DCF
Influent A	11.0	106	372	605.0
Influent B	6.9	67.1	250	271.7
Influent C	8.0	89.9	382	577.0
Mean	6.6 ± 5.1	87.7 ± 19.6	335 ± 73.7	484.6 ± 184.9
Effluent A	1.3	2.5	113	112.6
Effluent B	1.3	5.3	101	150.9
Effluent C	1.0	2.7	122	132.4
Mean	1.2 ± 0.2	3.5 ±1.6	112 ± 10.5	132.0 ± 19.2

* P4=Progesterone; FLX=Fluoxetine; PRPL=Propranolol; DCF=Diclofenac

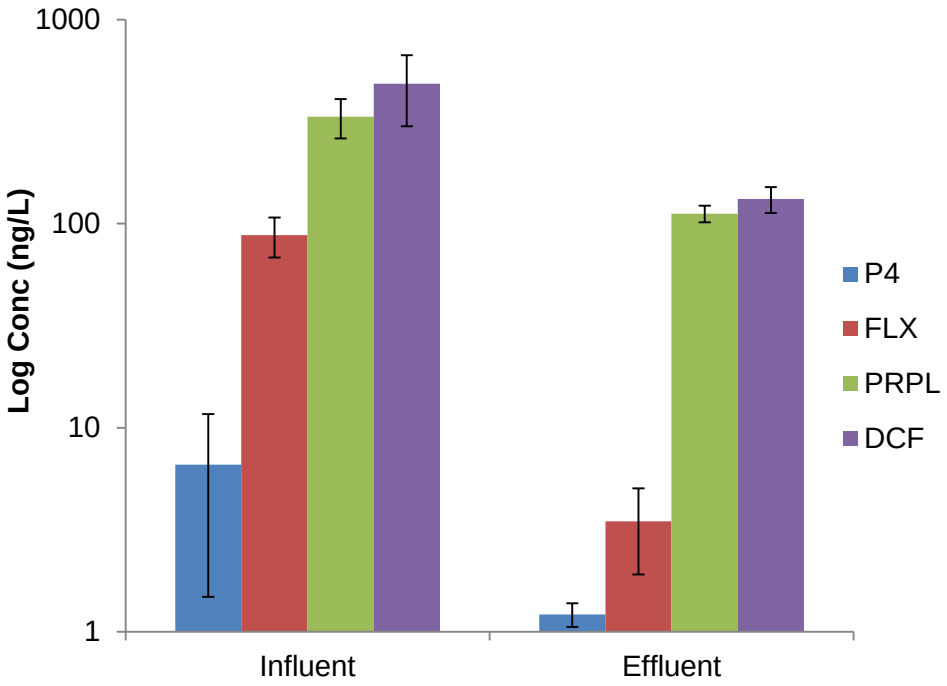


Figure 4.12: Occurrence of target compounds in influent and effluent of pilot reed beds (Error Bars – SD). Influent data were more varied when compared with effluent (P4=Progesterone; FLX=Fluoxetine; PRPL=Propranolol; DCF=Diclofenac)

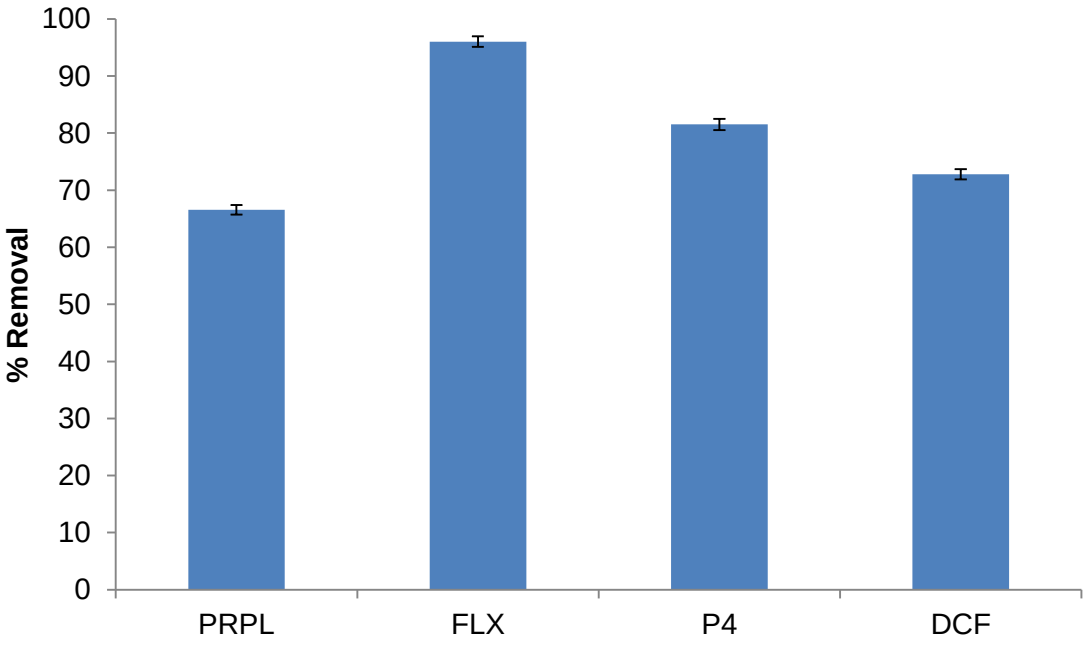


Figure 4.13: Performance of reed beds in the removal of target compounds. Removal was above 65% for all model compounds (n=3; P4=Progesterone; FLX=Fluoxetine; PRPL=Propranolol; DCF=Diclofenac).

4.3 Progesterone Degradation Study

4.3.1 Target Transformation Products Monitored

A summary of target TPs predicted by the EAWAG BBD is shown in Fig. 4.14 and compounds selected from the literature and that were produced through algal degradation of P4 are shown in Fig. 4.15. Some of the information provided in this section had been mentioned in chapter 2, but repeated here to put the results context. The details of compounds monitored in the suspect analysis is summarised in Table 4.4. Progesterone details are also included in this table for easy reference, especially when comparing the properties of individual TP with the parent molecule. Table 4.4 also details the chromatographic retention times (RT) of the target compounds and their relative retention times (R/RT) to that of the $P4d_9$ internal standard.

The biodegradation database predicted products (TP-BBD) resulting from the cleavage of the 'B' ring in the P4 C21 pregnane skeletal backbone (Fig. 4.14). The aerobic likelihood of the formation of the predicted intermediate product (TP-BBD-1) was *neutral* indicating that there was a 50% chance of its formation in aerobic and anaerobic systems. The implication is that, where the odds are against its formation in a given system (aerobic or anaerobic), subsequent TPs (TP-BBD 2-5) resulting from its breakdown, would not be produced.

Literature search, however, identified algal products (TP-ALG) (Fig. 4.15) that do not show ring cleavage, but the hydroxylation, oxidation, hydrogenation, dehydrogenation and breakdown of attached side chain. Thus, the biodegradation database predicted 'B' ring cleavage may be related more to anaerobic degradation pathways. Anaerobic transformation pathways are relevant as they are probably the dominant reaction pathways in the breakdown of pharmaceuticals in sewers and in many conventional treatment processes such as primary sedimentation tanks, consolidation tanks and anaerobic digesters.

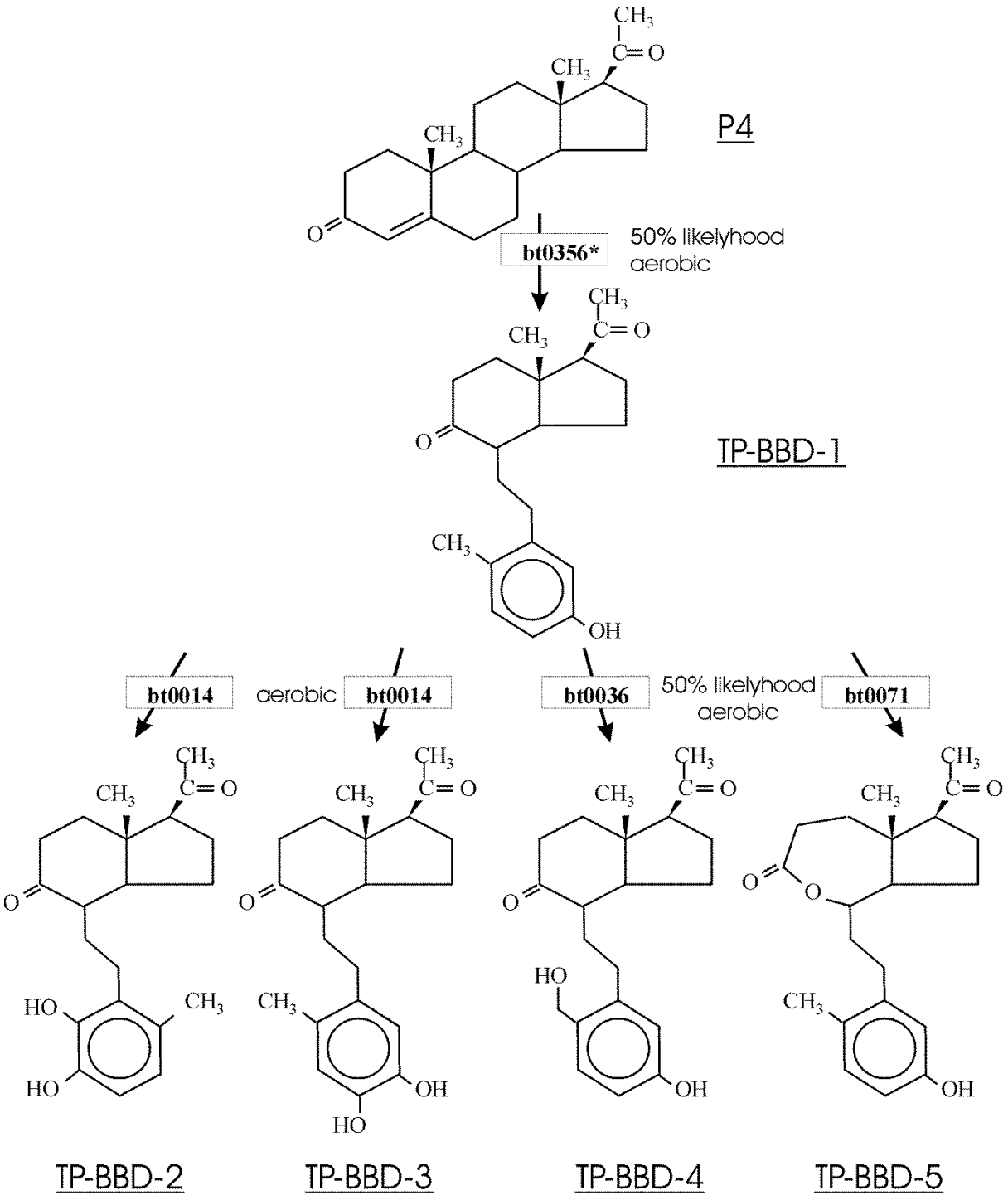


Figure 4.14: The EAWAG BBD prediction for the environmental transformation of P4. The predictions all show ring cleavage which may be related to anaerobic pathways in the biodegradation database

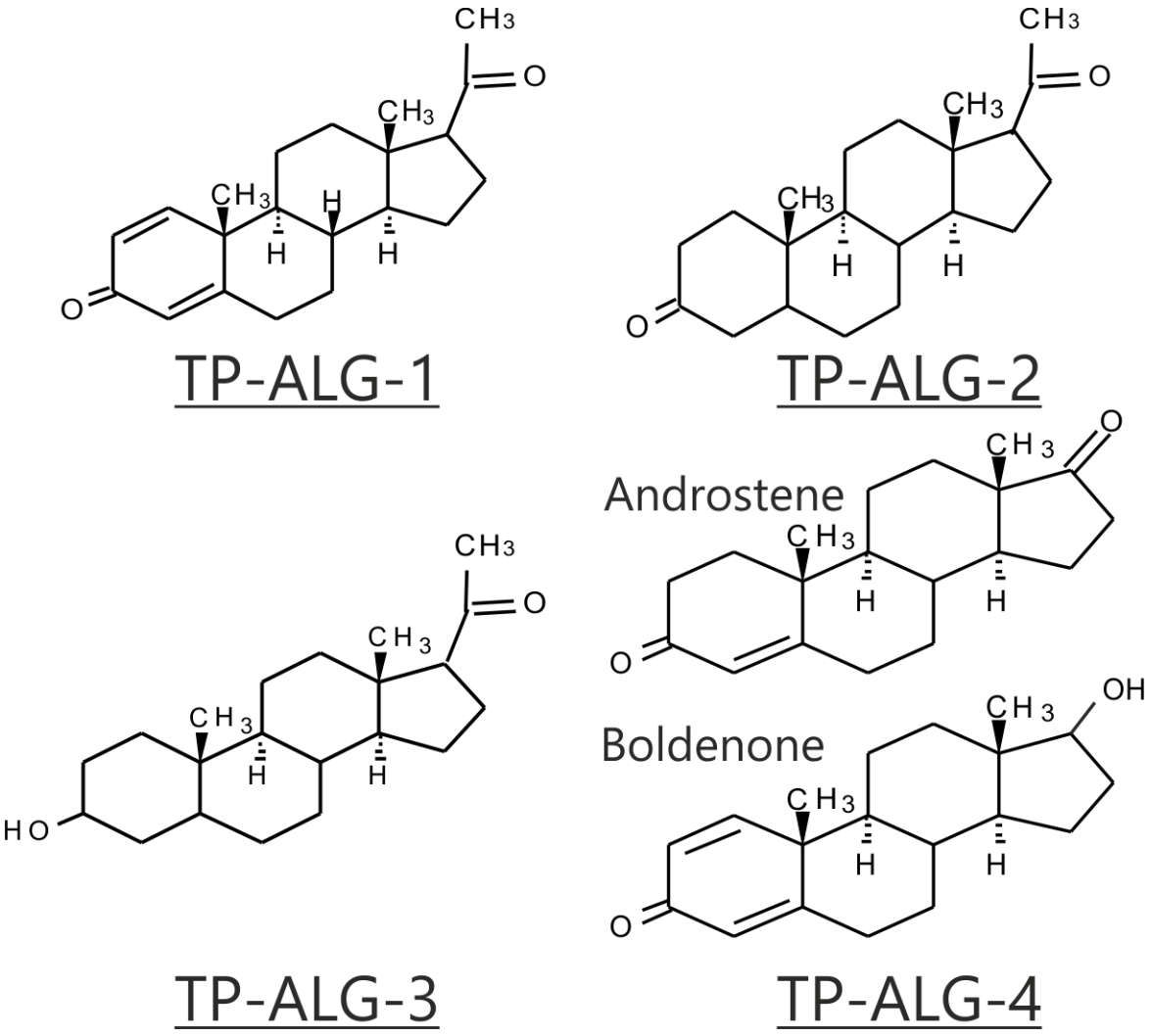


Figure 4.15: The predicted transformation products from the literature. Peng et al. 2014 identified these products from their algal studies (TP-ALG). There are two possible compounds identified for TP-ALG-4, see also Table 4.2.

Table 4.4: Characteristics of the BBD and algal metabolites monitored using unit resolution mass spectrometer in this study

Name	Abbr.	Mol. Wt. (Da)	CAS	m/z	RT* (%RSD)	R/RT*
Progesterone	P4	314.5	57-83-0	315.3	25.4 (0.6)	1
1-acetyl-4-[2-(5-hydroxy-2-methylphenyl)ethyl]-7a-methyl-octahydro-1H-inden-5-one	TP-BBD-1	328.5		329.3	12.4 (<0.1)	0.49
1-acetyl-4-[2-(2,3-dihydroxy-6-methylphenyl)ethyl]-7a-methyl-octahydro-1H-inden-5-one	TP-BBD-2	344.5		345.3	14.8	0.58
1-acetyl-4-[2-(4,5-dihydroxy-2-methylphenyl)ethyl]-7a-methyl-octahydro-1H-inden-5-one	TP-BBD-3	344.5		345.3		
1-acetyl-4-{2-[5-hydroxy-2-(hydroxymethyl)phenyl]ethyl}-7a-methyl-octahydro-1H-inden-5-one	TP-BBD-4	344.5		345.3		
6-acetyl-1-[2-(5-hydroxy-2-methylphenyl)ethyl]-5a-methyl-octahydro-1H-cyclopenta[c]oxepin-3-one	TP-BBD-5	344.5		345.3		
1,4-Pregnadiene-3,20-dione	TP-ALG-1	312.5	1162-54-5	313.3	23.3 (0.8)	0.92
3,20-Allopregnanedione	TP-ALG-2	316.5	566-65-4	317.3	21.5 (0.8)	0.85
3b-Hydroxy-5a-pregnan-20-one	TP-ALG-3	318.5	516-55-2	319.3	21.5 (0.6)	0.85
Androstene or 17b-Boldenone	TP-ALG-4	286.5	846-48-0	287.3	18.5 (0.1)	0.73

* Information for degradation products is only available if they were detected. m/z: parent ion

4.3.2 Progesterone Degradation in Reed Beds

Initial work on identifying putative degradation products in the reed bed samples was done using the unit resolution mass spectrometer at Brunel. Unit resolution mass spectrometry was performed using the selected ion recording mode that targeted the unit masses of suspect transformation products identified in Table 4.2. Presence of a suspected TP in samples was determined by extracting chromatogram for that product in sample and overlaying it with corresponding extracted chromatogram in the control (blank: double deionised water). All peaks found in samples that were also present in the control, were excluded. Response had to occur only in the spiked sample for peaks to be considered as possible degradation products.

In the reed bed study, none of the algae products were detected. It was the expectation that algal products will be present in at least the influent samples. The thick biofilm layer in the trickling filters applied for treatment of post primary sedimentation sewage at Barston, consisted of green algal layer. However, the m/z 329.3 corresponding to one of the predicted bacterial product, TP-BBD-1, was found (Fig. 4.16). This mass was consistently detected in all replicate samples. The height of the peak in effluent samples was smaller relative to the corresponding height in influent samples, indicating the possible removal of this product by the reed beds. The peak to the right of the one marked 'TP-BBD-1' in Figure 4.16 was excluded from consideration because, a similar peak was found in the blank. The detection of TP-BBD-1 and absence of the algal products was thought to be that bacterial mediated degradation process was the dominant metabolic process occurring in the reed beds. The non-detection of the algal-based products could also be because of their low concentration and/or perhaps reed beds conditions were not suitable for their formation. The application of the concentration step (SPE) to TPs whose sorption properties are unknown, may have added some uncertainty, as if the products were present, but not retained by cartridges, resulting in a false negative outcome. Generally, the work on the reed beds indicated that unit resolution work would not be enough to confirm the presence of target transformation products as commonly reported algal products were not detected (see Table 2.1).

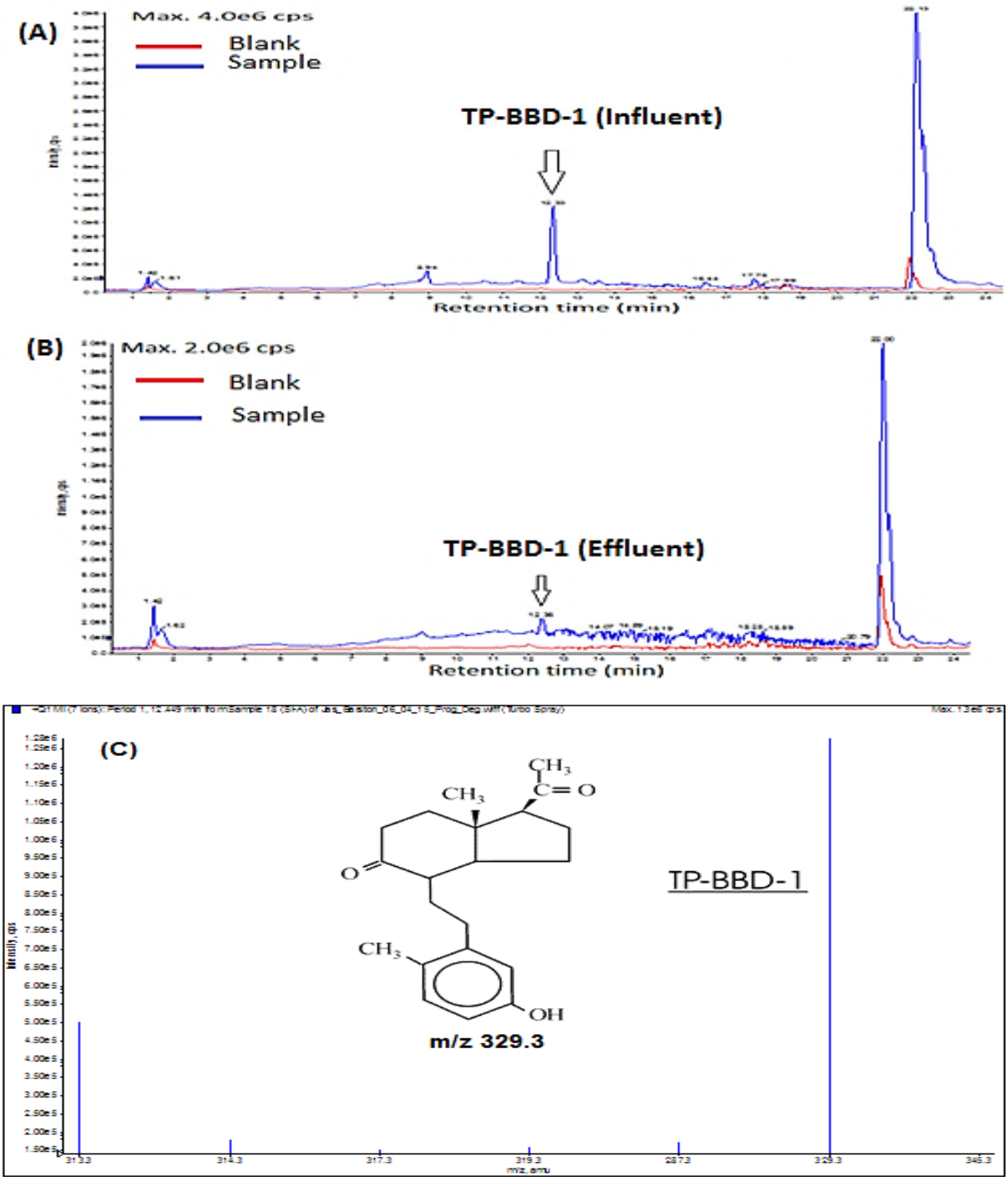


Figure 4.16: Overlaid extracted chromatograms of product TP-BBD-1 in influent (A) and effluent (B) of reed beds and associated mass spectra (C). Red (Blank: MilliQ water). Peak to the right was not considered because a corresponding peak was found in the blank.

4.3.3 Monitoring the Degradation of Progesterone in Laboratory Conditions

Following on from the assessment of samples from the reed beds, where perhaps low concentration was thought to lead to uncertainty in determining the presence or absence of degradation products, it was decided that a laboratory based degradation study in which river water was spiked with progesterone be undertaken (refer to section 3.3 for details of sampling and laboratory degradation set-up). In addition, these studies would be supported with the use of high resolution data from a time-of-flight instrument at Cranfield University.

Conditions within the spiked and control tanks were relatively stable for the entire study period. Of the parameters measured, Dissolved oxygen (DO) was stable at 8.1 to 8.9 mg/L, pH started at 8.7 in each tank, and decreased to 8.2 in the spiked and 8.5 in the control. Decrease in pH possibly due to metabolic activity in the closed degradation system. Temperature in both tanks ranged from 20 to 22°C (Table 4.5). There were no significant differences ($p > 0.05$) between all these parameters in the spiked and control tanks.

Table 4.5: Physical-chemical conditions in degradation tank

Sample	Spiked			Control (Unspiked)		
	Dissolved Oxygen (mg/L)	pH	Temp. (^{0}C)	Dissolved Oxygen (mg/L)	pH	Temp. (^{0}C)
A	8.61	8.72	20.9	8.61	8.72	20.9
0hr	8.60	8.58	20.7	8.69	8.57	19.8
4hrs	8.59	8.56	20.8	8.75	8.51	19.8
8hrs	8.53	8.55	21.3	8.79	8.50	20.0
12hrs	8.63	8.58	20.9	8.80	8.67	19.8
24hrs	8.46	8.46	20.9	8.89	8.48	19.7
48hrs	8.09	8.24	22.0	8.61	8.54	20.8
72hrs	8.01	8.22	22.3	8.57	8.48	21.1

*A: Conditions in degradation tanks prior to spiking

Overall, the outcome of the degradation study was that progesterone degraded rapidly from an initial 1,000 µg/L concentration (Fig. 4.17), with the concentration falling to <1.05 µg/L (method limit of quantification (LOQ)) after 72 h. This highlighted that it was a useful compound to study due to the time period for the reduction of concentration and likely formation of degradation products. Unit resolution results showed the formation of six putative transformation products of the nine target products monitored, as indicated by the relative retention times provided in Table 4.4.

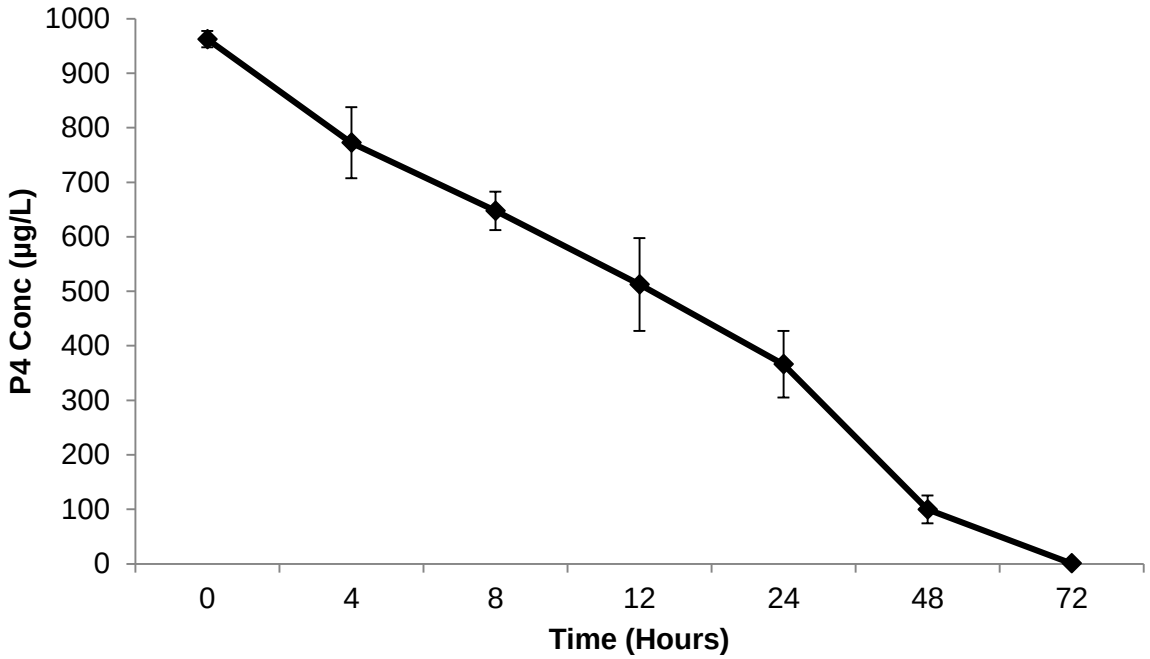


Figure 4.17: Progesterone concentrations with time (Instrument LOD 0.35 µg/L). There was a decrease in P4 concentration (1000 µg/L) with increasing incubation time, with concentration falling to <1.05 µg/L (methods LOQ) by 72 hours (n=3 (three batch of samples).

Unit Resolution Detection of Putative Transformation Products

Under unit resolution monitoring, a number of masses corresponding to suspected TPs were detected as the progesterone concentration declined. TPs were observed as peaks in chromatograms. A typical outcome of this work is shown in Fig 4.18, where a m/z 287.3 corresponding to TP-ALG-4 was observed at both 12.56 and 18.46 minutes. For this example,

the two peaks observed were compared to those found in the control tank (blank). The blank also had a response at RT 12.56 minutes, so the peak at that time point was dismissed as being linked to spiking with P4. The response at RT 18.46 minutes was recorded as no similar response was seen in the blank. In all instances where *m/z* responses linked to possible TPs were identified, target ions eluted before the parent (Table 4.2). In reversed-phase chromatography this is indicative of increasing polarity, which is reflected in the structures shown in Figs. 4.14 and 4.15.

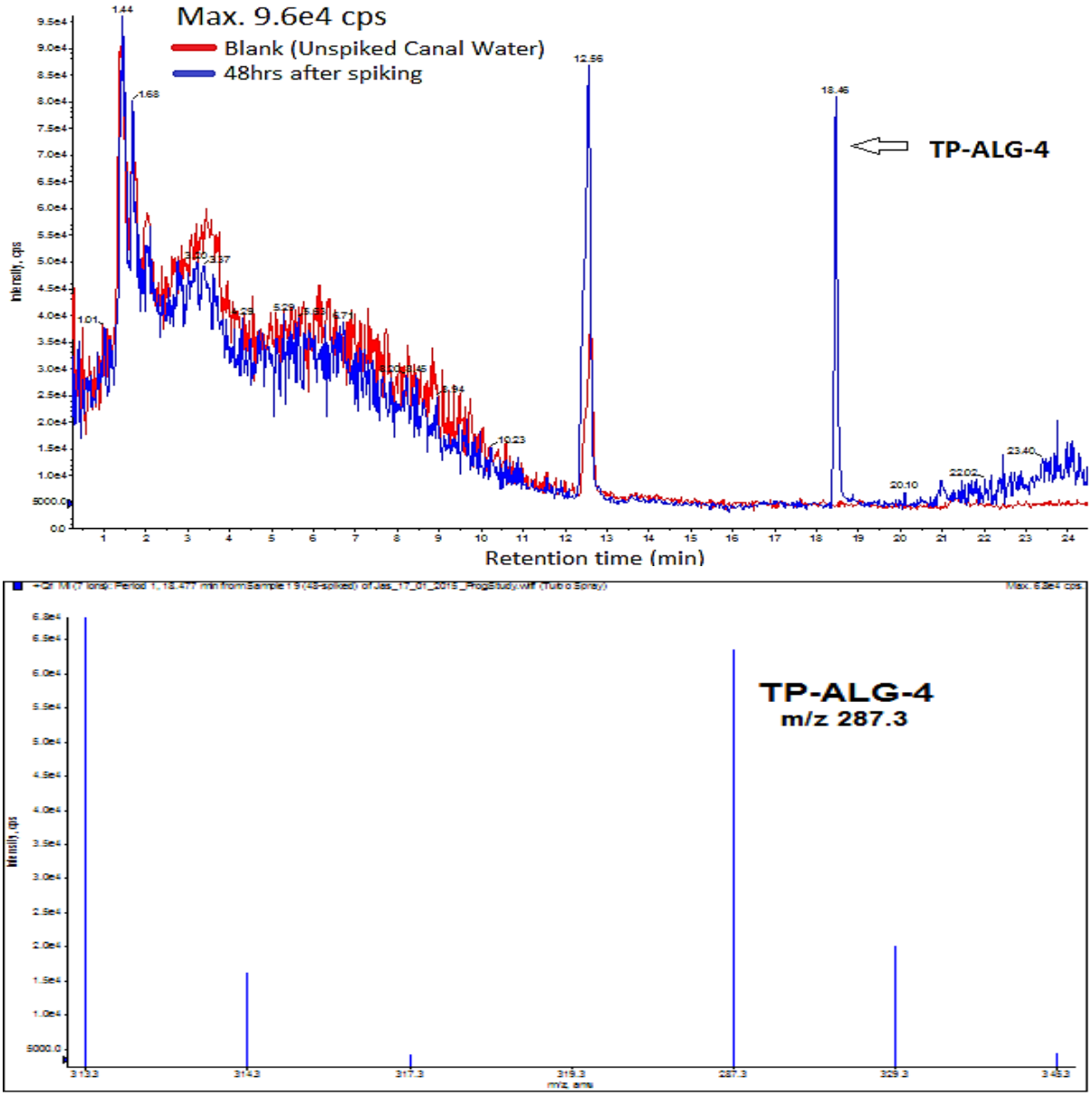


Figure 4.18: Overlaid chromatograms (top) and mass spectra (bottom) showing the formation of TP-ALG-4 by side chain breakdown (-2C and -4H) of the parent progesterone molecule.

The algal metabolic reactions were the dominant pathways observed in the degradation tanks, with fewer occurrences of the bacterial degradation products predicted by the BBD. In order to have a picture of the formation of these TPs with time, their corresponding peak areas were plotted against time (Fig. 4.19). All suspected TPs except TP-ALG-1 were formed four hours into the degradation study and degraded after 8 hours. TP-ALG-4 degraded after 12 hours. However, the formation of TP-ALG-1 peaked at 4 hours and build up to a maximum level in 48 hours and then also rapidly degraded in 72 hours. Of the potential products predicted by the biodegradation database, only TP-BBD-1, predicted as a first level product of progesterone degradation, was detected in the study. The *m/z* associated with this TP was detected only in the 4 h spiked sample (Fig. 4.14). The products predicted by the BBD have all undergone ring cleavage, and TP-BBD-1 eluted earlier than other TPs observed, with a R/RT of 0.58 (Table 4.4). Using the peak area approach, the associated pathways responsible for the formation of TPs is presented in Figure 4.20.

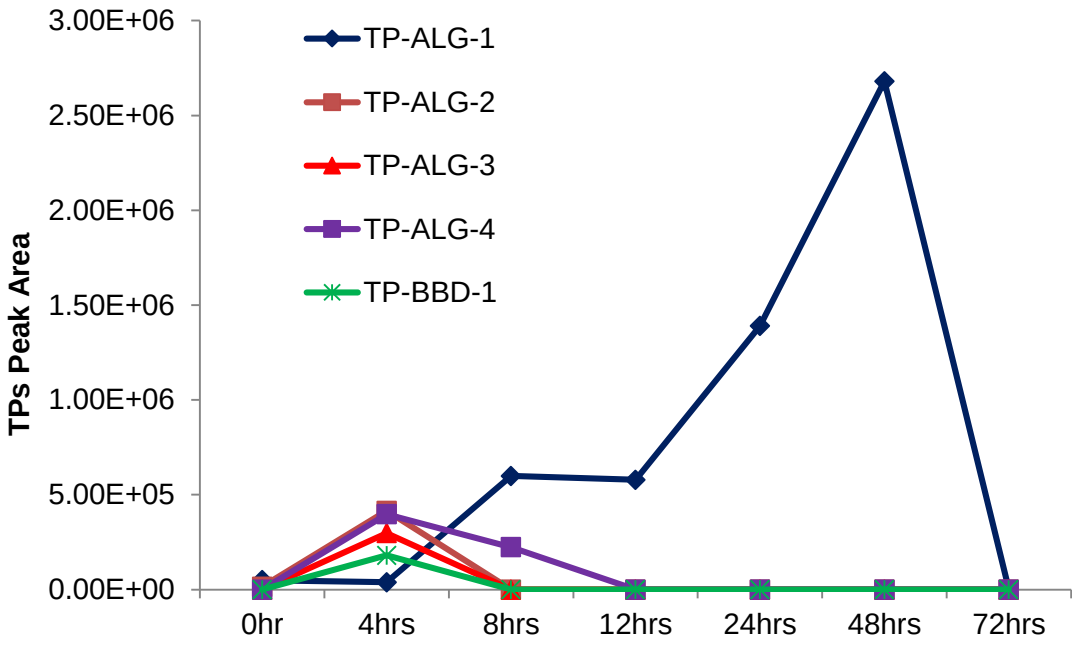


Figure 4.19: Formation of target P4 TPs with time, using peak area as a measure of occurrence

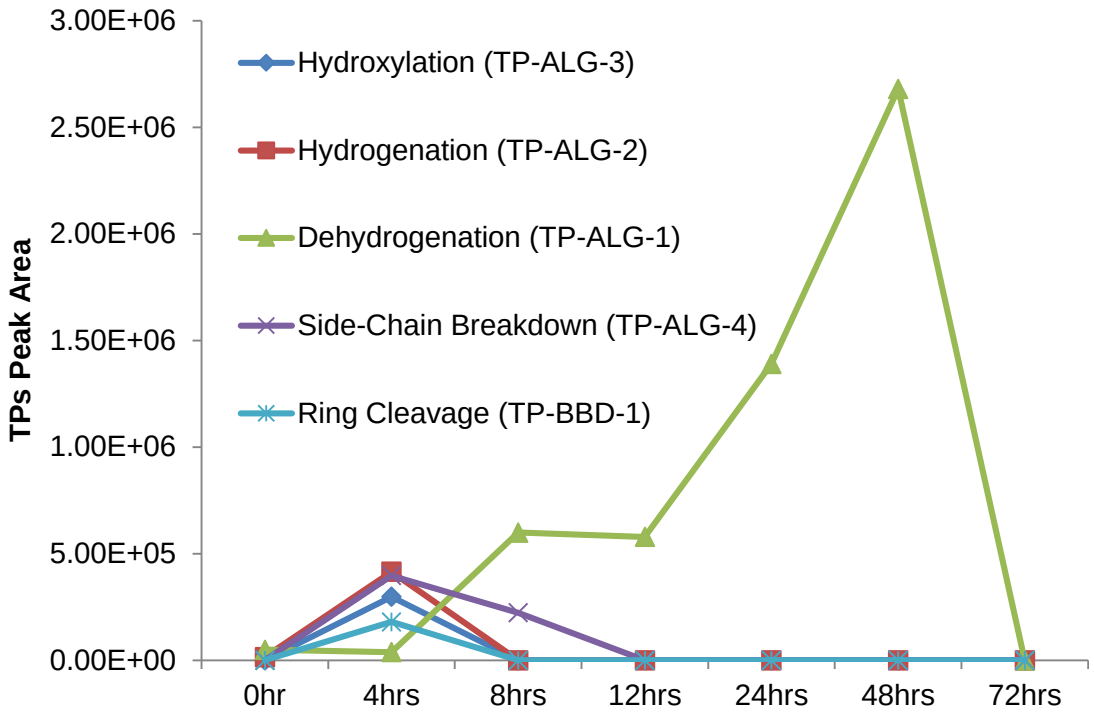


Figure 4.20: Reaction pathways that yielded the transformation products identified in this study. Based on peak area, dehydrogenation was the dominant pathway

High Resolution Detection of Putative Transformation Products

An Agilent Technologies ToF mass spectrometer associated data processing software, MassHunter, was used for processing of data acquired during HRMS run. Steps taking for data processing involved peak picking, involving the comparison of peaks in the sample with control and the exclusion of irrelevant peaks. This was followed by the elimination of noise peaks, mass recalibration and breakdown of isotopes and adducts into well-defined and easily identifiable units. Characteristic fragmentation pattern of TPs relative to P4, was used in the identification of TPs. Fragmentation pattern technique involved searching for specific fragment ions in MS/MS spectra throughout the chromatographic run and applying mass-defect filtering. See section 2.2.1.2 for the reputation and reliability of the process followed. **Note**: The range of other software comparison steps mentioned in section 2.2.1.2 were not followed in this study due to the limited booking time (duration) on the instrument at Cranfield during each run.

Following unit resolution analysis, high resolution time-of-flight results confirmed the presence of two of the products detected in unit resolution with their chemical formulae and accurate masses determined to four decimal places (Table 4.6). A third product seen under unit resolution was also detected in HRMS (TP-ALG-2; mass: 316.2402), however, this was with a lower degree of confidence, and lacked a molecular formula, as the intensity was very low. Detected TPs were seen in the 4, 24 and 48 hours samples. High resolution MS work could not be done for the reed bed study because, further access to site was not possible and previous samples collected were too old (over a year) at the time access to HRMS analysis was possible at Cranfield University.

By applying a mass defect filter (0.2246 $\pm$ 0.05 Da) in the identification of TPs in HRMS, two features were found at *m/z* 313.2170 (TP-ALG-1) and *m/z* 287.1997 (TP-ALG-4). The former corresponded to a mass of 312.2097 (-2.48 ppm error), a double bond equivalent of 8 and proposed molecular formula $C_{21}H_{28}O_2$. The latter resulted in a mass of 287.1997 (0.28 ppm error), double bond equivalent of 7 with $C_{19}H_{26}O_2$ being its molecular formula (see Table 4.6). Collision induced dissociation (CID) fragmentation of the aforementioned features showed similar fragment ions at *m/z* 121.0648 and 121.0643 respectively (Table 4.7 and Fig. 4.21). Fragmentation of both TP-ALG-1 and TP-ALG-4 was believed to have occurred in the A-ring, most probably due to protonation of the ketone group attached to C_3. Subsequent relocation of the positive charge either on carbon 1 or 5 due to resonance resulted in the dissociation of the bond between C_9 and C_{10}. CID experiments for *m/z* 287.1997 helped to identify this feature as boldenone since its two main product ions *m/z* 121.0643 and 135.1161 (Fig. 4.21) have been previously reported elsewhere. Thus, the identity of TP-ALG-4 as confirmed by high resolution mass spectrometry was boldenone and not androstene previously proposed in Fig 4.15.

The greater number of transformation products detected in unit resolution was probably a result of operating the triple quadrupole in selected ion monitoring mode, increasing sensitivity for the specific ions. The high-resolution work was undertaken in non-scanning mode that only

target specific ions for the collision induced fragmentation. However, other, instrument specific factors, such as the ionisation source, may also play a role in determining sensitivity.

Table 4.6: High resolution MS data (Accurate mass measurements)

Targets	Monoisotopic mass	Measured mass	*m/z*	Putative formula	Score (MFG)	DBE	Error
TP-ALG-1	312.2089	312.2097	313.2170	$C_{21}H_{28}O_2$	90.42	8	-2.48
TP-ALG-4	286.1932	286.1932	287.1997	$C_{19}H_{26}O_2$	99.8	7	0.28

* *m/z*: mass-to-charge ratio; MFG: molecular formula generator; DBE: double bond equivalents

Table 4.7: Fragmentation pattern of the putative transformation products

	Precursor Ion		Base Peak			
Targets	*m/z*	Putative formula	*m/z*	Putative formula	Loss mass	Loss formula
TP-ALG-1	313.2173	C_{21} H_{28} O_2	121.0648	C_8 H_9 O	192.1514	C_{13} H_{20} O
TP-ALG-4	287.2001	C_{19} H_{26} O_2	121.0643	C_8 H_9 O	166.1358	C_{11} H_{18} O

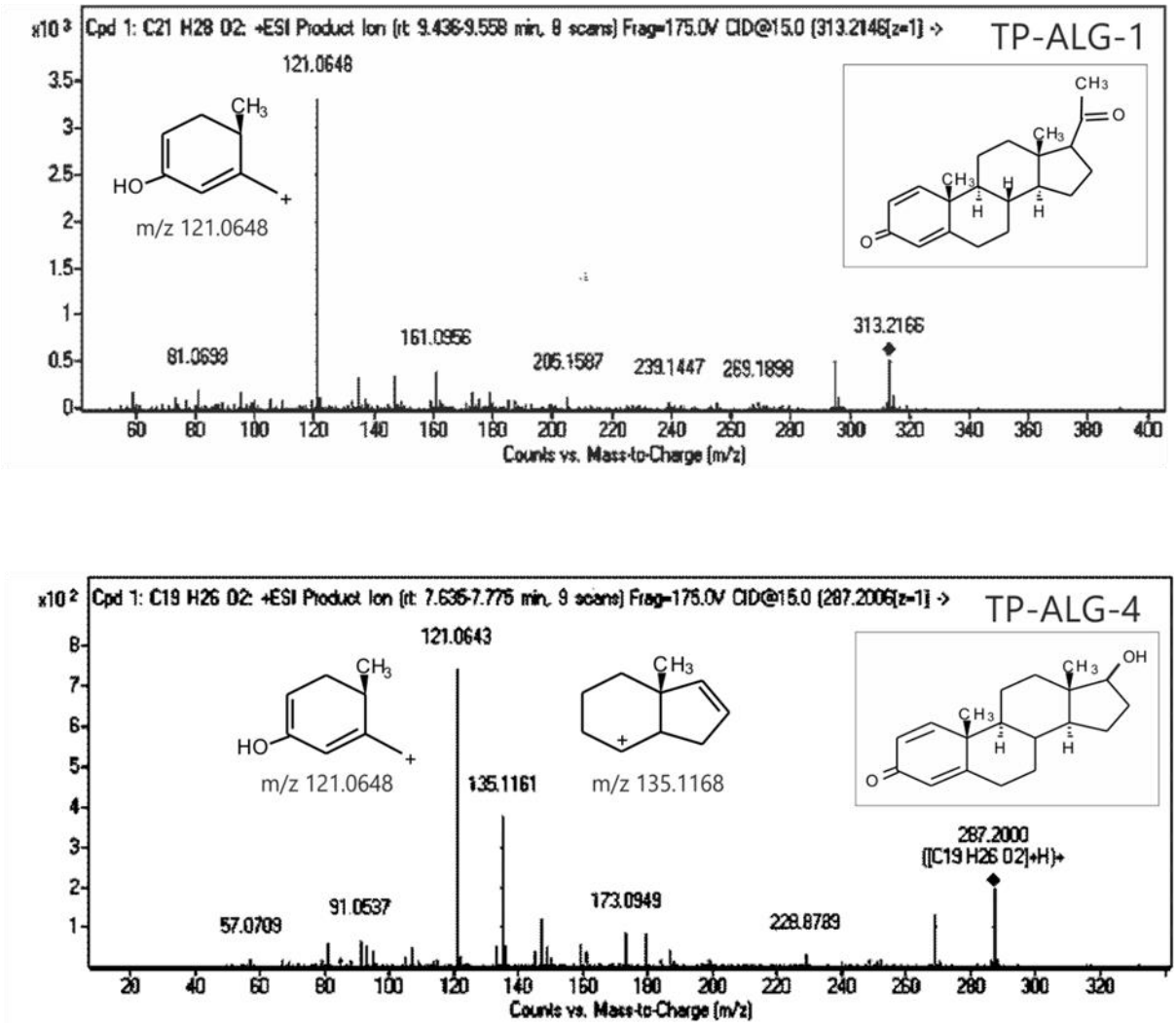


Figure 4.21: ESI-CID mass spectra for putative transformation products TP-ALG-1 (top) and TP-ALG-4 (bottom).

4.3.4 Second Progesterone Degradation Study

Following the initial degradation study, a second study was performed using a water sample collected from the same location as the first study, one kilometre downstream of the effluent discharge point of the Maple Cross STW, and at the same time of the year. The second study was aimed at determining the reproducibility of progesterone degradation and undertaking yeast based biological assays to determine if resultant TPs were biologically active and to establish the nature (progestogenic or androgenic) of their activity. Similar to the first study, conditions within the spiked and control tanks in the second study were relatively stable for

the entire study period. DO was stable at 8.1 to 8.2 mg/L in both tanks for up to 12 h and then dropped to 7.0 to 7.7 mg/L in the spiked tanks. Of the other parameters, pH started at 8.4 in each tank and dropped to 8.0 in the spiked tanks. Temperature in both tanks ranged from 23 to 24^{0}C (Table 4.8). There were no significant differences (p > 0.05) between all these parameters in the spike and control tanks.

Table 4.8: Physical-chemical conditions in degradation tank in second study

Sample	Spiked			Control (Unspiked)		
	Dissolved Oxygen (mg/L)	pH	Temp. (^{0}C)	Dissolved Oxygen (mg/L)	pH	Temp. (^{0}C)
A	8.11	8.38	24.0	8.07	8.42	23.9
0hr	8.16	8.38	23.4	8.14	8.39	23.3
4hrs	8.11	8.36	23.9	8.09	8.38	23.8
8hrs	8.13	8.37	24.0	8.12	8.40	24.0
12hrs	8.11	8.39	24.3	8.12	8.43	24.2
24hrs	7.51	8.23	24.6	8.10	8.44	24.3
48hrs	7.21	8.04	24.3	8.19	8.40	24.1
72hrs	7.35	7.99	24.0	8.22	8.41	24.0

*A: conditions in degradation tanks prior to spiking

The sensitivity of the analytical method (LOD) was determined at each run by calculating the signal-to-noise-ratio of the progesterone peak recorded in the lowest calibration standard. In the present study, the instrument recorded a distinct peak for progesterone in the 0.25 ng/mL standard compared to 1 ng/mL which was the lowest standard in which a peak was recorded in the first study. The overall outcome of the study was that P4 again degraded rapidly from an initial 1,000 µg/L concentration (Fig. 4.22), with the concentration falling to <0.81 µg/L (method LOQ) after 48 h. Comparing only the total time it took for spiked progesterone concentration to be completely degraded in both studies, it would appear P4 degraded faster in the second study relative to the first as nominal concentration fell to <0.81 µg/L just after 48 h (Fig. 4.22).

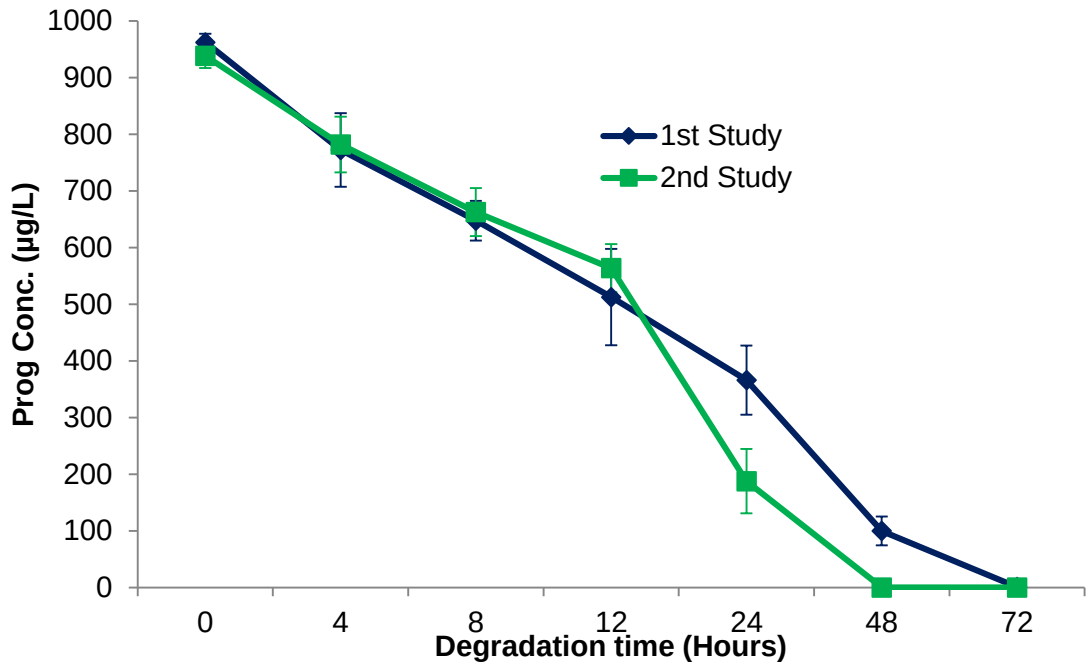


Figure 4.22: Comparison of the rate of degradation for P4. Similar to the first study, P4 concentration decreased with increasing incubation time, with concentration falling below 0.81 µg/L (method LOQ) by 48 hours (Instrument LOD (0.27 ng/ml). Total degradation time was 24 hrs quicker in the second study

Low Resolution Detection of Putative Transformation Products

Similar to the first study results, a number of suspected masses corresponding to TPs were detected under both low-resolution monitoring, as the P4 concentration declined. Prior to the second study mass spectrometry suspect screening, a further review of literature (Peng et al. 2014; Carson et al. 2008; Jenkins et al. 2004) had identified three additional transformation products (testosterone, 5α-dihydroxytestosterone and epi-androsterone) resulting from progesterone degradation, compounds which are also known to exhibit androgenic effects. The molecular weights of 5α-dihydroxytestosterone and epi-androsterone were the same (290 Da). Thus, the list of suspect masses monitored in unit resolution was updated in the second study by the addition of two more masses (289.5 (TP-ALG-5) and 290.5 (TP-ALG-6)) (Table 4.9). Eight putative TPs of the eleven target products monitored in unit resolution were detected, some just 20 minutes after spiking. Peaks corresponding to TP-ALG-5 and TP-ALG-6 in the second study are presented in Figures 4.23 and 4.24 respectively.

Table 4.9: Additional masses monitored under unit resolution

Name	Abbr.	Mol. Wt.	CAS	m/z	RT* (%RSD)	R/RT*
Testosterone	TP-ALG-5	288.5	58-22-0	289.3	20.6 (0.2)	0.79
Dihydroxytestosterone or Epi-androsterone	TP-ALG-6	290.5	521-18-6 or 481-29-8	291.3	15.7 (0.1)	0.61

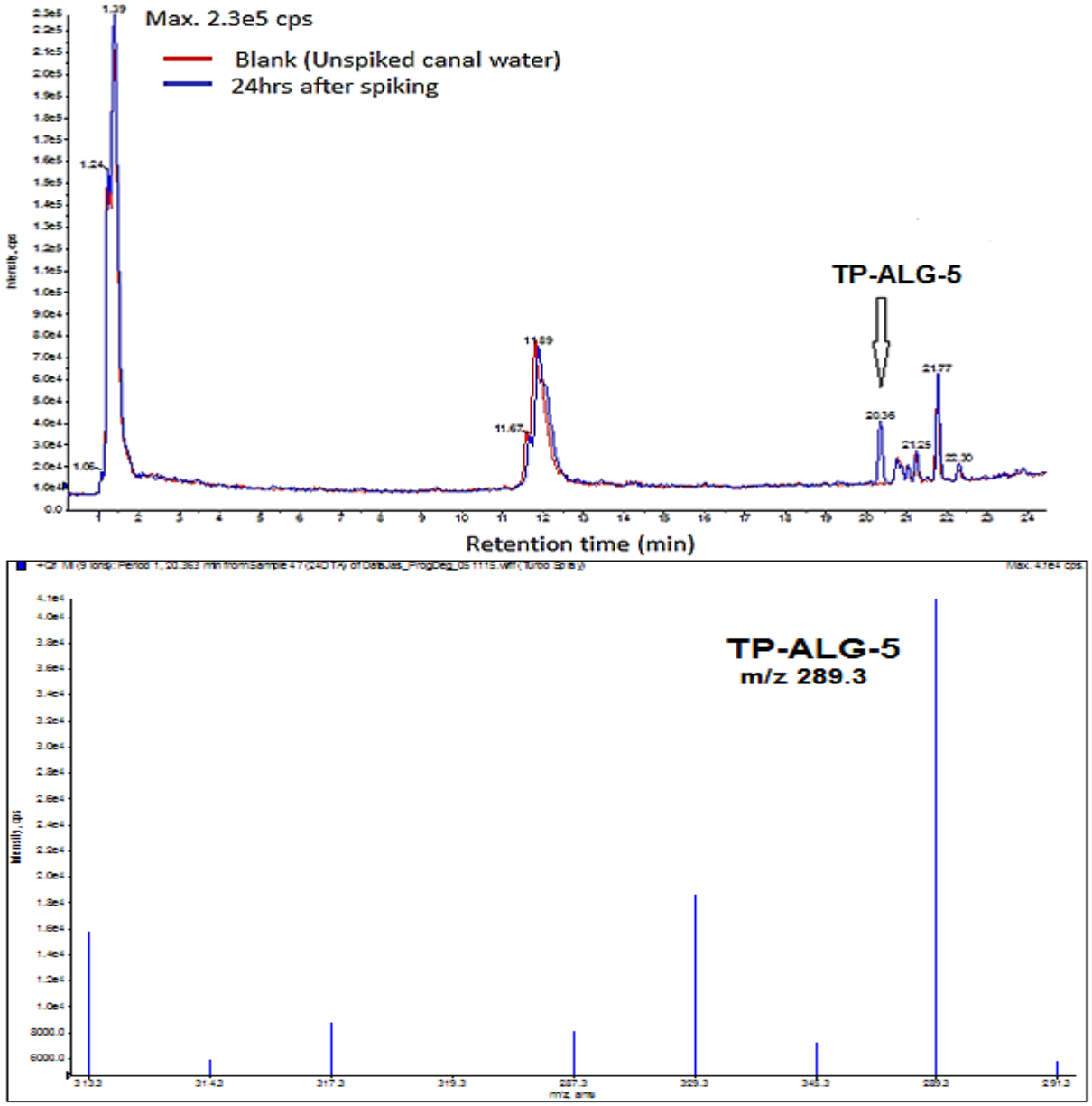


Figure 4.23: Overlaid chromatograms (top) and mass spectra (bottom) showing the formation of TP-ALG-5 formed by a side chain breakdown (-2C and -4H) of the parent progesterone molecule. TP-ALG-5 appeared 4hrs after spiking. Peak height progressively increased in size until the 24th hour.

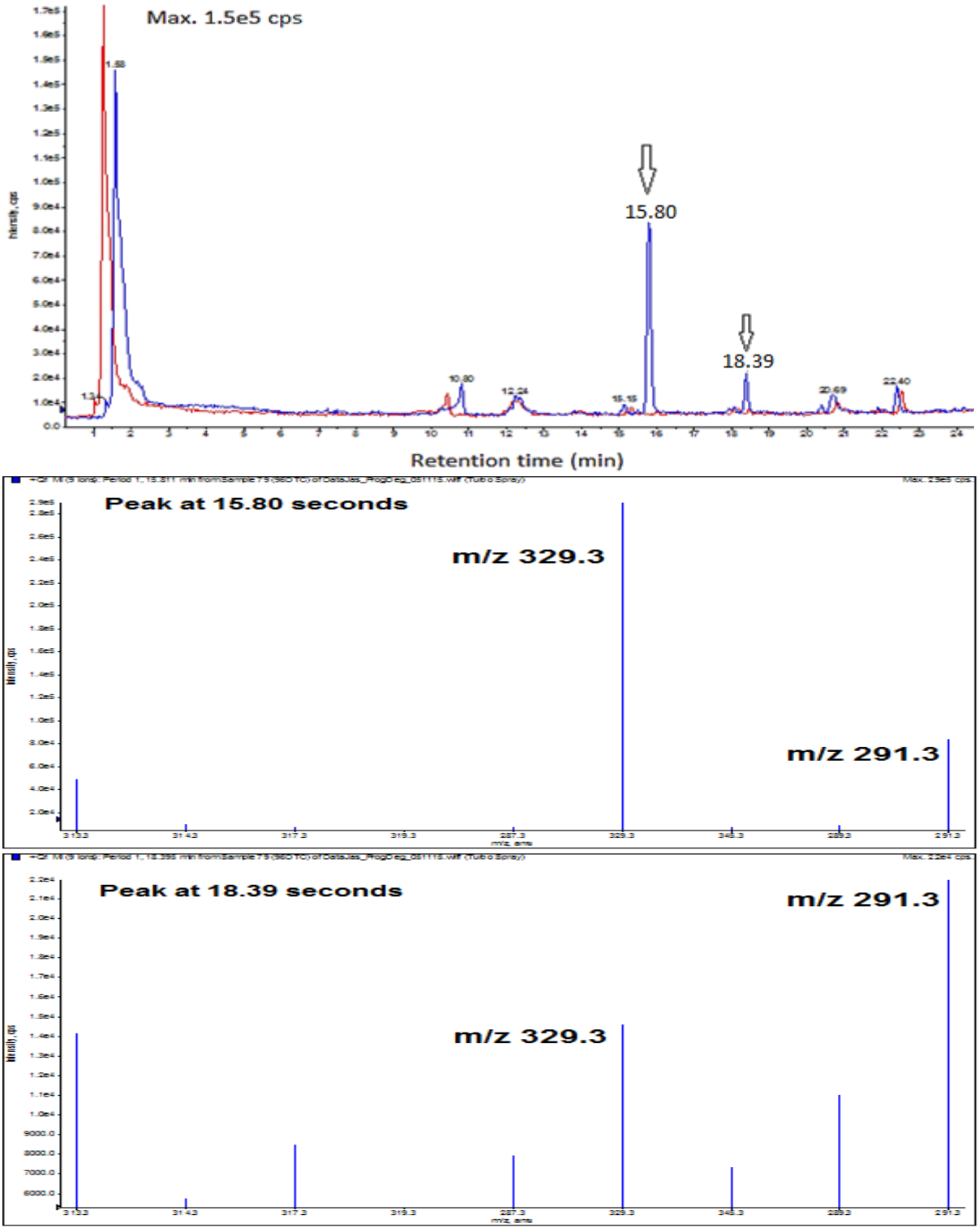


Figure 4.24: Overlaid chromatogram and mass spectra for the two peaks corresponding to TP-ALG-6. Mass for the peak at 15.80 seconds shows it is most likely m/z 329.3 (TP-BBD-1) as it recorded the highest response relative to m/z 291.3. Conversely, the peak at 18.39 seconds is most probable peak for TP-ALG-6 (m/z 291.3 had the highest response.

High Resolution Detection of Putative Transformation Products

High resolution mass spectrometry confirmed the presence of four of the products detected in unit resolution. These products were seen only in the 24 h samples, indicating that they degraded afterwards. Two of the products, TP-ALG-1 and TP-ALG-4 were present in the initial degradation study. However, two new products (TP-ALG-2 and TP-ALG-5) not found in the first study were confirmed to be present in the second study. The chemical formulae of these new products and their accurate mass determined to four decimal places are presented in Table 4.10. CID fragmentation of these new products was attempted, but concentrations were too low to work with.

Table 4.10: Second degradation study high resolution MS data (Accurate mass measurements)

Targets	Monoisotopic mass	Measured mass	*m/z*	Putative formula	Score (MFG)	Error
TP-ALG-2	316.2387	316.2402	317.2466	$C_{21}H_{32}O_2$	75.7	-4.61
TP-ALG-5	288.2088	288.2089	289.2160	$C_{19}H_{28}O_2$	97.9	0.28

4.3.4.1 Linking the Degradation of Progesterone to Biological Activity

It was deemed important to assess not only chemical degradation, but also evaluate any possible biological activity associated with degradation products. This was particularly important as putative products such as testosterone, may exhibit activity and cause effects in the environment. Sampling for ecotoxicological work was done by measuring 5 mL of sample from the spiked and non-spiked tanks followed by the addition of 5 mL of ethanol, representing a 50:50 dilution. Volume of sample taken was such that there was enough sample stored at -80^{0}C for multiple confirmatory yeast screen. Sample dilution with ethanol was done following established protocol for sample fixing. Three replicate samples were taken at each sampling time.

4.3.4.1.1 Progesterone Screen

For the yeast screen, all calculations are done relative to a standard curve (P4 curve in Figure 4.19 (now Figure 4.25)). The P4 concentration used for the screen ranged from <0.1 to 78 µg/L. Concentration of P4 in test tanks at different sampling times relative to P4 equivalent concentrations are shown in Figure 4.25. The analysis of samples using the yeast screen supported chemical analysis results. The P4 concentration decreased with increasing degradation time. Figure 4.25 shows a decrease in spiked P4 potency with time. No activity was recorded in the control tanks. No progestogenic activity was noticed in spiked tanks after 24 h of incubation. After 24 h there was a reduction in progestogenic activity compared with P4 standard curve (no dose response was seen in the other times (curves flatten out). This is most probably because residual P4 concentrations if any in samples beyond 24 h were not high enough to elicit a dose-response curve and/or that degradation products formed did not retain the parent compound's biological activity. Chemical analysis results indicated that residual P4 concentrations in spiked tanks were <0.81 µg/L at 48 h, lower than the minimum concentration (3.15 µg/L LOD and literature reported P4 concentration below which no activity is seen. See section 2.6.1)) needed to activate the progesterone receptor in the assay. Absorbance correction is a standard literature supported protocol performed to eliminate assay associated reagents' absorbance to focus on absorbance from dead cells.

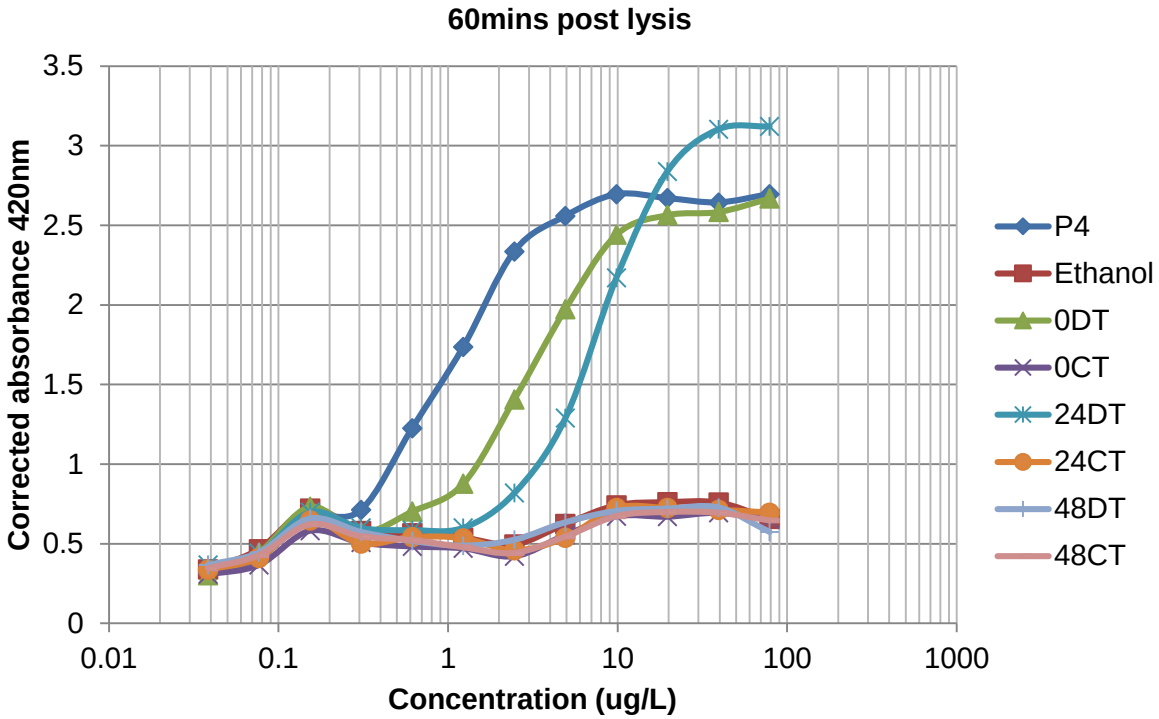


Figure 4.25: Yeast assay for P4 receptor activity. Potency of spiked P4 decreased with time until the 24 h, after which concentrations were too low to elicit a dose-response curve. (DT: Degradation Tank; CT: Control Tank)

4.3.4.1.2 Androgen Screen

The androgen screen response curve for the positive control dihydrotestosterone (DHT) relative to other degradation time points is shown in Figure 4.26. Absorbance was plotted against the equivalent concentration of DHT in assay wells to give an indication of the relative concentration of androgenic TPs. Androgenic response for the positive control was observed to start at a low concentration of 0.45 ng/L DHT, showing the high sensitivity of the assay. The responses observed at 0 h and 24 h, are similar to those seen in the progesterone screen and probably do not represent androgenic activity, but cross reactivity of progesterone with the androgen receptor, a condition reported in the literature. Reported in vivo experiment shows that progesterone can interact with other steroidal nuclear receptors especially the androgen receptor. Reported control for the observed cross reactivity is to run the screen on a test sample solely spiked with a given androgen. This is however, not possible in the present study as the main intent is to observe spiked progesterone degrading into androgens. The

implication therefore is, P4 will always be present in test tanks until it is degraded below the minimum concentration needed to activate the androgen receptor. No activity was recorded in the control tanks.

No similar or close response was seen in the samples for other time point as P4 degraded. These results show that as P4 degraded, resultant androgenic TPs concentration were below 0.03 µg/L, the minimum concentration of androgenic compounds needed to activate the androgen receptor. If 20% of progesterone were to degrade into an androgen over 48 hours, this would be equivalent to 200 µg/L of that androgen in the present study considering that tanks were spiked at relatively high concentration of P4 (1,000 µg/L). Such concentration would have been high enough to cause an observable response in the 48 hours samples. But this was not the case. Samples used for the present assay were in aqueous phase compared to the conventional use of concentrated samples. As noted earlier, samples were not concentrated up because of the uncertainty involved if a given SPE method is applied in the recovery of TPs whose chemical identity and properties are unknown. The use of these aqueous solutions is relevant considering that any androgen occurring in the environment as a result of progesterone degradation will exist in a similar phase. Thus, it is reasonable to conclude in the light of the present results that though P4 degrades to androgen as shown by chemical analysis, the concentrations in the environment are unlikely to represent any immediate threat to aquatic resources.

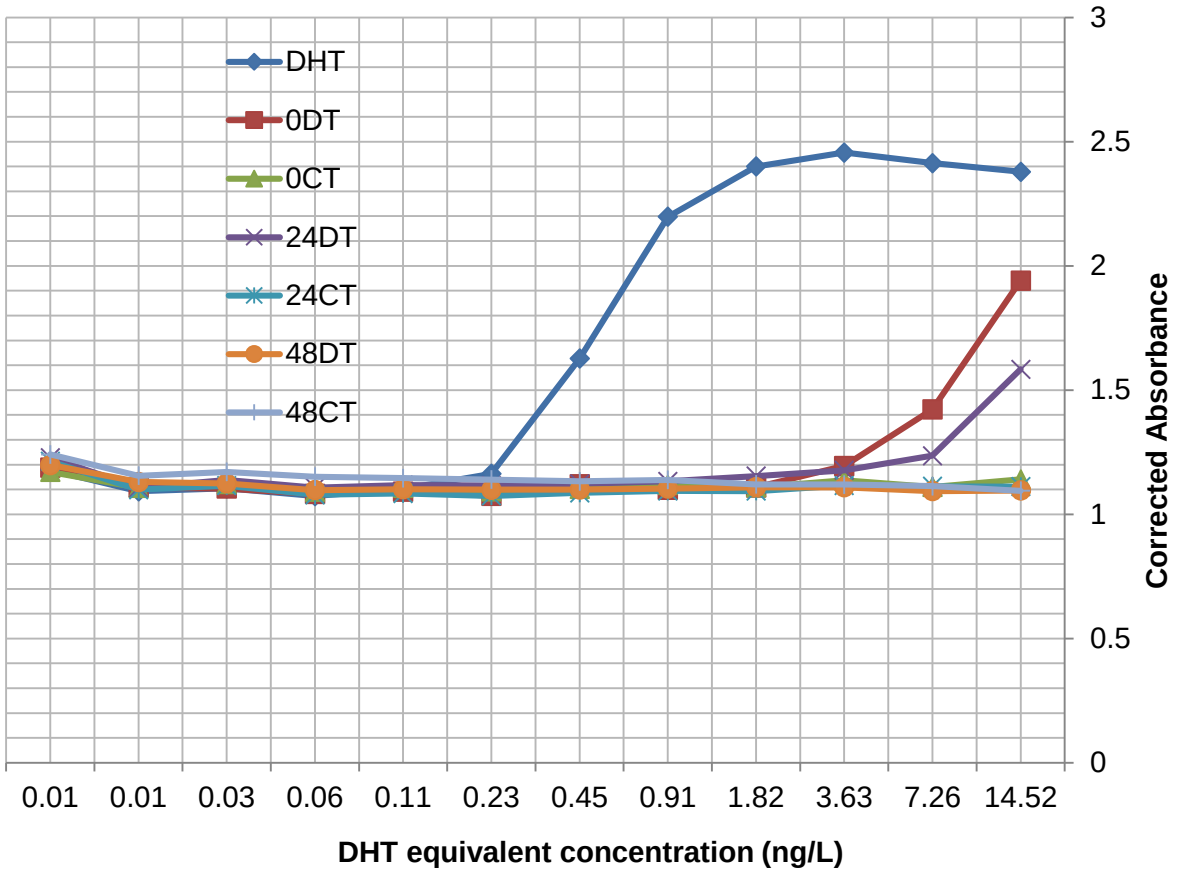


Figure 4.26: Androgen screen response curve. Response seen at 0 h (15 minutes after spiking) and 24 h probably does not represent androgenic activity, but cross-reactivity of P4 with the androgen receptor. High concentration of P4 is reported in the literature to activate the androgen receptor.

4.3.5 Repeatability of P4 Degradation across multiple sites

Following the second degradation study that confirmed the reproducibility of progesterone degradation in the Maple Cross location, a third study was undertaken. As noted in section 3.3.2, this third study was designed to observe and compare P4 degradation across multiple sites. Sampling time and conditions were kept the same to minimize temporal variability in conditions and allow for effective comparison of results.

The conditions within the control and degradation tanks for all sites were stable for the entire study period. Temperature was stable from 22 to 24^{0}C and pH at 8.1 to 8.8 (Table 4.11). Overall, dissolved oxygen (DO) was stable at 8.1 to 8.3 mg/L in all control tanks and in the

High Wycombe degradation tanks. However, DO dropped at 24 hours to 7.1 and 7.8 in the other sites and remained below 8.0 mg/L for the remaining study period. There were no significant differences ($p > 0.05$) between all these parameters in the spike and control tanks.

Table 4.11: Physical-chemical conditions in degradation tanks across sites

Sample	Degradation Tanks (DT) (Spiked)									Control Tanks (CT) (Non-Spiked)								
	High Wycombe			Chesham			Blackbirds			High Wycombe			Chesham			Blackbirds		
Time	DO (mg/L)	pH	Temp. (^{0}C)	DO (mg/L)	pH	Temp. (^{0}C)	DO (mg/L)	pH	Temp. (^{0}C)	DO (mg/L)	pH	Temp. (^{0}C)	DO (mg/L)	pH	Temp. (^{0}C)	DO (mg/L)	pH	Temp. (^{0}C)
A	8.2	8.4	23.4	8.1	8.8	24.1	8.1	8.5	24.0	8.2	9.7	23.6	8.1	8.7	23.7	8.0	8.5	24.2
0	8.1	8.5	23.0	8.0	8.7	24.7	8.0	8.4	23.6	8.2	8.7	23.3	8.1	8.8	23.4	8.0	8.5	24.0
4	8.1	8.4	23.1	8.1	8.8	24.8	8.1	8.5	23.4	8.2	8.8	23.4	8.1	8.7	23.5	8.1	8.6	24.0
8	8.2	8.4	22.5	8.1	8.8	23.3	8.1	8.5	23.2	8.3	8.7	22.7	8.3	8.8	22.9	8.2	8.6	23.0
12	8.3	8.3	22.0	8.2	8.8	22.6	8.1	8.6	22.5	8.3	8.5	22.6	8.3	8.8	22.5	8.2	8.6	23.0
24	8.3	8.3	22.6	7.1	8.4	23.4	7.9	8.3	23.2	8.3	8.4	23.2	8.3	8.7	23.0	8.2	8.5	23.4
48	8.4	8.4	21.6	7.5	8.1	22.8	8.1	8.3	22.4	8.4	8.1	22.4	8.4	8.8	22.4	8.3	8.6	22.7
72	8.3	8.4	21.9	7.6	8.2	22.7	8.1	8.3	22.5	8.5	8.3	22.3	8.5	8.8	22.3	8.3	8.7	22.8

*A: Starting conditions in tanks prior to spiking

Overall, spiked progesterone concentrations (1,000 µg/L nominal) degraded rapidly such that concentrations at the Chesham and Blackbirds sites were below instrument limit of quantification (0.96 µg/L) (Fig 4.27) after 48 hours of degradation. As noted in section 4.3.4, the sensitivity of the analytical method (LOD) was determined at each run by calculating the signal-to-noise-ratio of the progesterone peak recorded in the lowest calibration standard. However, approximately 100 µg/L of the spiked P4 concentration was left after 72 hours of degradation in the High Wycombe site, representing 90% breakdown of the nominal progesterone concentration. Measured starting concentrations were below (Fig 4.27) nominal concentration (1,000 µg/L) in all sites in this study relative to the previous studies. This observation may be because volume of P4 used for spiking in the third study or solvent volumes measured when preparing calibration standard, were not exactly right. However, the degradation trend was the same for all studies. The High Wycombe location was the only location in all the degradation studies that had residuals of P4 detected after 72 hours of degradation. This observation can be attributed to two possible factors, namely; variation in microbial community in the High Wycombe site relative to the other sites or that existing similar microbial population in this site were less metabolically active relative to the Maple Cross, Chesham and Blackbirds sites. Progesterone was degraded below analytical method limit of quantification in four (Blackbirds, Chesham, Maple Cross 1 and 2) out of the five studies.

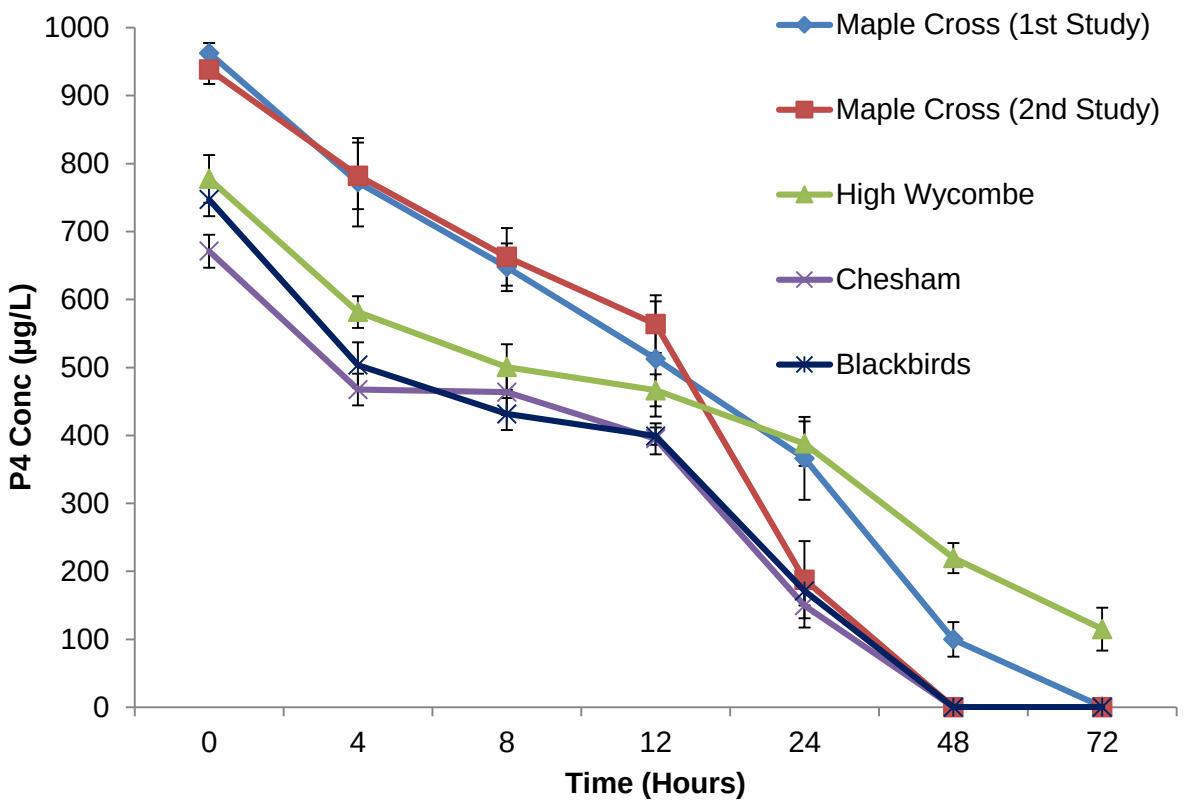


Figure 4.27: Comparison of P4 rate of degradation across degradation sites. Like the first and second studies, P4 nominal concentration (1000 µg/L) decreased with increasing incubation time, with concentration falling below 0.96 µg/L (methods LOQ) in 48th hour at all sites except in High Wycombe where remnant (approx. 100 µg/L) of P4 were detected at 72nd Hour of degradation (Instrument LOD (0.32 µg/L)).

Low Resolution Detection of Putative Transformation Products

Like the first and second studies, peaks corresponding to monitored suspected masses, were detected as progesterone concentration decreases. Masses detected and their corresponding transformation products codes are illustrated with m/z 313 (TP-ALG-1) (Fig. 4.28) and 287 (TP-ALG-4) (Fig. 4.29). Detected transformation products were similar to those reported in the two previous studies.

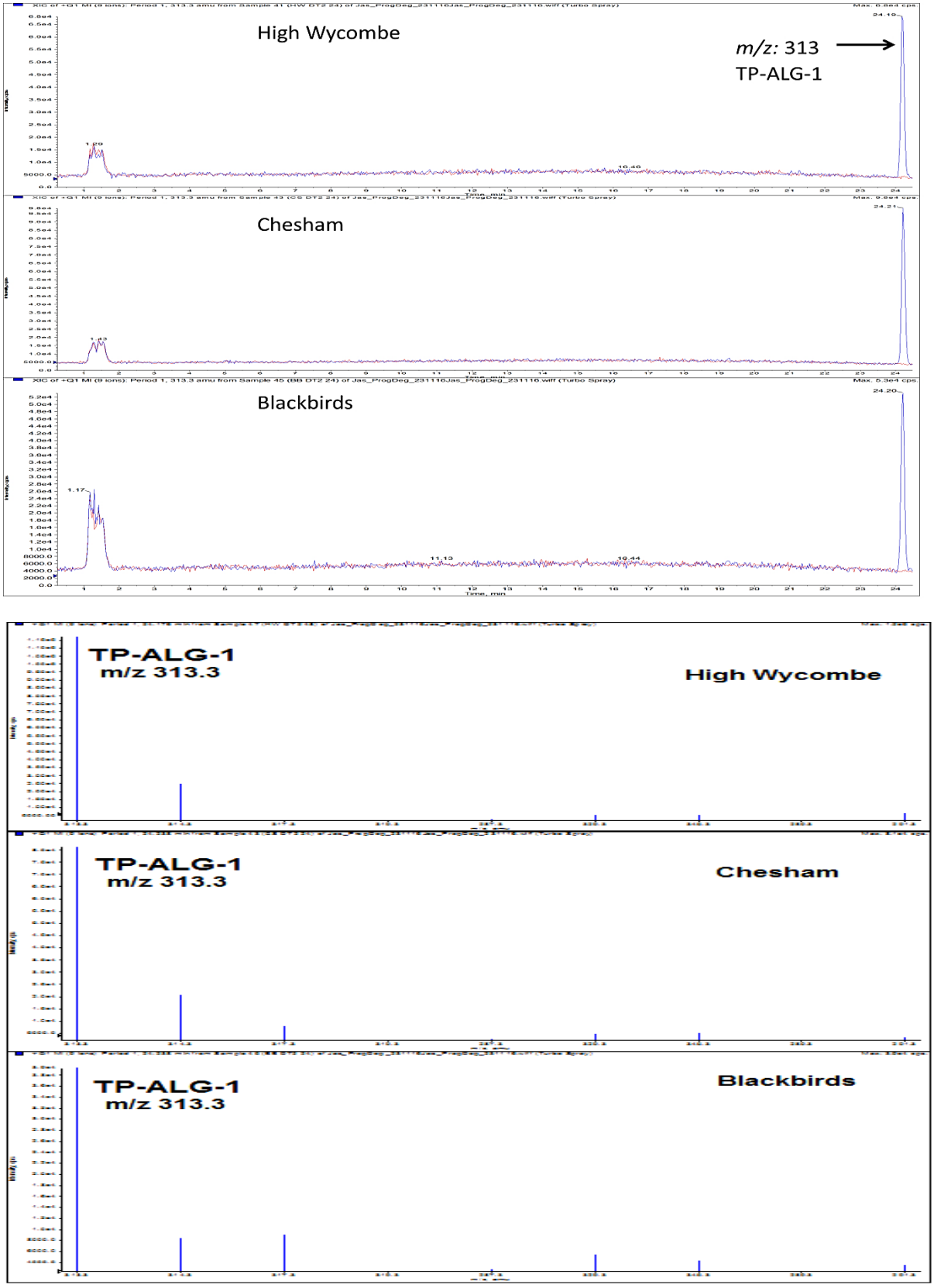


Figure 4.28: Overlaid chromatogram (top) and mass spectra (bottom) showing detected peak corresponding to m/z 313 across sites under low resolution.

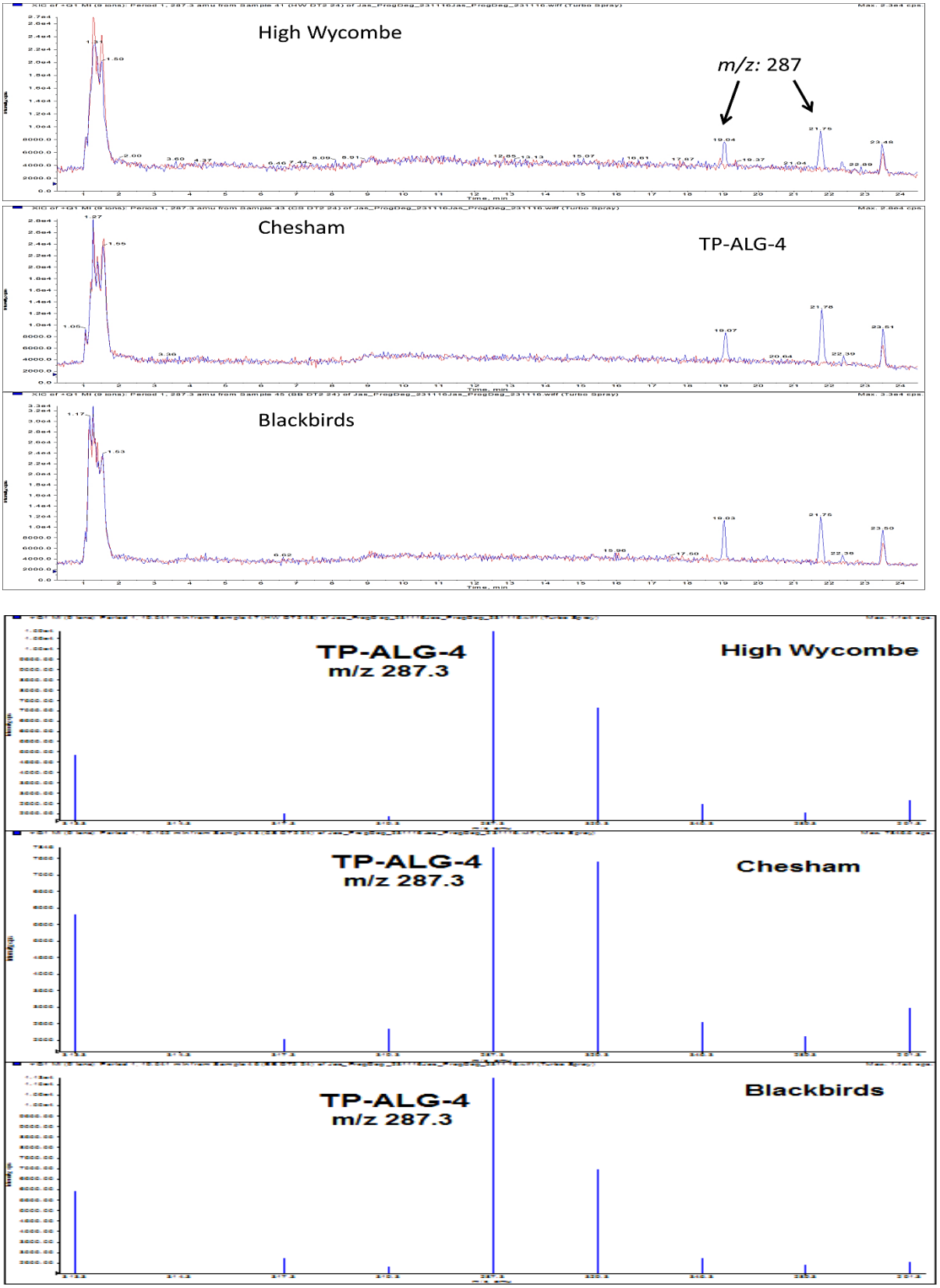


Figure 4.29: Overlaid chromatogram (top) and mass spectra (bottom) showing detected peak corresponding to m/z 287 across sites under low resolution. Mass spectra for peaks highlighted in chromatogram for all sites were like the ones presented here.

High Resolution Detection of Putative Transformation Products

High resolution mass spectrometry confirmed the presence of three of the products (TP-ALG-1, TP-ALG-4 and TP-ALG-5) detected in unit resolution and one of the ones predicted by the biodegradation database, m/z 329 (TP-BBD-1). TP-BBD-1 was found at *m/z* 329.2115 which corresponded to a mass of 328.2038 (0.72 ppm error) and proposed molecular formula $C_{21}H_{28}O_3$. Product TP-ALG-4 was present in samples from all study sites including Maple Cross (Table 4.12). While this product was degraded after 48 h in samples from Maple Cross, Chesham and Blackbirds, it was still seen at 72 hours in samples from the High Wycombe site. Product TP-ALG-5 was also detected at 72 h in the High Wycombe site and not in the others. Product TP-BBD-1 was detected only in the High Wycombe, Chesham and Blackbirds sites and not in Maple Cross. This product was also seen at the 72 h in the High Wycombe samples relative to the other sites. TP-BDD-1 corresponded to a molecular mass of 328.2038 (monoisotopic mass was 324.2038), a 2.32 ppm error and a proposed molecular formula of $C_{21}H_{28}O_3$, which matches with the database predicted formula for this product. Two of the products, TP-ALG-1 and TP-ALG-4 were present in the initial degradation study.

Table 4.12: Summary of High Resolution Data for Detected TPs at all Sites

TPs	Locations				
	Maple Lodge		High Wycombe	Chesham	Blackbirds
	1st Study	2nd Study			
TP-ALG-1	+	+	+	+	+
TP-ALG-2		+			
TP-ALG-3					
TP-ALG-4	+	+	+	+	+
TP-ALG-5		+	+		
TP-ALG-6					
TP-BBD-1			+	+	+
TP-BBD-2					

*+: Present in samples from that site

4.4 The Half-life ($t_{1/2}$) and Kinetic Rate Constant of Progesterone Degradation Across Sites

The interest in half-life and kinetic rate constant was to further understand the empirical variation in degradation rate seen in Figure 4.27 and to compare results of the present study with those reported in the literature. Such comparison is aimed at putting the results obtained in perspective and of establishing the reliability of degradation approach adopted in this study. A review of literature shows that no study till date had investigated P4 degradation in surface waters. Thus, a comparison of kinetic rate and half-life reported for batch experiment with the present study result, will fill a gap in knowledge.

The degradation of P4 followed the first-order reaction model (Figure 4.30a and b). Half-life and kinetic rate constant were calculated using the relation presented in equations 3 and 4 (section 3.3.2). The kinetic parameters including the kinetic rate constant (k) and the half-life ($t_{1/2}$) are given in Table 4.13. Degradation rate was faster in the Chesham (0.1024 h) and Blackbirds (0.1025 h) locations relative to the other two (Maple Cross and High Wycombe). This is also reflected in their similar half-life of 6.77 h and 6.76 h respectively. This observation may be due to similarity in microbial community and metabolic rate in the two locations. The second study at the Maple Cross was had a higher degradation rate (0.1106 h) relative to the first (0.0459 h) even though sampling was done at the same location and at the same season. Perhaps, the microbial community were more diverse or if they were the same as the first, they are more active than before. Progesterone degraded least (0.0236 h) in the High Wycombe location with a half-life of 29.39 h (Table 4.13).

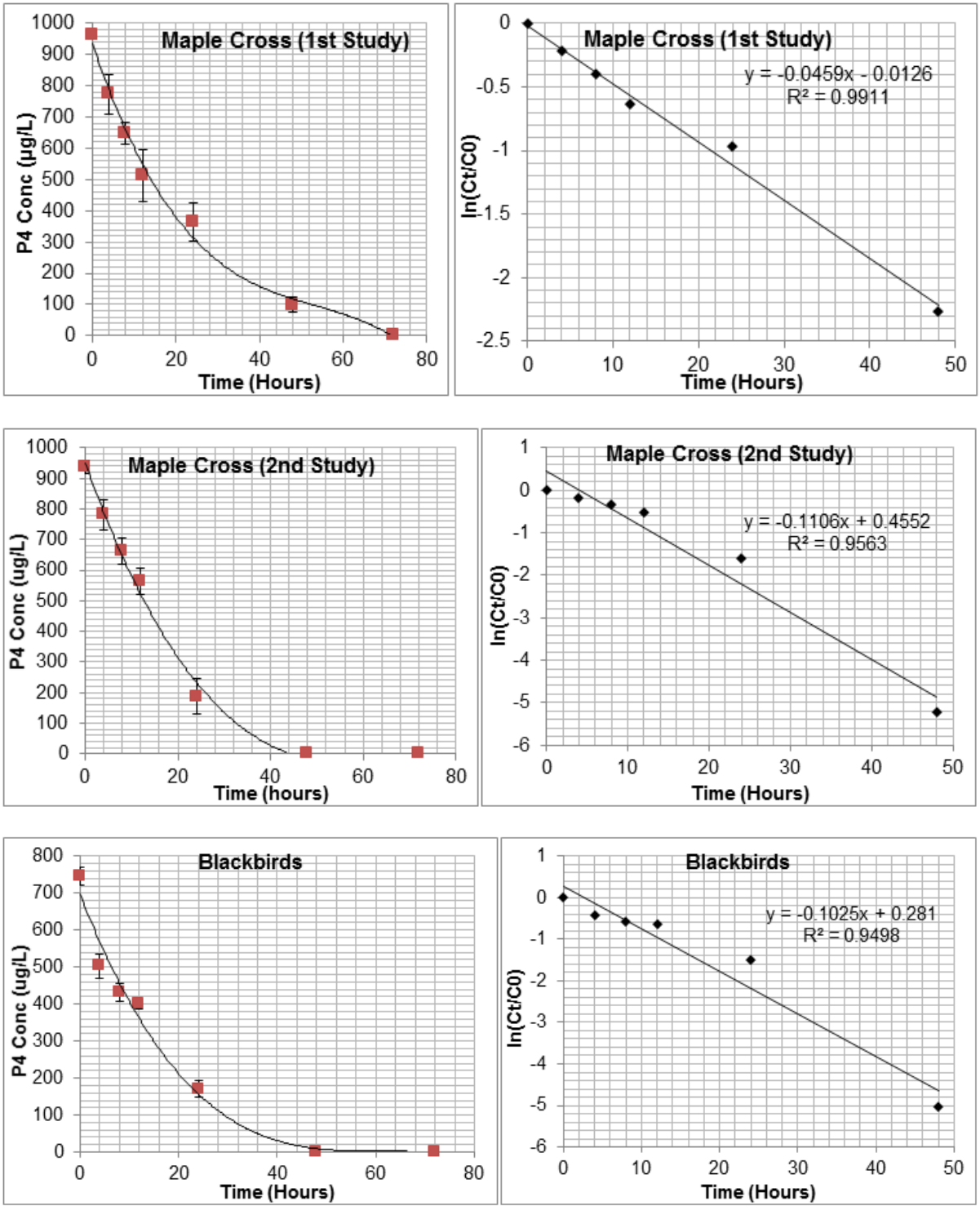


Figure: 4.30a: Comparison of progesterone degradation kinetic rate constant and half-life across different sites. Plots to the left represent first order degradation curve and the one to the right, rate constant plot. Progesterone degradation rate and half-life were similar for the Chesham and Blackbirds sites. Degradation rate was least in the High Wycombe and half-life was longer (29.39 h).

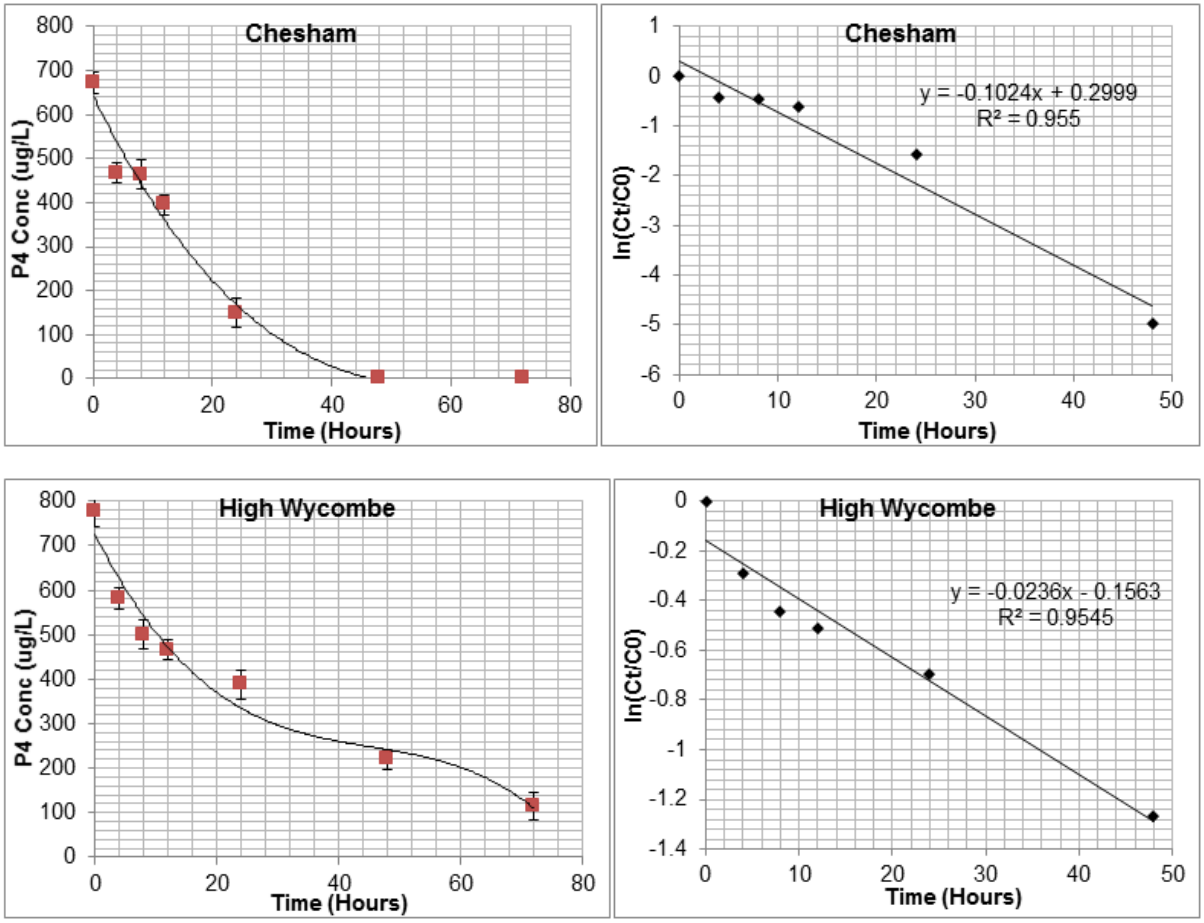


Figure: 4.30b: Comparison of progesterone degradation kinetic rate constant and half-life across different sites. Plots to the left represent first order degradation curve and the one to the right, rate constant plot. Progesterone degradation rate and half-life were similar for the Chesham and Blackbirds sites. Degradation rate was least in the High Wycombe and half-life was longer (29.39 h).

Table 4.13: Kinetic Parameters and Half-life of Progesterone Degradation

Location	Rate constant (k)(h)	R^2	Half-life ($t_{1/2}$) (h)
Maple Cross 1	0.0459	0.99	15.11
Maple Cross 2	0.1106	0.95	6.27
Chesham	0.1024	0.95	6.77
Blackbirds	0.1025	0.95	6.76
High Wycombe	0.0236	0.95	29.39

*h: Hours; k: Kinetic rate constant, calculated using the first-order reaction kinetic model ($C = C_0 * \exp(-kt)$); R^2: Correlation coefficient, which represents the fitness of the data; Half-life, calculated as (ln 2)/k.

Chapter Five: General Discussion

5.0 General Discussion

5.1 Analytical Methods

5.1.1 Analysis of Model Compounds and Suspected Transformation Products in Reed Bed

As discussed in section 3.2 and 4.3.2, a relatively simple multi-residue analytical method involving an SPE extraction step, was developed and applied briefly to detect model compounds and suspected TPs in influent and effluent from reed beds. The determination of the presence or absence of TPs in samples, involved the extraction of the chromatogram for a given product in a sample and overlaying it with the corresponding extracted chromatogram in the control (blank: double deionised water). All peaks found in samples that were also present in the control, were excluded. Response had to occur only in the spiked sample for peaks to be considered as possible degradation products. The quantitation of transformation products was not undertaken. Suspected TPs of P4 were the sole focus in reed bed sample analysis owing to progesterone rapid degradability relative to the other model compounds (see section 3.2.2). It was thought that, to validate developed SPE method in the recovery of degradation products, a compound that was known to readily degrade in the environment was ideal as its associated TPs were expected to be present in reed bed samples.

Multi-residue methods involving SPE procedure are popular in analytical chemistry especially for parent compound, and involve the application of a single analytical method for the determination of a range of compounds in aqueous environmental samples. For example, 81 pharmaceuticals covering several therapeutic classes were determined in surface water by the application of a multi-residue analytical method that was based on automated off-line SPE and followed by high performance liquid chromatography coupled to a quadrupole linear ion trap mass spectrometer (Gros et al., 2012). As noted in section 2.2.1, some workers have applied extraction procedures in the analysis of TPs in environmental matrices, but mostly in target screening were the identity of TPs are known and associated standards are available.

For example, a sample extraction step was used in the target analysis of metabolites of the analgesic dipyrone in effluent samples to avoid the assignment of erroneous identifications to detected TPs (Ibáñez et al. 2012). However, because most TPs are unknown, analytical standards are largely unavailable and the understanding of their binding properties to sorbent materials is limited, extraction procedures that have been applied by some researchers in transformation studies, are usually not complicated, but generic and robust involving mostly the use of Oasis HLB (Pico et al. 2015; Al-Odaini et al. 2010; Kasprzyk-Hordern et al. 2007; 2009a, b). See section 2.2.1.1 for reasons for the preferred use of Oasis HLB relative to other sorbents. A major drawback highlighted in the literature for the application of extraction methods to suspect and non-target screen, is the potential for loss of TPs due to breakthrough, because of the absence of analytical standards for most TPs and the lack of knowledge on their binding properties (Pico et al. 2015; Krauss et al. 2010; Kern et al. 2009).

As noted in section 4.3.2, only one peak corresponding to the biodegradation database predicted ring cleaved product, TP-BBD-1, was detected. None of the commonly reported products relating to side-chain modification of the P4 structure, were detected (see Table 2.1). Because, literature recommended hydrophilic-lipophilic balance reversed-phase sorbents, Oasis HLB, was used in this study and suspected TPs were largely undetected, it is believed that suspected TPs monitored and commonly reported for P4, could have been present in samples, but lost due to breakthrough effect. The application of the concentration step (SPE) of unknown efficiency was thought to have added some uncertainty, as if the products were present, but not retained by cartridges. This may have resulted in a false negative outcome.

A further sampling of reed beds was planned to compare results. However, because of limited access to the site, it was not possible to do further sampling of the reed beds. Thus, the present study moved on to spiking river water in the laboratory with high concentration of progesterone such that concentrations of transformation products formed can be detected in aqueous solution without the application of an SPE process whose efficiency for unknown TPs could not be assessed.

5.1.2 Removal of Parent Compounds by the Reed Bed

As discussed in section 4.2.1 of this work, a further aim of the sampling done at Barston STW was the measurement of the concentrations of four model parent compounds including progesterone, to briefly assess reed beds performance in removing these compounds. Reed beds have been reported to have varying removal efficiency for a range of pharmaceuticals depending on individual compound's properties (e.g. Koc, Kow, hydrophobicity, planarity, lipophilicity, etc.), reed beds characteristics (type of reed beds and operational design including effluent (hydraulic) retention time), associated flora and extent of colonization, environmental factors and soil properties (Verlicchi & Zambello, 2014; Li et al. 2014).

Concentration of individual compounds in influent was compared with their corresponding concentrations in effluent following influent polishing by the reed bed. Comparison of concentration pharmaceuticals in influent samples with their corresponding concentration in effluent is a common approach applied in measuring reed beds performance. For example, Verlicchi & Zambello, 2014 reported a removal efficiency of 88 and 99% for sulfamethoxazole and acetaminophen in horizontal subsurface flow and surface flow reed beds when the concentrations of the two pharmaceuticals in influent and effluent samples were compared. Diclofenac is reported to be among most readily removed pharmaceuticals by reed beds with over 70% removal efficiency in both surface and subsurface flow systems (Kucerak 2014; Verlicchi & Zambello, 2014; Li et al. 2014).

In the present study, removal was highest for fluoxetine, followed by progesterone, diclofenac and propranolol. Removal of target compounds was generally above 65% for all model compounds. Observed 73% removal of DCF in reed beds is similar to those reported in the literature (Kucerak 2014; Verlicchi & Zambello, 2014; Li et al. 2014). Progesterone influent concentration of 7 ng/L was removed to 1 ng/L in effluent, representing 90% removal efficiency. This result was similar to that reported by Vymazal et al. (2015), in their study of steroids removal efficiency by horizontal subsurface flow system. Progesterone influent concentration of 20 ng/L in that study, was removed to below analytical method limit of quantification (LOQ=0.5 ng/L) in effluent. Removal efficiency similar to P4 was further reported

by Vymazal et al. (2015), for the other steroids, estrone, estriol, 17β-estradiol, 17α-ethinylestradiol and testosterone. Similar high removal efficiency was reported when the proportion of estrogenic and androgenic activities in influent and effluent samples treated with reed beds were compared. Cai et al. (2012), reported a 95% and 92% removal efficiency for estrogen and androgen respectively when dairy wastewater was treated with surface flow reed beds. Overall, reed beds appear to be an effective tertiary treatment option.

At the time of sampling reed beds, reeds diversity could not be determined as reeds had not fully colonized the bed surface (very sparse reed distribution). Associated reed bed soil properties such as organic carbon content (critical for model compounds' partitioning behaviour to soil) was not known and hydraulic retention time was zero as effluent sample (outlet of reed bed) was taking immediately after flooding with influent (spot sampling). These are factors reported (Verlicchi & Zambello, 2014; Li et al. 2014) to influence organic compounds removal by reed beds. The plan was to determine these factors in subsequent visit to the site. However, because further access to site was not possible, the reed beds characteristics (factors mentioned above), could not be determined and subsequent modification of sampling protocol to compare results, could not be achieved. Furthermore, initial work in reed beds used $P4d_9$ as a sole IS, with the intention that further work will use compound specific IS, which were not available at the time the first sampling was carried out. The sole use of one IS for compound quantification, was thought to lead to some bias, in that we were correcting for recovery using a compound with different characteristics and which may respond differently in different matrices. The use of compound specific IS may not always eliminate problems associated with ionisation efficiency in MS analysis (Gomez et al. 2004), let alone when they are dissimilar to the compound they quantify. Thus, the results presented for concentrations of model compounds in reed beds and their performance in removing model compounds, will require further study to verify their reliability.

5.2 Progesterone Degradation

5.2.1 Chemical Analysis and Biological Assays

Results of progesterone degradation were the same in both chemical and yeast assays. Progesterone concentration and associated progestogenic activity decreased with increasing incubation time. Results of the present study are similar to chemical analysis results reported by Janna (2011), who monitored the degradation of a range of progestogens including P4. Janna (2011) reported a decrease from 410 ng/L nominal progesterone concentration to 6 ng/L after 24 hours of incubation. Cwiertny et al. (2014) noted that there are situations were decrease in a parent compound's concentration in a degradation system, is marked by corresponding increase in biological activity, showing the formation of bioactive TP(s). This report, informed the inclusion of biological screens in the present study design to determine if degradation of P4 was associated with increased progestogenic or androgenic activity and to what extent. The observed decrease in progestogenic activity in the present study was not marked by a corresponding increase in androgenic activity seeing that androgens were detected in chemical analysis. The responses observed at 0 h and 24 h, were similar to those seen in the progesterone screen and may not represent androgenic activity, but cross reactivity of progesterone with the androgen receptor, an effect previously reported in the literature (Runnalls et al., 2013). Runnalls et al. (2013) in vivo experiment on the relative potency of synthetic progestogens on the fathead minnow indicated that these compounds can interact with other steroidal nuclear receptors especially the androgen receptor. Recommended control in the literature (Runnalls et al. 2013) for the observed cross reactivity of progesterone in androgen screen, is to run the screen on a test sample solely spiked with a given androgen. This was however, not possible in the present study as the main intent is to observe spiked progesterone degrading into androgens. The implication therefore was, P4 was always present in test tanks until it was degraded below the minimum concentration needed to activate the androgen receptor.

5.2.2 The Efficiency of the EAWAG Biodegradation Database Prediction

As mentioned in section 4.3.1, in addition to literature reported TPs of P4 that were selected for monitoring under low resolution, the EAWAG Biodegradation Biocatalysis Database pathway prediction system was used to predict other potential products of progesterone transformation in the environment. The efficacy of the BBD in predicting real time metabolism of pharmaceuticals and other contaminants (including pesticides) has been reported by Prasse et al. (2011) and Helbling et al. (2010).

In the present study, the database predicted five bacteria mediated products (TP-BBD) (Fig. 4.11) resulting from the cleavage of the 'B' ring in the P4 C21 pregnane skeletal backbone through a two-step aerobic prediction system. The ring cleavage of P4 predicted by the BBD may hardly have occurred as only m/z 329.3 corresponding to one (TP-BBD-1) of the five BBD predicted masses was seen in all study sites. Biotransformation rule 0356 that facilitated the formation of TP-BBD-1 was reported to facilitate several steps in steroid metabolism including aromatization, spontaneous ring cleavage and oxidation (Philipp, 2011; Horinouchi et al., 2005). However, it has a 50% likelihood of occurrence in an aerobic system. The assumption on the possible factors that may have accounted for this observation were:

A. The aerobic condition of the degradation tank may not have favoured reactions that yield BBD-predicted transformation products.
B. BBD-predicted transformation products are rapidly formed and degraded at a rate that the present study sampling regime is unable to detect
C. The microbial community available in degradation tank may be less diverse/active and thus not include bacterial strains predicted by the database to carry out reactions that yield its predicted TPs.
D. Cleaving of the P4 ring may require too high an input of metabolic energy; instead, degrading microorganisms favour the alternative carbon source available in side chains and that requires relatively less energy.

The low occurrence of the database predicted TPs observed is not specific to the present study alone. In the Prasse et al. (2011) study mentioned earlier, the database predicted 58 possible products out of which just four were observed in their batch experiment. Furthermore, the database accessory pathway prediction system has been reported to predict irrelevant TPs (low selectivity) that are unlikely to be seen in real environmental situations (Fenner et al., 2008). These performance issues were the reason for the development of the new tool (enviPath) designed as an improvement and replacement for the EAWAG BBD (Wicker et al. 2016).

Using this new tool (enviPath), the degradation of P4 was modelled. The structures and names of predicted TPs are presented in Figure 5.1. The molecular formulae and isotopic masses of the predicted TPs are presented in Table 5.1. The information on progesterone has been included in the Table 5.1 for easy reference. Side-chain modification of peripheral atoms relative to ring-cleavage predicted by the EAWAG BBD, was responsible for the formation of enviPath predicted TPs. Hydroxylation was the main pathway responsible for the formation of predicted TPs. Product TP-ALG-2 detected in the second study in the Maple Cross location (see Table 4.12) corresponds to enviPath predicted Pregnane-3,20-dione. It would be recalled that this product (TP-ALG-2) was detected following HRMS in the first study, but with lower degree of confidence as its intensity was low and molecular formula could not be generated. The peak corresponding to this product was however stronger in the second study at Maple Cross and molecular formula could be generated. The predicted molecular formula and isotopic mass by enviPath, corresponds to HRMS measured mass and formula for TP-ALG-2 (compare Table 4.10 and Table 5.1). A review of literature on evidence of enviPath application with a view to comparing other workers report on its predictive power was not successful. Publications available either referred to the Eawag-Soil public database developed in enviPath, and containing all freely accessible regulatory data on pesticide degradation in laboratory soil simulation studies for pesticides registered in the EU (Latino et al. 2017), or applied EAWAG BBD and only passively mentioned enviPath as a new tool (Tratnyek et al. 2017; Çeçen, 2017; Letzel et al. 2016).

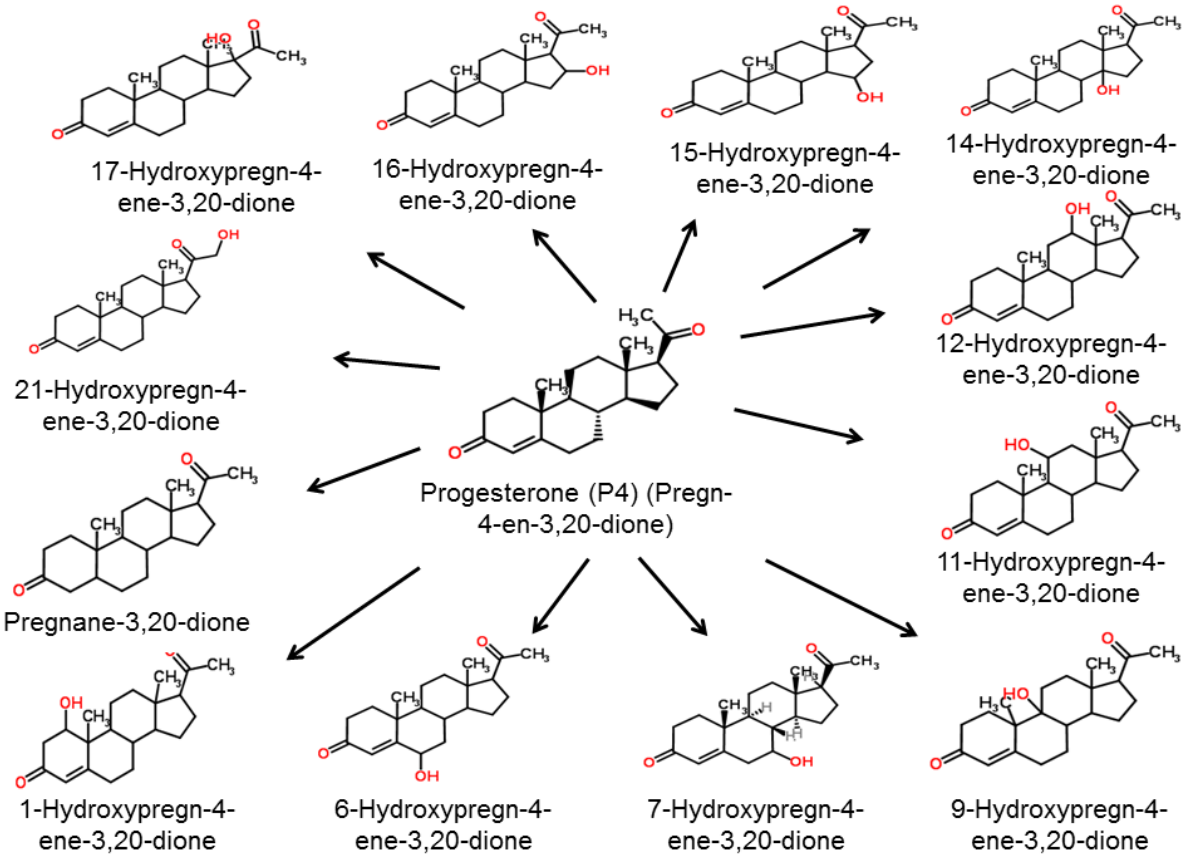


Figure 5.1: enviPath Predicted transformation products of progesterone. Side-change modification rather than EAWAG BBD predicted ring-cleavage resulted in the products predicted

Table 34: enviPath Predicted Transformation Products of Progesterone

Predicted TPs Name	Molecule Formula	Isotopic Mass (Da)
P4 (Pregn-4-en-3,20-dione)	$C_{21}H_{30}O_2$	314.2246
Pregnane-3,20-dione	$C_{21}H_{32}O_2$	316.2402
11-Hydroxypregn-4-ene-3,20-dione	$C_{21}H_{30}O_3$	330.2195
12-Hydroxypregn-4-ene-3,20-dione	$C_{21}H_{30}O_3$	330.2195
15-Hydroxypregn-4-ene-3,20-dione	$C_{21}H_{30}O_3$	330.2195
14-Hydroxypregn-4-ene-3,20-dione	$C_{21}H_{30}O_3$	330.2195
21-Hydroxypregn-4-ene-3,20-dione	$C_{21}H_{30}O_3$	330.2195
16-Hydroxypregn-4-ene-3,20-dione	$C_{21}H_{30}O_3$	330.2195
7-Hydroxypregn-4-ene-3,20-dione	$C_{21}H_{30}O_3$	330.2195
17-Hydroxypregn-4-ene-3,20-dione	$C_{21}H_{30}O_3$	330.2195
6-Hydroxypregn-4-ene-3,20-dione	$C_{21}H_{30}O_3$	330.2195
(5β)-5-Hydroxypregnane-3,20-dione	$C_{21}H_{32}O_3$	332.2351
1-Hydroxypregn-4-ene-3,20-dione	$C_{21}H_{30}O_3$	330.2195
9-Hydroxypregn-4-ene-3,20-dione	$C_{21}H_{30}O_3$	330.2195

5.2.3 Degradation Pathways

The reactions that resulted in detection of most *m/z* linked to the TPs observed in the degradation study were hydroxylation, hydrogenation, dehydrogenation, ketone and hydroxyl functional group interconversion and side-chain breakdown. Sometimes, individual pathways proceeded alone to form products. But most often, several pathways acted together to produce a given TP. Of these pathways, dehydrogenation and side–chain breakdown were the dominant pathways as determined empirically by a combination of their peak area and the prevalence of their TPs across study sites. For example, the major transformation product of the first study was TP-ALG-1, as determined by comparison of products peak areas (Fig. 5.2).

It was not possible to calibrate the instrument on unknowns, so, peak area was used. A given compound's peak area is affected by factors such as ionisation efficiency, such that a higher size area may not mean more of that compound is present than another. For the degradation products and their associated pathways, however, peak area provided an empirical assessment of their possible significance and their change with time. The metabolic reactions identified for P4 degradation have earlier been reported as the main transformation pathways for progesterone degradation by some strains of fungi, the cyanobacterium *Microchaeta tenera* and two freshwater microalgae, *Scenedesmus obliquus* and *Chlorella pyrenoidosa* (Polio *et al.,* 1994; 1996, Safiarian *et al.*, 2012 and Peng *et al.*, 2014). Peng et al. (2014) study spiked with an initial P4 concentration of 503 µg/L and 250 mg/L by Safiarian et al. (2012). Accordingly, it is possible that P4 transformation observed in this study may have been carried out by a consortium of microorganisms using P4 as a carbon source and utilizing diverse reaction pathways.

TP-ALG-1 was formed by a dehydrogenation reaction at C1 and C2 resulting in the formation of a double bond between these carbon atoms. This is a common bacterial and algal mediated transformation pathway in natural and engineered treatment systems. For example, boldenone was reported to be formed from testosterone by bacteria-mediated dehydrogenation at C1 and C2 of the parent molecule (Cwiertny et al., 2014; Yang et al., 2011). Microbial (*Nocardia sp.*) mediated dehydrogenation of ring D of 17β-estradiol at C17 was a contributory pathway in the formation of estrone (Yu et al. 2013; 2007). Dihydrotestosterone was reported to have also been formed from testosterone by dehydrogenation at C4 and C5 of the parent compound (Peng et al., 2014). TP-ALG-2 was formed by the hydrogenation reaction at C4 and C5 resulting in the loss of the double between those carbon atoms in the parent molecule. A similar pathway was reported for the formation of 4,5-dihydronorgestrel from algae-mediated hydrogenation of C4 and C5 of norgestrel by Peng et al. (2014).

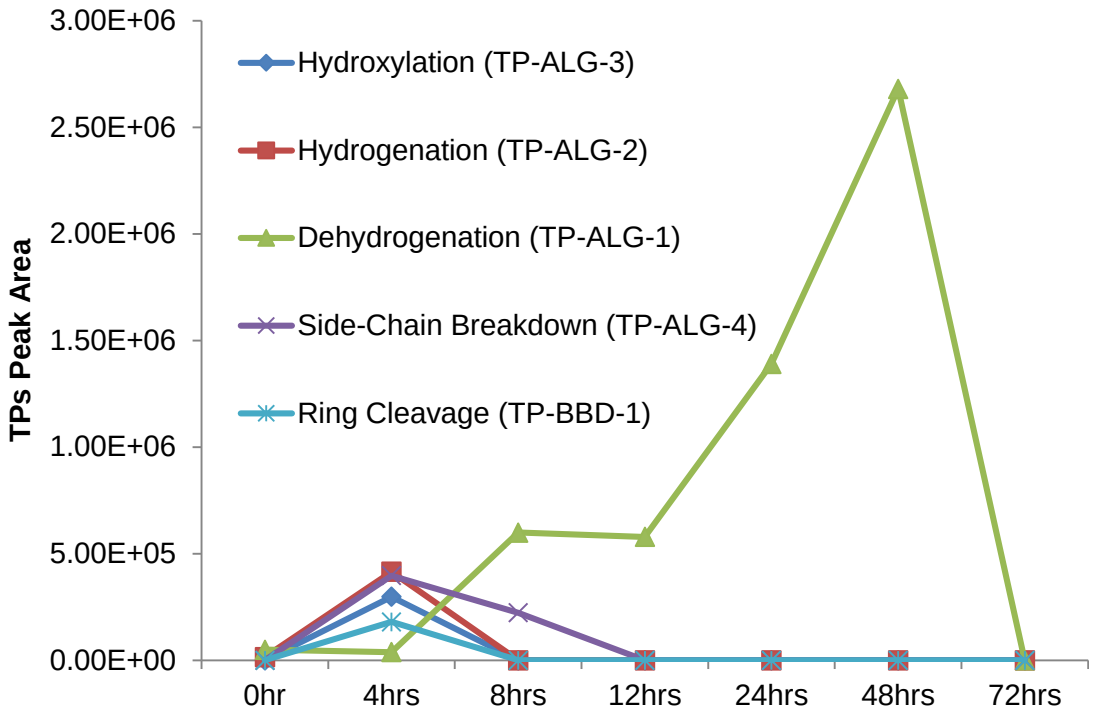


Figure 5.2: Chemical reactions that yielded the transformation products identified in this study. Based on peak areas, dehydrogenation was the most dominant reaction.

Hydroxylation is another important pathway commonly reported in the literature for the biological transformation of pharmaceuticals. For example, degradation of 17β-estradiol into 4-hydroxyl-17β estradiol by the aerobic bacteria *Sphingomonas sp.* was reported by Kurisu et al. (2010), to proceed by hydroxylation at C4. Similarly, hydroxylation of C2 in 17α-ethinyl estradiol by nitrifying bacteria was reported to result in the formation of 2-hydroxyl-17α-ethinyl estradiol (Yi & Harper, 2007). Also, the hydroxylation of C6 of 17α-ethinyl estradiol by the microalgae *Ankistrodesmus braunii* was reported to yield 6-hydroxyl-17α-ethinyl estradiol (Della Greca et al., 2008). Hydroxylation, reduction and the side-chain degradation, were also reported as the dominant pathway for P4 degradation by microalgae (Safiarian et al. 2012; Polio et al. 1994; 1996). In the present study, hydroxylation of the ketone group at C3 of P4 was a contributory pathway in the biotransformation of progesterone to TP-ALG-3. Side-chain breakdown was an important pathway in the formation of TPs suspected to be androgens in this study. The formation of TP-ALG-4 was partly by side chain breakdown, the cleavage of -

$COCH_3$ at C17 of progesterone and subsequent hydroxylation at C17. This could be followed by dehydrogenation at C1 and C2 of P4 to form boldenone or oxygenation of C17 into ketone to form androstene (Peng et al., 2014). TP-ALG-5 was formed by similar side chain breakdown and subsequent hydroxylation of C17. Dehydrogenation at C4 and C5 of P4 following side-chain cleavage and hydroxylation at C17 resulted in the formation of one of the possible compounds depicted as TP-ALG-6 in this study, dihydrotestosterone. Hydroxylation would account for the formation of epi-androsterone (the other form of TP-ALG-6) from TP-ALG-4, assuming TP-ALG-4 was 4-androstene-3,17-dione as reported by Peng et al. (2014).

5.2.4 Identification of the Transformation Products

The identification of transformation products was achieved by applying a mass defect filter in HRMS. This was followed with fragmentation by collision induced dissociation of any feature recorded. The systematic approach followed in this study for the identification TPs have been reported previously. For example, Ruff et al. (2015) applied mass defect filtering and collision induced dissociation in the target and non-target analysis of polar compounds in a major river in central Europe. Guan et al. (2006) applied collision induced dissociation in the elucidation of the fragmentation pathways of anabolic steroids, including boldenone, trenbolone and tetrahydrogestrinone. Similarly, analysis of chromatograms for peaks of an exact mass that are derived from the known molecular formula of suspect TPs, comparison of their structure, retention behaviour and MSn fragmentation are common approaches used in the literature for the identification and confirmation of suspect TPs (Bletsou et al. 2015; Hug et al. 2014; Gómez-Ramos et al., 2011; Kern et al., 2009). In all degradation studies, five features were found; *m/z* 313.2170 (TP-ALG-1), *m/z* 317.2466 (TP-ALG-2), *m/z* 287.1997 (TP-ALG-4), *m/z* 289.2160 (TP-ALG-5) and *m/z* 329.2115 (TP-BBD-1). TP-ALG-2 that was detected with a lower degree of confidence in the first study because of the lack of a molecular formula and very low intensity was seen in the other studies with high confidence (Refer to Tables 4.6 and 4.10 for HRMS data). Collision induced dissociation (CID) fragmentation of the detected features was however successful only for m/z 313.2170 and 287.1997 and not the others

because their concentrations were too low to work with. CID MS^n fragmentation of the *m/z* corresponding to TP-ALG-1 and TP-ALG-4, showed similar fragment ions at *m/z* 121.0648 and 121.0643 respectively (Table 4.7 and Fig. 4.17). Fragmentation of both TP-ALG-1 and TP-ALG-4 was believed to have occurred in the A-ring, most probably due to protonation of the ketone group attached to C_3 (Fig. 5.3). Subsequent relocation of the positive charge either on carbon 1 or 5 due to resonance resulted in the dissociation of the bond between C_9 and C_{10}. CID experiments for *m/z* 287.1997 helped to identify this feature as boldenone since the two major fragments of 121.0643 and 135.1161 are likely to be produced via the fragmentation pathway for boldenone proposed by Guan et al. (2006).

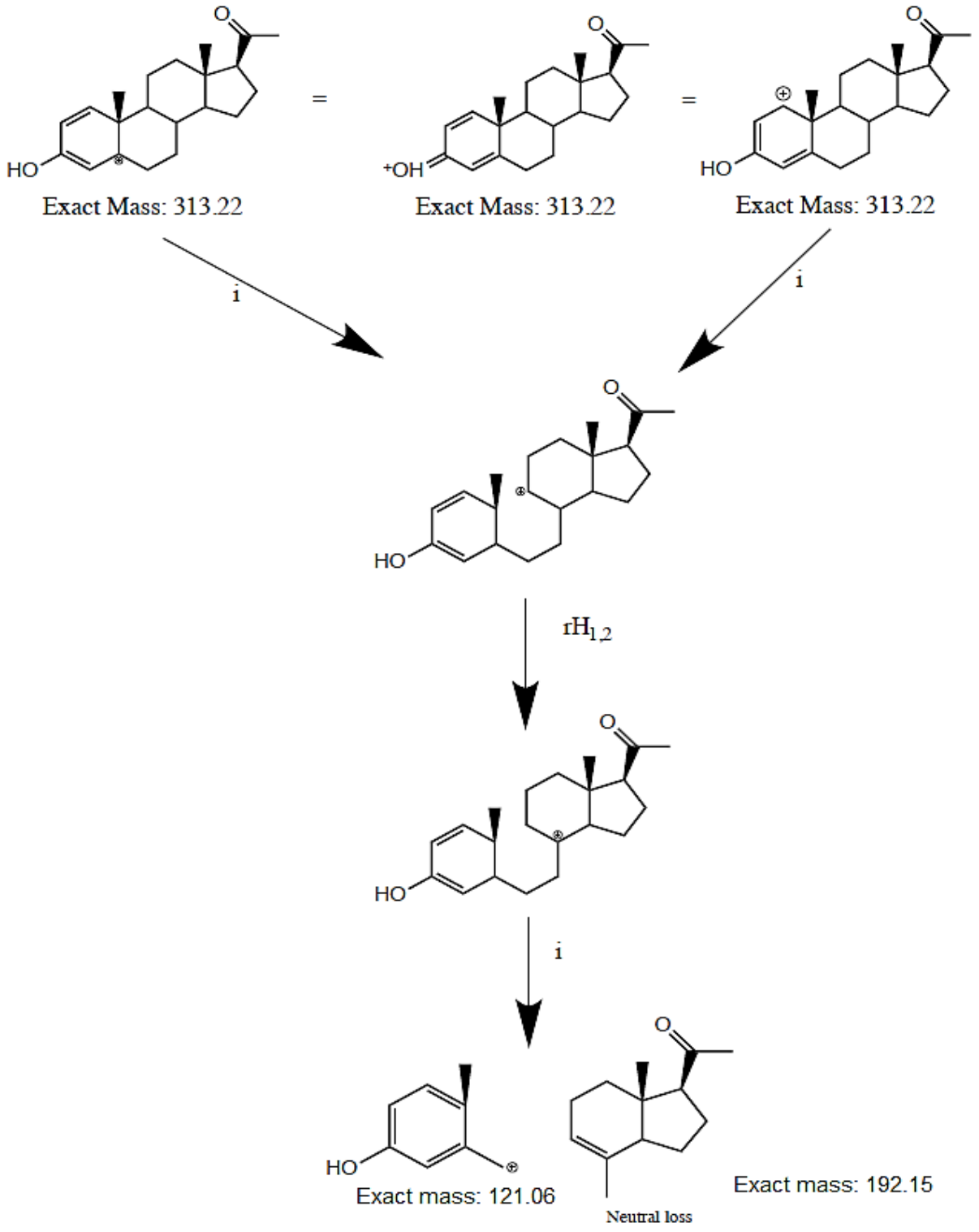


Figure 5.3: Proposed fragmentation scheme for the generation of the fragments ions of m/z 313 (TP-ALG-1). Similar scheme was reported by Guan et al. (2006) for the generation of the major fragment (m/z 121 and 135) of boldenone (i: inductive cleavage; $rH_{1,2}$: rearrangement of a hydrogen atom to an adjacent carbon with concurrent site arrangement of the charge).

The high-resolution data provided accurate masses for TP-ALG-1, TP-ALG-2, TP-ALG-4, TP-ALG-5 and TP-BBD-1 as 312.2097, 316.2402, 286.1933, 288.2089 and 328.2038 respectively. For TP-ALG-1 this matches the theoretical monoisotopic mass/formula for 1, 4-pregnadiene-3, 20-dione, a reported derivative of P4 degradation. The accurate mass of 286.1933 for TP-ALG-4, fits the theoretical monoisotopic mass/formula for androstenedione and boldenone, both reported derivatives of P4 (Peng et al., 2014). Also, the accurate masses of 216.2402 for TP-ALG-2 and 288.2089 for TP-ALG-5 matche the theoretical monoisotopic masses/formulae for 3,20-allpregnanedione and testosterone respectively. The CID fragmentation of TP-ALG-1 and TP-ALG-4 peaks shows that both TPs had the base peak m/z 121.064 in common. This fragment has been previously reported as the major fragment resulting from collision-induced dissociation of a number of microbially synthesized monohydroxylated progesterones (2α-hydroxyP4, 7β-ydroxyP4 and 9α-hydroxyP4), compounds recognized for their pharmacologically active properties (Kang et al., 2004).

5.2.5 Half-life of Progesterone Degradation

The transformation of progesterone in all sites in the present study followed the first-order reaction model (Fig 4.30a, b). First order degradation kinetics like those observed in this study have been reported for the biotransformation of progesterone and norgestrel by two microalgae, *Scenedesmus obliquus and Chlorella pyrenoidosa* (Peng et al. 2014). Unlike the Peng et al. (2014) study where 95% of spiked P4 concentration (503 µg/L) was degraded in 120 h (5 days), spiked P4 concentration in the present study (1,000 µg/L) was degraded below instrument detection limit (representative 100% degradation) in 72 h (3 days) for all locations except High Wycombe where 90% degradation occurred. A comparison of the progesterone degradation parameters recorded in this study with those reported by other workers are presented in Table 5.1. Degradation rate was more rapid in the present study than those reported for algae, thus, indicating that a more diverse microbial community may have been responsible for the breakdown of P4 observed. The data for norgestrel has been included in the table to compare the relative ease of biodegradation of the natural progesterone, relative

to the synthetic progestogen. According to Hu et al. (1998), the relative persistence of norgestrel is due to the presence of 17α-ethynyl group in the compound's structure. The 17α-ethynyl group is resistant to microbial breakdown (Hu et al. 1998.

Table 5.1: Comparison of degradation parameters reported for Progesterone

Study	Compound	Degrader/System	Parameters	
			Rate Constant (k)(h)	Half-life (h)
Present	P4	River	0.046	15.1
Present	P4	River	0.111	6.27
Present	P4	River	0.102	6.77
Present	P4	River	0.103	6.76
Present	P4	River	0.024	29.4
Peng et al. 2014	P4	*S. obliquus*	0.044	16.0
Peng et al. 2014	P4	*C. pyrenoidosa*	0.018	39.0
Lui et al. 2013	P4	Activated Sludge	-	4.30
Peng et al. 2014	Norgestrel	*S. obliquus*	0.017	40.0
Peng et al. 2014	Norgestrel	*C. pyrenoidosa*	0.009	88.0

*h=hours

The term "biodegradation" as used in this study refers to the loss of the parent compound in surface water samples relative to a sterile double deionised water (control). It is not known if sorption of P4 or TPs in suspended solids happened as river water was not filtered prior to the study. Biodegradation and sorption are components of the natural attenuation process, during which parent compounds are lost from the aqueous phase. It is difficult at best and often nearly impossible to separate the influence of biodegradation from that of sorption. Therefore, the experiments were designed to simplify the complexities of natural systems and to focus only on water phase.

5.3 Significance of the Results of the Present Study

This study has highlighted the significance of the bioconversion of one class of steroid into another. The result presented will help the water industry appreciate the possibility that there

are diverse sources of steroids in surface waters as there are multiple transformation pathways in the water environment. That this is the case is highlighted by the Peng et al. (2014) study. Furthermore, this study indicates that in addition to the natural excretion from humans and animals, androgens present in the aquatic environment could be produced from the transformation of progesterone in surface water. A range of synthetic progestogens are used clinically, primarily in hormonal contraceptives. These synthetic progestogens differ appreciatively in structure from each other, and from P4. For example, quite a few, such as the widely used levonorgestrel, is a C19 steroid, not a C21 steroid like P4. These significant differences in structure mean that it is impossible to know whether or not results presented here are applicable to the biodegradation of synthetic progestogens. Nevertheless, it may be likely that the main finding, that as a steroid hormone degrades, it can lead to the formation of other steroids with different biological activities, will be applicable to the degradation of other steroid hormones, both natural and synthetic.

The yeast screen results demonstrated that P4 degradation in the environment is likely to result in a corresponding decrease in associated progestogenic activity. Though P4 degrades to androgen as shown by chemical analysis, the concentrations in the environment are unlikely to represent any immediate threat to aquatic resources, as results of the androgen screen did not show any androgenic activity when working at elevated (1,000 µg/L) concentrations. Knowledge of degradation half-lives and pathways of degradation of progesterone in surface waters will be useful in understanding the fate of this compound in the environment and will be relevant in the development and application of fate models.

Chapter Six: General Conclusion

6.0 General Conclusion

- A number of compounds were initially identified for study on the basis of their high and regular use in the United Kingdom, constant detection in wastewater influent, effluent and surface water, reported toxicity effects on aquatic biota and inclusion in the EU priority chemical watchlist.
- However, based on the degradation potential reported for identified compounds in the literature, progesterone was selected for laboratory based degradation study based on its rapid biodegradability relative to the other model compounds.
- Degradation products and pathways for progesterone, were identified from the literature and from predictions by the EAWAG Biodegradation Biocatalysis Database (BBD). The biodegradation database predicted products (abbreviated as TP-BBD) resulting from the cleavage of the 'B' ring in the progesterone C21 pregnane skeletal backbone through a two-step aerobic prediction system. The literature search, however, identified algal products (TP-ALG) that did not show ring cleavage. Reported algal TPs resulted from reaction pathways such as hydroxylation, oxidation, hydrogenation, dehydrogenation and breakdown of side chains.
- It was apparent that the BBD accompanied pathway predicting system gave different products and pathways to those reported in the literature. The BBD predicted ring cleavage may follow anaerobic degradation pathways.
- Using the new tool, enviPath, predicted TPs were observed to be different from those predicted by the EAWAG BBD. Instead of ring cleavage predicted by the latter, peripheral rings modification mainly by hydroxylation was predicted by the former (enviPath).
- Reed beds at a wastewater treatment works were identified as a suitable system for studying the degradation and removal of selected compounds.
- An analytical methodology was set up using HPLC and initially low resolution tandem mass spectrometry (LC-MS/MS). Methods were applied and data obtained from reed beds at Barston wastewater treatment works.

- The developed method could detect the selected compounds at environmentally relevant concentrations. Influent concentrations (ng/L) in reed beds were 8.6±2.1 (Progesterone), 87.7±19.6 (Fluoxetine), 335±73.7 (Propranolol) and 5,704±4,700 (Diclofenac). Effluent concentrations (ng/L) were 1.2±0.2 (Progesterone), 3.5±1.6 (Fluoxetine), 112±10.5 (Propranolol) and 3,570±2,924 (Diclofenac). Limits of detection (LODs) were 0.27 µg/L (Progesterone), 0.22 µg/L (Fluoxetine), 0.25 µg/L and 1.0 µg/L (Diclofenac). It was apparent, however, that using low resolution mass spectrometry alone would limit identification of transformation products.
- Removal of target compounds was generally above 65% for all model compounds except DCF that was 37%.
- Further access to reed bed site was not possible to determine certain reed beds removal dependent characteristics and implement needed modification to sampling protocol. Thus, results presented for concentrations of model compounds in reed beds and their performance in removing model compounds, will require further study to verify their reliability.
- Following the assessment of reed beds samples for degradation products, low concentration was thought to lead to uncertainty in determining the presence or absence of TPs. It was apparent that environmental concentrations of model compounds in the real environment would prove challenging for detection and identification of transformation products.
- Specific putative degradation products were identified in the laboratory studies; 1,4-Pregnadiene-3,20-dione (TP-ALG-1), 3,20-Allopregnanedione (TP-ALG-2), boldenone (TP-ALG-4), testosterone (TP-ALG-5) and 1-acetyl-4-[2-(5-hydroxy-2-methylphenyl)ethyl]-7a-methyl-octahydro-1H-inden-5-one (TP-BBD-1) (Tables 4.4 and 4.9).
- The reactions that resulted in detection of TPs observed in the degradation study were hydroxylation, hydrogenation, dehydrogenation, ketone and hydroxyl functional group interconversion and side-chain breakdown. Of these pathways, dehydrogenation and side–chain breakdown were estimated to be the dominant pathways

- Sometimes, individual pathways proceeded alone to form products. But most often, several pathways acted together to produce a given TP.
- It was apparent that some degradation products were biologically active. However, there was no evidence of activity in either progesterone or androgen assays resulting from their formation from a 1,000 µg/L initial progesterone concentration. Thus, it would seem unlikely that the degradation products of progesterone pose a risk in the environment through pathways monitored in this work.
- The degradation of progesterone was complete in three of the four sites studied after 48 hours. Progesterone degradation half-life was approximately 7 hours in all sites except High Wycombe (29 hours) where some residual progesterone (100 µg/L) was present after 72 hours. The study therefore indicates that degradation of progesterone is relatively rapid in surface waters, following similar pathways as those identified.

6.1 Recommendation for Future Work

- Carry out multiple sampling of reed beds with modified sampling protocol to allow for literature recommended HRT coupled with monitoring of reed bed dependent compound removal characteristics.
- Purchase and use each model compound specific internal standard and compare removal results.
- Model progesterone degradation using a range of predictive models and compare results.
- Use a range of more sensitive analytical instruments (e.g. Fourier Transform Ion Cyclotron Resonance (FT-ICR) and Orbitrap) and data processing software to compare results and reach more definite conclusion on identity of TPs.
- Carry out degradation study in the summer and microbial profiling of sampling sites to compare results with those of the present study (winter) and link observed degradation rate with microbial diversity and metabolic activity.

- Design study such that any degradation due to hydrolysis or volatilization is determined to account for degradation based sole on biological breakdown. This was not done in the present study owing to literature reports (Peng et al. 2014; Liu et al. 2013) that degradation due to hydrolysis and volatilization was negligible for progesterone.
- Determine insitu physiochemical conditions (e.g. temperature) of surface waters at the time of sampling and compare such with observed conditions in the laboratory. This will help put results obtained in context of what happens in the field.

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Appendix