# REMOVAL AND TRANSFORMATION OF GEMFIBROZIL, A PHARMACEUTICALLY ACTIVE COMPOUND, IN WASTEWATER TREATMENT

by

Wendy Helen Krkošek

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# ABSTRACT

Pharmaceutically active compounds (PhACs) have been found in wastewater effluents and receiving waters around the world. As yet there are no jurisdictions that regulate their release, or their impact on receiving water ecosystem health. The issue is complex due to the number of PhACs that exist, the variability in their structure and function, the variability in removal during different wastewater treatment processes, the potential for formation of metabolites and transformation products, and a lack of information on the impacts due to their presence on receiving waters. Gemfibrozil is a lipid regulating drug that is commonly found in wastewater effluents and receiving waters. It has been shown to partially degrade during biological wastewater treatment processes and has also been shown to produce reaction products through reactions with free chlorine.

This thesis investigated the removal and transformation of gemfibrozil through several different wastewater treatment processes, namely biological removal and chlorination. Reactions between gemfibrozil and free chlorine led to the identification of four reaction products. The structures of three of the four reaction products were elucidated. The kinetics of formation of these reaction products were then investigated at a range of pH values, and in two wastewater matrices. One reaction product, 4'-ClGem was shown to form under conditions relevant to wastewater treatment. The impacts of gemfibrozil and 4'-ClGem presence on the abundance of suspended and biofilm bacteria in a simulated receiving water experiment were evaluated. It was shown that changes in the water matrix had more of an impact on bacterial abundance than presence of gemfibrozil or 4'-ClGem. A bacterial dose-response experiment showed a negative response at 10 mg/L exposure to 4'-ClGem, which is orders of magnitude higher then what would be found in receiving waters.

In order to prevent the formation of chlorinated reaction products, it is necessary to remove gemfibrozil prior to disinfection. Recirculating biofilters (RBS), a biological technology for onsite or small-scale wastewater treatment, were explored as a potential treatment process for gemfibrozil removal. Results indicate that RBFs show promise as a robust technology to remove greater than 50% of influent gemfibrozil.

# LIST OF ABBREVIATIONS AND SYMBOLS USED

| | |
|---|---|
| °C | degrees Celsius |
| $^{13}C$ | Carbon Isotope 13 |
| 3′,4′,6′-triClGem | 5-(3,4,6-trichloro-2,5-dimethylphenoxy)-2,2-dimethylpentanoic acid |
| $^{35}Cl$ | Chlorine Isotope 35 |
| $^{37}Cl$ | Chlorine Isotope 37 |
| 4′-ClGem | 5-(4-chloro-2,5-dimethylphenoxy)-2,2-dimethylpentanoic acid, |
| 4′,6′-diClGem | 5-(4,6-dichloro-2,5-dimethylphenoxy)-2,2-dimethylpentanoic acid |
| 6′-ClGem | 5-(6-chloro-2,5-dimethylphenoxy)-2,2-dimethylpentanoic acid |
| ANOVA | Analysis of variance |
| AOB | Ammonia oxidizing bacteria |
| APHA | American Public Health Association |
| atm | Atmosphere |
| AWWA | American Water Works Association |
| BOD | Biochemical oxygen demand |
| bp | Base pairs |
| BSTFA | (*N,O-bis*-[trimethylsilyl]trifluoroacetamide with 1% trimethylchlorosilane) |
| CA | California |
| $CaCO_3$ | Calcium carbonate |
| $C_{15}H_{22}O_3$ | Gemfibrozil |
| ClGem | Mono chlorinated gemfibrozil |
| cm | Centimetre |
| C:N:P | Carbon: Nitrogen: Phosphorus ratio |
| CWWA | Canadian Water and Wastewater Association |
| d | Day |
| $D_{10}$ | Effective size |
| DBP | Disinfection by-products |
| DCDMS | Dichlorodimethylsilane |

| | |
|---|---|
| diClGem | 5-(4,6-dichloro-2,5-dimethylphenoxy)-2,2-dimethylpentanoic acid |
| DNA | Deoxyribonucleaic acid |
| DOC | Dissolved organic carbon |
| EC50 | Half maximal effective concentration |
| EI | Electron ionization |
| EPS | Extracellular polymeric substance |
| ESI | Electrospray ionization |
| ESI-MS | Electrospray ionization mass spectrometry |
| ESI-MS/MS | Electrospray ionization mass spectrometry/mass spectrometry |
| ESI-TOF | Electrospray ionization – time of flight |
| eV | Electron volt |
| g | Grams |
| g/L | Grams per litre |
| GC | Gas chromatography |
| GC-MS | Gas chromatography mass spectrometry |
| GEM | Gemfibrozil |
| h, hr | Hour |
| $H^+$ | Hydrogen ion |
| $H_2O$ | Water |
| HAA | Haloacetic acids |
| HOCl | Hypochlorous acid |
| Hz | Hertz |
| K | Degree kelvin |
| $K_d$ | Dissociation constant |
| $K_{ow}$ | Octanol-water partition coefficient |
| $KH_2PO_4$ | Potassium dihydrogen phosphate |
| $K_2HPO_4$ | Dipotassium hydrogen phosphate |
| $km^2$ | Square kilometres |
| LC-MS | Liquid chromatography-mass spectrometry |
| log | Logarithm |
| ln | Natural logarithm |

| | |
|---|---|
| M | Moles per Litre |
| m/z | Mass to charge ratio |
| $M^{+\bullet}$ | Molecular ion |
| MD | Maryland |
| mg | Milligram |
| mg/mL | Milligram per millilitre |
| mg/L | Milligram per litre |
| MHz | Mega hertz |
| mL | Millilitre |
| MLD | Million litres per day |
| mm | Millimeter |
| mmol/L | Millimoles per litre |
| mM | Millimoles per litre |
| mol | Mole |
| mp | Melting point |
| MS/MS | Tandem mass spectrometry |
| NaOCl | Sodium hypochlorite |
| $Na_2S_2O_3$ | Sodium thiosulfate |
| ng/L | nanogram per litre |
| $NH_3$-N | Ammonia- Nitrogen |
| NL | Newfoundland and Labrador |
| NMR | Nuclear magnetic resonance |
| NOESY | Nuclear Overhauser Enhancement Spectroscopy |
| $NO_3^-$-N | Nitrate nitrogen |
| $OCl^-$ | Hypochlorite ion |
| ON | Ontario |
| PBS | Phosphate buffer solutions |
| PCR | Polymerase chain reaction |
| PEI | Prince Edward Island |
| PhACs | Pharmaceutically active compounds |
| $pK_a$ | Acid Dissociation constant |
| PNEC | Predicted no-effect concentration |

| | |
|---|---|
| QPCR | Quantitative polymerase chain reaction |
| ® | Registered trademark |
| RBF | Recirculating biofilter |
| Rpm | Revolutions per minute |
| RQ | Risk quotient |
| SRT | Solids retention time |
| TOC | Total organic carbon |
| THM | Trihalomethane |
| TMCS | Trimethylchlorosilane |
| TMS | Trimethylsilyl |
| triClGem | 5-(3,4,6-trichloro-2,5-dimethylphenoxy)-2,2-dimethylpentanoic acid |
| TSA | Tryptic soy agar |
| TSB | Tryptic soy broth |
| TSS | Total suspended solids |
| USEPA | United States Environmental Protection Agency |
| UV | Ultraviolet |
| V | Volt |
| WEF | Water Environment Federation |
| WWTP | Wastewater treatment plant |
| ± | Plus or minus |
| α | Significance level |
| µg/L | Microgram per litre |
| µL | Micro litre |
| µL/min | Micro litre per minute |
| µL/s | Micro litre per second |
| µm | Micrometer |
| µM | Micromoles per litre |

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# CHAPTER 1 INTRODUCTION

## 1.1 RESEARCH RATIONALE

In the late 1990's, advances in analytical chemistry enabled the detection of pharmaceutically active compounds (PhACs) at ng/L concentrations in wastewater and receiving waters (Ternes et al., 1998). Ternes (1998) published the first seminal paper on the occurrence of drugs in sewage treatment plants, which has now been cited over 1200 times. In 1999, Daughton and Ternes published a landmark paper in Environmental Health Perspectives (over 1400 citations) on pharmaceuticals and personal care products in the environment and the potential risks and challenges associated with their presence. Since then, researchers have taken on many different facets of the issue, including but not limited to:

- Improvements in detection and method development;
- Occurrence in the environment;
- Evaluation of removal efficiencies of different drinking water and wastewater unit processes;
- Identification of transformation products formed through different treatment processes;
- Determination of toxicity of single compounds and mixtures on non-target organisms;
- Implications for regulatory frameworks

Although there has been significant effort directed towards this topic area, there are still many questions that remain unanswered, namely due to the vast number of PhACs prescribed, their different modes of action, the impacts of water quality and process

configuration on removal and transformation potential, and the complexity of determining impacts on non-target organisms to a varying, site-specific combination of PhACs and transformation products.

As yet there is no jurisdiction in the world that regulates the release of PhACs into the environment; this is due to a lack of both scientific research related to contaminant risk and fiscal capacity to treat and regulate these contaminants. The implications are largely on environmental, not human health and there is difficulty understanding the implications of PhAC presence on environmental health, and what concentration/negative response requires remedial action. There would be costs associated with monitoring PhACs, and it would not be practical to monitor all of them, but prioritizing becomes a challenge due to the complexities and variability in occurrence, removal and impacts. There would also be a high cost associated with upgrading treatment facilities to remove PhACs to acceptable levels. There is some discussion of using risk management approaches that would include monitoring of substances but also the changes to health of the receiving water; however this type of approach also comes with considerable costs (Boxall et al., 2012).

There is still a significant amount of research that needs to be conducted to understand the full extent of the issue of PhACs entering the environment before appropriate decisions can be made regarding the need for monitoring requirements and/or the establishment of maximum acceptable concentrations. This thesis addresses one small aspect of the larger picture. Specifically, this thesis involves expanding the understanding

of gemfibrozil removal and transformation during different wastewater treatment processes (chlorination and biological removal), and providing an evaluation of potential implications on organisms in receiving waters.

Gemfibrozil was chosen as the model PhAC for this thesis for several reasons:

- Gemfibrozil is a PhAC commonly found in wastewater influent and effluent surveys (Verlicchi et al., 2012; Metcalfe et al., 2003a);
- Gemfibrozil has shown some promise for removal using biological processes, however results are highly variable (Onesios et al., 2009);
- There is evidence of formation of gemfibrozil chlorinated reaction products but there is little information on these products and their behavior in the environment (Bulloch et al., 2012; Glassmaker and Shoemaker, 2005).

Chlorination was chosen as a treatment process to study, because it is a widely used, cost-effective disinfection option, and it is well documented that reactions between organic compounds and chlorine can lead to the formation of byproducts that can be harmful to human health (Inaba et al., 2006; Kanokkantapong et al., 2006; Westerhoff et al., 2004).

Biofilm processes can offer a robust and cost effective option for both small and large-scale wastewater treatment. Most studies related to biofilm processes for the removal of PhACs have focused on large-scale treatment; however, a large portion of the population relies on small-scale or onsite treatment. Recirculating biofilters are a technology that can be implemented in a small-scale or onsite system, however there is a literature gap in

understanding their use for PhAC removal (Camacho-Munoz et al., 2001; Matamoros, et al., 2009), which is why they were chosen as a process to study for this thesis.

## 1.2 Research Hypothesis

Many wastewater treatment processes can lead to the reduction or removal of PhACs, however removal does not necessarily lead to the elimination of risk. Transformation of the parent compound can lead to the presence of transformation products with unknown, and potentially higher, toxicity than the parent compound. The central hypothesis of this thesis is that **reactions between chlorine and gemfibrozil during wastewater disinfection can lead to the formation of chlorinated reaction products, which could lead to negative impacts on organisms in receiving waters.**

## 1.3 Research Objectives

The research hypothesis is addressed through four main objectives:

**Objective l:** Identify the structure of products formed from the reaction of gemfibrozil and chlorine.

**Objective 2:** Determine the kinetics of gemfibrozil degradation and formation of identified reaction products during chlorination under controlled conditions at a range of pH values and in wastewater matrices.

**Objective 3:** Investigate the impacts of exposure to gemfibrozil and the main chlorinated reaction product on bacterial abundance in a simulated receiving water experiment.

**Objective 4:** Examine the removal of gemfibrozil from wastewater using biological processes, to minimize the potential of chlorinated reaction product formation during disinfection.

## 1.4 Organization of Thesis

This thesis was organized in a way to present results from each of the four objectives in one chapter in the style of a refereed journal paper. Therefore each of chapters 4, 5, 6 and 7 contain their own abstract, introduction, materials and methods, results, discussion and conclusions.

**Chapter 1** discusses the research rationale, thesis hypothesis, objectives and organization of the thesis.

**Chapter 2** provides a brief summary of the current state of knowledge on PhACs in wastewater and the environment. Discussed in this chapter are the presence, removal mechanisms, reaction product formation, and environmental impacts of PhACs, specifically gemfibrozil.

**Chapter 3** discusses the materials and methods that are common between the experimental work in chapters 4, 5, 6 and 7. Materials and methods specific to a chapter are discussed in the respective chapter.

**Chapter 4** presents the findings of objective 1, which involved identifying chlorinated gemfibrozil reaction products. This work has been published in the journal *Water Research.*

**Chapter 5** presents the findings of objective 2, which involved investigating the kinetics of gemfibrozil degradation and formation of reaction products under a variety of pH values. Reaction kinetics were also evaluated for two different spiked wastewater matrices. Daily mass loads of the main chlorinated reaction product were then calculated for two wastewater treatment facilities that use chlorination for their disinfection step and have been found to have gemfibrozil in influent and effluent samples. This work was accepted for publication on December 15, 3013 in *Water Environment Research.*

**Chapter 6** presents the findings of objective 3, which involved studying the impacts of exposure to gemfibrozil and the main chlorinated byproduct on biofilm and suspended bacterial abundance in receiving waters. This was studied through the use of a bench-scale batch system and a dose-response trial.

**Chapter 7** Presents the findings of objective 4, which was to reduce the potential for chlorinated reaction product formation by removing gemfibrozil prior to disinfection. This was accomplished by evaluating the removal of gemfibrozil and three other PhACs in a bench-scale recirculating biofilter, which is a type of intermittent sand filter. This work has been submitted to the *Journal of Hazardous Materials.*

**Chapter 8** Provides overall conclusions for the thesis and synthesizes key findings from this research. Recommendations for future work with respect to the removal and transformation of gemfibrozil during wastewater treatment are provided.

# CHAPTER 2 BACKGROUND

## 2.1 INTRODUCTION

With recent advances in analytical chemistry, it is now possible to trace low-level organic compounds through sewage treatment plants, and furthermore, into the environment through sewage outfalls (Lin et al., 2005; Paxeus, 2004; Rodriguez et al., 2003; Reddersen and Heberer, 2003; Ollers et al., 2001; Ternes et al., 1998; Ternes, 1998). One such group of compounds that are an emerging concern is pharmaceutically active compounds (PhACs). PhACs can enter the environment from both human and animal sources, however this thesis only considers PhACs produced for human consumption. PhACs are ingested, but not always fully metabolized within the human body, thus leading to excretion and discharge of both the parent and metabolites to the waste stream (Heberer, 2002). The process of PhAC metabolism (or biotransformation) generally converts a lipophilic compound into a more polar, hydrophilic metabolite, enabling excretion from the system (Plant, 2003). The parent compound refers to the ingested PhAC, which may or may not be the active form of the pharmaceutical. Some pharmaceuticals require bioactivation through metabolic pathways to convert the inactive parent compound into the active metabolite responsible for the therapeutic effect (Caira and Ionescu, 2005).

When passing through the wastewater treatment process, PhACs may be: fully degraded, converted to metabolites, conjugated to polar molecules (which can be cleaved during wastewater treatment processes), transformed to transformation/reaction products through

chemical/physical treatment, or they can pass through the treatment plant unchanged (Daughton and Ternes, 1999; Heberer, 2002). Verlicchi et al., (2012) and Pomiès et al., (2013) both provided reviews of papers published on the occurrence and removal of PhACs through wastewater treatment processes. The results for each PhAC vary depending on which treatment process is used, the water quality, operating conditions, etc. One thing in common with all removal studies is that many PhACs are not fully removed and thus have been detected in receiving waters in the low ng/L to µg/L range (Boyd et al., 2003; Brun et al., 2006; Gross et al., 2004; Kolpin et al., 2002; Daughton and Ternes, 1999; Halling-Sorensen et al., 1998; Ternes, 1998).

## 2.2 Regulation Of PhAC Release

Awareness of contaminants of emerging concern, including PhACs, personal care products and pesticides, and their presence in wastewater effluents, the environment and drinking water has been on the rise (Dohle et al, 2013). Despite the increased attention, there are no formal regulations as of yet for controlling concentrations of PhACs discharged from wastewater treatment plants. The issue is complex; difficulties arise from: analytical detection at low concentrations, the variety of compounds, their metabolites and transformation products, and the lack of understanding of how the presence of these compounds may affect biota and sensitive endpoints downstream (Clouzot et al., 2013, Verlicchi et al., 2012; Escher and Fenner, 2011).

## 2.3 Fate During Wastewater Treatment

Due to the low volatility of most PhACs, it is likely they will remain in the aqueous phase and potentially lead to aquatic organism exposure (Fent, 2006). In general there are two

main mechanisms of removal during wastewater treatment: biodegradation and sorption to solids, both of which are discussed in the following sections.

As previously mentioned, studies of PhAC removal within WWTPs show large variability in results (Verlicchi et al., 2012). PhAC removal may be affected by treatment processes, environmental factors (temperature, pH), quality of effluent, season, and many other factors (Fernandez-Fontaina et al, 2012; Hyland et al., 2012; Stevens-Garmon et al., 2011; Zorita et al. 2009). Some compounds such as salicylic acid and ibuprofen are nearly completely removed (99%) whereas carbamazepine shows very little removal across studies (7-8%) (Pomiès, et al., 2013; Verlicchi et al., 2012; Heberer, 2002; Ternes, 1998).

### 2.3.1 Adsorption and Partitioning to Sludge

When relating log $K_{ow}$ (Octanol-water partition coefficient) to sorption potential through wastewater treatment, the following general rule is sometimes adopted: Log $K_{ow}$ <2.5 yields low sorption potential, 2.5< log $K_{ow}$ <4.0 yields medium sorption potential and log $K_{ow}$ >4.0 yields high sorption potential (Jones-Lepp and Stevens, 2007).

Acidic pharmaceuticals, including gemfibrozil, naproxen, ibuprofen, and diclofenac (pKa 3.2-4.8) are all negatively charged and in dissolved form at neutral pH, and have low to moderate tendency to sorb to solids (Ternes et al, 2004). For more basic PhACs at ambient operating pH, sorption to solids and presence in sewage sludge is a more common process (i.e. ethinyl estradiol) (Fent et al., 2006). Antibiotics also tend to sorb readily to solids and thus end up in the biosolids stream (Jones-Lepp and Stevens, 2007).

Increasing the solids retention time generally increases the sorption potential of PhACs by ensuring that the compounds are present for long enough to adjust to adsorption equilibrium (Kreuzinger et al., 2004).

### 2.3.2 Biodegradation

As many PhACs do not sorb well to solids, biodegradation often becomes the main mechanism by which they are removed from solution. PhAC biodegradation can occur in both aerobic and anaerobic processes and is often assumed to be governed by first order, pseudo-first order, or Monod kinetics reaction (Pomiès et al., 2013, Rittmann and McCarty, 2001). Many studies have been conducted on the removal of PhACs within bench-scale, pilot-scale and full-scale systems, and using a variety of treatment processes, from membrane bioreactors to activated sludge, however there is little information on small-scale or onsite system removal of PhACs (Camacho-Munoz et al., 2011; Matamoros et al., 2009).

It is difficult to comment on general biodegradability of PhACs due to the wide range of structures, and range of treatment technologies. Most reviewers group PhACs by class (Pomiès et al., 2013; Verlicchi et al; 2012; Santos et al., 2010; Onesios et al., 2009), but even within classes there are great differences in biological removal between compounds. For example, within the group of non-steroidal anti-inflammatory drugs (NSAIDs), ibuprofen can often be readily biodegraded (>99% removal by biological processes), whereas diclofenac removal is moderate (<50% removal by biological processes) (Verlicchi et al., 2012; Onesios et al., 2009). There is also large variability in biological

removal results for each PhAC depending on treatment technology used, scale, and experimental conditions.

### 2.3.3 PhAC Mass Balance – Metabolites and Transformation Products

In studying the removal of PhACs within wastewater treatment, it is important to consider mass balances. Thus far, much of the literature has followed the path of parent compounds through the sewage treatment plant and into receiving waters. However, active and inactive metabolites, along with the parent compound, are also excreted and enter the waste stream (Writer et al., 2013; Bonvin et al., 2013). The reactions of PhACs within wastewater treatment (chlorine, ozone, UV) may lead to the creation of transformation products (Escher and Fenner, 2011). Thus, removal of the parent compound may not indicate the removal of any potentially toxic metabolites or transformation products that may have formed, both within the treatment process and within the human body (Escher and Fenner, 2011; Rosal et al., 2009; Isidori et al., 2007; Bedner and MacCrehan, 2006; Mompelat et al., 2009; Xagoraraki et al., 2008; Dodd and Huang, 2007; Westerhoff et al., 2005; Boyd et al., 2005; Glassmeyer and Shoemaker, 2005). Typically, PhAC analytical methods involving GC-MS or LC-MS are designed to target specific compounds and anything that exists outside the narrow range of analytical capability would not be detected, thus performing complete mass balances becomes analytically challenging.

#### *2.3.3.1 Chlorination Reaction Products*

A survey by the Canadian Water and Wastewater Association indicated that 184 of 738 Canadian WWTPs used chlorine for disinfection (CWWA, 2001). Free chlorine is a

strong, non-specific oxidant that reacts with most organic material and can form disinfection by-products (DBPs) (Sirivedhin and Gray, 2005; Pinkston and Sedlak, 2004). The reactions of natural organic matter and dissolved organic carbon with free chlorine, resulting in the formation of trihalomethanes (THMs) and haloacetic acids (HAAs) through drinking water treatment are well documented (Inaba et al., 2006; Kanokkantapong et al., 2006; Westerhoff et al., 2004). THMs, such as chloroform ($CHCl_3$), containing only one carbon atom, correspond to the final degradation products resulting from the chlorination of complex organic molecules when exposed to free chlorine (Singer, 2006).

The use of chlorine as a disinfectant also could lead to reactions with organic matter that form other chlorinated products. Reactions of chlorine with PhACs have been identified by several studies (Bulloch et al., 2012; Quintana et al., 2010; Benotti et al., 2009; Xagoraraki et al., 2008; Gibs et al., 2007; Dodd and Huang, 2007; Pinkston and Sedlak, 2004; Bedner and MacCrehan, 2006a; Bedner and MacCrehan, 2006b; Glassmeyer and Shoemaker, 2005; and Boyd et al., 2003). These reports suggest that some PhACs may not be fully oxidized to carbon dioxide and water during chlorine disinfection. The disinfection byproducts may or may not have greater adverse human and environmental effects than the parent compounds (e.g., Boyd et al., 2005; Vogna et al., 2004), and their formation will result in the discharge of a mixture of unknown toxicity into the environment.

## 2.4 PHACs In The Environment

Traditional acute and chronic toxicity tests have been conducted on individual compounds and, in some cases, on mixtures of compounds. Another approach has been to focus on specific biological responses to whole municipal wastewater effluent exposure, rather than targeting specific PhACs or other contaminants (Ings et al., 2011; Metcalfe et al., 2010). Three major reviews of ecotoxicological literature have been conducted (Verlicchi et al., 2012; Santos et al., 2011; and Fent, 2006). Santos et al. (2011) found that most literature to date focused on endpoints such as growth, survival, reproduction and immobilization of species. There was very little literature available on transgenerational and population level studies, which are likely outcomes due to constant low-level exposure to PhACs (Boxall et al., 2012; Verlicchi et al., 2012; Santos et al., 2010, Ferrari et al., 2003). Even slight insignificant effects on single organisms that would not cause acute toxicity, could lead to population effects through reduced levels of fitness, disturbances in hormonal homeostasis, changes in immunological status, interferences in signal transduction or gene activation (Medina et al., 2007; Jos et al., 2003). One consequence of continuous low-level exposure is the potential for development of genetically inherited tolerance through selection of less-sensitive organisms. The toxicity of transformation products of different treatment processes is an on-going research question in the literature (Boxall et al., 2012; Escher and Fenner, 2011; Santos et al., 2010; Sanderson et al., 2004).

### 2.4.1 Biofilms

Biofilms are characterized as attached communities of microorganisms and their extracellular polymeric substances (EPS). Biofilms are often a major component of

biochemical oxygen demand removal within biological wastewater treatment processes, and also play an important role in nutrient cycling at the sediment/water interface in receiving waters. Bacteria are also at the base of the food web and thus negative impacts on the biofilm could affect higher trophic levels and nutrient cycling in an ecosystem (Rosi-Marshall et al., 2013). Interaction of biofilms with toxicants can result in two main responses: short-term physiological alterations and long-term changes in community structure (Rosi-Marshall et al., 2013; Sabater et al., 2007; Lawrence et al., 2007). These effects could occur rapidly in comparison to effects higher up the food chain due to the fast growth rate and life cycle of bacteria.

Exposure to selected PhACs has been shown to alter microbial activity in biofilms (Aristilde et al., 2013, Bonnineau et al., 2010). Biofilm and planktonic exposure experiments of anti-inflammatory drugs to the fungi *Candida albicans* (known to produce prostaglandins) were conducted by Alem and Douglas (2004). Aspirin and diclofenac in mM concentrations both inhibited biofilm growth significantly. Lawrence et al., (2005, 2007) conducted biofilm experiments on river water and assessed changes in community structure and function. Results showed reductions in population and species shifts resulting in changes in community composition. Yergeau et al. (2012) also examined exposure of biofilms to PhACs, including gemfibrozil, in river water. They observed some significant shifts in the active community. Naproxen, in the presence of chlorine, was found to have an adverse effect on biofilm in a bioreactor (Boyd et al., 2005).

## 2.5 GEMFIBROZIL

### 2.5.1 Background

Gemfibrozil (CAS 25812-30-0), is a lipid regulating drug with a molecular formula of $C_{15}H_{22}O_3$, a molecular weight of 250, a melting point of 61-63 °C, a boiling point of 158-159 °C, pKa of 4.79 and log $K_{ow}$ of 4.77. Gemfibrozil has a low solubility in water (19 mg/L) and is metabolized in the liver with approximately 70% being excreted in urine as a combination of the parent compound and metabolites (Zimetbaum et al., 1991). In 2009, gemfibrozil's prescription rate in Canada and the United States was 24 and 182 prescriptions per 100,000 people, respectively, as surveyed through the IMS Health database (Jackevicius et al., 2011).

### 2.5.2 Fate During Wastewater Treatment

In a survey of literature of raw and secondary biological treated wastewater, Verlicchi et al., (2012) found a range of gemfibrozil of 0.3 to 11 µg/L for raw influent wastewater and 0.003 to 7 µg/L for secondary biologically treated wastewater. Average removal rates through full-scale biological treatment processes range from approximately 50 – 70% (Verlicchi et al., 2012; Camacho-Munoz et al., 2011; Miège et al., 2008; Lishman et al., 2006; Paxeus, 2004; Stumpf et al., 1999; Ternes, 1998).

The partition coefficient kd have been shown to be variable with values ranging from 19 to 327 L/kgMLSS (Radjenovic et al., 2009; Urase and Kikuta, 2005). Joss et al. (2006) found that activated sludge processes resulted in greater removals of gemfibrozil compared to a membrane bioreactor, with calculated $K_{biol}$ for activated sludge of 6.4-9.6 L/ gss/d versus 0.5-1.8 L/gss/d for a membrane bioreactor, However, Radjenovic et al.

(2009), found that the opposite with membrane bioreactors achieving higher removal of gemfibrozil compared to activated sludge (90% versus 39 %.). Falås et al. (2012) found higher removal rates of gemfibrozil in reactors containing biofilm carriers versus reactors sludge reactors. A longer solids retention time and nitrification conditions were found to increase the removal of gemfibrozil in membrane bioreactors (Maeng et al., 2013).

Reactions between gemfibrozil and chlorine are pH dependent and up to 100% transformation of gemfibrozil through chlorine oxidation can be achieved (Westerhoff et al., 2005; Pinkston and Sedlak, 2004).

### 2.5.3 Presence in Receiving Waters

Brun et al., (2006) and Metcalfe et al., (2003b) conducted surveys of selected PhACs in Canadian wastewater treatment plants. Gemfibrozil was detected in effluent samples at concentrations of up to 1.4 μg/L. Furthermore, gemfibrozil was detected in receiving water up to 5 km downstream of the wastewater discharges. Araugo et al. (2011) found gemfibrozil to be resistant to photolysis and persistent for up to 150 days in simulated natural water and Grenni et al., (2013) found a biodegradation half-life for gemfibrozil of 70 days in a simulated river water ecosystem.

Using standardized toxicity tests, the $EC_{50}$ of gemfibrozil was determined to be in the low mg/L range using the organisms *Vibrio fischeri, Daphnia magna, Anabaena* sp. 4337, *Pseudokirchneriella subcapitata and L. minor* (Quinn et al., 2011; Rosal et al., 2010). However, with more sensitive biomarker tests using a zebra mussel, Quinn et al. (2011) showed that concentrations of gemfibrozil in the environmentally relevant range and as

low as 1μg/L produced signs of oxidative stress with elevated lipid peroxidation levels. Gemfibrozil and all fibrates are considered to be peroxisome proliferators, and there is evidence that gemfibrozil can inhibit *in vitro* testosterone production in rat Leydig cells (Liu et al., 1996), and in goldfish (5-fold decrease at nearly environmentally relevant concentrations) (Mimeault et al., 2005). In a study of four different aquatic systems, organism response to gemfibrozil was found to have the following sensitivities: *D. magna* > *V. fischeri* >PLHC-1 cells > *C. vulgaris*, the most sensitive bio-indicator being *D. magna* with an EC50 of 120 μM (Zurita et al., 2007).

In a study on the impact of gemfibrozil on lipid metabolism, steroidogenesis and reproduction in the fathead minnow, Skolness et al. (2012) found that exposure to gemfibrozil does affect lipid metabolism in fish, however no effect on sex steroids or reproduction was observed over a 21 day period.

### 2.5.4 Gemfibrozil Transformation Products

Photolysis of gemfibrozil in distilled water produced one photoproduct at 9% by weight (Isidori et al., 2007). The acute and chronic toxicity of both gemfibrozil and its reaction product were evaluated using several different organisms, and the genotoxicity and mutagenicity were evaluated using the SOS Chromotest and Ames test respectively. Their results indicate no acute or chronic effect to the bacterium *V. fischeri* (EC50) up to 200 mg/L for the photoproduct and an EC50 of 85 mg/L for gemfibrozil. Gemfibrozil showed no mutagenicity, and a genotoxicity of 2.5-5 mg/L, while the photoproduct

showed a mutagenicity concentration of 0.625 – 10 mg/L and genotoxicity of 1.25 – 5.0 mg/L.

Glassmeyer and Shoemaker (2005) indicated that gemfibrozil was susceptible to chlorination and their liquid chromatography-mass spectrometry (LC-MS) analysis of a 48-h reaction mixture containing gemfibrozil and free chlorine (1:2 molar ratio) showed the formation of a product at longer retention time. Bulloch et al., (2012) also confirmed the formation of this chlorinated reaction product and identified the formation of a brominated reaction product in the presence of bromide ions. The impacts on testosterone and 11-ketotestoterone levels in the Japanese medaka over 21-day exposure to gemfibrozil, the chlorinated and brominated reaction products were investigated (Bulloch et al., 2012). Exposure to the chlorinated reaction product at 55.1 μg/L showed significant reductions in the levels of 11-ketotestosterone, and exposure to 58.8 μg/L of the brominated reaction product resulted in significant reduction in testosterone levels.

## 2.6 SUMMARY

The literature review has shown that gemfibrozil undergoes transformations during wastewater treatment and in the environment. However there is still a paucity of information related to gemfibrozil and its transformation products during wastewater treatment processes and in the environment. This thesis will aim to contribute to the literature and better understand gemfibrozil during specific wastewater treatment processes (i.e., chlorination; biofilm processes).

# CHAPTER 3 COMMON MATERIALS AND METHODOLOGY

## 3.1 REAGENTS AND STANDARDS

The four pharmaceutically active compounds: gemfibrozil, naproxen, ibuprofen and diclofenac, as well as the surrogate standard meclofenamic acid and the derivatization agent BSTFA + TMCS, 99:1 (*N,O-bis*-[trimethylsilyl]trifluoroacetamide with 1% trimethylchlorosilane) and 5% dichlorodimethylsilane (DCDMS) in toluene used for glassware silylation were obtained from Sigma-Aldrich. HPLC grade methanol, acetone, ethyl acetate, toluene (all >99.9%), ascorbic acid and sodium thiosulphate used for quenching chlorine were purchased from Fisher Scientific. Aqueous 6% sodium hypochlorite (NaOCl) was purchased from VWR and diluted as necessary. The free chlorine concentration of stock solutions was determined prior to use by the *N,N*-diethyl-*p*-phenylenediamine (DPD) colorimetric method (APHA, AWWA, WEF, 1999). Oasis HLB solid phase extraction cartridges (3 $cm^3$, 6 mg sorbant) were purchased from Waters (Milford, US).

Stock solutions of the pharmaceuticals and surrogate standard in methanol (0.8 g/L) were stored in amber flasks at –18 °C and diluted as necessary for working solutions. Ultrapure water was obtained from a Milli-Q water generator (Millipore, USA).

## 3.2 Analysis of Pharmaceutically Active Compounds

### 3.2.1 Sample Preparation And Extraction Of Acidic Pharmaceuticals

Extraction and processing of samples was modeled after Yu et al., (2007) with deviations used to optimize recovery for PhACs used in this study. Significant method optimization was conducted on the sample extraction and derivatization procedures, but is described elsewhere (Koziar, 2007). Samples were acidified to pH 2 with 1.0 N sulphuric acid and stored at 4 ºC until extraction was performed. 40 µg/L of meclofenamic acid was added to each sample as a surrogate standard, and samples were filtered through a 0.75 µm glass fibre filter to remove suspended solids if required (i.e. for wastewater samples).

Extraction was conducted on a 20 position Waters vacuum extraction manifold using 3 $cm^3$, 60 mg Oasis HLB cartridges, used as received from Waters. Vacuum was adjusted according to desired flow rate through cartridges. Fresh cartridges were conditioned sequentially with 3 mL ethyl acetate, 3 mL acetone and 3 mL ultrapure water adjusted to pH 2. The sample was passed through the cartridge at a flow rate of approximately 10 mL/min. Cartridges were eluted with 6 mL of 50:50 (v:v) ethyl acetate:acetone. The eluate was retained in conical glass vials and evaporated under a gentle stream of nitrogen until complete dryness. The conical glass vials were silylated prior to use to minimize adsorption to glass surfaces with 1.5 mL of 5% DCDMS in toluene, followed by toluene (1.5 mL) and acetone (2 x 1.5 mL) rinses. Silylated vials were then fired in an oven at 104 °C for 12 h prior to use.

### 3.2.2 Derivatization Of Pharmaceuticals

150 µL of derivatizing agent BSTFA was added to residual eluate in the derivatization vial, and allowed to react for 90 minutes at 100 ºC. After derivatization, a clean Pasteur pipette was used to transfer the solution to a 200 µL glass insert inside a 2 mL GC vial. Samples were placed immediately on the autosampler tray and processed using GC-MS.

### 3.2.3 Gas Chromatography-Mass Spectrometry Of Acidic Pharmaceuticals

There was significant optimization carried out on the GC-MS methodology in order to increase sensitivity in the method and reduce the instrument detection limit. This optimization work is described elsewhere (Koziar, 2007). The following describes the optimized method.

Samples were analyzed on a Varian CP-3800 GC and Saturn 2200 Ion Trap Mass Spectrometer (MS) with CP-8400 autosampler. Syringe fill speed was 2.0 µL/s and injection speed was 0.5 µL/s. Pre-injection delay was 6.0 s, and the post-injection delay was 0.5 s. Total injected sample volume was 2.0 µL. The CP-1177 injector was held constant at 250°C (523 K). Helium of >99.999% purity was used as the carrier gas. The capillary column was a Varian Factor Four™ VF-5ms, with 29.5 m length x 0.25 mm internal diameter x 0.25 µm film thickness, preceded by a 5 m x 0.25 mm deactivated fused silica guard column. The guard column and Deactivated Universal Presstight connectors were purchased from Chromatographic Specialties (division of Restek). The GC temperature ramping program was as follows: 120 ºC [2 min] → (20 ºC /min) 215 ºC [2 min] → (2 ºC /min) 230 ºC [0 min] → (30 ºC /min) 290 ºC [2 min]. The total time for the method was 20.25 min. Retention times were 6.6 min for ibuprofen, 8.95 min for

gemfibrozil, 11.0 min for naproxen, 14.6 min for diclofenac, and 16.4 min for meclofenamic acid, 10.9 min for the postulated 6′-ClGem, 11.5 min for 4′-ClGem, 14.0 min for 4′,6′-diClGem, and 17.5 min for 3′,4′,6′-triClGem. A constant column flow of 1.0 mL/min was used. The ion trap was kept at a temperature of 185°C, and the transfer line was at 200°C. Electron ionization conditions of 70 eV were employed. The quantifier and qualifier ions used for the four PhACs and surrogate standard are presented in Table 3-1.

Table 3-1 - Quantifier and qualifier ions used for GC/MS analysis

| | **Quantifier Ion *m/z*** | **Qualifier Ions *m/z*** |
|---|---|---|
| Ibuprofen | 160 | 117,263 |
| Gemfibrozil | 201 | 55, 83 |
| Naproxen | 243 | 185,302 |
| Diclofenac | 242 | 214, 367 |
| Meclofenamic Acid | 242 | 244, 367 |

The instrument detection limit (IDL) for the GC-MS was determined by analyzing eight replicates at spiked concentrations of 20 ng/L and multiplying the standard deviation by the t-statistic for 6 degrees of freedom and $\alpha = 0.01$ (Berthouex and Brown, 2002). IDLs were as follows:

- Ibuprofen = 5.6 ng/150 uL derivatized sample
- Gemfibrozil = 10.0 ng/150 uL derivatized sample
- Naproxen = 5.9 ng/150 uL derivatized sample
- Diclofenac = 7.4/150 uL derivatized sample

Since this is a calculation of the IDL, the volume of the original sample pre-SPE was not relevant as SPE concentrates the analytes, and thus the IDL is reported as ng/volume of derivatized sample in the GC vial. Five-point calibration curves in the concentration range required for the experiment were developed using the same methodology as processed samples (filtration if required, SPE, drying, derivatization, GC/MS). Only

calibrations with an $R^2$ value greater than 0.9 were used, and only results with a signal to noise ration >10 were quantified, to ensure that results were above the reporting detection limit of the method.

### 3.2.4 Physicochemical Analysis Of Water

Samples were analyzed using Standard Methods for the examination of Water and Wastewater (American Public Health Agency (APHA), American Water Works Association (AWWA), Water Environment Federation (WEF), 1999). Ammonia was measured by Method 8038 on the HACH DR/2500 Spectrophometer. Orthophosphate was measured by Method 8039 on the HACH DR/2500 Spectrophometer. The free chlorine concentration was determined by the *N,N*-diethyl-*p*-phenylenediamine (DPD) colorimetric method (APHA, AWWA, WEF, 1999). pH was measured using an Orion Model 230A pH meter.

For chloride analysis, 10 mL samples were collected in 12 mL plastic Ion Chromatography tubes and analysis was performed on a Metrohm 761 Compact Ion Chromatograph with chemical suppression of eluent conductivity. The column used was a Metrosep A Supp 5-250/4.0 with eluent consisting of 3.2 mmol/L of $Na_2CO_3$ and 1.0 mmol/L $NaHCO_3$ and a flow rate of 0.7 mL/min.

# CHAPTER 4 IDENTIFICATION OF REACTION PRODUCTS FROM REACTIONS OF FREE CHLORINE WITH THE LIPID-REGULATOR GEMFIBROZIL

## 4.1 ABSTRACT

High global consumption rates have led to the occurrence of pharmaceutically active compounds (PhACs) in wastewater. The use of chlorine to disinfect wastewater prior to release into the environment may convert PhACs into uncharacterized chlorinated by-products. In this investigation, chlorination of a common pharmaceutical, the antihyperlipidemic agent gemfibrozil, was documented. Gemfibrozil (2,2-dimethyl-5-(2,5-dimethylphenoxy)pentanoic acid) was reacted with sodium hypochlorite and product formation was monitored by gas chromatography-mass spectrometry (GC-MS). The incorporation of one, two or three chlorine atoms into the aromatic region of gemfibrozil was demonstrated using negative-ion electrospray ionization mass spectrometry (ESI-MS) and tandem mass spectrometry (ESI-MS/MS). Further analysis using $^{1}$H nuclear magnetic resonance (NMR) spectroscopy identified the reaction products as 4′-ClGem (5-(4-chloro-2,5-dimethylphenoxy)-2,2-dimethylpentanoic acid) 4′,6′-diClGem (5-(4,6-dichloro-2,5-dimethylphenoxy)-2,2-dimethylpentanoic acid), and 3′,4′,6′-triClGem (5-(3,4,6-trichloro-2,5-dimethylphenoxy)-2,2-dimethylpentanoic acid), products consistent with electrophillic aromatic substitution reactions. The rapid reaction of gemfibrozil with free chlorine at pH conditions relevant to water treatment indicates that a mixture of chlorinated gemfibrozils is likely to be found in wastewater disinfected with chlorine.[*]

## 4.2 INTRODUCTION

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[*] Note: This Chapter is published in *Water Research:*

Krkošek, W. H.; Koziar, S. A.; White, R. L.; Gagnon, G. A. (2011) Identification of reaction products from reactions of free chlorine with the lipid-regulator gemfibrozil. *Water Res*., 45(3): 1414-1422.

Pharmaceutically active compounds (PhACs) enter the environment through a variety of pathways, including human or livestock excretion (treated or untreated), PhAC manufacturing discharge, or improper disposal of unused prescriptions into sewers or landfill sites (Ternes, 1998). Typically 12-20% of people in the United Kingdom dispose of unfinished prescriptions in household liquid wastes (Bound et al., 2006; Slack et al., 2005). In North America, PhAC concentrations in surface and ground waters have been quantified in the ng/L-µg/L range (Boyd et al., 2005; Brun et al., 2006; Kolpin et al., 2002; Metcalfe et al., 2003a and 2003b), and two Canadian surveys of wastewater treatment plants (WWTPs) detected gemfibrozil (a lipid regulating drug) in 7 out of 8 (Brun et al., 2006) and 3 out of 18 (Metcalfe et al., 2003a) WWTP effluents. Furthermore, literature reports have shown that PhACs are not fully degraded or removed through conventional WWTPs (Brun et al., 2006; Miao et al., 2002; Thomas and Foster, 2005).

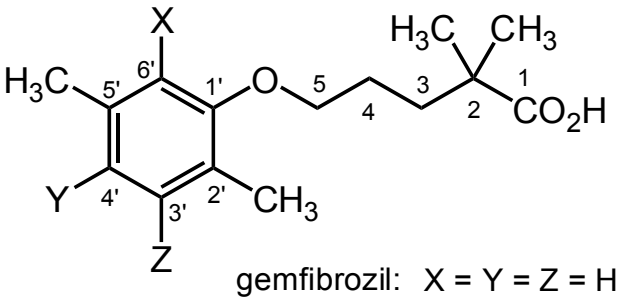
A survey (CWWA, 2001) by the Canadian Water and Wastewater Association indicated that 184 of 738 Canadian WWTPs used chlorine for disinfection. Free chlorine is a strong, non-specific oxidant, that reacts with most organic material (Sirivedhin and Gray, 2005; Pinkston and Sedlak, 2004) to form disinfection by-products (DBPs) (Sirivedhin and Gray, 2005). The reactions of natural organic matter and dissolved organic carbon with free chlorine to produce trihalomethanes (THMs) and haloacetic acids (HAAs) through drinking water treatment are well-documented (Inaba et al., 2006; Kanokkantapong et al., 2006; Westerhoff et al., 2004). THMs, such as chloroform ($CHCl_3$), containing only one carbon atom, correspond to the final degradation products

resulting from the chlorination of complex organic molecules when exposed to free chlorine. Chloroform is used in the water industry as a generic indicator for DBPs, and signifies the current or past presence of other chlorinated by-products in the sample (Singer, 2006).

The use of chlorine as a disinfectant also could lead to reactions with organic matter that form other chlorinated products. Reactions of chlorine with PhACs have been indicated by several studies (Benotti et al., 2009; Gibs et al., 2007; and Pinkston and Sedlak, 2004), while other investigations have provided evidence for the formation of chlorinated products from PhACs, such as acetominophen, gemfibrozil, naproxen and trimethoprim (Boyd et al., 2003; Bedner and MacCrehan, 2006a; Bedner and MacCrehan, 2006b; Dodd and Huang, 2007; Glassmeyer and Shoemaker, 2005; Quintana et al., 2010: and Xagoraraki et al., 2008). These reports suggest that PhAC contaminants in the waste stream may not be fully oxidized to carbon dioxide and water during chlorine disinfection. The disinfection byproducts may have greater adverse human and ecosystem effects than the parent compounds (e.g., Boyd et al., 2005; Vogna et al., 2004), and their formation will result in the discharge of a mixture of unknown toxicity into the environment.

The lipid regulator gemfibrozil (Figure 4-1) is frequently detected in drinking water, surface water and wastewater in the ng/L to low μg/L range (Batt et al., 2008; Benotti et al., 2009; Bueno et al., 2007; Gibs et al., 2007; Gros et al., 2006; Miao et al., 2002; Mompelat et al., 2009; Wu et al., 2009). Glassmeyer and Shoemaker (2005) indicated

that gemfibrozil was susceptable to chlorination and in their liquid chromatography-mass spectrometry (LC-MS) analysis of a 48-h reaction mixture containing gemfibrozil and free chlorine (1:2 molar ratio) showed the formation of a product at longer retention time. The presence of chlorine in the product was inferred by comparing *m*/*z* values of fragment ions formed from the product and gemfibrozil in the particle beam interface. The present investigation was conducted to identify the structure of this product as well as others formed upon reaction of gemfibrozil with free chlorine. The structures of three of the four possible ring chlorinated reaction products (Figure 4-1) were assigned using direct evidence collected by electrospray ionization mass spectrometry (ESI-MS) and $^{1}H$ nuclear magnetic resonance (NMR) spectroscopy.




gemfibrozil: X = Y = Z = H
4'-ClGem: Y = Cl; X = Z = H
6'-ClGem: X = Cl; Y = Z = H
4',6'-diClGem: X = Y = Cl; Z = H
3',4',6'-triClGem: X = Y = Z = Cl

Figure 4-1 Structure of gemfibrozil and the structures deduced for the gemfibrozil-HOCl reaction products.

## 4.3 MATERIALS AND METHODS

### 4.3.1 Standards And Reagents

Details are provided in Chapter 3.

### 4.3.2 Analytical Methods

Sample extraction and GC-MS analysis is detailed in Chapter 3. Negative-ion electrospray ionization mass spectroscopy (ESI-MS and ESI-MS/MS) was performed on a Thermo-Finnigan LCQ Duo ion trap using flow-injection analysis (20 μL/min) as described by Grossert et al., (2006). Collision energies for MS/MS are given in parentheses in the arbitrary units (%) supplied by the software. Solutions of gemfibrozil and the reaction products were prepared in methanol (1 mg/mL); the ESI needle was maintained at 3000-4000 V and the capillary at 200 °C. Accurate masses were determined on a Bruker microTof Focus orthogonal ESI-TOF. $^{1}H$, $^{13}C$ and NOESY (Nuclear Overhauser Enhancement Spectroscopy) NMR spectra were acquired on Bruker/Tecmag AC-250 (250 MHz) and Bruker Avance (500 MHz) spectrometers.

### 4.3.3 Initial Chlorination Reaction Conditions

Experiments were performed at a pH ranging from 3 to 9 in order to determine potential reaction products formed from the reaction between gemfibrozil and free chlorine. These experiments were conducted by reacting sodium hypochlorite with 40 μg/L of gemfibrozil in ultrapure water in an 850:1 molar ratio for various reaction times ranging from 0-60 min. Experiments were conducted in a 200 mL volume. At the end of the reaction period, blanks and samples were quenched with sodium thiosulphate in a 5:1 molar ratio to initial chlorine concentration. Samples were processed and analyzed by GC-MS as described in Chapter 3 for the presence of gemfibrozil as well as any potential

reaction products. Based on the results of the pH trials, several different conditions were then established where each of the reaction products was produced selectively, with all other reaction products and the parent compound constituting less than 5% of total peak area on the chromatogram.

### 4.3.4 Preparation Of Chlorinated Products

To produce adequate quantities of the individual reaction products for structural determinations, gemfibrozil was reacted under different conditions. Because of the low solubility of gemfibrozil in water, and the mass of chlorinated product required for identification purposes, gemfibrozil (200 mg) was first dissolved in methanol and the reactions were performed in aqueous methanol at ambient temperature. The minimum 55% methanol concentration required to keep 1 g/L of gemfibrozil in solution was experimentally determined. Molar ratios of NaOCl:gemfibrozil varied from 2:1 to 20:1 and the formation of the product was confirmed by GC-MS as described above. Comparisons of retention time and mass spectra of the products produced in the methanol mixutures with those found in the pH trials in ultrapure water described in section 4.3.3 confirmed that the same products were formed. Upon depletion of gemfibrozil, the reactions were quenched using ascorbic acid (2:1 molar ratio to initial NaOCl). The products were isolated as described below, and their structures were determined from ESI-MS and NMR data.

#### *4.3.4.1 Gemfibrozil*

δ (250 MHz, $CDCl_3$) 6.97 (1H, d, $J$ = 7 Hz), 6.66 (1H, d, $J$ = 7 Hz), 6.61 (1H, s), 3.93 (2H, t, $J$ = 6 Hz), 2.31 (3H, s), 2.18 (3H, s), 1.88-1.70 (4H, m), 1.26 (6H, s).

#### 4.3.4.2 4′-ClGem (5-(4-chloro-2,5-dimethylphenoxy) -2,2-dimethylpentanoic acid)

Two quenched 200-mL reaction mixtures (pH 8.0; ratio for NaOCl:gemfibrozil of 2:1; 3 h reaction time) were concentrated by rotary evaporation and filtered. The collected solid (366 mg) was recrystallized from aqueous methanol, yielding 348 mg: mp 82-83 ºC.

$^{1}$H NMR (250 MHz, $CDCl_3$) δ: 7.07 (1H, s), 6.62 (1H, s), 3.90 (2H, t, $J$ = 5.6 Hz), 2.31 (3H, s), 2.15 (3H, s), 1.85-1.67 (4H, m), 1.25 (6H, s); $^{13}$C NMR (125.8 MHz, $CDCl_3$) δ: 184.7, 155.7, 133.8, 130.7, 126.1, 125.0, 113.6, 68.4, 42.1, 36.9, 25.2, 25.1, 20.2, 15.7.

ESI-MS *m*/*z* (relative intensity) 285 (34), 283 (100, $[M - H]^-$), 157 (1), 155 (5), MS/MS (17%) of *m*/*z* 285 and 283 ($[M - H]^-$): *m*/*z* 157 and 155, respectively.

ESI-TOF *m*/*z* (relative intensity; calculated mass) 285.1073 (28; 285.1077, calc. for $C_{15}H_{20}{}^{37}ClO_3$), 283.1098 (100; 283.1106, calc. for $C_{15}H_{20}{}^{35}ClO_3$), $[M - H]^-$), 157.0244 (31; 157.0240, calc. for $C_8H_8{}^{37}ClO$), 155.0266 (100; 155.0269, calc. for $C_8H_8{}^{35}ClO$).

#### 4.3.4.3 4′,6′-diClGem (5-(4,6-dichloro-2,5-dimethylphenoxy) -2,2-dimethylpentanoic acid)

An unquenched 200-mL reaction mixture (pH 6.0; ratio for NaOCl:gemfibrozil of 5:1; 1 h reaction time) was concentrated by rotary evaporation and extracted with ether (2 x 25 mL). The ether was dried (anhydrous $MgSO_4$) and removed by rotary evaporation. The resulting yellow oil was dissolved in methanol/water (2:1) and applied to a $C_{18}$ extraction column (10 x 15 mm). The column was washed successively with methanol/water (2:1; 12 mL) and methanol (3 mL). Rotary evaporation of the methanol fraction yielded 32 mg of colorless oil.

$^{1}$H NMR (500 MHz, $CDCl_3$) δ: 7.13 (1H, s), 3.88 (2H, t, *J* = 5.7 Hz), 2.45 (3H, s), 2.28 (3H, s), 1.90-1.81 (4H, m), 1.30 (6H, s);$^{13}$C NMR (125.8 MHz, $CDCl_3$) δ: 184.8, 152.5, 133.0, 131.0, 129.55, 129.53, 129.3, 73.0, 42.1, 36.8, 25.9, 25.1, 17.8, 16.3.

ESI-MS *m*/*z* (relative intensity) 321 (11), 319 (64), 317 (100, $[M - H]^-$), 193 (11), 191 (63), 189 (100); MS/MS (15%) of *m*/*z* 321: *m*/*z* 285 (52), 283 (50), 193 (100); *m*/*z* 319: *m*/*z* 283 (31), 191 (100); *m*/*z* 317($[M - H]^-$): *m*/*z* 281 (3), 189 (100).

ESI-TOF *m*/*z* (relative intensity; calculated mass) 321.0657 (9; 321.0658, calc. for $C_{15}H_{19}{}^{37}Cl_2O_3$), 319.0674 (62; 319.0687, calc. for $C_{15}H_{19}{}^{37}Cl^{35}ClO_3$), 317.0706 (100; 317.0717, calc. for $C_{15}H_{19}{}^{35}Cl_2O_3$, $[M - H]^-$), 192.9821 (9; 192.9820, calc. for $C_8H_7{}^{37}Cl_2O$), 190.9844 (61; 190.9850, calc. for $C_8H_7{}^{37}Cl^{35}ClO$), 188.9877 (100; 188.9879, calc. for $C_8H_7{}^{35}Cl_2O$).

#### *4.3.4.4 3′,4′,6′-triClGem (5-(3,4,6-trichloro-2,5-dimethylphenoxy) -2,2-dimethylpentanoic acid)*

The quenched 200-mL reaction mixture (pH 6.0; ratio for NaOCl:gemfibrozil of 20:1; 24 h reaction time) was filtered. The solid collected (33 mg) was recrystallized from aqueous methanol, yielding 12 mg: mp 93-94 ºC.

$^{1}$H NMR (500 MHz, $CDCl_3$) δ: 3.83 (2H, t, *J* = 6.0 Hz), 2.50 (3H, s), 2.36 (3H, s), 1.84-1.79 (4H, m), 1.27 (6H, s);$^{13}$C NMR (125.8 MHz, $CDCl_3$) δ: 184.3, 152.5, 134.2, 132.1, 130.4, 129.1, 127.9, 73.5, 42.1, 36.8, 25.9, 25.1, 19.1, 15.2.

ESI-MS *m/z* (relative intensity) 355 (16), 353 (60), 351 (77, $[M - H]^-$), 227 (28), 225 (100), 223 (100).

ESI-TOF *m/z* (relative intensity; calculated mass) 357.0253 (2; 357.0238, calc. for $C_{15}H_{18}{}^{37}Cl_3O_3$), 355.0266 (27; 355.0268, calc. for $C_{15}H_{18}{}^{37}Cl_2{}^{35}ClO_3$), 353.0289 (95; 353.0297, calc. for $C_{15}H_{18}{}^{37}Cl{}^{35}Cl_2O_3$), 351.0317 (100; 351.0327, calc. for $C_{15}H_{18}{}^{35}Cl_3O_3$, $[M - H]^-$), 228.9412 (2; 228.9401, calc. for $C_8H_6{}^{37}Cl_3O$), 226.9427 (24; 226.9431, calc. for $C_8H_6{}^{37}Cl_2{}^{35}ClO$), 224.9455 (92; 224.9460, calc. for $C_8H_6{}^{37}Cl{}^{35}Cl_2O$), 222.9483 (100; 222.9490, calc. for $C_8H_6{}^{35}Cl_3O$).

## 4.4 Results And Discussion

### 4.4.1 GC-MS Evidence Of Gemfibrozil Chlorination

BSTFA derivatization of gemfibrozil-HOCl reaction mixtures and subsequent GC-MS analysis revealed the formation of three different reaction products during pH trials (Figure 4-2, upper chromatogram). The products eluted at longer GC retention times (10.9, 11.5 and 14.0), and their formation correlated with the depletion of gemfibrozil (GC ret. time 8.9 min). A fourth reaction product Figure 4-2, lower chromatogram, GC ret. time 17.5 min) was discovered when determining conditions to produce chlorinated products in methanol-water mixtures.

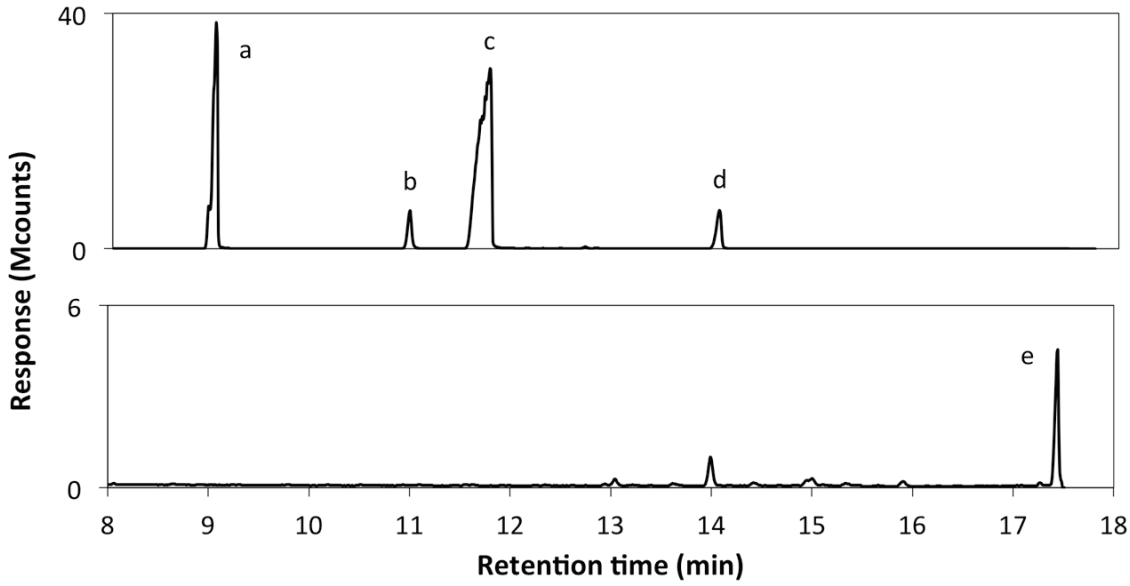



Figure 4-2 GC-MS analysis of a NaOCl:gemfibrozil reaction mixture (850:1 molar ratio). The upper chromatogram is a 30-min sample, showing the presence of gemfibrozil (a) and the formation of three reaction products (4′-ClGem (b), 6′-ClGem (c) and 4′,6′-diClGem(d)) at pH 8. The lower chromatogram is a 24-h sample, showing the accumulation of 3′,4′,6′-triClGem (e) at pH 6.

The electron ionization (EI) mass spectra of trimethylsilyl (TMS) derivatives of gemfibrozil and the reaction products each lacked a molecular ion ($M^{+\bullet}$), to assess the incorporation of chlorine, but displayed a base peak at *m*/*z* 201, indicating the formation of a common, abundant fragment ion. A similar fragmentation for the *tert*-butyldimethylsilyl derivative of gemfibrozil is indicated by the presence of the analogous ion at *m*/*z* 243 in its EI mass spectrum (Durán-Alvarez et al., 2009). Formation of the *m*/*z* 201 ion ($C_{10}H_{21}SiO_2$) from ionized TMS gemfibrozil is consistent with loss of the substituted aromatic ring ($C_8H_9O$) by C-O bond cleavage and the retention of charge on the TMS aliphatic portion of gemfibrozil. Formation of the same *m*/*z* 201 ion from the TMS reaction products indicates that reaction with HOCl does not incorporate chlorine

into the aliphatic region of gemfibrozil, leaving the aromatic portion of gemfibrozil as the site of reactions.

As noted by Sebök et al. (2009), a low intensity peak at *m*/*z* 307 was present in the EI mass spectrum of TMS gemfibrozil. The formation of the *m*/*z* 307 ion was attributed to the loss of $CH_3$ from the TMS group in the molecular ion of TMS gemfibrozil (*m*/*z* 322)). The *m*/*z* 307 peak was not present in the mass spectra of the reaction products, but corresponding low intensity peaks at *m*/*z* 341 and 375 indicated the incorporation of one chlorine into two reaction products (GC ret. times 10.9 and 11.5 min) and two chlorine atoms into the third reaction product (GC ret. time 14.0 min). The correlation of GC retention time with chlorine content suggested that the fourth reaction product eluting at 17.5 min was trichlorinated. The process used to determine the structures of the reaction products is described in the following sections.

### 4.4.2 Isolation Of Reaction Products

Reaction conditions were controlled to produce the three products separately. However, no conditions were found that produced one of the monochlorinated products in excess of the other products. 4′-ClGem formed selectively at pH 8 with a 2:1 molar ratio of hypochlorite to gemfibrozil and a reaction time of 3 h. At pH 6, the dichloro and trichloro reaction products (4′,6′-diClGem and 3′,4′,6′-triClGem) formed using higher molar ratios of hypochlorite to gemfibrozil, 5:1 and 20:1, and reaction times of 1 h and 24 h, respectively. The mixture containing 3′,4′,6′-triClGem was quenched with a 2:1 molar ratio of ascorbic acid to the initial sodium hypochlorite. Ascorbic acid was used rather then sodium thiosulphate to eliminate sulphur buildup in dried samples and interference

with further analytical procedures. The mixture containing 4′,6′-diClGem was not quenched as ascorbic acid interfered with the recovery process; however, it was processed immediately after the 1-h reaction time. The presence of a major product in each sample was confirmed by GC-MS. The isolated products were crystallized, except for the dichloro product, which was extracted from the reaction mixture and purified using a $C_{18}$ column to yield a colourless oil; structures were determined using ESI-MS and NMR spectroscopy.

### 4.4.3 Confirmation Of Gemfibrozil Chlorination

When analyzed by electrospray ionization mass spectrometry (ESI-MS) in the negative-ion mode (Figure 4-3A), the acidic pharmaceutical gemfibrozil ($C_{15}H_{22}O_3$) readily loses a proton to form a carboxylate anion at *m*/*z* 249 ($[C_{15}H_{21}O_3]^-$ or $[M - H]^-$) (Batt et al., 2008; Bueno et al., 2007; Gros et al., 2006; Hernando et al., 2004; Miao et al., 2002; Murai et al., 2004; Ramirez et al., 2007), whereas the ESI mass spectrum of the reaction product (GC ret. time 11.5 min) showed a predominant peak at *m*/*z* 283 (Figure 4-3B). The mass difference between these ions indicates that one hydrogen atom in gemfibrozil is substituted by chlorine during the reaction with HOCl. Further support for the incorporation of chlorine into the reaction product is provided by a peak at *m*/*z* 285 that is approximately one-third the intensity of the *m*/*z* 283 peak (Figure 4-3B, expanded view) reflecting the approximately 3:1 natural abundances of the $^{35}Cl$ and $^{37}Cl$ isotopes (de Laeter et al., 2003).

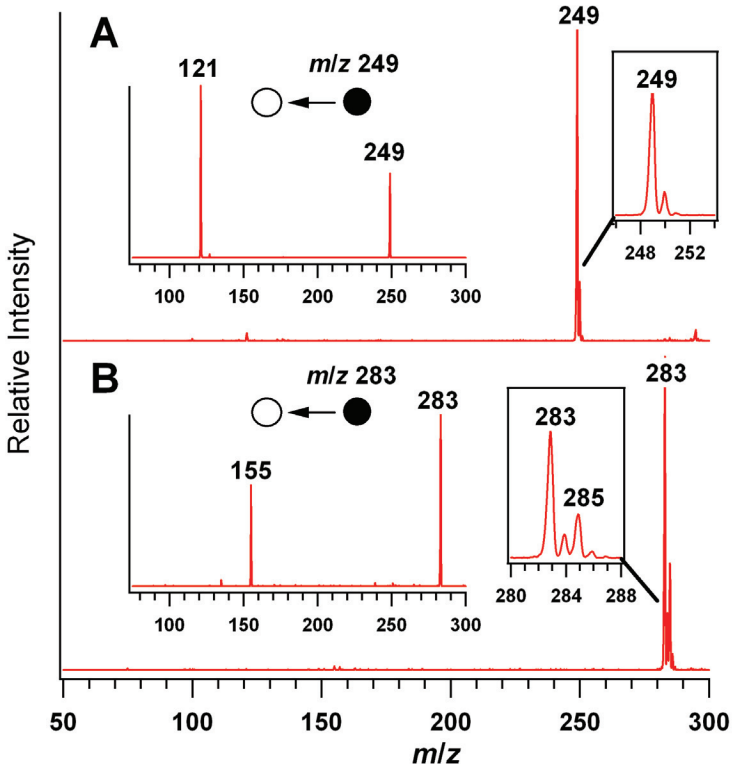



Figure 4-3 Negative ion ESI-MS and ESI-MS/MS (inset) spectra of gemfibrozil (A) and the 4′ monochlorinated reaction product (B) at 22 h reaction time. Note the 3:1 relative peak intensities for *m/z* 283 and 285 (B: expanded view).

ESI-MS/MS of deprotonated gemfibrozil (*m/z* 249) yielded a product ion at *m/z* 121 (Figure 4-3A inset) (Murai et al., 2004; Xia et al., 2003). The established precursor-product relationship between these anions has been used as a characteristic MS/MS transition for selected reaction monitoring determinations of gemfibrozil in surface water, wastewater, river sediments and fish (Batt et al., 2008; Bueno et al., 2007; Gros et al., 2006 and 2009; Löffler and Ternes, 2003; Miao et al., 2002; Ramirez et al., 2007; Wu et al., 2009). Accurate mass measurements (Farré et al., 2008; Petrovic et al., 2006) and isotopic labeling results (Batt et al., 2008) establish the *m/z* 121 ion as the 2,5-dimethylphenolate anion ($C_8H_9O^-$) formed from the aromatic portion of gemfibrozil.

Similar accurate mass determinations and MS/MS experiments (e.g., Figure 4-3B inset) on the reaction product ions at *m*/*z* 283 ($C_{15}H_{20}O_3{}^{35}Cl$) and 285 ($C_{15}H_{20}O_3{}^{37}Cl$) yielded product ions at *m*/*z* 155 ($C_8H_8O^{35}Cl$) and 157 ($C_8H_8O^{37}Cl$), respectively, confirming the aromatic substructure of gemfibrozil as the site of chlorine incorporation.

The ESI mass spectra of the two compounds (GC ret. times of 14.0 and 17.5 min) isolated from the pH 6 reaction mixtures showed clusters of peaks at the *m*/*z* values expected for the di- and trichlorogemfibrozil anions (Figure 4-4, expanded views). ESI-MS/MS of the dichlorogemifibrozil anion (e.g., Figure 4-4A, inset) yielded dichloro-2,5-dimethylphenolate product ions, while trichloro-2,5-dimethylphenolate anions were obtained upon electrospray ionization of the trichlorogemfibrozil anion (Figure 4-4B). The relative intensities of the peaks in each cluster were consistent with the natural abundances of the $^{35}Cl$ and $^{37}Cl$ isotopes (de Laeter et al., 2003), and accurate mass determinations (ESI-TOF) support the assigned compositions. Thus both the initial and subsequent chlorinations occurred on the aromatic portion of gemfibrozil.

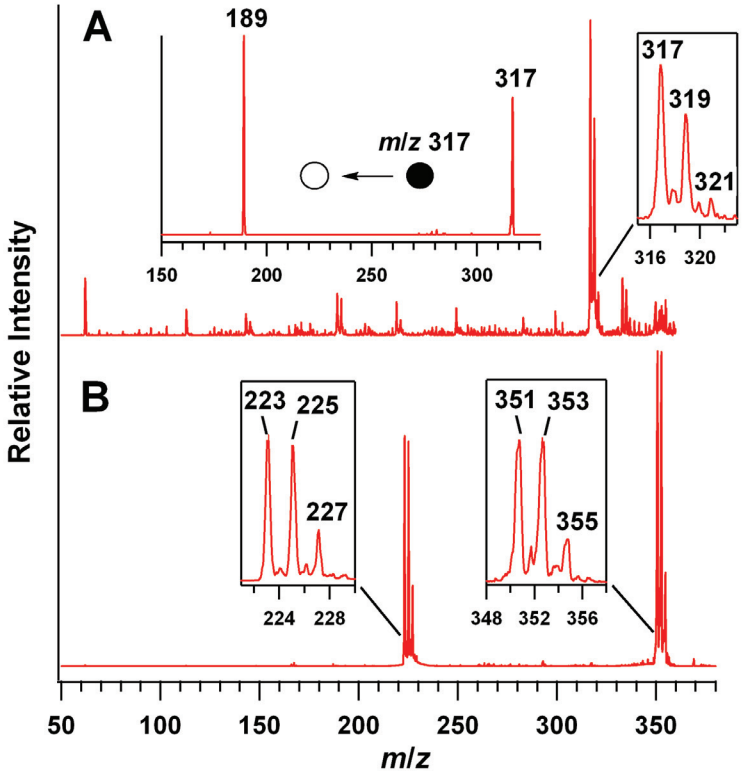



Figure 4-4 Negative ion ESI-MS of the 4',6'-diClGem (A) and 3',4',6'-triClGem (B) reaction products and the ESI-MS/MS spectrum of 4',6'-diClGem (A:insert). The peak clusters (expanded views) show the expected relative intensities for the di- and trichlorinated ions.

### 4.4.4 Location Of Chlorination Sites

Metabolic oxidation in the aromatic substructure of gemfibrozil has been documented (Baer et al., 2009; Murai et al., 2004; Thomas et al., 1999; Xia et al., 2003), specifically at C4′, and the C2′ and C5′ methyl groups. To distinguish among the several different possible chlorination sites, the $^{1}$H NMR spectra of gemfibrozil and the reaction products were acquired. The $^{1}$H NMR spectrum of the reaction products only differed from the gemfibrozil spectrum in the aromatic chemical shift range (Figure 4-5). While three distinct resonances were observed for the aromatic protons of gemfibrozil (Figure 4-5A), only two resonances were located in the aromatic region in the $^{1}$H NMR spectrum (GC

retention time 11.5 min) of the reaction product (Figure 4-5B), establishing the presence of chlorine on the aromatic ring.

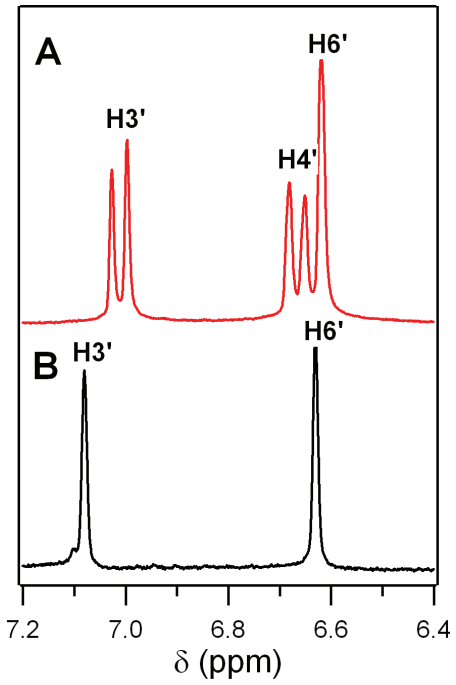



Figure 4-5 Partial $^{1}$H-NMR (250 MHz) spectra showing the aromatic proton resonances of gemfibrozil (A) and 4′-ClGem (B). The remainder of the spectra showed similar patterns of resonances.

Given the similar shielding effects experienced by aromatic protons ortho and para to an oxygen substituent (Pretsch et al., 2000), it is likely that the H4′ and H6′ protons in gemfibrozil have similar chemical shifts, assigning the doublets at δ 6.97 and 6.66 to H3′ and H4′, respectively, and the singlet at δ 6.61 to H6′. This is consistent with literature assignments (Murai et al., 2004; Thomas et al., 1999); the different relative chemical shifts reported for the H4′ doublet and the H6′ singlet are attributed to solvent effects. The coupling observed between the adjacent H3′-H4′ aromatic protons in gemfibrozil is absent in the $^{1}$H NMR spectrum of the reaction product (Figure 4-5), locating chlorine at

C3′ or C4′. Because a chlorine substituent on an aromatic ring does not have a large influence on the chemical shifts of protons on the ring (Pretsch et al., 2000), the resonance at δ 7.07 was assigned to H3′ of the chlorinated product. In the NOESY spectrum of the chlorinated product, the resonance at δ 7.07 (H3′) only correlated with one at δ 2.31 (2′ $CH_3$), whereas resonances at δ 2.14 (5′ $CH_3$) and δ 3.90 (H5) correlated with the resonance at δ 6.62 (H6′), establishing the proximity of H6′ (δ 6.62) and the aliphatic chain. Therefore, the spectral data are most consistent with 5-(4-chloro-2,5-dimethylphenoxy)-2,2-dimethylpentanoic acid (Figure 4-1; 4′-ClGem) as the structure of the major monochlorinated product. Moreover, the location of chlorine at C4′ is consistent with the directing effect of oxygen (Eğe, 2004) in the electrophilic aromatic substitution reaction between free chlorine and the aromatic ring of gemfibrozil.

Using LC-MS with a particle beam interface, Glassmeyer and Shoemaker (2005) observed the degradation of gemfibrozil and formation of a single product when gemfibrozil was treated with hypochlorite. The different masses of fragment ions at *m/z* 122 and *m/z* 156 in the mass spectra of gemfibrozil and the reaction product, respectively, were attributed to the incorporation of chlorine. The location of chlorination was not determined; however, the reaction product is most likely the same as 4′-ClGem, the main monochlorinated reaction product isolated and identified in this investigation.

For the di- and trichloro products formed in the pH 6 reaction mixtures, the resonances in the aliphatic region of their $^1$H NMR spectra had similar chemical shifts and coupling patterns to those in the spectra of gemfibrozil (Murai et al., 2004; Thomas et al., 1999)

and 4′-ClGem. Six distinct resonances between δ 155 and δ 125 in the $^{13}C$ NMR spectra are consistent with asymmetrically substituted aromatic rings. In the $^{1}H$ NMR spectra, a one-hydrogen singlet at δ 7.13, was found for the product of GC ret. time of 14.0 min and no aromatic resonances were observed for the product of GC ret. time of 17.5 min. The latter is consistent with the trichlorinated aromatic ring in 3′,4′,6′-triClGem (Figure 4-1), while the single aromatic hydrogen resonance (δ 7.13) is most consistent with H3′ in 4′,6′-diClGem.

In the reaction of free chlorine with gemfibrozil, GC-MS analysis (Figure 4-2) indicated a second monochlorinated reaction product (GC ret. time 10.9 min) eluting just before the main 4′ monochlorinated product (GC ret. time 11.5 min), characterized above as 4′-ClGem (Figure 4-1). The formation of a second monochlorinated gemfibrozil product would be consistent with the similar retention times and the oxygen-directed reactivity at the ortho position in electrophillic aromatic substitution reactions (Eğe, 2004). In gemfibrozil, the ortho position available for chlorination (C6′) is located between the oxygen substituent and the C5′ methyl group, and this steric crowding could account for a slower rate of chlorination at C6′ leading to the formation of a small amount of a second monochlorinated product. As mentioned previously it was not possible to create experimental conditions promoting the formation of this second monochlorinated reaction product in excess of the other reaction products. Thus it was not possible to confirm the location of the chlorine. The proposed formation of 5-(6-chloro-2,5-dimethylphenoxy)-2,2-dimethylpentanoic acid is based on initial GC-MS evidence and

the logical assumption that it corresponds to the fourth aromatic substitution product (Figure 4-1).

The gemfibrozil concentration of 40 μg/L used in the pH trials to initially identify the formation of gemfibrozil chlorinated reaction products was slightly higher than the concentrations expected in raw or treated wastewater (Brun et al., 2006; Paxeus, 2004; Metcalfe et al., 2003a and 2003b). However, the presence of chlorinated reaction products for several other pharmaceutical compounds have been confirmed in wastewater effluents (Quintana et al., 2010; Bedner and MacCrehan, 2006a, b) and salicylic acid reaction products have been found in finished drinking water and tap water samples (Quintana et al., 2010). Thus, it is possible for gemfibrozil chlorination reaction products to exist in finished drinking water and wastewater samples, and further research needs to be conducted to confirm their presence, pathways, kinetics of formation, and toxicological relevance in order to evaluate the potential risks to human and environmental health.

## 4.5 Conclusion

In conclusion, reactions between gemfibrozil and sodium hypochlorite under different experimental conditions revealed the formation of four chlorinated reaction products through GC-MS analysis. Three of the four compounds were formed under controlled reaction conditions and isolated either as crystals or oil. Characterization by GC-MS, ESI mass spectrometry and NMR spectroscopy led to the assignment of their structures as 5-(4-chloro-2,5-dimethylphenoxy)-2,2-dimethylpentanoic acid (4′-ClGem), 5-(4,6-dichloro-2,5-dimethylphenoxy)-2,2-dimethylpentanoic acid (4′,6′-diClGem) and 5-(3,4,6-

trichloro-2,5-dimethylphenoxy)-2,2-dimethylpentanoic acid (3′,4′,6′-triClGem). Through GC-MS evidence alone, 5-(6-chloro-2,5-dimethylphenoxy)-2,2-dimethylpentanoic acid (6′-ClGem) was proposed as the fourth reaction product. Based on the reaction conditions used in this paper, it is possible that during wastewater chlorination, gemfibrozil may react to form one or more of these byproducts, which would then be discharged to a receiving water. There is no toxicological data to date for these byproducts and thus the potential impact on biota in receiving environments is unknown.

# CHAPTER 5 FORMATION KINETICS OF GEMFIBROZIL CHLORINATION REACTION PRODUCTS: ANALYSIS AND APPLICATION

## 5.1 ABSTRACT

Aqueous chlorination kinetics of the lipid regulator gemfibrozil and the formation of reaction products were investigated in ultrapure water over the pH range 3-9, and in two wastewater matrices. Chlorine oxidation of gemfibrozil was found to be highly dependent on pH. No statistically significant degradation of gemfibrozil was observed at pH values greater than 7. Gemfibrozil oxidation between pH 4 and 7 was best represented by first order kinetics. At pH 3, formation of three reaction products was observed. 4'-ClGem was the only reaction product formed from pH 4-7 and was modeled with zero order kinetics. Chlorine oxidation of gemfibrozil in two wastewater matrices followed second order kinetics. 4'-ClGem was only formed in wastewater with pH below 7. Ultrapure water rate kinetic models were applied to two wastewater effluents with gemfibrozil concentrations reported in literature in order to calculate potential mass loading rates of 4'-ClGem to the receiving water.[†]

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[†] Note: A version of this chapter is in press at the journal *Water Environment Research:*

Krkošek, W. H.; Peldszus, S.; Huck, P. M. ; Gagnon, G. A. (2014) Formation Kinetics of Gemfibrozil Chlorination Reaction Products: Analysis and Application. *Water Env. Res.*, *In Press.*

## 5.2 INTRODUCTION

Pharmaceutically active compounds (PhACs) have been detected in wastewater effluent, surface water and drinking water (raw and treated) (Westerhoff et al., 2005; Kolpin et al., 2002; Brun et al., 2006; Metcalfe et al. 2003a, b; Ternes, 1998). Their removal within engineered and natural systems depends on many physical and chemical parameters. Elimination of the parent compound during treatment does not necessarily eliminate risk. PhACs may undergo metabolism or transformation within treatment processes that lead to formation of metabolites or transformation products of unknown toxicity (Mompelat et al., 2009; Xagoraraki et al., 2008; Dodd and Huang, 2007; Westerhoff et al., 2005; Boyd et al., 2005; Glassmeyer and Shoemaker, 2005).

Chlorination for wastewater disinfection is still a widely used process in North America, (Pinkston and Sedlak, 2004; Canadian Water and Wastewater Association (CWWA), 2001) mainly due to the low cost and availability of chlorine. During chlorination organic matter is oxidized, however; complete mineralization of organic matter to carbon dioxide and water is often not achieved. Incomplete mineralization may lead to the formation of known disinfection byproducts including trihalomethanes and haloacetic acids, which are regulated compounds in drinking water in many jurisdictions (Krasner et al., 2006; Singer, 2006; Richardson, 2005), but not wastewater. Furthermore, unknown reaction products may also be formed, some of which have been found to be biologically active, in ultrapure water (Moriyama et al., 2004), drinking water (Hu et al., 2002a, b, 2003) and wastewater (Bedner and MacCrehan, 2006). These reaction products can persist for long periods of time, even in the presence of residual chlorine in drinking water (Deborde and

von Gunten, 2008; Dodd and Huang, 2007) and wastewater matrices (Dodd and Huang, 2007; Inaba et al., 2006).

Gemfibrozil (CAS 25812-30-0), a fibrate (a class of amphipathic carboxylic acids) is a lipid regulating drug with a molecular formula of $C_{15}H_{22}O_3$, a molecular weight of 250, a melting point of 61-63 °C, a boiling point of 158-159 °C, and pKa of 4.7. It has low solubility in water (19 mg/L) and is metabolized in the liver, with approximately 70% being excreted in urine as a combination of the parent compound and metabolites (Zimetbaum et al. 1991). Chlorine oxidation of gemfibrozil is dependent on pH, and removals of up to 100% of the parent compound can be achieved (Westerhoff et al., 2005; Pinkston and Sedlak, 2004).

In a previous study (Krkošek et al., 2011), chlorination of gemfibrozil led to the formation of four different reaction products. The formation of one of these products, 4'-ClGem, following chlorination in laboratory experiments, was confirmed by Bulloch et al. (2012) in ultrapure water and a wastewater matrix. However, the rate and formation potential of these compounds is as yet undetermined. The objectives of this paper were to determine the formation kinetics of gemfibrozil reaction products upon oxidation of gemfibrozil with hypochlorous acid/hypyochlorite ion over a wide pH range in ultrapure water and also within wastewater matrices. The kinetic models were then applied to real world examples to determine the potential mass loading of gemfibrozil reaction products to receiving waters.

## 5.3 Materials And Methods

### 5.3.1 Standards And Reagents

General standards and reagents are presented in Chapter 3. 4'-ClGem (5-(4-chloro-2,5-dimethylphenoxy)-2,2-dimethylpentanoic acid) was produced in the laboratory by reacting sodium hypochlorite with gemfibrozil at a 2:1 molar ratio at pH 6 for 3 hrs as described in Krkošek et al. (2011). The quality of the crystallized reaction product was confirmed using GC-MS. Sodium thiosulphate ($Na_2S_2O_3$) solutions in ultrapure water were used to quench free chlorine after desired contact times.

### 5.3.2 Ultrapure Water Chlorination Reaction Conditions

For ultrapure water kinetic trials, water was buffered (0.02 mM) to establish pH values ranging from 3-9. Boric acid ($pK_a$ 9.24) was used to buffer to pH 9.0 and 8.0, phosphate buffer ($pK_{a2}$ = 6.82) was used for pH 7.0 and 6.0, acetic acid ($pK_a$ = 4.76) for pH 5.0 and 4.0 and formic acid for pH 3.0 ($pK_a$ 3.7). pH was measured throughout the trials and did not deviate from the buffered values by more than 0.2 units.

Experiments were performed with 200 mL of buffered ultrapure water. All contact times: 0, 0.5, 2, 5, 10, 15, 30, and 60 minutes were conducted in triplicate. 40 µg/L of gemfibrozil was added to each flask. Sodium hypochlorite stock solution was added to the 200-mL flask to obtain a measured concentration of 10 mg/L of free chlorine (sum of hypochlorous acid and hypochlorite ion). These concentrations are consistent with other pharmaceutical chlorination studies (Acero et al., 2010; Xagorarki et al., 2008), and were chosen so that chlorine was in excess and not a rate limiting component, creating pseudo first order conditions.

After the reaction period, samples were quenched with a 5:1 molar ratio of $Na_2S_2O_3$:free chlorine. 40 µg/L of meclofenamic acid was then added as the surrogate standard, pH was adjusted to 2 and samples were processed according to the extraction procedures described in Chapter 3.

### 5.3.3 Wastewater Chlorination Reaction Conditions

Wastewater was gathered from two separate wastewater treatment facilities that employ chlorine disinfection processes. Wastewater was collected prior to disinfection, stored at 4 °C, and processed within 2 weeks of collection. Wastewater Treatment Plant A (WWTPA) is a secondary treatment plant with a capacity of 0.075 ML/day and treatment by primary clarification, trickling filters, secondary clarification and chlorine disinfection prior to discharge to a wetland. WWTPB is a primary treatment plant with a design flow rate of 15 ML/day and water treatment through bar screen and grit removal followed by clarification and chlorine disinfection with discharge to a marine environment. Physical and chemical analysis of the wastewaters is presented in Table 5-1. Chlorine demand curves were completed on both wastewaters to determine breakpoint chlorination, which was found to be 18 mg/L and 250 mg/L for WWTPA and WWTPB, respectively.

Table 5-1 Water quality analysis for effluent of the two wastewater treatment plants studied and free chlorine dose used for kinetic experiments.

| Type of Treatment | **Wastewater A** Trickling Filter, Clarification, Chlorine Disinfection | **Wastewater B** Primary Clarification, Chlorine disinfection |
|---|---|---|
| pH | 6.38 | 7.58 |
| TOC (mg/L) | 6.17 | 13.21 |
| DOC (mg/L) | 5.75 | 12.35 |
| $NH_3$-N (mg/L) | 1.75 | 34.5 |
| $PO_4^{3-}$(mg/L) | 8.2 | 2.4 |
| BOD | 22 | 76 |
| Dose $Cl_2$ (mg/L) | 25 | 270 |
| Residual free $Cl_2$ (mg/L) | 3.5 | 9.4 |

Wastewater trials were conducted using 200 mL of wastewater filtered through a 0.75 µm glass fiber filter, with chlorine contact times of 0, 0.5, 2, 5, 10, 15, 30 and 60 minutes conducted in triplicate. Wastewater samples were not pH buffered, but pH did not change significantly over the course of the trial. 40 µg/L of gemfibrozil was added to each flask. Sodium hypochlorite was then added to achieve a free chlorine concentration above breakpoint chlorination to ensure a residual, resulting in spiked chlorine dosages of 25 mg/L and 270 mg/L, and free chlorine residuals of 3.5 mg/L and 9.4 mg/L for WWTPA and WWTPB, respectively. After the reaction period, samples were quenched with a 5:1 molar ratio of $Na_2S_2O_3$:free chlorine. 40 µg/L of meclofenamic acid was added and the pH was lowered to 2 and samples were processed according to the extraction procedures described in Chapter 3.

## 5.4 Results And Discussion

### 5.4.1 Chlorine And Gemfibrozil Chemistry

Free chlorine is the sum of chlorine species $Cl_2$, hypochlorous acid (HOCl) and hypochlorite ($OCl^-$). The equilibrium distribution of chlorine species is described by (Wang and Margerum, 1994; Morris, 1966):

$$HOCl \rightleftharpoons OCl^- + H^+ \qquad pK_a=7.6 \text{ @ } 25°C \qquad (1)$$

Hypochlorous acid is 80-100 times more reactive than hypochlorite (Droste, 1997) and thus pH has a strong impact on reactions between chlorine and organic micropollutants, such as gemfibrozil. At pH values below 7, nearly 100 % of free chlorine is present as HOCl, with $OCl^-$ dominating at pH greater than 8 (Droste, 1997).

At lower pH, increases in PhAC reaction rate have been observed in several studies under both ultrapure water (Dodd and Huang, 2007; Deborde et al., 2004; Rule et al., 2005; and Gallard and von Gunten, 2002) and wastewater conditions (Dodd and Huang, 2007) and it has been attributed to the presence of a strong oxidant species other than HOCl. Further research has shown that for solutions containing high chloride concentrations, the main oxidant species present below a pH of 5 is $Cl_2$ (Eq. (2)) (Cherney et al., 2006; Deborde and von Gunten, 2008). Chlorine intermediates, including $Cl_3^-$, $Cl_2O$ and $H_2OCl^+$, may also be present at low concentrations. The presence of $Cl_2$ is a function of temperature, pH and chloride concentration (Deborde and von Gunten 2008):

$$Cl_2 + H_20 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} HOCl + H^+ + Cl^- \qquad K_{Cl2} = k_1/k_{-1} \qquad (2)$$

At the point where [HOCl]=[$Cl_2$], the equilibrium reaction for Eq. (2) can be written as:

$$pK_{Cl_2} = -\log[Cl^-] + pH \qquad (3)$$

Several estimates for $K_{Cl2}$ from literature include 5.1 x $10^{-4}$ $M^2$ (Wang and Margerum, 1994), and 2.56 x $10^{-4}$ $M^2$ (Cherney et al., 2006). The Chloride:HOCl molar ratio in the NaOCl stock solution used for this research was determined to be approximately 1:1 at a pH of 11 and room temperature. Based on this concentration, using the two $K_{Cl2}$ constants given above, the pH at which [HOCl]=[$Cl_2$] for this study would be approximately 0, which is well below the pH used in these experiments. Thus it can be concluded that $Cl_2$ is not a dominant species at the pH values used, and oxidation at pH values below 7.5 would occur either by HOCl or the chlorine intermediates $Cl_3^-$, $Cl_2O$ and $H_2OCl^+$.

Gemfibrozil is a carboxylic acid that can be present either in its protonated or deprotonated form:

$$GEM \rightleftharpoons GEM^- + H^+ \qquad \text{pKa=4.7 @ 25°C} \qquad (4)$$

A previous study (Krkošek et al. 2011) has shown the formation of four gemfibrozil reaction products under varying chlorination conditions. Chlorine was incorporated into

the aromatic portion of gemfibrozil for these four reaction products. Two of the reaction products are singly chlorinated, one is dichlorinated and the final is trichlorinated:

4′-ClGem - 5-(4-chloro-2,5-dimethylphenoxy)-2,2-dimethylpentanoic acid,

6′-ClGem - 5-(6-chloro-2,5-dimethylphenoxy)-2,2-dimethylpentanoic acid,

4′,6′-diClGem - 5-(4,6-dichloro-2,5-dimethylphenoxy)-2,2-dimethylpentanoic acid

3′,4′,6′-triClGem - 5-(3,4,6-trichloro-2,5-dimethylphenoxy)-2,2-dimethylpentanoic acid

The pKa's of the reaction products are as yet unknown, however they would all be involved in acid/base equilibrium similar to that described for gemfibrozil in Eq. (4). The chlorine substitutions occur on the aromatic portion of gemfibrozil (and the reaction products), which are at a distance from the carboxylic functional group. Hence, it is anticipated that the form (protonated/deprotonated) of the original drug and reaction products is not expected to have a great effect on the reaction rate (Pinkston and Sedlak, 2004).

### 5.4.2 Hypothesized Reaction Pathway

At pH below 4.7, reactions would occur between the protonated form of gemfibrozil and HOCl (total gemfibrozil ($GEM_T$) in Eq. (5) is predominantly GEM). At pH values between 4.7 and 7.5 (the pKa's for gemfibrozil and hypochlorous acid, respectively), reactions would occur with deprotonated forms of gemfibrozil (i.e. $GEM_T$ in Eq. (5) would be predominantly in the form $GEM^-$). Similar protonated/deprotonated pH relationships would exist for the reaction products (shown in Eq. (6)-(8)) but their $pK_a$'s are as yet unknown.

$$[GEM_T] = [GEM] + [GEM^-] \quad (5)$$

$$[ClGEM_T] = [ClGEM] + [ClGEM^-] \quad (6)$$

$$[diClGEM_T] = [diClGEM] + [diClGEM^-] \quad (7)$$

$$[triClGEM_T] = [triClGEM] + [triClGEM^-] \quad (8)$$

For pH greater than 7.5, with $OCl^-$ as the main oxidizing species, it is anticipated that there will be little gemfibrozil oxidation as HOCl is generally a stronger oxidant than $OCl^-$, and previous studies of gemfibrozil reactions with chlorine showed an increase in reaction rate with decrease in pH (Pinkston and Sedlak, 2004). The following equations for reactions between gemfibrozil and reaction products with free chlorine were written with HOCl as the dominant oxidizing species. Hypochlorous acid may react with gemfibrozil to form either 4'-ClGem or 6'-ClGem (depicted by $ClGEM_T$):

$$HOCl + GEM_T \rightleftharpoons ClGEM_T + H_2O \quad (9)$$

Similarly, hypochlorous acid can react with gemfibrozil to produce the dichlorinated reaction product 4',6'-DiClGem, or hypochlorous acid can react with either of the singly chlorinated reaction products produced in Eq. (9) to form the dichloro product:

$$2HOCl + GEM_T \rightleftharpoons diClGEM_T + 2H_2O \quad (10)$$

$$HOCl + ClGEM_T \rightleftharpoons diClGEM_T + H_2O \quad (11)$$

The triClGem product can be formed by similar processes, it may be formed directly through reactions with gemfibrozil, or through intermediate reactions involving mono or dichloro species.

### 5.4.3 Effect Of pH On Reaction Kinetics

#### *5.4.3.1 Gemfibrozil Degradation*

Results of chlorine oxidation of gemfibrozil in ultrapure water varied significantly with pH. At pH 3 (Figure 5-1), gemfibrozil underwent 88% degradation in the first 0.5 minutes and no model or rate constant was applied due to lack of a sufficient number of data points in this region. This result is consistent with kinetic pH studies of gemfibrozil (Pinkston and Sedlak, 2004) and of other compounds (e.g., naproxen, metoprolol, and phenol), where an increase in reaction rate is often observed at lower pH (Acero et al., 2010; Deborde and von Gunten, 2008; Gallard and von Gunten, 2002). As mentioned above, this may be attributed to minor amounts of other oxidizing chlorine species ($Cl_3^-$, $Cl_2O$ and $H_2OCl^+$) in addition to HOCl (Dodd and Huang, 2007; Pinkston and Sedlak, 2004).

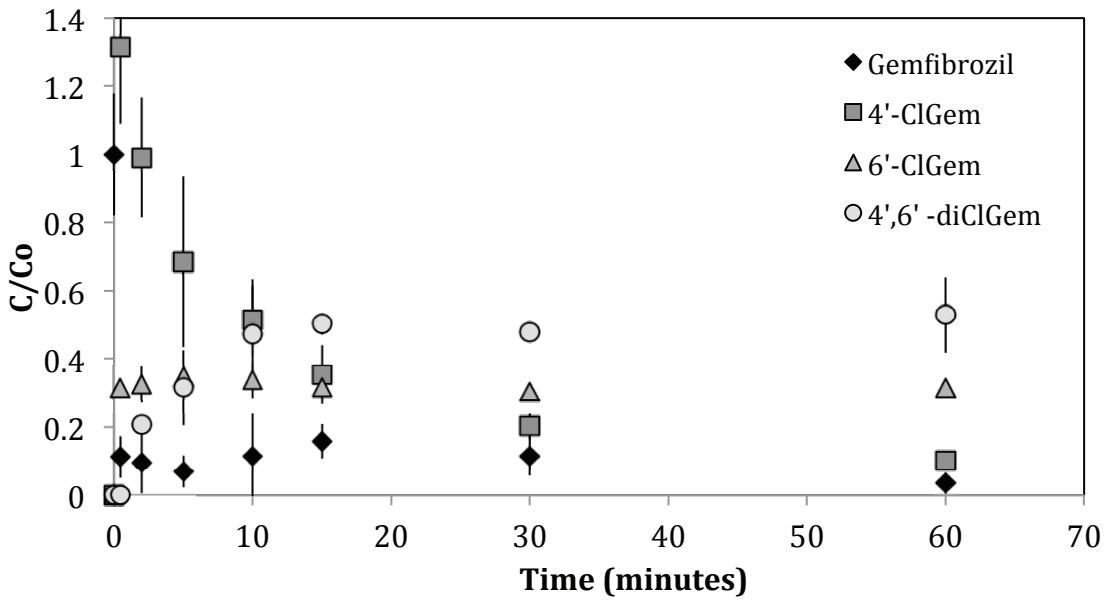


Figure 5-1 Degradation of gemfibrozil and formation of three reaction products in ultrapure water at pH 3. Data for 4'-ClGem are normalized to the initial concentration of gemfibrozil on a mass basis, whereas 6'-ClGem and 4',6'-diClGem concentrations were calculated based on chromatogram peak area relative to gemfibrozil peak area because standards were not available. Error bars represent one standard deviation of three replicate samples.

A pseudo-first order model Eq. (12) was found to describe the degradation of gemfibrozil from time 0-60 minutes for pH 4-7 Eq. (Figure 5-2), where HOCl is the dominant chlorine species. Half lives ranged from 4.7 minutes for pH 4 to 86.9 minutes for pH 7 (Table 5-2). Similarly, Pinkston and Sedlak (2004) reported a half-life for gemfibrozil of 93 minutes at a pH of 7 and chlorine concentration of 10 mg/L. Consistent with this result, Westerhoff et al. (2005) observed gemfibrozil degradation rates close to 100% after a 24 hour contact time in river water with free chlorine residuals around 3-3.5 mg/L at a pH of 5.5, whereas removals at ambient pH (7-8) were reduced by 20%.

$$\frac{d[GEM_T]}{dt} = -k[GEM_T][HOCl] = -k_{obs}[GEM_T] \qquad (12)$$

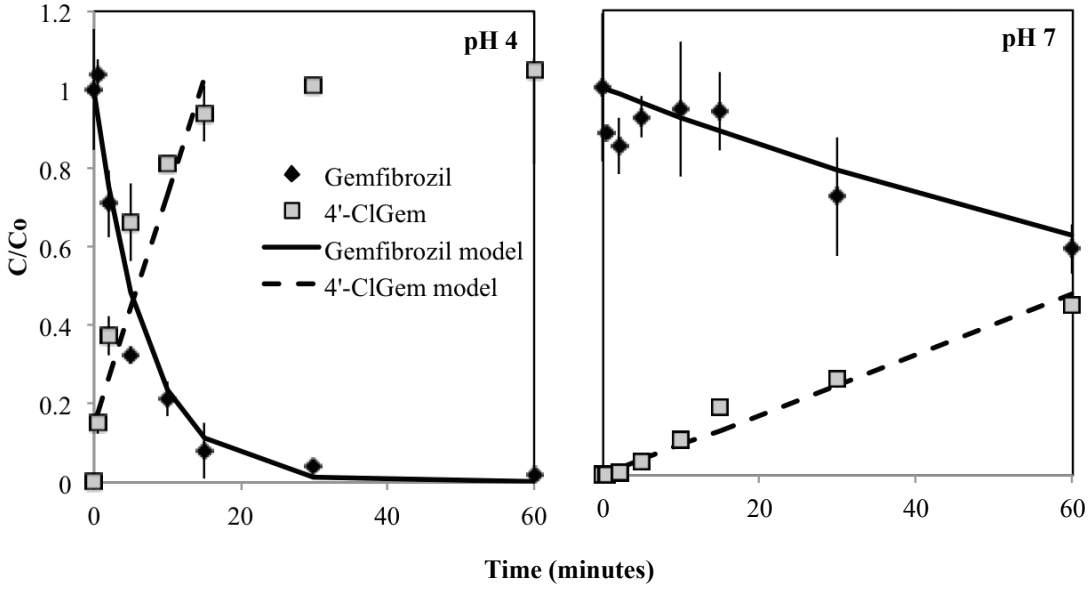


Figure 5-2 Degradation of gemfibrozil and formation of 4'-ClGem at pH 4 (left) and pH 7 (right) in ultrapure water. Pseudo first order models for degradation of gemfibrozil and zero order models for 4'-ClGem formation are included. Data are normalized to the initial gemfibrozil concentration. Error bars represent one standard deviation of three replicate samples.

Table 5-2 Summary of calculated rate constants for degradation of gemfibrozil in ultrapure water for pH values ranging from 3-9. The constant for pH 4 was calculated based on the time series 0-15 minutes whereas pH 5-9 were calculated based on the full 0-60 minute time series.

| pH | Order | $k_{obs}$ | $R^2$ | Half Life (min) |
|---|---|---|---|---|
| 3 | - | [1] | - | < 0.5 |
| 4 | 1st | $1.5x10^{-1}$ ($min^{-1}$) | 0.96 | 4.7 |
| 5 | 1st | $4.5x10^{-2}$ ($min^{-1}$) | 0.98 | 16 |
| 6 | 1st | $1.5x10^{-2}$ ($min^{-1}$) | 0.94 | 48 |
| 7 | 1st | $8.0x10^{-3}$ ($min^{-1}$) | 0.84 | 87 |
| 8 | - | [2] | - | |
| 9 | - | [2] | - | |

[1] – 88% depletion of gemfibrozil after 0.5 minutes

[2] - Slopes of model linear regressions were equal to zero ($\alpha$=0.05) indicating no degradation of gemfibrozil at pH 8 and 9.

As previously mentioned, it is anticipated that the protonated/deprotonated form of gemfibrozil will not have an affect on chlorination reactions due to the distance between

the carboxylic group and the aromatic ring at which chlorination occurs. This is shown through the difference in reaction rate between pH 3 and 4. At both of these pH values the protonated form of gemfibrozil is present, however pH 4 can be modelled as a first order reaction similar to pH 5-7, whereas reactions at pH 3 occurred so rapidly that it is indicative that other processes are involved, as described above. At pH 8 and 9, where $OCl^-$ is the dominant chlorine species, there was no significant degradation of gemfibrozil as determined by comparing linear regression modelled slopes to zero ($\alpha = 0.05$).

#### *5.4.3.2 4'-ClGem Formation And Degradation*

The formation of 4'-ClGem was also affected by pH and speciation of free chlorine. The rate of formation for 4'-ClGem was best described by zero order kinetics for the time range of 0-15 minutes for pH 4 and 5 and time range of 0-60 minutes for pH 6 and 7 (pH 4 and 7 shown in Figure 5-2, pH 5 and 6 not shown), with rate constants presented in Table 5-3.

Table 5-3 Summary of calculated rate constants for reaction products in ultrapure water.

| pH | Reaction Product | Formation or Degradation | Order | $k_{obs}$ | $R^2$ |
|---|---|---|---|---|---|
| 3 | 4'-ClGem | Degradation | 1st | 0.12 $(min^{-1})$[1] | 0.95 |
| 3 | 4',6'-DiClGem | Formation | 0 | 0.03 $(\mu g/L \cdot min)^2$ | 0.79 |
| 4 | 4'-ClGem | Formation | 0 | 3.0 $(\mu g/L \cdot min)^2$ | 0.87 |
| 5 | 4'-ClGem | Formation | 0 | 3.3 $(\mu g/L \cdot min)^2$ | 0.96 |
| 6 | 4'-ClGem | Formation | 0 | 0.59 $(\mu g/L \cdot min)^3$ | 0.99 |
| 7 | 4'-ClGem | Formation | 0 | 0.34 $(\mu g/L \cdot min)^3$ | 0.97 |
| 8 | 4'-ClGem | - | - | [4] | - |
| 9 | 4'-ClGem | - | - | [4] | - |

[1] 4'-ClGem at pH 3 was formed between 0 and 0.5 min, the constant for the subsequent degradation was calculated from 0.5-60 min.
[2] Constants for 4',6'-DiClGem at pH 3 and 4'-ClGem at pH 4 and 5 were calculated for the time series of 0 - 15 minutes.
[3] Constants for 4'-ClGem for pH 6 and 7 were calculated for time series 0-60 min.
[4] – Slopes of model linear regressions were equal to zero ($\alpha = 0.05$) indicating no formation of 4'-ClGem at pH 8 and 9.

For pH 4, 4'-ClGem reached steady state by 60 minutes. For pH 5 to 7, it was observed that 4'-ClGem formation continued to increase up to 60 minutes, which is consistent with results by Bulloch at al. (2012) in unbuffered Ultrapure water. No significant formation of 4'-ClGem occurred with $OCl^-$ as the dominant species at pH 8 and 9 (Table 5-3) based on statistical analysis of modelled linear regression slopes ($\alpha = 0.05$). At pH 3 there was a 1:1 molar conversion of gemfibrozil to 4'-ClGem in the first 0.5 minutes, but 4'-ClGem was not stable at this pH and followed first order degradation kinetics from 0.5-60 minutes ($k = 0.119\ min^{-1}$). For pH 4-7, the conversion between gemfibrozil and 4'-ClGem followed a 1:1 molar ratio (taking into account standard deviations) up to 60 minutes for each pH value. Results for 4'-ClGem formation in Figure 5-1 and Figure 5-2 are normalized on a mass basis to the initial gemfibrozil concentration at time zero.

It is possible that other oxidizing free chlorine species present in small quantities ($Cl_3^-$, $Cl_20$ or $H_2OCl^+$) at pH 3 (Deborde and von Gunten, 2008; Pinkston and Sedlak, 2004) led to the rapid formation of 4'-ClGem and its subsequent degradation.

#### *5.4.3.3 Formation Of Other Reaction Products*

At pH 3, there was evidence of formation of 6'-ClGem and 4',6'-diClGem, but there was no evidence of the formation of 3',4',6'-triClGem. 6'-ClGem formed and reached a steady state concentration within the first 0.5 minutes (Figure 5-1). The formation of 4',6'-diClGem followed zero order formation from 0-15 minutes after which time it reached a steady state concentration. The formation of the dichloro product can likely be attributed to degradation of 4'-ClGem as described by Eq. (11) rather than through a reaction with gemfibrozil, as described by Eq. (10). This is because gemfibrozil

concentrations were depleted 88% in the first 0.5 minutes, while diClGem continued to form until 15 minutes, and during this time 4'-ClGem concentrations were declining.

There was no measured formation of 6'-ClGem, 4',6'-diClGem, or 3',4',6'-triClGem in any samples from pH 4-9. It is possible that over a longer contact time or higher chlorine concentration that some of these reaction products may form at pH values greater than 3. The trichloro reaction product was observed to be the dominant reaction product at a pH of 6 after 24 hours (Krkošek et al., 2011). Further, Quintana et al., (2010) and Xagoraraki et al., (2008) found the concentration of free chlorine to be an important factor for PhAC degradation and reaction product formation potential and kinetics.

It was not possible to isolate either 6'-ClGem or 4',6'-diClGem as crystallized neat compounds, and thus concentrations of these compounds were not calculated based on mass concentrations. Instead results were normalized by comparisons of chromatogram peak area of the reaction products, compared to the chromatogram peak area of gemfibrozil. Concentrations of 4'-ClGem were calculated on a mass basis using a calibration curve generated with solutions created from the crystallized neat compound.

### 5.4.4 Degradation Of Gemfibrozil And Formation Of Reaction Products In Wastewater Effluents

#### *5.4.4.1 Gemfibrozil Degradation*

The two wastewaters used in this study were chosen as they had varying water quality (Table 5-1). WWTPA is a secondary treatment plant serving a small community with a low ammonia concentration of 1.75 mg/L and pH of 6.38. In contrast, WWTPB employs

only primary treatment, which results in a high ammonia concentration of 34.5 mg/L and a pH of 7.58. Chlorine demand curves were established resulting in breakpoint chlorination close to the expected 7.6 times the ammonia concentration at 18 and 250 mg/L for WWTPA and WWTPB, respectively. Chlorine doses of 25 mg/L and 270 mg/L were used, resulting in free chlorine residuals of 3.4 mg/L and 9.4 mg/L for WWTPA and WWTPB, respectively.

Figure 5-3 shows the degradation of gemfibrozil and formation of 4'-ClGem for each of the two wastewaters. Degradation for gemfibrozil in both wastewaters was best modelled by second order kinetics, with rate constants presented in Table 5-4. The increased rate of degradation of gemfibrozil in both wastewaters, compared to the ultrapure water trials, indicated that there were processes other than chlorine oxidation involved in transformation of gemfibrozil within the wastewater matrix. These processes could include biodegradation, transformation by other oxidizing agents present, or sorption. Gemfibrozil has a log $K_{ow}$ of 4.77 (Meylan and Howard, 1995), and $k_d$ in sewage sludge ranging from 19 -75 L/kg (Radjenovic et al., 2009; Urase and Kikuta, 2005). Although the wastewater was filtered (0.75 μm) prior to processing, dissolved organic matter could have been involved in sorption processes. The increased ionic strength, alkalinity, inorganic constituents, etc. could also affect the reaction rate. Transformation of gemfibrozil in wastewater could lead to the formation of additional transformation products other than 4'ClGem that may or may not be chlorinated. However, none were detected by the methods used in this study.

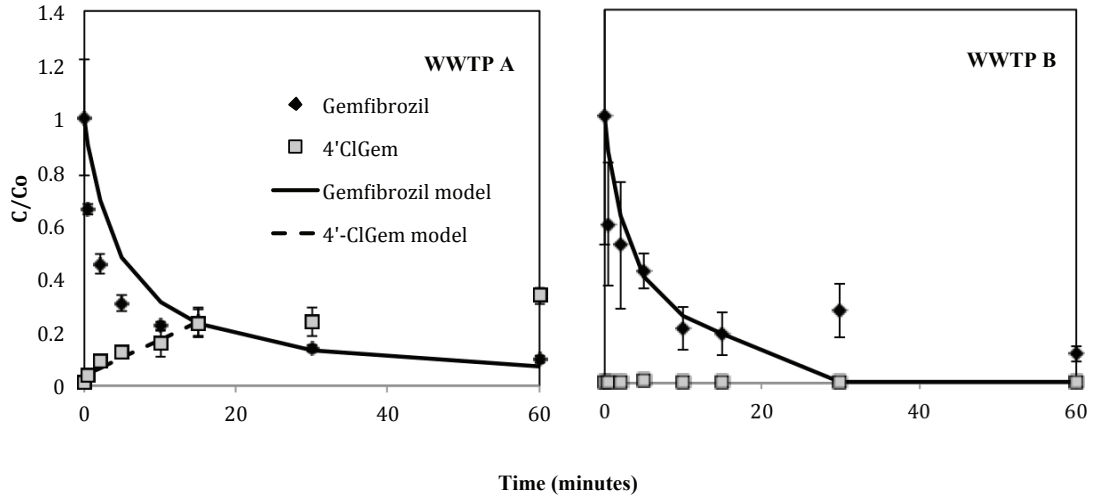


Figure 5-3 Degradation of gemfibrozil and formation of 4'-ClGem for WWTPA (left) with a pH of 6.38 and WWTPB (right) with a pH of 7.58. Second order models for degradation of gemfibrozil and zero order model (0-15 min) for 4'-ClGem formation for WWTPA are included. Data are normalized to the initial gemfibrozil concentration. Errors bars represent one standard deviation of three replicate samples.

Table 5-4 Summary of calculated rate constants for degradation of gemfibrozil and formation of 4'-ClGem in wastewater. Gemfibrozil degradation constants were calculated for the time series of 0-60 minutes, and 4'-ClGem formation in WWTPA was calculated for time 0-15 minutes.

| WW source and pH | Compound | Order | K | $R^2$ |
|---|---|---|---|---|
| A – 6.38 | Gemfibrozil degradation | 2nd | $3.5 \times 10^{-3}$ (L/μg·min) | 0.84 |
| A – 6.38 | 4'-ClGem formation | 0 | 0.82 (μg/L·min) | 0.94 |
| B – 7.58 | Gemfibrozil degradation | 2nd | $1.4 \times 10^{-3}$ (L/μg·min) | 0.95 |
| B – 7.58 | 4'-ClGem formation | - | [1] | - |

[1] - Slope of model linear regression was equal to zero (α=0.05) indicating no formation of 4'-ClGem for WWTPB.

#### *5.4.4.2 Reaction Production Formation*

As shown in Figure 5-3, there was some formation of 4'-ClGem in WWTPA (pH 6.38), but no significant formation in WWTPB (pH 7.58). This result is consistent with the results of the ultrapure water trials, where no formation of 4'-ClGem was found for pH greater than 7 (Table 5-3), and is also consistent with Bulloch et. al. (2012), who

observed 4'-ClGem formation in a 50/50 blend of primary/secondary wastewater with pH 7. The rate of formation of 4'-ClGem in WWTPA was best described by zero order kinetics from 0-15 minutes (as shown in Figure 5-3) and the rate constant (0.82 μg/L·min) was comparable to that found for pH 6 and 7 in the ultrapure water trials (0.74 and 0.51 μg/L·min, respectively). At 60 minutes the concentration of 4'-ClGem normalized to the initial gemfibrozil concentration is 0.33, whereas normalized 4'-ClGem in the ultrapure water trials reached 0.44 at pH 7. This decrease in overall formation would further substantiate the presence of alternative transformation pathways for gemfibrozil in wastewater matrices. The calculated mass-loading rate of 4'-ClGem to the environment for WWTPA, assuming the spiked gemfibrozil concentration of 40 μg/L, a plant flow rate of 0.075 ML/day and an assumed contact time of 60 minutes would be: 1.125 g/day of 4'-ClGem. There was no evidence of formation for the other mono chlorinated or the di and tri chlorinated reaction products within the wastewater matrices.

#### *5.4.4.3 Practical Application Of Kinetic Models*

Brun et al., 2006 conducted a survey of selected PhACs in Canadian wastewater treatment plants. Two of the plants used chlorination for disinfection: Charlottetown, PEI and Gander, NL. Gemfibrozil was detected in both effluent samples at concentrations of up to 1.4 μg/L and 0.620 μg/L, respectively. Furthermore, gemfibrozil was detected in receiving water up to 5 km downstream of the Gander, NL wastewater treatment plant. Similarly, Metcalfe et al. (2003b) detected gemfibrozil in sewage effluent and receiving water downstream of several plants that employ chlorine disinfection, including Windsor, ON. Based on the research presented herein, it is plausible that in addition to the presence of gemfibrozil, 4'-ClGem may exist in plant effluent and receiving water.

Furthermore, in situations where gemfibrozil is not detected in chlorinated effluents or receiving waters, it is possible that the parent compound has been fully transformed but there may still be transformation products such as 4'-ClGem present.

General water quality parameters for both the Windsor, ON (Lou Romano Water Reclamation Plant) and Gander, NL plants are provided in Table 5-5. Gemfibrozil concentrations in the effluent and receiving water were taken from Brun et al. (2006) for Gander, and Metcalfe et al., (2003b) for Windsor. Water quality and plant data were provided through personal communication with plant staff in Windsor and Gander. A high estimate of the potential load of 4'-ClGem entering the receiving waters can be determined using the kinetic models for de-ionized water trials to first back calculate the concentration of gemfibrozil entering the plant and then the subsequent formation of 4'-ClGem through chlorine oxidation. The effluent at Windsor has a pH of 7.0, so the rate constant of $7.98 \times 10^{-3}$ ($min^{-1}$) was used for this case. For Gander, the pH was 6.4. There is a linear relationship between the ln of the rate constant and pH between pH 4 and 7 for ultrapure water trials ($R^2 = 0.98$). Using this relationship, a rate constant for pH 6.4 is calculated as $1.21 \times 10^{-2}$ ($min^{-1}$). Using these rate constants, the back calculated concentrations of gemfibrozil entering the two plants were 0.52 and 2.80 μg/L for Windsor and Gander, respectively.

Table 5-5 General wastewater effluent water quality and chlorination conditions for plants in Gander, Newfoundland and Windsor, Ontario. Gemfibrozil concentrations are from Brun et al., 2006 and Metcalfe et al., 2003b. All other data were obtained directly from plant personnel.

| | **Gander, Newfoundland** | **Lou Romano Water Reclamation Plant, Windsor Ontario** |
|---|---|---|
| Type of Treatment | Hydrodynamic separators, Chlorine Disinfection | Primary Clarification, Chlorine disinfection |
| Average Flow Rate | 1.8 MLD | 130 MLD |
| Receiving water body | Headwaters Soulis Pond | Detroit River |
| Gemfibrozil in effluent | 620 ng/L | 43 ng/L |
| Gemfibrozil 100 m downstream | 580 ng/L[1] | 110 ng/L[2] |
| Effluent pH | 6.4 | 7.0 |
| $Cl_2$ Dose | 0.53 mg/L | 3.8 mg/L |
| $Cl_2$ Contact Time | 180 min | 60 min |
| $Cl_2$ Residual | 0.21 mg/L | 0.7 mg/L |
| Total Effluent Ammonia | 5.5 mg/L | 10.5 mg/L |

[1] Gemfibrozil detected as far downstream as 5 km.
[2] Gemfibrozil detected as far downstream as 400 m.

For Windsor with pH of 7, a rate constant of formation for 4'-ClGem of 0.34 (μg/L·min) was used (Table 5-3). This rate constant was corrected for the influent gemfibrozil concentration mentioned above, with a resulting rate constant of formation of $4.38 \times 10^{-3}$ (μg/L·min). Using the contact time of 60 minutes for Windsor gives an estimated effluent concentration of 4'-ClGem of 0.263 μg/L. The 180 minute chlorine contact time for Gander is beyond the 60 minutes used in the experimental trials presented in this paper. It is anticipated that the zero order formation reaction would not apply between 60 and 120 minutes for a pH of 6.4, as the maximum concentration of 4'-ClGem (representing full conversion of gemfibrozil to 4'-ClGem) would be achieved. The maximum concentration of 4'-ClGem in Gander, given molar conversions and an influent gemfibrozil concentration of 2.80 μg/L, is 3.18 μg/L. Based on the average flow rates of the two plants, this leads to an estimated daily loading to the receiving water of gemfibrozil of

5.59 g and 1.12 g; and 4'ClGem of 34.19 g and 5.73 g, for Windsor and Gander, respectively.

As shown in the wastewater kinetic trials above, there are other factors that can influence the reaction rate between gemfibrozil and chlorine, leading to a lower reaction rate than that calculated in the ultrapure water experiments. Additionally, for both the ultrapure and wastewater trials conducted in this study, the chlorine concentration was in excess of chlorine demand for breakpoint chlorination, whereas the chlorine dose for both the Gander and Windsor wastewater was not in excess of ammonia demand. Due to these limitations, the calculated mass loading of 4'-ClGem for these two treatment plants would represent a high estimate or worst-case scenario.

The toxicity of chlorinated gemfibrozil reaction products has yet to be determined. However, literature suggests that pharmaceutical reaction products produced through photolytic processes may be as biologically active as the parent product, if not more (Fatta-Kassinos et al., 2011; Jiao et al., 2008; Schmitt-Jansen et al., 2007). Thus using appropriate tests and endpoints to measure the toxicity of chlorinated gemfibrozil reaction products is an important next step to understand the impact of the environmental loading of these compounds.

## 5.5 Conclusion

In reactions between gemfibrozil and free chlorine, gemfibrozil degradation and formation of the reaction products 4'-ClGem, 6'-ClGem, and 4'-6'diClGem was highly dependent on pH and free chlorine speciation. At pH 8 and 9, with $OCl^-$ as the main

oxidizing species, there was no degradation of gemfibrozil or formation of reaction products. From pH 4-7 degradation of gemfibrozil followed first order kinetics and formation of 4'-ClGem followed zero order kinetics, with reaction kinetics increasing with decreasing pH. At pH 3, the majority of gemfibrozil degradation occurred within 0.5 minutes and three reaction products were formed. The formation and subsequent degradation of 4'-ClGem corresponded to an increase in 4'6'-ClGem.

Similar kinetic experiments performed on two wastewaters (one primary and one secondary effluent) showed the degradation of gemfibrozil to follow second order kinetics, which provides evidence to the existence of other transformation processes in these more complex matrices. 4'-ClGem was formed by zero order kinetics in the secondary wastewater with pH of 6.38, whereas no reaction products were detected in the primary wastewater with pH of 7.58, which can be explained by the speciation of free chlorine. The lower overall formation of 4'-ClGem compared to ultrapure water trials provided further evidence of alternative gemfibrozil transformation processes.

Oxidation of PhACs during wastewater treatment by chlorine does not necessarily lead to full mineralization to carbon dioxide and water. The results of this study indicate that at pH values relevant to water and wastewater treatment, chlorination processes could lead to the formation and subsequent discharge of chlorinated gemfibrozil reaction products to receiving water bodies.

The kinetic models developed in this chapter were used to calculate mass loading rates of gemfibrozil and 4'ClGem to the environment using effluent gemfibrozil concentrations from two wastewater treatment plants in Canada. Estimated daily loading to the receiving water of gemfibrozil of 5.59 g and 1.12 g and of 4'ClGem of 34.19 g and 5.73 g were calculated for Windsor and Gander, respectively. These are high estimates of the potential environmental loading.

# CHAPTER 6 GEMFIBROZIL AND ITS CHLORINATED REACTION PRODUCTS IN RECEIVING WATERS: IMPACTS ON ABUNDANCE OF SUSPENDED AND BIOFILM BACTERIA

## 6.1 ABSTRACT

Reactions between chlorine and PhACs during wastewater treatment can lead to the formation of chlorinated reaction products. The toxicity of chlorinated reaction products is not yet well understood. Reactions between chlorine and gemfibrozil, a lipid regulator, at pH values relevant to wastewater treatment have been shown to produce one major reaction product, 4'-ClGem. The objective of this study was to evaluate the impacts of the presence of gemfibrozil and 4'-ClGem on abundance of suspended and biofilm bacteria in a bench-scale simulated receiving water. Experiments were conducted at 1 μg/L and 100 μg/L using two different source waters: a highly urbanized stream with variable water quality, and a lake with consistent water quality. A subsequent dose-response experiment was carried out using *Pseudomonas fluorescens*, a ubiquitous bacterium in freshwater systems, to determine what concentration of gemfibrozil or 4'-ClGem, if any, would elicit a response.

Results indicate that, at the concentrations used, the water matrix and sediment type may play a more significant role in suspended and biofilm abundance than the presence of gemfibrozil or 4'-ClGem. For suspended population, there was evidence to suggest that both gemfibrozil and 4'-ClGem were being utilized as substrates, rather than providing an inhibitory response. The dose-response experiment showed no impact due to gemfibrozil exposure up to 100 mg/L, however exposure to 4'-ClGem led to a 2 log reduction in abundance at 10 mg/L.

## 6.2 INTRODUCTION

Pharmaceutically active compounds (PhACs) can enter the environment through a variety of different pathways, including: human medicinal use, veterinary use, and agricultural/livestock use. PhACs are often resistant to biodegradation since metabolic stability is required to accomplish their task (Jos et al., 2003). The concentrations these compounds are found in the environment are orders of magnitude lower than therapeutic levels due to human metabolism, dilution and removal within treatment systems. Researchers are taking steps to quantify the possible effects of PhACs on non-target organisms at sub-therapeutic doses. Traditional acute and chronic toxicity tests have been conducted on individual compounds and in some cases, on mixtures of compounds. The toxicity of transformation products of different treatment processes (e.g., photolysis, chlorination) is an on-going research question in the literature (Escher and Fenner, 2011; Santos et al., 2010; Sanderson et al., 2004).

In addition to traditional toxicity tests, researchers have identified the need for developing toxicity tests such as biomarker tests, genotoxicity and mutation studies, long-term exposure assessments, and multigenerational tests, which will quantify the more likely outcomes from constant low-level exposures to PhACs (Verlicchi et al., 2012; Santos et al., 2010, Ferrari et al., 2003). Even slight insignificant effects on single organisms that would not cause acute toxicity could lead to population effects through reduced levels of fitness, disturbances in hormonal homeostasis, changes in immunological status, interferences in signal transduction, or gene activation (Jos et al., 2003).

Often the exact modes of action of particular drugs in humans are uncertain. There can be many side effects and action in secondary pathways. This makes it difficult to predict what types of responses will be elucidated by exposure to particular drugs, especially for non-target organisms (Verlicchi et al., 2012; Daughton and Ternes, 1999). Furthermore, each wastewater effluent is a different cocktail of compounds, and thus the aquatic responses may be highly variable.

Gemfibrozil is a fibrate acid derivative, of which 70% generally pass through humans unmodified (Isidori et al., 2007). Gemfibrozil and all fibrates are considered to be peroxisome proliferators, and there is evidence that gemfibrozil can inhibit *in vitro* testosterone production in rat Leydig cells (Liu et al., 1996), and in goldfish (5-fold decrease at nearly environmentally relevant concentrations) (Mimeault et al., 2005). Gemfibrozil metabolism is mainly facilitated through glucuronidation, which is a key Phase II pathway in the metabolism and homeostasis of endogenous molecules and plays a key role in the detoxification and excretion of contaminants (Thibaut et al., 2006). In an examination of the effects of gemfibrozil on enzymatic activity in carp liver, it was found that gemfibrozil had a strong inhibitory effect on CYP2M-catalyzed activity. In a study of four different aquatic systems, organism response to gemfibrozil was found to have the following sensitivities: *D. magna* > *V. fischeri* >PLHC-1 cells > *C. vulgaris*, the most sensitive bio-indicator being *D. magna* with an EC50 of 120 μM (Zurita et al., 2007).

Borgmann et al., (2007) conducted experiments using 7 different PhACs, including gemfibrozil, maproxen, ibuprofen, diclofenac, acetaminophen, salycylic acid and triclosan. They did not observe any major effects on *H. azteca* as a result of exposure over multiple generations at a concentration of approximately 100 ng/L.

Photolysis of gemfibrozil in distilled water produced one photoproduct at 9% by weight. (Isidori et al., 2007). The acute and chronic toxicity as well as genotoxicity and mutagenicity of both gemfibrozil and its reaction product were evaluated. Their results indicate no acute or chronic effect to the bacterium *V. fischeri* (EC50) up to 200 mg/L for the photoproduct and an EC50 of 85 mg/L for gemfibrozil. Gemfibrozil showed no mutagenicity, and a genotoxicity of 2.5-5 mg/L, while the photoproduct showed a mutagenicity concentration of 0.625 – 10 mg/L and genotoxicity of 1.25 – 5.0 mg/L. Bulloch et al., (2012) confirmed the formation of a chlorinated reaction product and a brominated reaction product in reactions between gemfibrozil and free chlorine in the presence of bromide ions. The impacts on testosterone and 11-ketotestoterone levels in the Japanese medaka over 21-day exposure to gemfibrozil, the chlorinated and brominated reaction products were investigated (Bulloch et al., 2012). Exposure to the chlorinated reaction product at 55.1 μg/L showed significant reductions in the levels of 11-ketotestosterone, and exposure to 58.8 μg/L of the brominated reaction product resulted in significant reduction in testosterone levels.

Biofilms are characterized as attached communities of microorganisms and their extracellular polymeric substances. Biofilms are often a major component of biochemical

oxygen demand removal within biological wastewater treatment processes, and also play an important role in nutrient cycling at the sediment/water interface in receiving waters. Bacteria are also at the base of the food web and thus negative impacts on the biofilm could affect higher trophic levels and nutrient cycling in an ecosystem (Rosi-Marshall, 2013). Interaction of biofilms with toxicants can result in two main responses: short-term physiological alterations and long-term changes in community structure (Rosi-Marshall et al., 2013; Sabater et al., 2007).

Exposure to selected PhACs has been shown to alter microbial activity in biofilms (Aristilde et al., 2013; Bonnineau et al., 2010). Biofilm and planktonic exposure experiments of anti-inflammatory drugs to the fungus *Candida albicans* (known to produce prostaglandins) were conducted by Alem and Douglas (2004). Aspirin and diclofenac in mM concentrations both inhibited biofilm growth significantly. Lawrence et al., (2005) conducted a biofilm experiment on river water and assessed changes in community structure and function. Results showed reductions in population and species shifts resulting in changes in community composition. Yergeau et al. (2012) also examined exposure of biofilms to PhACs, including gemfibrozil, in river water. They observed some significant shifts in active community composition and abundance of transcript categories. Naproxen, in the presence of chlorine, was found to have an adverse effect on biofilm in a bioreactor (Boyd et al., 2005). One day after the addition of a chlorine and naproxen solution, significant biomass was discharged from the system.

The objective of this study was to investigate the impacts of exposure to gemfibrozil and its chlorinated reaction product (4'-ClGem) on abundance of suspended and biofilm bacteria in a bench-scale simulated receiving water system. This was accomplished through two sets of trials using an aerated batch reactor bench-scale set-up. Gemfibrozil and its chlorinated reaction product 4'-ClGem were spiked into the system at two different concentrations and both suspended and biofilm heterotrophic bacteria were enumerated. Additionally, a dose-response study was carried out in a similar bench-scale batch system for both gemfibrozil and 4'-ClGem.

## 6.3 Materials And Methods

### 6.3.1 Standards And Reagents

Reagents and standards for PhAC analysis are described in Chapter 3. Slow fade and SYBR gold 10,000x stock were purchased from Invitrogen. R2A agar, Pseudomonas agar base and CFC supplement were purchased from Sigma-Aldrich. *Pseudomonas fluorescens* culture was obtained from ATCC.

### 6.3.2 Microbiological Analysis

#### *6.3.2.1 Biofilm And Suspended Bacteria Sampling*

To process samples for suspended bacteria, 2 mL of supernatant from each flask was removed using a sterilized pipette and placed into sterilized microcentrifuge tubes from which dilution series were prepared as described below.

The remainder of the supernatant was extracted from the flasks using a 10 mL sterile pipette, being careful to not disturb the media. 30 mL of sterile phosphate buffer solutions

(PBS) was then added to each flask and flasks were placed on a shaker table at 300 rpm for 10 min to break up and detach biofilm from sediment; this is a modification of biofilm extraction methods from Gagnon and Slawson (1999). 1 mL of the shaken biofilm supernatant was then transferred to sterile microcentrifuge tubes from which a dilution series was prepared as described below.

#### *6.3.2.2 Enumeration Of Suspended And Biofilm Bacteria*

Heterotrophic bacteria were quantified through plate counts, herein referred to as heterotrophic plate counts (HPC). Samples were collected for both suspended and biofilm bacteria as described above. HPC analysis was conducted following procedures outlined in standard methods for the analysis of microbiological parameters (APHA, 2012). *Pseudomonas* agar with CFC supplement was used as the plating medium for *Psuedomonas fluorescens*. All plates and broths were prepared according to the manufacturer's directions and autoclaved before use to ensure sterility. The following is a brief description of the plating methods:

- A 10 fold dilution series from the neat sample down to $10^{-4}$ was prepared for both suspended and biofilm samples, using autoclaved phosphate buffer solution (PBS) as the diluent.
- 0.1 mL from each dilution tube was then spread across the surface of R2A agar. Plates were prepared in duplicate for each dilution in the series.
- Plates were incubated upside down at room temperature for 7 days in the dark.
- Plates containing between 30 and 300 colonies were used for enumeration. Colonies were counted using an automated colony counter with consistent settings used for contrast and sensitivity to ensure homogeneity in results.

#### 6.3.2.3 *Pseudomonas fluorescens Spiking Procedure*

*Pseudomonas fluorescens* culture (ATCC) was added to a sterile test tube of tryptic soy broth (TSB) and incubated for 24 hrs at 30 °C. The culture was then streaked onto Tryptic soy agar (TSA) and incubated for 24 hours. Colonies were then scraped off the agar with a sterile loop, and added to a sterile test tube containing TSB and incubated overnight. Tubes were centrifuged at 4000 rpm for 10 minutes, the supernatant was discarded and 10 mL of a 0.85% NaCl solution was added. The pellet was resuspended by vortexing. This wash process was repeated 3 times. The washed pellet was then resuspended into a 100 mL sterile flask containing 0.85% NaCl solution. 2 mL of this solution was spiked into each flask.

#### *6.3.2.4 Direct Cell Counts*

Direct cell counts were performed on biofilm samples only. The $10^{-2}$ dilutions used for biofilm HPC described above were used for direct cell counting. 1-mL of the diluted sample was added to a sterile 1.5 mL microcentrifuge tube. The sample was fixed by adding 0.1 mL of a 0.22 mg/mL paraformaldehyde stock solution. Fixed samples were stored at 4°C for a maximum of 1 week before analysis. To prepare samples for direct counts, 1 µL of SYBR gold solution (10,000x) was added to the sample and incubated at room temperature in the dark for 30 min. Cells that are stained using SYBR gold produce a green fluorescence under UV light when bound to double-stranded DNA.

The samples were then vacuum filtered through a black 0.22 µm filter. 2 mL of Autoclaved PBS was used to precondition the filter, followed by which 1 mL of SYBR gold stained sample was passed through the filter and finally, 3 x 10 mL of autoclaved

PBS was passed through the filter unit to rinse the sample. One drop of slow fade mounting media was added to a glass microscope slide. The filter membrane was aseptically removed from the filter unit and placed on top of the drop of slow fade. Another drop of slow fade was placed on top of the filter membrane followed by a coverslip.

Cells were then enumerated using a microscope equipped with a 100x oil immersion objective lens under a UV filter. Fluorescing cells were enumerated in 7 randomly selected fields of view for each sample and results were averaged to give a direct cell count per gram of media for biofilm samples.

### 6.3.3 Chlorination Reaction Conditions

At the time of these experiments, there was no access to the crystallized neat compound of 4'-ClGem, and thus a reaction mixture was used to spike into the reactors as described below. A 100 mL stock solution of the chlorinated equivalent of 15 mg/L gemfibrozil was created by adding 1.875 mL of a 0.8 mg/mL methanolic stock solution of gemfibrozil to a 100 mL volumetric flask. The methanol was left to evaporate for several hours. After evaporation, NaOCl was added in a 10:1 molar ratio from a 3000 mg/L NaOCl stock solution and ultrapure water was added up to 100 mL. This solution was allowed to react for 4 hours at which time sodium thiosulphate was added in a 15:1 molar ratio to chlorine to quench the reaction. The presence of 4'ClGem was confirmed by GC-MS analysis and a reaction time of 4 hours was chosen based on a kinetic experiment using the described reaction conditions shown in Figure 6-1. There was no significant formation of other reaction products as determined by the GC-MS method used. It is

possible that the chlorination conditions used in this study produced reaction products in addition to 4'-ClGem, that were not detected by the GC-MS. However, for this paper, the chlorinated reaction product treatments will be referred to as 4'-ClGem as this was the only reaction product observed. 1 mL of this solution was then added to each of the 100 µg/L chlorinated reaction product flasks in the experiment. For the 1 µg/L chlorinated reaction product equivalent flasks, 10 µL of the 4'-ClGem solution was added to the flasks. It is also possible that other products exist in the mixture due to the use of sodium thiosulphate to quench chlorine, therefore any impacts observed must be attributed to the mixture that was spiked into the reactors.

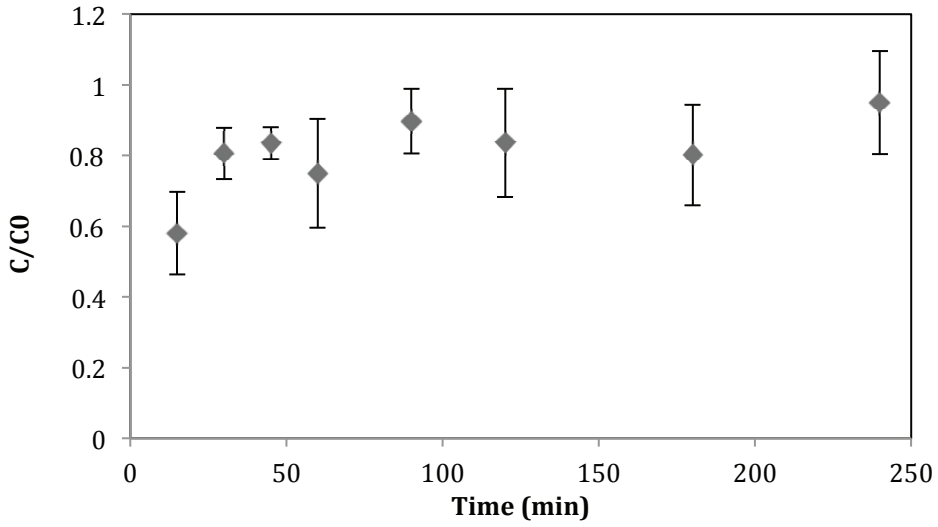


Figure 6-1 Formation of 4'- ClGem under experimental conditions used to create stock solution to spike into 4'-ClGem flasks. Data are normalized to the initial gemfibrozil concentration using peak area ratios between gemfibrozil and 4'-ClGem, as no standards were available for 4'-ClGem. Error bars represent one standard deviation of five replicate samples.

## 6.3.4 Experimental Design

### *6.3.4.1 Bench-Scale Setup For Receiving Water Impact Studies*

The bench-scale receiving water study experimental set-up was modeled after a modified version of the Environment Canada Biological Test method: Test for Survival and

Growth in Sediment Using the Freshwater Amphipod *Hyalella azteca* (EPS 1/RM/33) (Environment Canada, 1997).

The Environment Canada toxicity test has the following characteristics:

- 300 mL beakers
- 100 mL sediment, 200 mL overlying water
- 14 days
- Gentle aeration
- 16 hours of light and 8 hours of darkness per day

The setup for this study used 300 mL Erlenmeyer flasks, with 40 g of sediment and 150 mL of overlying water. Bench-scale tests were conducted for 14 days and the top of the flasks were covered in aluminum foil to minimize evaporation over the length of the trial. Air was supplied to the flasks using a 32-position air manifold. A series of 5-way aquarium gang valves, in-line air filters, polyethylene tubing and sterilized disposable glass Pasteur pipettes were used to carry air from a central air supply to the flasks. The gang valves allow for adjustment of airflow in order to ensure equal distribution across flasks. Air was supplied just above the sediment surface at a rate that would not fluidize the sediment, creating minimal disturbance and shear on the sediment water interface, while maintaining saturated dissolved oxygen levels. The flasks were positioned next to a window ensuring a consistent light/dark cycle. The bench-scale setup is shown below in Figure 6-2 and Figure 6-3.

Figure 6-2 - Bench-scale set-up of receiving water study

Figure 6-3 - Close-up of flask in the bench-scale receiving water study

Experimental trials were operated as a batch system such that the overlying water was not renewed throughout the length of the trial. It was important to operate the experimental trials in a batch system to allow the biofilm to establish and mature throughout the length of the trial. If new organisms and water are added to the system, it would be difficult to

determine bacteriological effects induced by the presence of PhACs, both due to dilution and change in bacterial community structure and age.

The Environment Canada toxicity test was developed for toxicity of sediments to amphipods, not for microbial impacts or characterization. Therefore, some factors were changed to optimize the protocol for bacterial processes, including:

- Sediment type
- Microbial sampling methodology, both suspended and biofilm
- Source water

A series of experiments were conducted (not presented in this thesis) to determine a sediment recipe that would mimic the natural environment while also facilitating extraction of fixed biofilm cells. The optimal sediment was found to be a combination of clay, silt, sand, peat and calcium carbonate in a ratio of 2.5:5:80:5:1 which is similar to the formulated sediment prepared for the Organization for Economic Cooperation and Development Test Guideline 218 (OECD, 2004).

There were two sets of bench-scale trials conducted to determine the effects of gemfibrozil and 4'-ClGem on both suspended and biofilm bacteria levels.

#### *6.3.4.2 Sackville River Receiving Water Impact Experiment*

Water was collected from the Sackville River in Bedford, Nova Scotia, which is an urban river susceptible to water quality changes due to land use and runoff. The river is approximately 40 km in length with a drainage area of about 150 $km^2$. The lower portions surrounding the river are mainly residential and the flows increase substantially in the spring and after heavy rains. A 10 L sample was taken for each trial near a footbridge

close to the mouth of the river. Two trials were completed using the batch system setup described above. Table 6-1 below summarizes the water quality characteristics, experimental conditions and endpoints measured. Each treatment was conducted in triplicate for a total of 15 flasks per experiment, including controls, with no gemfibrozil or 4'-ClGem.

Table 6-1 Summary of water quality and experimental conditions for Sackville River receiving water impact experiment.

| | **Trial 1** | **Trial 2** |
|---|---|---|
| Water Quality and Sediment Characteristics | | |
| TOC/DOC (mg/L) | 9.4/5.7 | 6.0/5.5 |
| Raw water pH | 6.85 | 6.63 |
| Sediment (clay:silt:sand:peat: $CaCO_3$) | 2.5:5:80:5:1 | 2.5:5:80:5:1 |
| Adjusted pH | 7.0 | 7.0 |
| Treatments | | |
| High Gemfibrozil | 100 µg/L | 100 µg/L |
| Low Gemfibrozil | 1 µg/L | 1 µg/L |
| High 4'-ClGem mixture | 100 µg/L* | 100 µg/L* |
| Low 4'-ClGem mixture | 1 µg/L* | 1 µg/L* |
| Control | No gem or 4'-ClGem | No gem or 4'-ClGem |
| Measured endpoints | | |
| Endpoints | HPC - Biofilm and suspended, P*seudomonas* spp. - Biofilm | HPC - Biofilm and suspended, P*seudomonas* spp. - Biofilm |

* Based on initial gemfibrozil concentration using chlorination conditions described in section 6.3.3. No standards were available for quantification of 4'-ClGem at the time of the study.

#### *6.3.4.3 Pockwock Lake Receiving Water Impact Experiment*

A second set of trials was conducted using Pockwock Lake water as the matrix. Pockwock Lake has much more stable water quality and is the water source for the J.D. Kline Water Supply Plant, which provides Halifax with drinking water. Halifax Water manages the land-use within the Pockwock watershed and there is very limited anthropogenic activity and no major short-term variations in water quality. The sediment

in these experiments was simplified to sand only to eliminate the impact that organic enrichment (peat) and buffering ($CaCO_3$) can have on bacterial populations. A summary of water quality, experimental conditions and measured endpoints is provided below in Table 6-2. Each treatment was conducted in triplicate for a total of 15 flasks per experiment, including controls, with no gemfibrozil or 4'-ClGem.

Table 6-2 Summary of water quality and experimental conditions for Pockwock Lake receiving water impact experiment

| | **Trial 1** | **Trial 2** |
|---|---|---|
| Water Quality and Sediment Characteristics | | |
| TOC/DOC (mg/L) | 2.61/2.46 | 3.2/2.5 |
| Raw water pH | 5.8 | 5.9 |
| Sediment | sand | sand |
| Adjusted pH | 7.0 | 7.0 |
| Treatments | | |
| High Gemfibrozil | 100 µg/L | 100 µg/L |
| Low Gemfibrozil | 1 µg/L | 1 µg/L |
| High 4'-ClGem mixture | 100 µg/L* | 100 µg/L* |
| Low 4'-ClGem mixture | 1 µg/L* | 1 µg/L* |
| Control | No gem or 4'-ClGem | No gem or 4'-ClGem |
| Measured endpoints | | |
| Endpoints | HPC - Biofilm and suspended, Total Direct Counts | HPC - Biofilm and suspended, Total Direct Counts |

* Based on initial gemfibrozil concentration using chlorination conditions described in section 6.3.3. No standards were available for quantification of 4'-ClGem at the time of the study.

#### *6.3.4.4 Dose – Response Experiment*

As a follow-up experiment to the receiving water trials, a dose-response experiment was carried out using a controlled matrix to investigate whether there was an inhibitory effect on a single suspended bacterial species due to a range of concentrations of gemfibrozil or 4'-ClGem. Flasks were prepared in triplicate at the following gemfibrozil concentrations or equivalent concentrations for 4'-ClGem: 0.1, 1, 10, 100, 1,000 and 10,000 µg/L. No sediment was used in this experiment. A synthetic nutrient feed water was used as the

matrix and was prepared with ultrapure water with a 5 mg/L concentration of a 100:20:5 C:N:P ratio stock nutrient solution (Camper 2006). Components of the nutrient stock solution included ethyl alcohol, propionaldehyde, parahydroxybenzoic acid, acetate, nitrogen ($NaNO_3$) and phosphorus ($K_2HPO_4$ and $KH_2PO_4$).

*Pseudomonas fluorescens* was chosen as the bacterium for these trials as it is a ubiquitous bacterium known to produce biofilm in freshwater ecosystems (Heydorn et al., 2000). *Pseudomonads* are known to be able to degrade a variety of organic compounds, including many that are toxic to other bacteria and higher organisms (Palleroni, 2010).

The flasks were then incubated in the dark for 6 days and suspended bacteria were enumerated.

## 6.4 Results and Discussion

### 6.4.1 Sackville River Receiving Water Impact Experiment

The results of the suspended and biofilm HPC samples from both Sackville River trials are presented below in Figure 6-4 and Figure 6-5. Statistical analysis of HPCs for both biofilm and suspended bacteria shows that there is no statistically significant difference between treatments within each trial for biofilm samples or for suspended HPC in the first of the two trials (one-way ANOVA, $\alpha = 0.05$). For the suspended HPC in the second trial, the only significant difference between treatments was between the high gemfibrozil treatment and the high 4'-ClGem mixture treatment; this trend was not observed in the first trial. Although not statistically significant at 95% confidence, there is a trend in the

suspended HPC results where the treatment with no gemfibrozil or 4'-ClGem has the lowest average concentration.

Statistical analysis does show that there was a significant difference in both suspended and biofilm HPCs between the trials (2-way ANOVA, $\alpha = 0.05$). This indicates that the matrix effects due to differences in water quality are greater than the impacts between treatments at the concentrations used in this study. *Psuedomonas* spp. in biofilm samples (Figure 6-6) showed a similar trend with significant difference between trials, but showed no statistically significant difference between treatments within each trial.

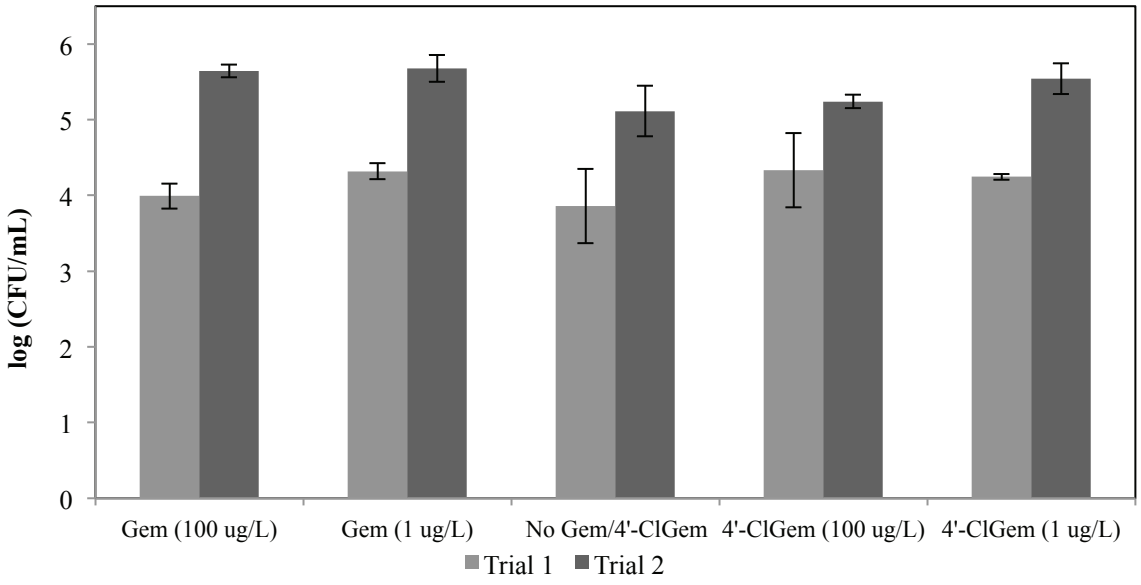


Figure 6-4 Heterotrophic plate count for suspended bacteria in the two Sackville River receiving water impact studies. Error bars represent the standard deviation between triplicate samples

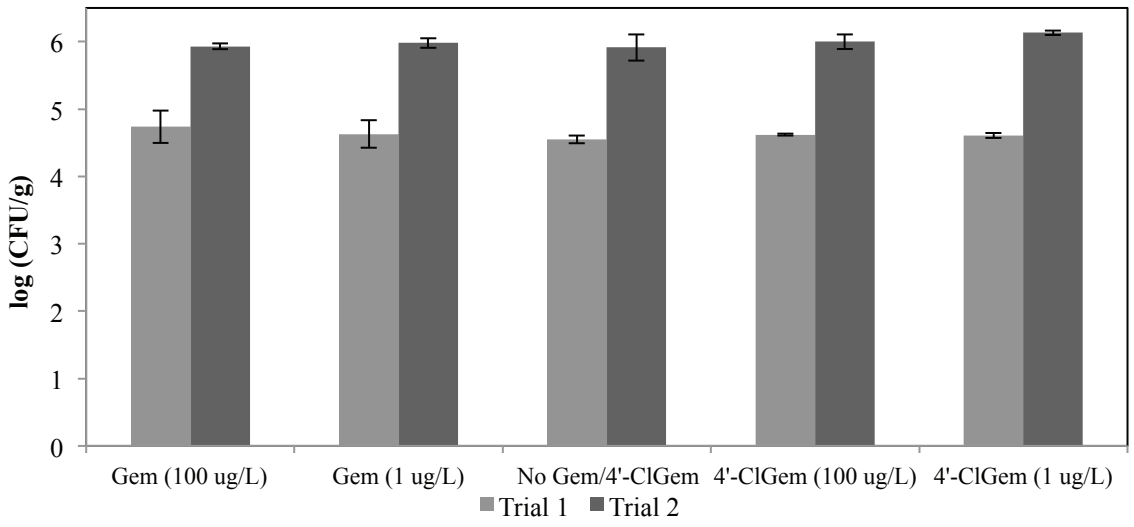


Figure 6-5 Heterotrophic plate count for biofilm bacteria in the two Sackville River receiving water impact studies. Error bars represent the standard deviation between triplicate samples

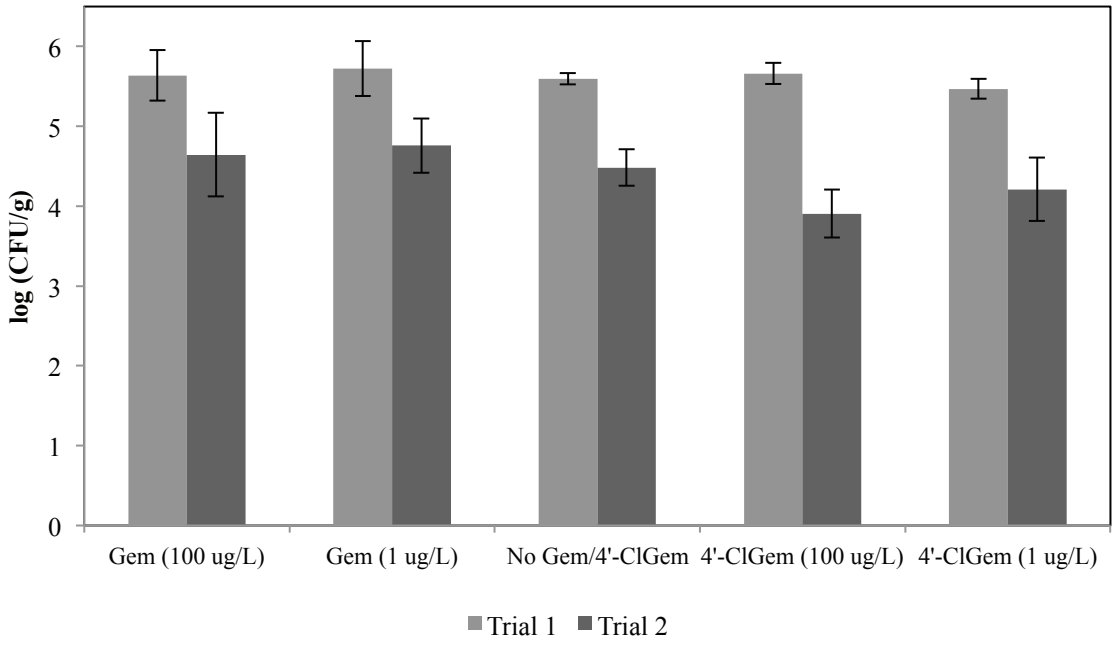


Figure 6-6 Biofilm *Pseudomonas* spp. in the two Sackville River receiving water impact studies. Error bars represent the standard deviation between triplicate samples

### 6.4.2 Pockwock Lake Receiving Water Impact Experiment

The suspended bacteria HPC results (Figure 6-7) show a statistically significant difference between treatments for both trials (one-way ANOVA, $\alpha = 0.05$), with the lowest average concentration being the treatment with no gemfibrozil or 4'-ClGem mixture. This is the same trend observed for the Sackville River, although it is more pronounced in the Pockwock trials. This result could indicate that both gemfibrozil and 4'-ClGem are being used as substrates, thus increasing biological activity rather than providing an inhibitory effect.

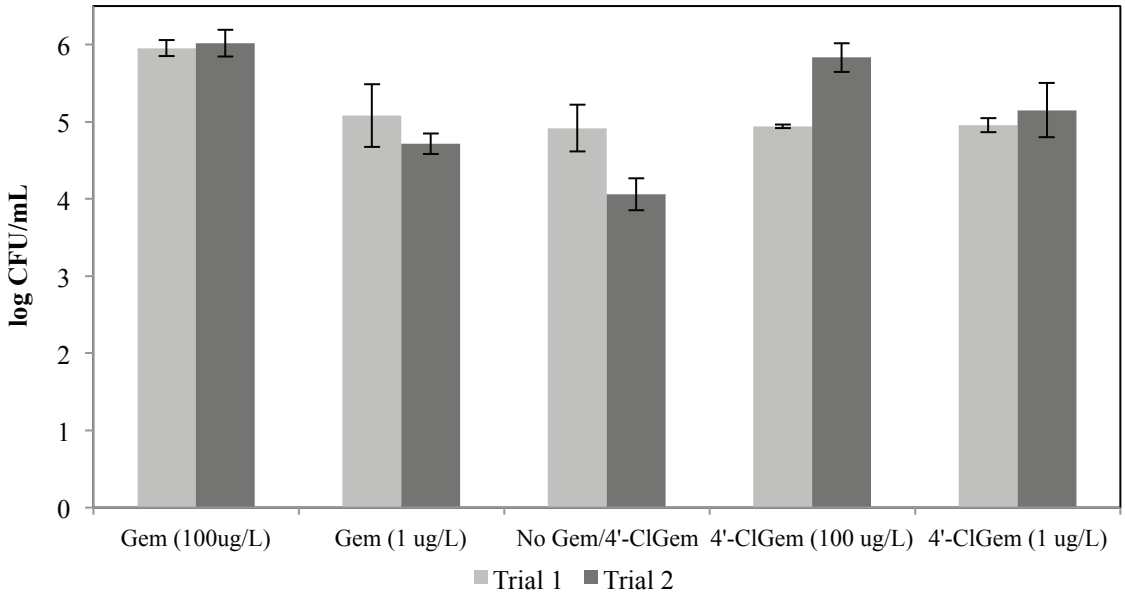


Figure 6-7 Heterotrophic plate count for suspended bacteria in the two Pockwock Lake receiving water impact studies. Error bars represent the standard deviation between triplicate samples.

Peat has long been used as an adsorbant material for water and wastewater treatment (Couillard, 1994), and recent studies have shown that PhACs can adsorb to peat material (Aristilde and Sposito, 2013; Ottmar et al., 2010). Fang et al., 2012 also showed that

gemfibrozil has a tendency to sorb to silty loam soils. Therefore, in the Sackville River trials, some gemfibrozil and 4'-ClGem could be adsorbing to the peat in the sediment, which would mean less substrate is available for bacterial growth thus confirming the smaller impact observed in the suspended bacteria results for Sackville River compared to the sand only (no adsorptive capacity) Pockwock Lake trials.

Biofilm HPC results (Figure 6-8) also show significant difference between treatments for both trials (2- one-way ANOVAs, $\alpha = 0.05$), although the difference in concentration is more subtle than for the suspended HPC results for these trials. Unlike the Sackville River trials, there was no statistically significant difference in either the suspended or biofilm HPCs between the two trials (2-way ANOVA, $\alpha=0.05$). Total direct counts (Figure 6-9) showed very similar trends to the biofilm HPC results, both between treatments and between trials.

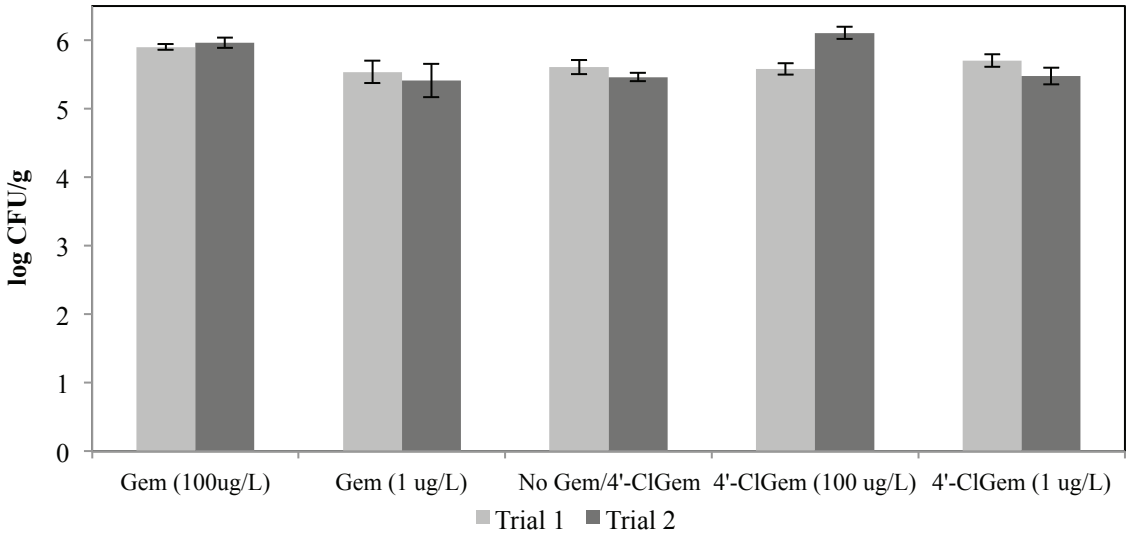


Figure 6-8 Heterotrophic plate count for biofilm bacteria in the two Pockwock Lake receiving water impact studies. Error bars represent the standard deviation between triplicate samples.

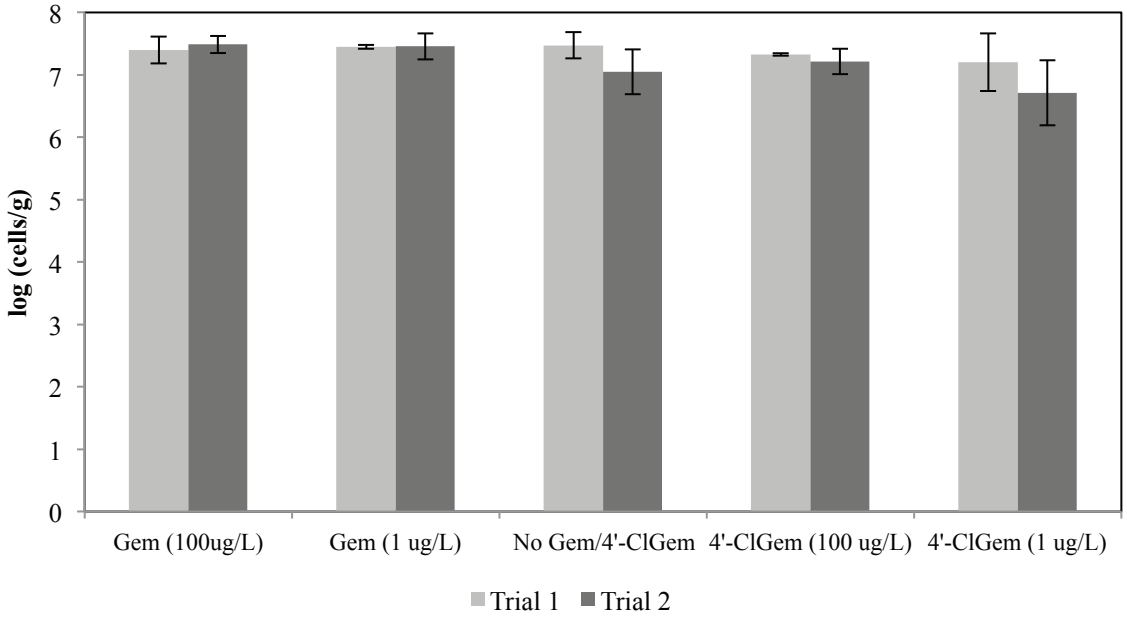


Figure 6-9 Total direct cell counts for biofilm bacteria in the two Pockwock Lake receiving water impact studies. Error bars represent the standard deviation between triplicate samples.

#### *6.4.2.1 Dose-Response Experiment*

The results of the dose-response experiment (Figure 6-10) show that there was no inhibitory effect on *Pseuodomonas fluorescens* concentration due to exposure to gemfibrozil at any of the concentrations used in the trial. However, the 4'-ClGem mixture did show an inhibitory effect on *Pseuodomonas fluorescens* concentration at 10 mg/L with a 2-log concentration reduction.

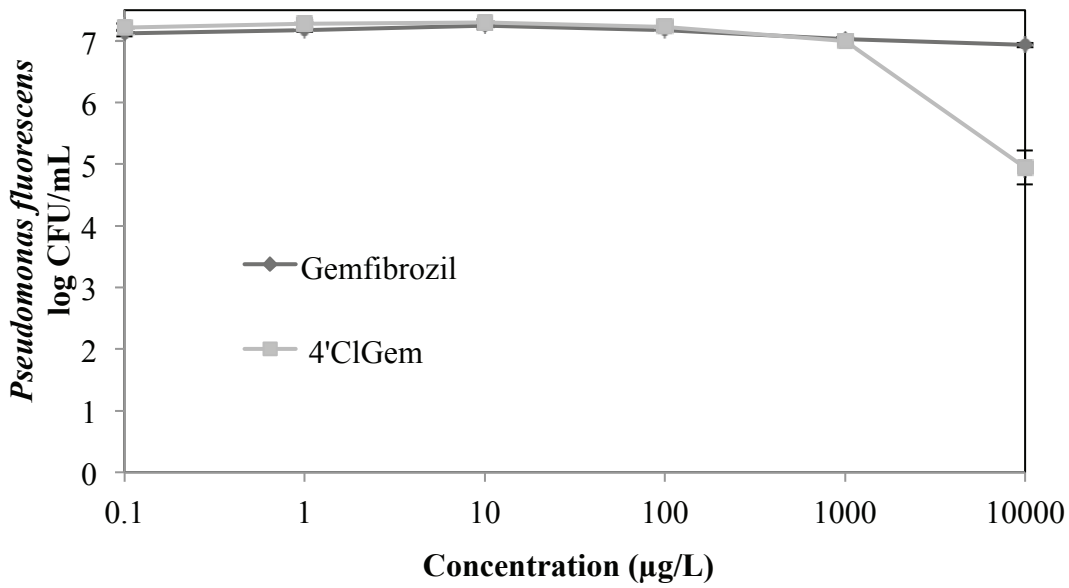


Figure 6-10 - Dose-response experiment for *Pseudomonas fluorescens* exposure to gemfibrozil and 4'-ClGem.

Studies conducted on the impact of gemfibrozil on the model bacterium *V. fischeri* showed that the half maximal effect concentration (EC50) (24 hr) was 64.6 mg/L (Zurita et al., 2007); the EC 50 (30 min) was 85.74 mg/L (Isidori et al., 2007); and EC50 (15 min) was 35.3 mg/L and 18.8 mg/L (Rosal et al., 2009; Farré et al., 2001). Toxicity studies on higher level organisms have found EC50 as low as 0.44 mg/L and 0.53 mg/L for the rotifer *B. calcyflorus* (48 hr) and the crustacean *C. dubia* (7 day), respectively (Isidori et al., 2007).

Verlicchi et al. (2012) conducted an evaluation of environmental risk posed by different PhACs by calculating a risk quotient (RQ). The RQ is the ratio between the average PhAC concentration measured in secondary effluent and its corresponding predicted no-effect concentration (PNEC). The resulting RQ can be placed into three different categories: low (<0.1), medium (0.1<RQ<1) and high risk (>1) (Hernando et al., 2006).

They used average secondary effluent concentrations and PNECs as reported in literature. Of their analysis, gemfibrozil was ranked 14/ 51 for the PhACs surveyed, and it fell within the high risk RQ. The concentrations of gemfibrozil used in the dose-response experiments in this study were not high enough to induce an effect on abundance of *Pseudomonas fluorescens*. A calculated RQ from this work for gemfibrozil would be much less than 0.1, however this is based solely on abundance of one species of bacteria, and does not take into account more chronic effects, such as changes in community composition and activity, or impacts on higher level organisms.

Bulloch et al., (2012) found that 21-day exposure of Japanese medaka to approximately 50 µg/L of 4'-ClGem and a brominated gemfibrozil reaction product led to decreases in testosterone 11-ketotestosterone, respectively. Isidori et al., (2007) examined a photoproduct of gemfibrozil and found no effects on *V. fischeri* at concentrations up to 100 mg/L, but did find chronic reproductive effects for several rotifers in the 0.1 – 1 mg/L range for gemfibrozil and its photoproduct. Chlorination of acetaminophen can result in the production of 1,4 - benzoquinone, which has been shown to have a higher toxicity (in mice) than acetaminophen (Bedner and MacCrehan, 2006). Similarly, Rosal et al. (2010) found higher toxicity to *V. fischeri* for ozonated reaction products of clofibric acid compared to the parent compound. Through a literature review, Escher and Fenner (2011) observed that in general, transformation products from a wide range of transformation pathways have lower toxicity than their parent compounds.

Using a PNEC of 4'-ClGem of 1 mg/L (the highest concentration to have no effect in the dose-response trial) and a calculated effluent concentration of 3.18 ug/L for Gander, NL wastewater (from Chapter 5), the calculated RQ for 4'-ClGem for *Pseudomonas fluorescens* would be less than 0.1 which would represent a low risk based on the RQ criteria.

There is evidence from this study and others that byproducts have different toxicity than their parent compounds (Boxall et al., 2012). Hazards can occur through elements other than toxicity, including persistence, bioaccumulation, and long-range transport potential. It is important to address each of these elements of toxicity in future studies using the reaction product 4'-ClGem (Escher and Fenner, 2011).

## 6.5 Conclusions

Two sets of batch system bench-scale studies were conducted to investigate the impacts of gemfibrozil and the chlorinated transformation product 4'-ClGem (as a mixture) exposure on the abundance of both suspended and biofilm bacteria. At the concentrations used in this study (1 and 100 μg/L), there was evidence to suggest that water matrix and sediment type may play a more significant role in heterotrophic bacterial abundance than exposure to gemfibrozil or 4'-ClGem. For suspended bacteria there is evidence to suggest that both gemfibrozil and 4'-ClGem may be used as a substrate thus resulting in an increase of suspended heterotrophic bacteria in these treatments. Biofilm bacteria abundance showed very little difference between treatments.

The dose-response trial carried out for gemfibrozil and 4'-ClGem on *Pseudomonas fluorescens* suspended bacteria showed no inhibitory effects due to gemfibrozil exposure up to the maximum concentration of 10 mg/L, whereas exposure to 4'-ClGem resulted in a 2-log reduction in abundance at 10 mg/L.

# CHAPTER 7 REMOVAL OF GEMFIBROZIL AND SELECTED ACIDIC PHARMACEUTICALS WITHIN A NITRIFYING RECIRCULATING BIOFILTER

## 7.1 ABSTRACT

The fate of pharmaceutically active compounds (PhACs) in wastewater systems is a concern to many communities; however, little research has been done on rural or decentralized communities. The objective of this study was to examine the ability of a bench scale nitrifying recirculating biofilter (RBF) to remove four several high use pharmaceuticals: gemfibrozil, naproxen, ibuprofen and diclofenac. The impacts of PhAC concentration, presence as mixtures, and response from system upsets on PhAC removal in RBFs was investigated through two experimental trials using secondary treated municipal wastewater.

The average removals in this study were between 92 – 99% for ibuprofen, 89 - 99% for naproxen, 62 – 92% for gemfibrozil and 40 - 76% for diclofenac, which is consistent with the high end of removals found in other studies for different biological wastewater treatment processes. Ibuprofen and naproxen were largely removed through microbial degradation processes; while gemfibrozil and diclofenac showed more variable removal, which may suggest sorption and desorption to the biofilm matrix. PhAC removal in the RBFs was: repeatable between trials, robust and responsive to system upsets, and PhAC presence (singularly or as a mixture) had no impact on removal. In summary, this study indicates that RBFs could be a viable technology for removal of some acidic pharmaceuticals in small onsite wastewater treatment facilities. ‡

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‡ Note: A version of this chapter has been submitted to the *Journal of Hazardous Materials:*

Krkošek, W. H.; Payne, S. J.; Gagnon, G. A. Removal of acidic pharmaceuticals within a nitrifying recirculating biofilter. Submitted to: *Journal of Hazardous Materials* October 2013.

## 7.2 INTRODUCTION

Onsite and small-scale wastewater treatment systems often utilize a combination of a septic tank and soil adsorption field to treat domestic wastewater (Loupasaki and Diamadopoulos, 2012). Approximately one-quarter of the households in the United States utilize onsite wastewater treatment systems (Conn et al., 2006), the majority of which are soil-treatment based systems (Hawkins et al., 2008). When properly constructed on suitable sites, these systems provide water of a high enough quality that can be discharged into surface or ground water. However, these systems can fail due to: poor construction practices (resulting in soil damage), installation on unsuitable sites with low soil permeability, a high water table during part of the year, or shallow rock depth. Large footprint requirements for soil adsorption fields are making onsite systems a less desirable wastewater treatment option, and therefore alternative small wastewater treatment technologies have become an attractive alternative.

Recirculating biofilters (RBFs), a modification of the intermittent sand filter, offer an alternative treatment approach and normally do not have the same footprint requirements that traditional on-site or septic systems. RBFs can be used for single households and small communities. According to USEPA (2008) RBFs are "extremely reliable treatment devices", and they are robust technologies resistant to flow variations. RBFs can be operated at higher hydraulic loading rates compared to single pass filters. Recirculation of wastewater can provide improved wastewater effluent quality (BOD, TSS) by diluting the influent wastewater, allowing for the higher application rates (Hu and Gagnon, 2006a). Traditionally, RBFs have been constructed with sand as the media; however

crushed glass, geotextile and peat have also been used successfully (Loupasaki and Diamadopoulos, 2012; Hu and Gagnon, 2006b). The treatment system consists of a septic tank and a lined underground filter bed. During treatment, settled wastewater flows from the top of the septic tank to the recirculation tank, where it is then fed though a distribution network onto the surface of the filter bed. Wastewater percolates through the filter bed and is gathered as effluent through a drain collection system. A portion of this effluent is then pumped to the recirculation tank and the remainder is discharged from the treatment system (treated wastewater effluent). Feeding frequency onto the filter bed is usually 1 to 3 times per hour and typical recirculating ratios are between 3:1 and 5:1 (USEPA, 2008).

Microorganisms form a biofilm on the surface of the filter media in a RBF and they absorb soluble and colloidal waste material (organic material and nutrients) in the wastewater. Normally, BOD and TSS effluent concentrations are less than 10 mg/l and nitrification is usually complete (Williamson, 2010; Hu and Gagnon, 2006a) due to the long solids retention time (SRT) that enables growth of ammonia oxidizing bacteria. The recirculation in an RBF promotes nitrification and denitrification processes which can result in 40 to 60 % removal of total nitrogen from the system through conversion to nitrogen gas (Loupasaki and Diamadopoulos, 2012; Hu and Gagnon, 2006a). Nitrification is the conversion of ammonia to nitrate through aerobic biological processes by ammonia oxidizing bacteria. Denitrification is the subsequent conversion of nitrate to nitrogen gas, which generally occurs under anoxic conditions at areas in the biofilter that have low dissolved oxygen.

There are many studies that report on the general occurrence and removal of PhACs within wastewater treatment plants (Lishman, et al. 2006; Servos et al. 2005; Poseidon, 2004; Metcalf and Eddy 2003); however, the removal of specific emerging contaminants through RBF treatment is not widely reported (Camacho-Munoz et al., 2012; Matamoros et al., 2009; Conn et al., 2006). Several studies have reported on the removal of pharmaceutically active compounds (PhACs) in wastewater systems that utilize conventional activated sludge treatment plants, membrane bioreactors or other process equipment that would be commonly found in centralized wastewater treatment systems (Pomiès et al., 2013; Verlicchi et al., 2012; Kreuzinger et al., 2004; Lishman et al., 2006). Similar to those systems, it is expected that the removal of PhACs within RBFs will be highly dependent on many factors: the microbial ecology in the treatment system (Wang et al. 2008), the type of treatment, water quality (e.g., pH, temperature), and flow rate.

In general, the mechanisms and pathways of removal are not completely understood for each PhAC; however, it is generally accepted that important removal processes are biodegradation of PhACs and sorption to wastewater biosolids (Pomiès et al., 2013; Verlicchi et al., 2012; Poseidon, 2004). Physical and chemical properties of the pharmaceutical compound and operational conditions (sludge retention time, biomass concentration, hydraulic retention time, pH and temperature) of the WWTP will collectively contribute to the efficiency of PhAC removal during wastewater treatment (Fernandez-Fontaina et al, 2012; Hyland et al., 2012; Stevens-Garmon et al., 2011; Zorita et al. 2009). Servos et al. (2005) also observed that WWTPs operating under nitrifying

conditions had higher removal of estrogens, compared to WWTPs with no nitrification. Fernandez-Fontaina et al. (2012) found that nitrification conditions increased the biodegradation rates of several PhACs including ibuprofen and naproxen, but had little impact on diclofenac. It has also been reported that aerobic conditions often result in higher PhAC removal rates compared to anaerobic systems (Fernandez-Fontaina et al., 2012; Suarez et al., 2010; Matamoros et al., 2009; Conn et al., 2006). Biofilters have been shown to have increased removal rates per unit biomass for certain pharmaceuticals when the system is at the same nutrient removal level as an activated sludge system (Falås et al., 2012; Radjenović et al., 2009; Poseidon, 2004).

The objective of this research was to examine the ability of bench scale RBF systems to remove gemfibrozil (a lipid-regulating drug), ibuprofen, naproxen and diclofenac (all non-steroidal anti-inflammatory drugs) under aerobic nitrifying conditions. These four acidic PhACs were chosen for this study based on their widespread use, existing literature on the behaviour of these pharmaceuticals in wastewater systems (Pomiès et al., 2013; Zorita et al., 2009; Urase and Kikuta, 2005), and the analytical capacity within the laboratory. Properties of the four PhACs are found in Table 7-1. The impacts of PhAC concentration, presence as mixtures and response from system upsets on PhAC removal in RBFs was investigated through two experimental trials using secondary treated municipal wastewater.

Table 7-1 - Properties of the four selected PhACs for this study

| | **Molecular Weight** | **Log $K_{ow}$** | **pKa** |
|---|---|---|---|
| Ibuprofen | 206 | 3.97 | 4.91 |
| Naproxen | 230 | 3.2 | 4.15 |
| Gemfibrozil | 250 | 4.77 | 4.79 |
| Diclofenac | 296 | 4.51 | 4.15 |

## 7.3 Materials And Methods

### 7.3.1 RBF Bench Scale Setup

Each RBF system setup consisted of a septic tank, a recirculation tank and a biofiltration column as shown in Hu and Gagnon (2006b) Figure 7-1. The treatability trial consisted of a total of six recirculating biofilter units that ran in parallel. The concentration trial consisted of four recirculating biofilter units also running in parallel. For both trials, wastewater (system influent) was added to the septic tank. Wastewater was then pumped to the recirculating tank and then into the biofilter column.

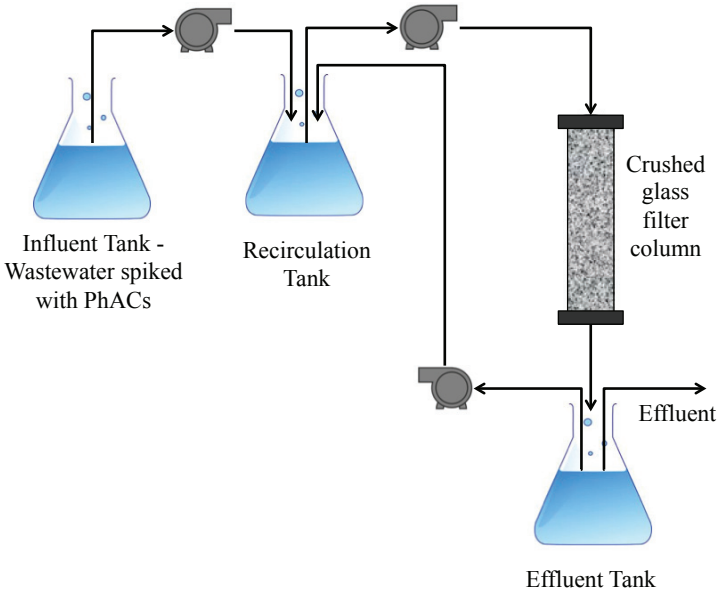


Figure 7-1 Schematic of RBF system setup, adapted from Hu and Gagnon (2006b).

The pharmaceuticals were spiked into the system in the septic tank. Biofilter effluent was gathered in the effluent tank where 80% of the water was pumped back to the recirculation tank and 20% was discharged from the system. Wastewater was fed into the system at a constant frequency and feeding was controlled by a computer based pumping and timing system. The feeding frequency was 96 times per day, based on a previous

evaluation by Hu and Gagnon (2006a). This translates to a cycle length of 15 minutes with 4 minutes of pumping wastewater onto the surface of the media followed by 11 minutes of no wastewater addition, allowing the wastewater to percolate through the media, leaving the biofilter in unsaturated conditions. Air holes (0.5 cm diameter) were drilled into the top of the filter units to keep the process under aerobic conditions.

RBF columns (diameter 2.5 cm, total length 20 cm) were filled with crushed glass media (15 cm of the total length) with effective size ($d_{10}$) of 1.5 mm. Filter media was supported at the base of the column by a layer of pea gravel, which helped to prevent clogging of the system. Columns were covered with aluminium foil to avoid contact with sunlight, eliminating effects due to photolysis and to prevent the growth of photosynthetic organisms. The septic, recirculating and effluent tanks were also covered with aluminium foil and both the septic tanks and recirculating tanks were sealed with parafilm and foil to simulate the conditions in the field. Water balance and loading rates for the system were calculated using experiences from previous studies and following USEPA (2008) recommendation. Following these recommendations, a hydraulic loading rate (HLR) was selected to be 0.20 $m^3/m^2/d$, resulting in a daily flow of 100 mL per column. The RBF system was operated at room temperature between 21 and 23ºC during the experiment.

### 7.3.2 Reagents And Standards

Reagents and standards for PhAC analysis are described in Chapter 3. Both raw and treated wastewater came from Mill Cove Wastewater Treatment plant in Bedford, Nova Scotia, Canada. Mill cove is a secondary activated sludge facility with UV disinfection

that treats 5 million gallons per day. The background concentrations of the four pharmaceuticals in both the treated and untreated wastewater were negligible.

### 7.3.3 Biofilm Analysis For Ammonia Oxidizing Bacteria

Biofilm attached to the biofilter media was sampled by aseptically removing some crushed glass media from the biofilter and placing it in a 50 mL sterile conical tube containing sterile phosphate buffered solution. The top and bottom of the biofilter were analyzed separately to capture aerobic and nitrifying conditions throughout the length of the biofilter. The biofilm was visible on the media, and was removed from the filter media by vortexing for 1 minute. The liquid suspension was removed and plated using the spread plate method on Tryptic Soy Agar (TSA) (Becton Dickinson and Companies, Sparks, MD). The plating was done to verify that there were viable bacteria in the biofilm.

For DNA analysis, 10 mL of the biofilm suspension was taken and centrifuged for ten minutes at 4,000 rpm. The supernatant was removed, and 1.5 mL of the sample from the bottom of the centrifuge tube was transferred to a 2 mL microcentrifuge tube, and was then centrifuged for 10 minutes at 10,000 rpm. The sampled was washed twice in phosphate buffer solution prior to DNA extraction. The DNA extraction was performed using an Ultra Clean Soil DNA kit (MoBio Laboratories Inc., Carlsbad, CA). QPCR was performed using the method detailed by Regan et al. (2007) for amoA real-time PCR. The amoA gene is exclusively found in ammonia oxidizing bacteria (Regan et al., 2007). The amoA primers were purchased through Sigma-Aldrich. The DNA of *Nitrosomonas europae* (ATCC 19718) was used as a positive control. The thermocycle was performed

using the Cepheid Smart Cycler II (Cepheid, Sunnyvale, CA). The resulting PCR products were then verified on 1.4% agarose gel using gel electrophoresis (PowerPac Basic, Bio-Rad, Hercules, CA). The agarose gel was then stained with ethidium bromide and photographed under UV light.

### 7.3.4 Experimental Design

Two trials were conducted in this study to investigate the removal of selected PhACs within an RBF system. The first was a short-term treatability trial of the removal of combined PhACs and gemfibrozil in isolation at 200 µg/L. The second was a longer-term concentration trial to investigate the reproducibility of the results from the short-term trial, and to compare removal potential at high (200 µg/L) and low (20 µg/L) concentrations. Specific details of trial setups and average water quality can be found in Table 7-2.

Table 7-2 Summary of conditions for the two RBF trials.

| | **Trial 1: Investigating treatability** | **Trial 2: Investigating concentration effects** |
|---|---|---|
| PhACs | Gemfibrozil, Ibuprofen, Naproxen, Diclofenac | Gemfibrozil, Ibuprofen, Naproxen, Diclofenac |
| # of RBFs | 6 | 4 |
| Treatments | 2 – Control<br>2 – Gemfibrozil<br>2 – Mixture of 4 PhACs | 2 – Control<br>2 – Mixture of 4 PhACs |
| Nitrifying conditions | Yes | Yes |
| Start-up with Raw Wastewater | 5 days | 5 days |
| Length of trial at 200 µg/L | 20 days | 73 days |
| Length of trial at 20 µg/L | 0 days | 25 days |
| Average Influent pH | 7.42 | 6.48 |
| Average Influent $NH_3$-N (mg/L) | 32.4 | 24.3 |
| Average Influent $BOD_5$ (mg/L) | 7 | 9 |
| Average Influent TSS (mg/L) | 10 | 12 |

#### *7.3.4.1 Treatability Trial Conditions*

In the treatability trial, the experimental setup consisted of six parallel RBFs operating for 25 days total. An initial five-day acclimation period was conducted by feeding raw wastewater into the system to establish a biofilm. After the acclimation phase, the feed water was changed to secondary treated effluent and PhACs were spiked into the system at 200 μg/L, marking the beginning of the trial (Time = 0), which lasted 20 days. Treated wastewater was used due to its decreased suspended solids content which helped to eliminate clogging of the bench-scale system. The Mill Cove wastewater treatment plant does not employ nitrification processes, thus the ammonia levels in raw and treated wastewater samples were similar. Two RBFs were set up as single PhAC RBFs and were fed secondary treated wastewater and gemfibrozil at 200 μg/L. Two combined PhAC RBFs were fed with secondary treated wastewater and a mixture of the four PhACs (gemfibrozil, ibuprofen, naproxen and diclofenac), each at 200 μg/L. The final two RBFs were set up as control filters and fed with secondary treated wastewater only. All six RBFs were set up under nitrifying conditions.

#### *7.3.4.2 Concentration Trial Conditions*

For the concentration trial, four RBFs were set up under nitrifying conditions. Two of these RBFs were fed only secondary treated wastewater whereas the other two were fed with secondary treated wastewater and a mixture of the 4 PhACs mentioned above. The long-term trial was divided into 4 phases that lasted a total 103 days. The first phase consisted of a 5-day acclimation period, during which all four RBFs were fed raw wastewater to establish a biofilm as in the treatability trial. After the acclimation period, the feed water was changed to secondary treated effluent and pharmaceutical loading at

200 μg/L started, marking the beginning of the trial (Time = 0) and Phase A. Phase B started at 65 days, at which point there was a power failure and the pumps were shut off for several hours. When the power returned, the pumps ran continuously for a few hours, which resulted in clogging of the RBFs. About 1 cm of media and biofilm was removed from the surface of each RBF and the system was restarted with PhAC concentration at 200 μg/L. The RBFs were given time to re-acclimate and then at day 73, the start of Phase C began where the concentration of PhAC entering the system was reduced to 20 μg/L to simulate more environmentally relevant concentrations. Phase C lasted 25 days.

For both trials, samples were taken from influent and effluent tanks every 2-3 days and analyzed for pH, ammonia ($NH_3$-N), nitrate nitrogen ($NO_3^-$-N) and pharmaceuticals,

## 7.4 Results and discussion

### 7.4.1 Confirmation Of Nitrifying Conditions

Both experimental trials were set up under nitrifying conditions as studies have shown that for many PhACs, removal rates are higher under nitrifying versus denitrifying conditions (Maeng et al., 2013; Fernandez-Fontaina et al., 2012; Falås et al., 2012; Miège et al., 2008). Falås et al. (2012) showed that ammonia oxidizing bacteria have a low ability to transform PhACs and thus the increased PhAC removal rates are due to processes other than degradation by ammonia oxidizing bacteria, likely through growth of other slow-growing organisms.

During the 5-day growth period at the beginning of the treatability trial, the system was fed with raw wastewater with a concentration of $NH_3$-N of 32.2 mg/L ± 1.98 and pH of

7.16 ± 0.35. The influent was changed to treated wastewater after five days, at which point PhAC addition started. The secondary treated wastewater that was used as influent for the RBFs had a $NH_3$-N concentration of 32.4 mg/L ± 2.12, and pH of 7.42 ± 0.48 for the treatability trial, and $NH_3$-N concentration of 24.3 mg/L ± 8.47, and pH of 6.48 ± 0.39 for the concentration trial. Figure 7-2 shows the concentration of ammonia in the influent and effluent of the combined PhAC RBFs in the treatability trial. Both gemfibrozil and control RBFs showed similar trends for ammonia removal with effluent concentrations being significantly lower than influent concentrations ($p<0.05$, paired t-test).

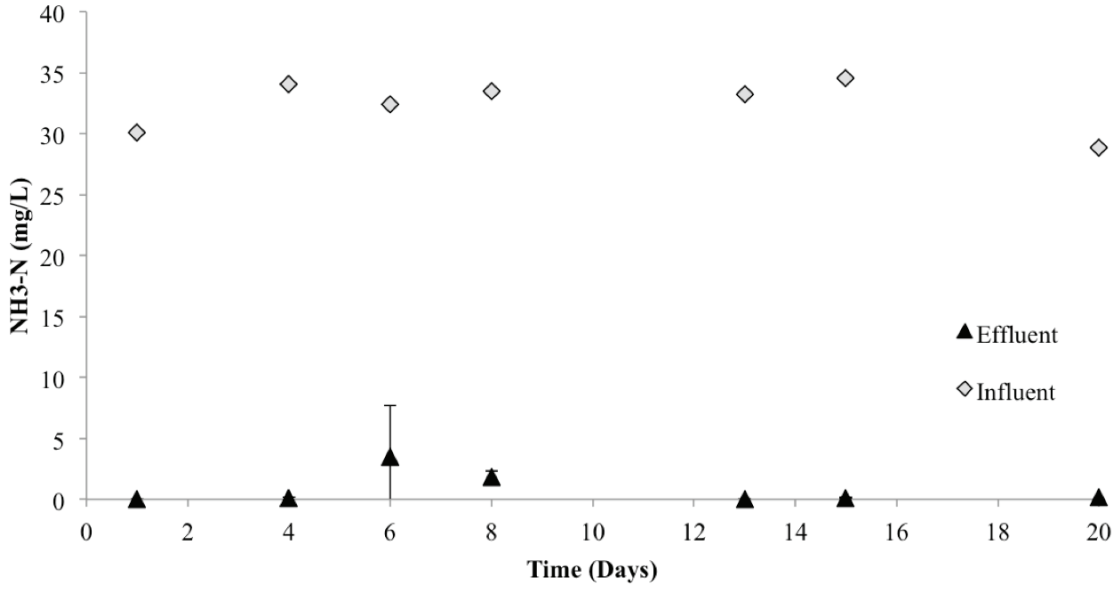


Figure 7-2 Average total ammonia nitrogen concentrations in influent and effluent of the PhAC mixture RBFs from the treatability trial.

Within the treatability trial, ammonia removal over the length of the trial was determined to be 98.1% ± 2.4 in single PhAC RBFs, 97.1% ± 4.4 in combined PhAC RBFs, and 91.8% ± 15.5 in control RBFs. The ammonia removal rates experienced in this trial were consistent with Hu and Gagnon, (2006a) and USEPA (2008).

Nitrification results in the conversion of ammonia to nitrite and subsequently nitrate. It would therefore be expected that nitrate concentration in RBF effluent should exceed that in the influent, which was the case in all RBFs ($p<0.05$, paired t-test) in the treatability trial. The pH was significantly lower in the effluent compared to the influent ($p<0.05$, paired t-test), confirming the occurrence of nitrification processes since ammonia conversion to nitrate releases $H^+$ ions, increasing the acidity of the solution.

The presence of ammonia oxidizing bacteria (AOB) in the treatability trial was confirmed using PCR and gel electrophoresis. The expected size of the amoA PCR product is 491bp. In Figure 7-3, lane 1 and lane 18 contain the DNA size ladder. The expected PCR product can be seen in lanes 2-7, 9-13, 16 and 17. Ammonia oxidizing bacteria were found to be present in the top and bottom of filter one, two, three, five and six, and in the bottom only of filter four. Lanes 16 and 17 show the PCR product for the DNA from *Nitrosomonas europae* ATCC 19718, the positive control. The confirmed presence of AOB suggests a plausible biological pathway for ammonia removal.

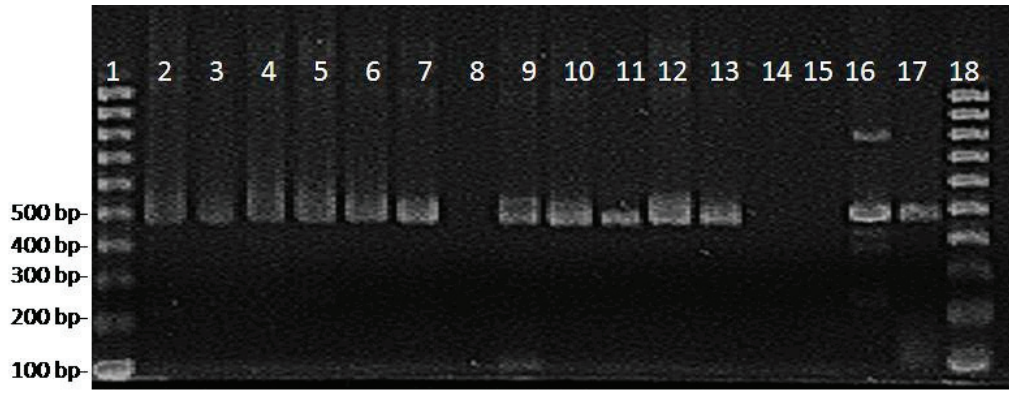


Figure 7-3 Agarose gel electrophoresis of PCR products of amoA primer for biomass extracted from treatability trial RBFs. (1) 100 bp EZ Load Molecular Ruler (BioRad), (2) top of Single PhAC Filter 1, (3) bottom of Single PhAC Filter 1, (4) top of Single PhAC Filter 2, (5) bottom of Single PhAC Filter 1, (6) top of PhAC Mixture Filter 1, (7) bottom of PhAC Mixture Filter 1, (8) top of PhAC Mixture Filter 2-no PCR product, (9) bottom of PhAC Mixture Filter 2, (10) top of control filter 1, (11) bottom of control filter 1, (12) top of control filter 2, (13) bottom of control filter 2, (16) *Nitrosomonas europaea* (ATCC 19718), (17) *Nitrosomonas europaea* (ATCC 19718) - duplicate, (18) 100 bp EZ Load Molecular Ruler (BioRad).

Nitrification conditions were slightly different within the concentration trial. Figure 7-4 shows the total ammonia nitrogen concentrations in the influent and effluent of the control RBFs in the concentration trial. It took approximately 8 days for the system to reach a steady state with respect to ammonia removal through nitrification after the initial 5-day acclimation period. In Phase A, from day 8 – 65 there was an average of 97.6% removal of ammonia. Following the system upset (power failure marking the start of Phase B) on day 65 that resulted in removal of the upper layer of media from the RBFs, there was a re-acclimation period during which there was decreased ammonia removal, likely due to the removal of ammonia oxidizing bacteria. During Phase C (day 75 and beyond), the system was again at steady state and achieved an average of 95.8% removal,

indicating a quick system recovery. As with the treatability trial, nitrate concentrations were higher in effluent of RBFs compared to influent (paired t-test, $p<0.05$).

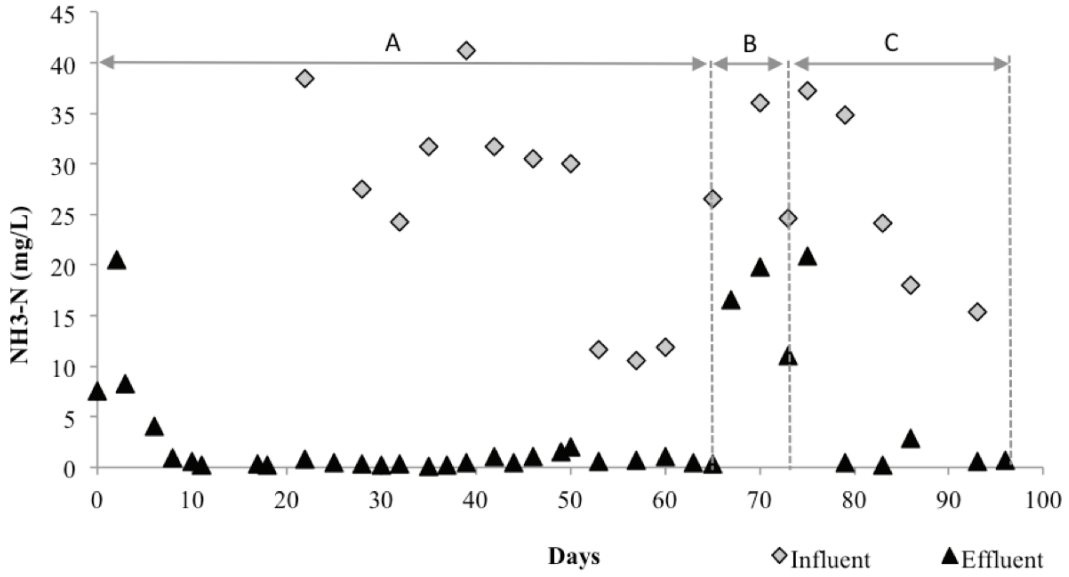


Figure 7-4 Average total ammonia nitrogen concentrations in influent and effluent of the Control RBFs from the concentration trial. (Phase A – initial acclimation and pseudo steady-state with PhAC concentration at 200 µg/L; Phase B – System upset; Phase C – Return to steady state, PhAC concentration reduced to 20 µg/L)

## 7.4.2 Removal Mechanisms

### *7.4.2.1 Potential Removal Pathways*

Two potential pathways of PhAC removal were identified in this study, a) sorption and b) biological transformation. Figure 7-5 depicts the potential removal and transformation pathways for the 4 PhACs within the RBF column. PhACs can adsorb to the extracellular polymeric substance (EPS), a bacteria-produced material that acts to hold the biofilm together, as shown by (a) in Figure 7-5. The adsorbed PhACs may then be transformed by bacteria in the biofilm (b) in Figure 7-5, or they may remain adsorbed to the biofilm.

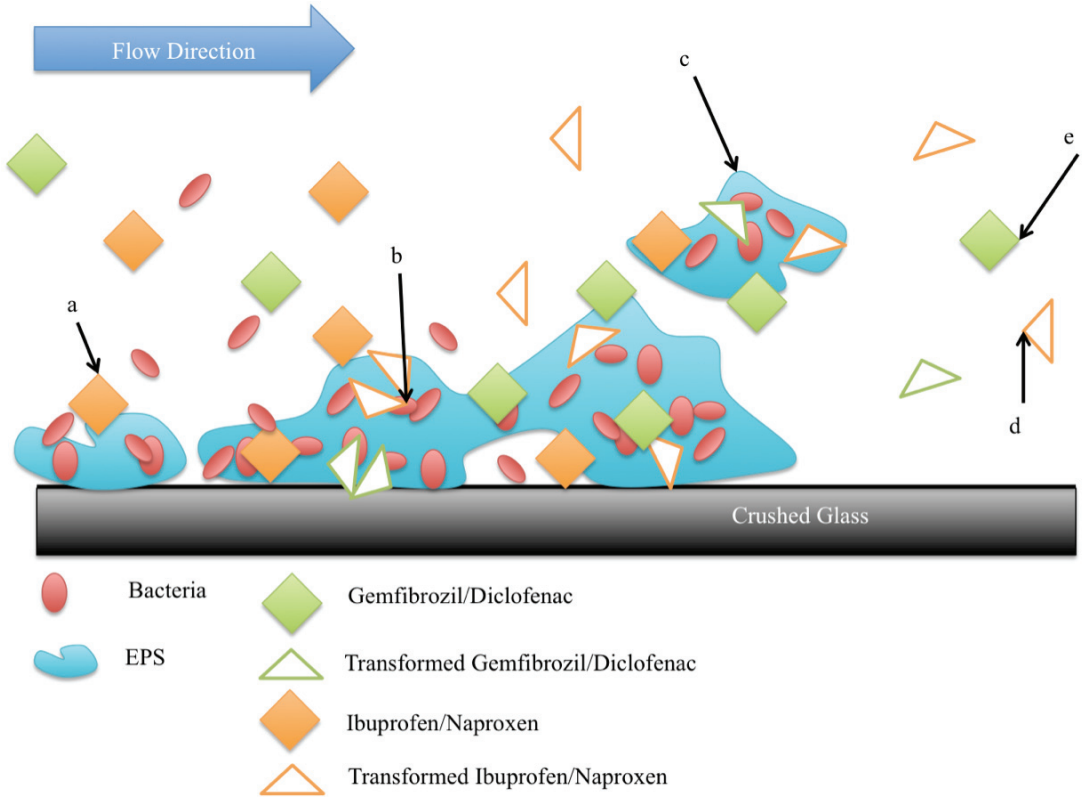


Figure 7-5 - Potential PhAC removal and transformation mechanisms within the RBF column. a) adsorbed PhAC, b) biologically transformed PhAC, c) detached biofilm fragment, d) desorbed dissolved biologically transformed product and e) desorbed dissolved PhAC.

PhACs can act as electron donors for oxidation reactions that produce energy for biomass maintenance and growth (Rittmann and McCarty, 2001). Free-floating planktonic bacteria may also oxidize PhACs; however it is more likely that oxidation occurs within the biofilm due to the amount of biofilm biomass present in the system, compared to planktonic bacteria (Urase and Kikuta, 2005). It is challenging to distinguish between planktonic and biofilm degradation, as PhACs are likely present in both dissolved and sorbed components and it is difficult to experimentally separate the components (Falås et al., 2012; Pomiès et al., 2013).

As biofilms mature, fragments disperse or detach from the biofilm (c) in Figure 7-5 and these fragments may travel through the column and end up in the RBF effluent. Within these biofilm fragments there may be oxidized PhAC transformation products or PhACs still adsorbed to the biofilm fragment. PhACs and oxidized PhAC products can also desorb from the biofilm surface and travel through the columns (d) and (e) in Figure 7-5. These PhACs and transformation products may eventually end in RBF effluent, which could cause sporadic elevated effluent concentrations. Previous studies have shown there is little adsorption of PhACs onto carrier surfaces within moving bed biofilm flow-through reactors without biofilm presence (Zupanc et al., 2013). Crushed glass was used as RBF media for this study to eliminate the potential for PhAC adsorption to media surface. Partitioning of PhACs to the air phase through volatilization was assumed to be minimal because the Henry's law constants for the 4 PhACs are less than $1.0 \times 10^{-3}$ atm $m^3$/mol (Urase and Kikuta, 2005; Namkung and Rittmann, 1987). Therefore the two most likely PhAC removal mechanisms for the RBFs are biological transformation and adsorption (and potential re-release) to EPS.

#### *7.4.2.2 Removal Mechanisms For Naproxen And Ibuprofen*

In the treatability trial, removal of ibuprofen and naproxen was nearly 100% over the 20-day trial (Figure 7-6). Similarly, the removal of naproxen and ibuprofen was consistently over 80% throughout Phases A and C in the concentration trial as shown in Figure 7-7, with several instances of decreased removals after 20 days. During the system upset in Phase B, where biomass was removed from the system following a power failure, both ibuprofen and naproxen experienced a significant decrease in removal compared to the average removals in Phase A. The removal of biomass could have resulted in a decrease

in biofilm capacity to oxidize these two PhACs. The system recovered within 7 days for these two PhACs, allowing time for the biofilm to re-establish itself. This response to biomass removal, combined with the relatively stable removals in Phases A and C, indicates that the governing removal process for ibuprofen and naproxen in the RBF system was biological transformation.

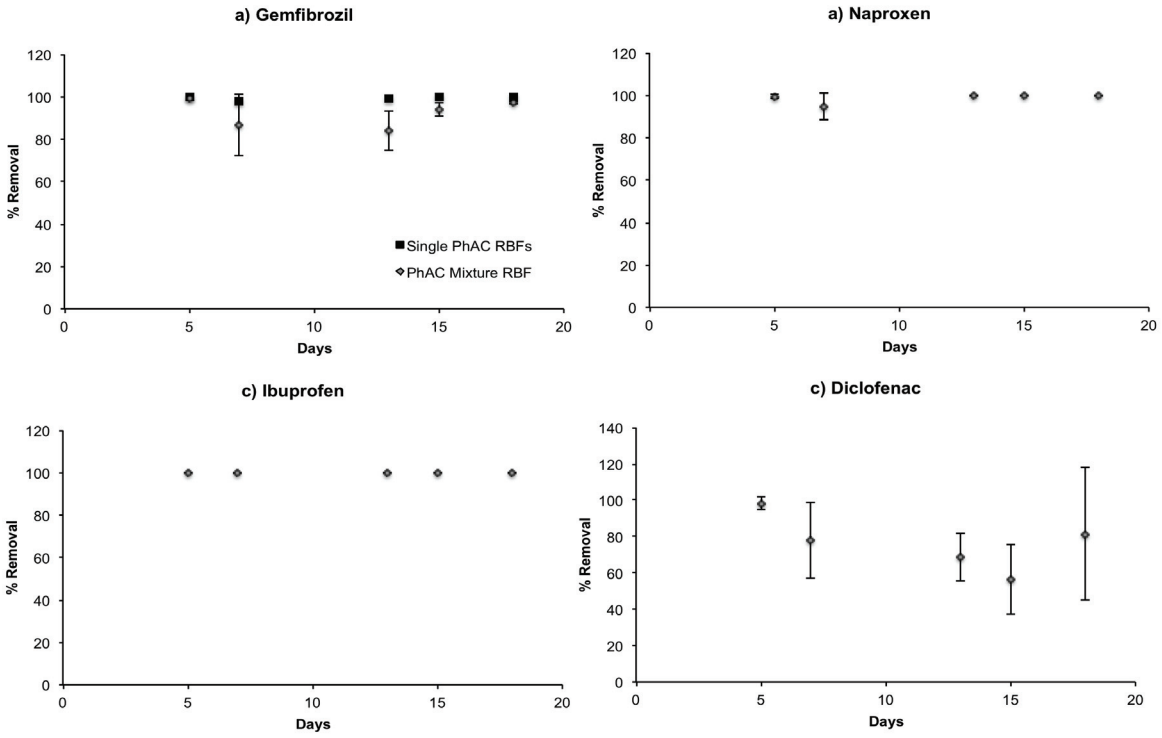


Figure 7-6 Removal of the four PhACs over the length of the experiment in the treatability trial. Error bars represent ± one standard deviation of the mean between the duplicate RBFs for each treatment.

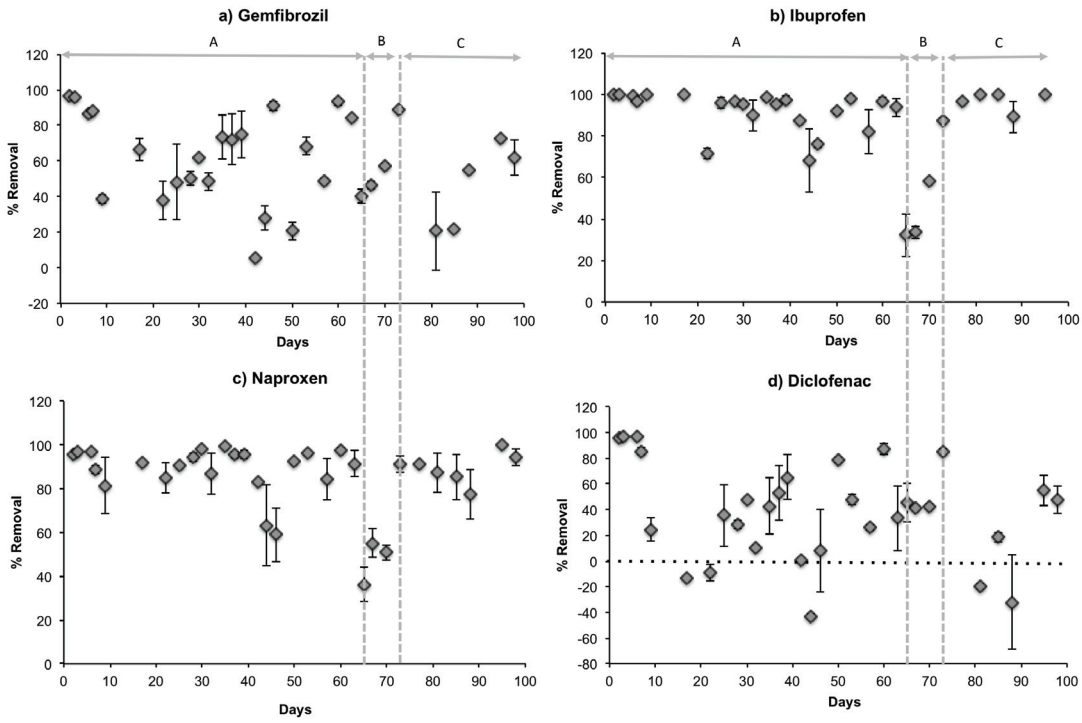


Figure 7-7 Removal percentages of the 4 PhACs over the length of the concentration trail. Phase A at 200 µg/L is shown from 0 to 65 days. The system upset and re-acclimation period at 20 µg/L is shown by Phase B (day 65 to 73). Phase C from day 73 to 98 represents when the concentration of PhACs was dropped to 20 µg/L. Error bars represent ± 1 standard deviation of the mean between the duplicate RBFs for each treatment.

Adsorption and subsequent release from detached biofilm fragments may account for the few data points that had decreased removal, however the majority of removal likely occurred due to biological transformation of ibuprofen and naproxen. Biological degradation rates for the 4 PhACs have been calculated through various studies, generally for activated sludge systems. The rates vary substantially based on treatment process configuration, water quality, loading rates, etc.; however, the biological degradation constants $K_{biol}$ for the 4 PhACs are consistent with the observations in this study with the highest biodegradation constant for ibuprofen followed by naproxen and gemfibrozil,

with diclofenac exhibiting the lowest biodegradation potential of the 4 PhACs (Pomiès et al., 2013; Fernandez-Fontaina et al. 2013; Joss et al., 2006; Urase and Kikuta, 2005).

Joss et al. (2005) found there was very little impact on removal of naproxen and ibuprofen due to changes in reactor configuration and SRT between conventional activated sludge, membrane bioreactors and Biostyr® fixed bed biofilm reactors. This indicates many organisms have the ability to transform ibuprofen and naproxen, their removal is insensitive to reactor design, and the presence of a microbial community is an important factor in the process.

#### *7.4.2.3 Removal Mechanisms For Gemfibrozil And Diclofenac*

For gemfibrozil and diclofenac removal within Phases A and C in the concentration trial, the results were more variable than those for naproxen and ibuprofen; some samples had a high removal and some a low or less than zero (diclofenac) removal (Figure 7-7) that could be achieved through release of adsorbed accumulated diclofenac. The high variability in gemfibrozil and diclofenac removal could indicate that, in addition to some biological transformation, these PhACs are being sorbed to the biofilm and subsequently released through detachment of biofilm fragments as the biofilm matures. Furthermore during the system upset in Phase B, gemfibrozil and dicloenac removals were not greatly affected, which indicates biological transformation of these PhACs is likely not as dominant a removal mechanism as it is for ibuprofen and naproxen.

The sorption coefficient ($K_d$) is an intrinsic physico-chemical parameter; however it will vary based on the type of solid matrix involved (Pomiès et al., 2013). Sorption depends

largely on hydrophobic interactions (i.e. $K_{ow}$) but can also be influenced by other factors such as electrostatic interactions, cationic exchanges, cationic bridges, surface complexation, and hydrogen bridges (Pomiès et al., 2013; Urase and Kikuta, 2005; Tolls, 2001). No studies have been conducted to date on the sorption of PhACs onto RBF biofilm; however, there have been several studies conducted on sorption of PhACs to other types of wastewater biosolids, including activated sludge and membrane bioreactors. The results are highly variable (Pomiès et al., 2013), but the general consensus is that ($K_d$) for the four PhACs is generally low, and $K_{ow}$ for gemfibrozil and diclofenac are slightly higher than for ibuprofen and naproxen (4.77 an 4.51 versus 3.97 and 3.2), suggesting they could have a slightly higher sorption affinity.

For both gemfibrozil and diclofenac for the first 7 days of the concentration trial and again on day 70 (several days after the removal of biomass), the removals were between 80 and 100%. In both these instances, removals decreased following these periods. This pattern further supports adsorption as a more dominant removal mechanism, as the PhACs would have a higher potential for adsorption at the beginning of the trial or as new biofilm was rapidly forming (more available binding sites).

Fernandez-Fontaina et al., (2012) found that solids retention time (SRT) was the only operational parameter to have an impact on diclofenac biodegradation under nitrifying conditions in an activated sludge process. Other factors (e.g., hydraulic retention time (HRT), temperature) contributed to less than 15% removal of diclofenac, while long SRT (150 days) resulted in 70% removal. Clara et al., (2005) and Lishman et al., (2006) found

similar results in lab scale reactors and full scale plants. It is possible that the increased SRT allows for the development of a slow-growing bacterial community that is diverse and capable of degradation of diclofenac (Fernandez-Fontaina et al., 2012). Since fixed film systems generally have a long SRT and high biomass concentration compared to activated sludge processes (Su and Ouyang, 1984), it is likely that the RBFs in this study were operating under conditions that could support a biofilm capable of degrading diclofenac. Since the trials were relatively short overall, there is a chance that removal of diclofenac could increase if the system were operated for a longer period of time, allowing for further establishment of a community that is able to transform the compound.

### 7.4.3 PhAC Removal – Impact Of Concentration

PhACs may be biodegraded either as a primary or as a secondary substrate. If acting as a primary substrate, there is a chance that environmentally relevant concentrations may be below the threshold at which the reaction speed is sufficient to provide the organism with adequate energy for growth, often termed $S_{min}$ (Rittmann and McCarty, 2001). During the treatability trial and Phase A of the concentration trial, an input concentration for PhACs of 200 µg/L was used to investigate general removal capabilities at concentrations where changes in concentration are easier to determine with confidence analytically.

In order to investigate the removal at more environmentally relevant concentrations, the concentration of PhACs was reduced to 20 µg/L in Phase C of the concentration trial. From Figure 7-7, it can be seen that for both naproxen and ibuprofen, the removal efficiencies remained near 100%, while gemfibrozil and diclofenac continued to have

lower and more variable removals. There was no statistically significant difference in removal efficiencies found for any of the 4 PhACs between the high and low concentrations ($P>0.05$, unpaired t-test). This indicates that even at environmentally relevant concentrations, removal is still occurring, likely because PhACs are being used as secondary rather than primary substrates.

### 7.4.4 PhAC Removal – Impact Of Mixtures

For the treatability trial, the % removals of ibuprofen, naproxen and diclofenac in the combined PhAC RBF over the length of the trial were: naproxen at 98.8% ± 2.7, ibuprofen at 99.9% ± 0.1 and diclofenac at 76.5% ± 18.6 (Figure 7-6). The average removal of gemfibrozil in the single PhAC RBFs was 99.4% ± 0.72 averaged over the 5 sampling days, while the average removal of gemfibrozil in combined PhAC RBFs was 92.2% ± 7.67. There was no statistically significant difference in gemfibrozil removal in the single PhAC RBFs versus the mixture RBFs ($p>0.05$, paired t-test), indicating the presence of multiple PhACs does not limit or inhibit the microbial degradation or sorption capacity within the system. This could be either due to the low concentration of PhACs compared to other organics and their use as a secondary substrate, the abundance of biofilm sorption sites, or the diversity and abundance of the microbial population able to degrade these substances.

### 7.4.5 PhAC Removal – Repeatability

The average % removal of the 4 PhACs in both the treatability and concentration trials are presented below in Figure 7-8. Data is presented for the concentration trial for all of Phase A at 200 µg/L and also for the first 20 days of the trial to compare to the results of the treatability trial (20 days long as well). The difference in removal between the

treatability trial and Phase A of the concentration trial was not statistically significant for ibuprofen, naproxen and diclofenac ($P>0.05$, unpaired t-test), but was statistically significant for gemfibrozil ($P<0.05$, unpaired t-test). However, when just considering the same timeframe for the two trials (first 20 days only), the average % removals in the concentration trial are much closer to those observed in the treatability trial.

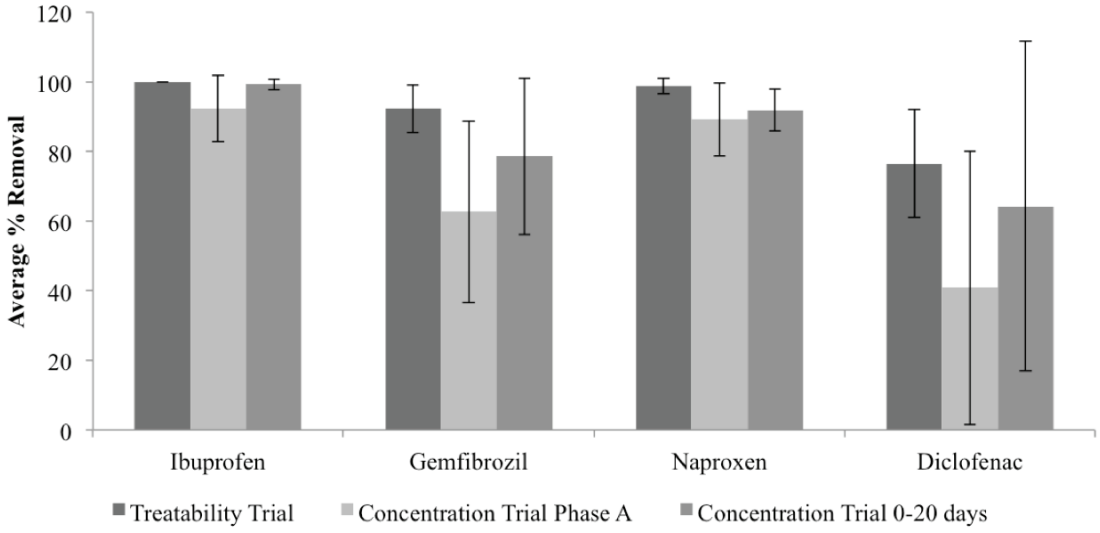


Figure 7-8 Comparison of average % removals of the four PhACs in the treatability trial and the concentration trial. For the concentration trial, one series of data is from Phase A (0-65 days) and the other series represents the first 20 days of Phase A. Error bars represent ± one standard deviation from the mean.

There are several possible explanations for the difference in removal over the course of Phase A of the concentration trial compared to the treatability trial. The first would be due to the length of the trial. Over time, if PhACs are being adsorbed to biofilm, there could be some detachment of biofilm fragments containing adsorbed PhACs leading to increased effluent concentrations and could explain sporadic decreases in removal efficiency for any or all four PhACs as shown in in Figure 7-7 and described in section 7.4.2.1.

A second explanation for the difference in removal between the two trials could be the difference in influent raw water quality between the two trials. The average pH within the RBFs in the treatability trial dropped from an average of 7.42 in the influent to 6.84 in the effluent. The reverse trend was observed in the concentration trial where the average influent pH was 6.48, while average effluent pH was 7.26. Since all four PhACs used in this study are acidic compounds, a difference in pH could change the electrostatic interaction between PhACs and biofilm within the system (Stevens-Garmon et al., 2011), which could affect adsorption processes. Using a two-phase model that accounts for sorption to sludge and subsequent biodegradation within the sludge, Urase and Kikuta (2005) found that acidic PhACs generally had higher removals at lower pH. They found that the limiting stage for removal was the transfer of PhACs from the water phase to the sludge. At neutral pH they found no linear relationship between the water-sludge partition coefficient ($K_d$) of the 4 PhACs and their log $K_{ow}$; however, at acidic pH there was a linear relationship between the two, because the acidic PhACs are more electrically neutral at lower pH. Since the average pH in the RBF effluent of the concentration trial was higher than the treatability trial, this could explain the slightly lower removals obtained in the concentration trial, as the higher pH may have resulted in less sorption to the biofilm.

### 7.4.6 Comparison Of PhAC Removal To Literature

Many studies (Lishman et al., 2006; Stumpf et al., 1999; Paxeus 2004; Ternes 1998; Kosjek et al., 2007; Kreuzinger et al., 2004; Zwiener and Frimmel 2003; Poseidon, 2004) have been conducted on the removal of PhACs within wastewater treatment plants. A

comparison of removal efficiencies becomes difficult, however, due to process configuration, treatment type, scale of the systems studied, and whether compounds are spiked into a laboratory system, or simply monitored through a full-scale plant. Table 7-3 presents a summary of selected papers reporting removal efficiencies of the four PhACs studied in this paper across several types of treatment systems. This is not an exhaustive list of studies on removal; it is meant to provide a snapshot of the trends in removal efficiency of the four PhACs studied.

Table 7-3 Summary of removal of selected PhACs within wastewater systems.

| PhAC | Type of Treatment plant | Average % Removal | Reference |
|---|---|---|---|
| Gemfibrozil | Full Scale WWTP | 38-69% | Lishman et al., 2006 |
| | Full Scale WWTP | 49% | Stumpf ef al., 1999 |
| | Full Scale WWTP | 55% | Paxeus, 2004 |
| | Full Scale WWTP | 69% | Ternes, 1998 |
| | Trickling Filter | 25% | Camacho-Munoz et al., 2011 |
| | Full Scale WWTP | 50% | Camacho-Munoz et al., 2011 |
| Naproxen | Full Scale WWTP | 79-98% | Lishman et al., 2006 |
| | Full Scale WWTP | 78% | Stumpf ef al., 1999 |
| | Full Scale WWTP | 80% | Paxeus, 2004 |
| | Full Scale WWTP | 66% | Ternes, 1998 |
| | Pilot WWTP | 87-94% | Kosjek et al., 2007 |
| | Sand Filter | 65% | Matamoros et al., 2009 |
| | Horizontal Flow Wetland | 45% | Matamoros et al., 2009 |
| | Vertical Flow Wetland | 92% | Matamoros et al., 2009 |
| | Trickling Filter | 45% | Camacho-Munoz et al., 2011 |
| | Full Scale WWTP | 80% | Camacho-Munoz et al., 2011 |
| Diclofenac | Full Scale WWTP | -143-77% | Lishman et al., 2006 |
| | Full Scale WWTP | -7.9-69% | Kreuzinger et al., 2004 |
| | Full Scale WWTP | 75% | Stumpf ef al., 1999 |
| | Full Scale WWTP | 39% | Paxeus, 2004 |
| | Full Scale WWTP | 69% | Ternes, 1998 |
| | Full Scale WWTP | 15-40% | Poseidon, 2004 |
| | Membrane Pilot Plant | -8-51 | Kreuzinger et al., 2004 |
| | Pilot WWTP | 1-6% | Zwiener and Frimmel, 2003 |
| | Biofilm Reactor (Oxic) | 1-4% | Zwiener and Frimmel, 2003 |
| | Pilot WWTP | 49-59% | Kosjek et al., 2007 |
| | Sand filter | 82% | Matamoros et al., 2009 |
| | Horizontal Flow Wetland | 21% | Matamoros et al., 2009 |
| | Trickling Filter | 0% | Camacho-Munoz et al., 2011 |
| | Full Scale WWTP | 0% | Camacho-Munoz et al., 2011 |
| Ibuprofen | Full Scale WWTP | 91-98% | Lishman et al., 2006 |
| | Full Scale WWTP | -1-99% | Kreuzinger et al., 2004 |
| | Full Scale WWTP | 75% | Stumpf ef al., 1999 |
| | Full Scale WWTP | 96% | Paxeus, 2004 |
| | Full Scale WWTP | 90% | Ternes, 1998 |
| | Full Scale WWTP | >90% | Poseidon, 2004 |
| | Membrane Pilot Plant | 97-99% | Kreuzinger et al., 2004 |
| | Pilot WWTP | 57-60% | Zwiener and Frimmel, 2003 |
| | Biofilm Reactor (Oxic) | 64-70% | Zwiener and Frimmel, 2003 |
| | Pilot WWTP | 90.8-91.5% | Kosjek et al., 2007 |
| | Sand Filter | 86% | Matamoros et al., 2009 |
| | Horizontal Flow Wetland | 65% | Matamoros et al., 2009 |
| | Vertical Flow Wetland | 89% | Matamoros et al., 2009 |
| | Trickling Filter | 85% | Camacho-Munoz et al., 2011 |
| | Full Scale WWTP | 95% | Camacho-Munoz et al., 2011 |

As shown in Table 7-3, ibuprofen generally exhibits the highest removal efficiency of the four PhACs, most often with a removal >90%. Naproxen was next highest with removals generally between 65 and 95%, followed by gemfibrozil with removals between 50 and 70%. Diclofenac shows the lowest and most variable removal rates averaged at approximately 15-50%.

In summary, this study shows that RBFs operating under nitrifying conditions are capable of removing PhACs at the higher end of the range of removals reported in literature for pilot and full scale plants as shown in Table 7-3. The average removals in this study between the two trials were between 92 – 99% for ibuprofen, 89 and 99% for naproxen, 62 – 92% for gemfibrozil and 40 and 76% for diclofenac.

## 7.5 CONCLUSIONS

In general, there is a paucity of information on the effectiveness of small-scale wastewater treatment technologies for the removal of PhACs. This is the first study to date to focus on nitrifying RBFs and their ability to remove selected PhACs. Bench-scale trials showed that, on average, RBFs show promise as a technology to remove acidic PhACs and were able to remove ibuprofen, naproxen, gemfibrozil and diclofenac at the high end of the range found in other pilot and full-scale biological technologies. The average removals in this study were between 92 – 99% for ibuprofen, 89 and 99% for naproxen, 62 – 92% for gemfibrozil and 40 and 76% for diclofenac. The main conclusions from this study are:

- Removal mechanisms vary for the four PhACs. Ibuprofen and naproxen are likely removed mainly through biological transformation, whereas gemfibrozil and

diclofenac are less readily biologically transformed and their main removal mechanism is likely adsorption to the EPS.

- RBFs were robust, responded quickly and were able to recover from major system upsets.
- Removal of PhACs was repeatable between RBF trials with slightly different water quality. However, removal of PhACs is dependent on raw water quality, with pH potentially playing a significant role in adsorption potential for acidic PhACs.
- PhACs were likely transformed as secondary substrates, as there was no impact on removal of gemfibrozil when it was the only PhAC in the system versus presence as a mixture of 4 PhACs. This is further confirmed as the 4 PhACs experienced similar removals at both high (200 μg/L) and more environmentally relevant (20 μg/L) concentrations.

# CHAPTER 8 CONCLUSIONS AND RECOMMENDATIONS

## 8.1 Conclusions

The central hypothesis for this thesis was that reactions between chlorine and gemfibrozil during wastewater disinfection can lead to the formation of chlorinated reaction products, whose presence could lead to negative impacts on organisms in receiving waters.

This hypothesis was addressed through four main objectives.

1. Identify the structure of products formed from the reaction of gemfibrozil and chlorine.
2. Determine the kinetics of gemfibrozil degradation and formation of reaction products during chlorination.
3. Investigate the impacts of exposure to gemfibrozil and the main chlorinated reaction product on bacterial abundance in a simulated receiving water experiment.
4. Examine the removal of gemfibrozil from wastewater in a small-scale treatment technology to minimize the potential of chlorinated reaction product formation during disinfection.

**The main contribution from this thesis is the identification of 4 gemfibrozil chlorination reaction products and evidence to show that the main reaction product, 4'-ClGem, can be formed in typical wastewater conditions.** A summary of the key findings for each of the four objectives is provided below in Table 8-1.

Table 8-1 Summary of key findings

| Chapter | Objective | Key Findings |
|---|---|---|
| 4 | Identification of chlorinated reaction products | • **Reactions between gemfibrozil and chlorine revealed four chlorinated reaction products.**<br>• **Structures for three products were identified using GC-MS, ESI-MS, and NMR. The structure for the fourth was hypothesized based on GC-MS evidence.**<br>• Two products were mono chlorinated, one was di-chlorinated and the last one was tri-chlorinated, all chlorination occurred on the ring structure. |
| 5 | Kinetics of chlorinated reaction product formation | • **Formation of chlorinated reaction products is highly dependent on pH.**<br>• In buffered ultrapure water at pH 3, three reaction products were formed. Between pH 4 and 7, only 4'-ClGem was formed. Above pH 7, there were no reaction products formed, and no degradation of gemfibrozil.<br>• **Kinetic experiments in two wastewater matrices showed formation of 4'-ClGem at pH < 7.**<br>• Application of kinetic models to two wastewaters with known gemfibrozil effluent concentrations provided estimates of daily mass loads of 4'-ClGem to the environment. |
| 6 | Receiving water impacts on bacterial abundance due to gemfibrozil and 4'-ClGem exposure | • Bench-scale batch reactor receiving water experiments revealed that both suspended and biofilm bacterial abundance are more impacted by changes in water quality than by spiked concentrations of gemfibrozil and 4'-ClGem.<br>• Suspended bacteria populations showed evidence of the use of gemfibrozil and 4'-ClGem as a substrate.<br>• A dose-response experiment using *P. fluorescens* showed no effects due to gemfibrozil exposure up to 10 mg/L, but there was a 2-log reduction in population in response to exposure to 10 mg/L of 4'-ClGem. |
| 7 | Gemfibrozil biological removal | • Bench-scale nitrifying recirculating biofilters show promise as a robust technology to reduce acidic PhACs and are comparable to other studied biological removal processes.<br>• Removals of gemfibrozil were in the range of 62-92%.<br>• There was evidence to show that removal mechanisms for gemfibrozil are a combination of adsorption to EPS and, to a lesser extent, biological transformation. |

In summary, this thesis identified four different products formed from reactions between gemfibrozil and chlorine. The formation of these reaction products is highly dependent

on pH and other water quality parameters. Of the four reaction products, 4'-ClGem, a mono-chlorinated reaction product, was found to be the most likely reaction product to form under conditions found in wastewater treatment operations.

Experiments on the impacts of gemfibrozil and 4'-ClGem on the abundance of suspended and bacterial biofilm in a simulated receiving water system showed that the compounds are more likely to be used as a substrate for bacterial growth than to induce any negative impacts on bacterial abundance. Negative impacts were observed on *P. fluorescens* suspended bacterial populations at a concentration of 4'-ClGem of 10 mg/L, which is orders of magnitude higher than would be found in the environment. Typical environmental PhAC concentrations entering wastewater treatment plants are generally in the ng/L to low μg/L range, and concentrations in receiving waters after dilution are even lower. The experiments on the impact of 4'-ClGem in receiving water are only a first step towards understanding the toxicity of this compound and potential implications on environmental health.

To prevent the formation of gemfibrozil (or any PhAC) chlorination reaction products, it is necessary to remove gemfibrozil prior to disinfection with chlorine. For large-scale centralized treatment facilities there are numerous unit operations that can be used to remove PhACs to varying extents, including, but not limited to: coagulation/flocculation, activated sludge, membrane processes, sedimentation, anaerobic digestion, ozonation, UV disinfection and chlorination. Many studies have been conducted on removal of gemfibrozil in large-scale unit operations. Some of these processes may also lead to the

formation of additional reaction products (ozone, UV, etc.). A large portion of the population is served by onsite or small-scale wastewater treatment systems. Due to the cost of some of the unit operations, small-scale and onsite systems are often reliant on biological processes with minimal chemical or energy requirements. Nitrifying recirculating biofilters are one technology that can be used for these small-scale systems. In this thesis, an experiment using bench-scale recirculating biofilters showed that >60% of gemfibrozil can be removed within the recirculating biofilters through a combination of adsorptive and biological processes, thus reducing the potential for formation of chlorinated reaction products and subsequent release to the environment.

## 8.2 RECOMMENDATIONS

### 8.2.1 Formation Of Reaction Products

This research identified four gemfibrozil chlorination reaction products. Three of these products had their structures identified, however it was not possible to determine conditions that would produce the fourth product, 6'-ClGem in isolation and thus its structure was hypothesized. It is recommended that further examination of this product be undertaken so that its structure can be confirmed. It is also recommended that standards be produced for these reaction products so that future studies can quantify the compounds as opposed to using estimates based on peak areas.

### 8.2.2 Reaction Kinetics

A reaction time of 60 minutes was chosen for the kinetic experiments in this thesis to represent contact time for chlorine disinfection within the plant. However, many plants do not de-chlorinate their effluent and thus reactions could continue after release to the environment. Data from chapter 4 suggests that the diClGem and triClGem products may

form at longer retention times. Therefore it is recommended that kinetic experiments be repeated at longer retention times.

Experiments in this thesis showed it is possible to form 4'-ClGem in a wastewater matrix; further experiments should be done on pilot or full-scale wastewater from treatment plants that chlorinate their effluent and are known to have gemfibrozil in their wastewater to determine whether there is any 4'-ClGem formed after disinfection. The application of the developed kinetic models could be applied to refine the model and to predict potential contamination.

### 8.2.3 Behaviour In The Environment

This thesis presented results from a preliminary experiment used to determine the impacts of exposure to gemfibrozil and 4'-ClGem on bacterial abundance. There are numerous standard acute and chronic toxicity tests that can be conducted on 4'-ClGem to generate more information about its characteristics and potential impacts. More importantly, biomarker tests, genotoxicity and mutagenicity studies, long-term exposure assessments and multigenerational tests could be used to determine the more likely subtle outcomes from a constant low-level exposure.

It is also recommended to look at the persistence of 4'-ClGem in the environment. Virtually nothing is known about this reaction product and whether it is stable, or subject to removal processes such as biodegradation, photolysis and adsorption in the environment.

### 8.2.4 Gemfibrozil Removal During Wastewater Treatment

To further understand the removal processes and applicability of RBFs for the removal of gemfibrozil, it is recommended that the experiment be repeated at the bench-scale with a higher strength wastewater (i.e. primary effluent) to provide the organic loading that would typically be observed in RBFs in practice. It would then be recommended to take the experiments beyond the bench-scale and use a pilot-scale setup to investigate the removal of gemfibrozil. In any future RBF studies, it is recommended to monitor the bacterial populations more closely (HPCs, ATP analysis) to obtain a more accurate measure of the health and response of the biofilm in the presence of PhACs.

# REFERENCES


Acero, J. L.; Benitez, F. J.; Real, F. J.; Roldan, G. (2010) Kinetics of aqueous chlorination of some pharmaceuticals and their elimination from Water Matrices. *Water Res*., 44, 4158-4170.

Alem, A. S.; Douglas, L. J. (2004) Effects of aspirin and other nonsteroidal anti-inflammatory drugs on biofilms and planktonic cells of *Candida albicans. Antimicrob. Agents Ch.,* 48(1), 41-47.

American Public Health Agency; American Water Works Association; Water Environment Federation (1999) *Standard Methods for the Examination of Water and Wastewater, 20th Edition*. Method 4500-Cl.

American Public Health Agency, American Water Works Association, Water Environment Federation. (2012) *Standard Methods for the Examination of Water and Wastewater,* 21st Edition.

Araujo, L.; Villa, N.; Camargo, N.; Bustos, M.; Carcia, T.; Preito, A. J. (2011) Persistence of gemfibrozil, naproxen and mefanamic acid in natural waters. *Environ. Chem. Lett.,* 9, 13-18.

Aristilde, L.; Sposito, G. (2013) Complexes of the antimicrobial ciprofloxacin with soil, peat, and aquatic humic substances. *Environ. Toxicol. Chem.,* 32(7), 1467-1478.

Baer, B. R.; DeLisle, R. K.; Allen, A. (2009) Benzyllic oxidation of gemfibrozil-1-O-β-glucuronide by P450 2C8 leads to heme alkylation and irreversible inhibition. *Chem. Res. Toxicol*., 22, 1298-1309.

Batt, A. L.; Kostich, M. S.; Lazorchak, J. M. (2008) Analysis of ecologically relevant pharmaceuticals in wastewater and surface water using selective solid-phase extraction and UPLC-MS/MS. *Anal. Chem*., 80, 5021-5030.

Bedner, M.; MacCrehan, W. A. (2006a) Reactions of the amine-containing drugs fluoxetine and metoprolol during chlorination and dechlorination processes used in wastewater treatment. *Chemosphere*, 65, 2130-2137.

Bedner, M.; MacCrehan, W. A. (2006b) Transformation of acetaminophen by chlorination produces the toxicants 1,4-benzoquinone and N-acetyl-p-benzoquinone imine. *Environ. Sci. Technol*., 40, 516-522.

Benotti, M. J.; Trenholm, R. A.; Vanderford, B. J.; Holady, J. C.; Stanford, B. D.; Snyder, S. A. (2009) Pharmaceuticals and endocrine disrupting compounds in U.S. drinking water. *Environ. Sci. Technol*., 43, 597-603.

Berthouex, P. M.; Brown, L. C. (2002) *Statistics for Environmental Engineers*. Lewis Publishers: Boca Raton, Florida, pp. 512.

Bonnineau, C.; Gausch, H.; Proia, L.; Ricart, M.; Geiszinger, A.; Romani, A. M.; Sabater, S. (2010) Fluvial biofilms: a pertinent toll to assess beta-blockers toxicity. *Aquat. Toxicol.,* 96, 225-233.

Bonvin, F.; Omlin, J.; Rutler, R.; Schwiezer, W. B.; Alaimo, P. J.; Strathmann, T. J.; McNeill, K.; Kohn, T. (2013) Direct photolysis of human metabolites of the antibiotic sulfamethoxazole: evidence for abiotic back-transformation. *Environ. Sci. Technol.,* 47(13), 6746-6755.

Borgmann, U.; Bennie, D. T.; Ball, A. L.; Palabrica, V. (2007) Effect of a mixture of seven pharmaceuticals on *Hyalella azteca* over multiple generations. *Chemosphere,* 66, 1278-1283.

Bound, J. P.; Kitsou, K.; Voulvoulis, N. (2006) Household disposal of pharmaceuticals and perception of risk to the environment. *Environ. Toxicol. Pharmacol*., 21, 301-307.

Boxall,A.B.A; Rudd, M.A.; Brooks, B.W.; Caldwell, D.J.; 3 Choi, K.; Hickmann, S.; Innes, E.; Ostapyk, K.; Staveley, J.P.; Verslycke, T.; Ankley, G.T.; Beazley, K.F.; Belanger, S.E.; Berninger, J.P.; Carriquiriborde, P.; Coors, A.; DeLeo, P.C.; Dyer, S.D.; Ericson, J.F. Gagné, F.; Giesy, J.P.; Gouin, T.; Hallstrom, L.; Karlsson, M.V.; Larsson, J.; Lazorchak, J.M.; Mastrocco, G.; McLaughlin, A.; McMaster, M.E.; Meyerhoff, R.D.; Moore, R.; Parrott, J.L.; Snape, J.R.; Murray-Smith, R.; Servos, M.R.; Sibley, P.K.; Straub, J.O.; Szabo, N.D.; Topp E.; Tetreault, G.R.; Trudeau, V. L.; Van Der Kraak, G. (2012) Pharmaceuticals and personal care products in the environment. What are the big questions? *Environ. Health. Persp.,* 120(9), 1221-1229.

Boyd, G. R.; Reemtsma, H.; Grimm, D. A.; Mitra, S. (2003) Pharmaceuticals and personal care products (PPCPs) in surface and treated waters of Louisiana, USA and Ontario, Canada. *Sci. Tot. Environ.,* 311, 135-149.

Boyd, G. R.; Zhang, S.; Grimm, D. A. (2005). Naproxen removal from water by chlorination and biofilm processes. *Water Res.,* 39, 668-676.

Brun, G. L.; Bernier, M.; Losier, R.; Doe, K.; Jackman, P.; Lee, H. B. (2006) Pharmaceutically active compounds in atlantic Canadian sewage treatment plant effluents and receiving waters, and potential for environmental effects as measured by acute and chronic aquatic toxicity. *Environ. Toxicol. Chem.,* 25(8), 2163-2176.

Bueno, M. J. M.; Agüera, A.; Gómez, M. J.; Hernando, M. D.; García-Reyes, J. F.; Fernández-Alba, A. R. (2007) Application of liquid chromatography/quadrupole-linear ion trap mass spectrometry and time-of-flight mass spectrometry to the determination of pharmaceuticals and related contaminants in wastewater. *Anal. Chem*., 79, 9372-9384.

Bulloch, D. N.; Lavado, R.; Forsgren K. L.; Beni, S.; Schlenk, D.; Larive, C. K. (2012) Analytical and biological characterization of halogenated gemfibrozil produced through chlorination of wastewater. *Environ. Sci. Technol*., 46, 5583-5589.

Caira, M. R.; Ionescu, C. (2005) *Drug Metabolism: Current concepts*. Springer Press: The Netherlands, pp. 422.

Camacho-Munoz, D.; Martin, J.; Santos, J. L.; Aparacio, I.; Alonso, E. (2011) Effectiveness of conventional and low-cost wastewater treatments in the removal of pharmaceutically active compounds. *Water Air Soil Pollut.,* 223, 2611-2621.

Camper, A. K. (1996) Factors limiting growth in distribution systems: Laboratory and pilot-scale experiments. *AwwaRF*

Canadian Water and Wastewater Association (2001) *Canadian Water and Wastewater Association National Survey of Wastewater Treatment Plants*. Final Report, 1-15.

Cherney, D. P.; Duirk, S. E.; Tarr, J. C.; Collette, T. W. (2006) Monitoring the speciation of aqueous free chlorine from pH 1 to 12 with raman spectroscopy to determine the identity of the potent low-pH oxidant. *Appl. Spectrosc*., 60, 764-772.

Clara, M.; Kreuzinger, N.; Strenn, B.; Gans, O.; Kroiss, H. (2005) The solids retention time – a suitable design parameter to evaluate the capacity for wastewater treatment plants to remove micropollutants. *Water Res.,* 39 (1), 97-106.

Clouzot, L.; Chouber, J-M.; Cloutier, F.; Goel, R.; Love, N.; Melcer, H.; Ort, C.; Patureau, D.; Plosz, B. G.; Pomiès, M,; Vanrolleghem, P. A. (2013) Perspectives on modelling micropollutants in wastewater treatment plants. *Wat. Sci. Technol.,* 68(2), 448-461.

Conn, K. E.; Barber, L. B.; Brown, G. K.; Siegrist, R. L. (2006) Occurrence and fate of organic contaminants during onsite wastewater treatment. *Environ. Sci. Technol.,* 40, 7358-7366.

Couillard, D. (1994) The use of peat in wastewater treatment. *Water Res.,* 28(6), 1261-1274.

Daughton, C. G.; Ternes, T. A. (1999) Pharmaceuticals and personal care products in the environment: agents of subtle change? *Environ. Health Perspect.,* 107(suppl 6), 907-938.

Deborde, M.; Rabouan, S.; Gallard, H.; Legube, B. (2004) Aqueous chlorination kinetics of some endocrine disruptors. *Environ. Sci. Technol*., 38, 5577-5583.

Deborde, M.; von Gunten, U. (2008) Reactions of chlorine with inorganic and organic compounds during water treatment – kinetics and mechanisms: a critical review. *Water Res*., 42, 13-51.

de Laeter, J. R.; Böhlke, J. K.; De Bièvre, P.; Hidaka, H.; Peiser, H. S.; Rosman, K. J. R.; Taylor, P. D. P. (2003) Atomic weights of the elements: review 2000. *Pure Appl. Chem*., 75, 683-800.

Dodd, M. C.; Huang, C. H. (2007) Aqueous chlorination of the antibacterial agent trimethoprim: Reaction kinetics and pathways. *Water Res*., 41, 647-655.

Dohle, S.; Campbell, V. E. A.; Arvai, J. L. (2013) Consumer-percieved risks and choices about pharmaceuticals in the environment: A cross-sectional study. *Environ. Health,* 12, 45-58.

Droste, R. L. (1997) *Theory and Practice of Water and Wastewater Treatment*. John Wiley & Sons, Inc.: New York, pp. 800

Durán-Alvarez, J. C.; Becerril-Bravo, E.; Castro, V. S.; Jiménez, B., Gibson, R. (2009) The analysis of a group of acidic pharmaceuticals, carbamazepine, and potential endocrine disrupting compounds in wastewater irrigated soils by gas chromatography-mass spectrometry. *Talanta*, 78, 1159-1166.

Eğe, S. (2004) *Organic Chemistry: Structure and Reactivity*, 5th Ed. Houghton Mifflin Company: Boston, pp. 366-377.

Eimers, M. C.; Evans, R. D.; Welbourn, P. M. (2002) Partitioning and bioaccumulation of cadmium in artificial sediment systems: Application of a stable isotope tracer technique. *Chemosphere,* 46, 543-551.

Environment Canada. (1997) Biological test method: test for survival and growth of sediment using the freshwater amphipod *Hyalella azteca* (EPS 1/RM/33).

Escher, B.; Fenner, K. (2011) Recent advances in environmental risk assessment of transformation products. *Environ. Sci. Technol.,* 45(9), 3835-3847.

Falås, P.; Baillon-Dhumez, A.; Andersen, H. R.; Ledin, A.; la Cour Jansen, J. (2012) Suspended biofilm carrier and activated sludge removal of acidic pharmaceuticals. *Water Res.,* 49, 1167-1175.

Fang, Y. ; Karnjanapiboonwong, A. ; Chase, D.A. ; Wang, J. ; Morse, A.N. ; Anderson, T.A. (2012) Occurrence, fate, and persistence of gemfibrozil in water and soil. *Environ. Toxicol. Chem.,* 31, 550-555.

Farré, M.; Ferrer, I.; Ginebreda, A.; Figueras, M.; Olivella, L.; Tirapu, L.; Vilanova, M.; Barcelo, D. (2001) Determination of drugs in surface water and wastewater samples by liquid chromatography-mass spectrometry: methods and preliminary results including toxicity studies with *Vibrio Fischeri,. J. Chromatog A.,* 938, 187-197

Farré, M.; Gros, M.; Hernández, B.; Petrovic, M.; Hancock, P.; Barceló, D. (2008) Analysis of biologically active compounds in water by ultra-performance liquid chromatography quadrupole time-of-flight mass spectrometry. *Rapid Commun. Mass Spectrom*., 22, 41-51.

Fatta-Kassinos, D.; Vasquez, M.; Kummerer, K. (2011) Transformation products of pharmaceuticals in surface waters and wastewater formed during photolysis and advanced oxidation processes – degradation, eludication of byproducs and assessment of their biological potency. *Chemosphere*, 85, 693-709.

Fent, K.; Weston, A. A.; Caminada, D. (2006) Ecotoxicology of human pharmaceuticals. *Aquat. Toxicol.,* 76, 122-169.

Fernandez-Fontaina, E.; Pinho, I.; Carballa, M.; Omil, F.; Lema, J. M. (2013) Biodegradation kinetic constants and sorption coefficients of micropollutants in membrane bioreactors. *Biodegradation,* 24, 165-177.

Ferrari, B.; Paxeus, N.; Lo Guidice, R.; Pollio, A.; Garric, J. (2003) Ecotoxicological impact of pharmaceuticals found in treated wastewaters; study of Carbamazepine, clofibric acid, and Diclofenac. *Ecotox. Environ. Safe.,* 55, 359-370.

Gagnon, G. A.; Slawson, R. M. (1999) An efficient biofilm removal method for bacterial cells exposed to drinking water. *J. Microbiol. Meth.,* 34, 203-214.

Gallard, H.; von Gunten U. (2002) Chlorination of phenols: kinetics and formation of chloroform. *Environ. Sci. Technol*., 36, 884-890.

Gibs, J.; Stackelberg, P. E.; Furlong, E. T.; Meyer, M.; Zaugg, S. D.; Lippincott, R. L. (2007) Persistence of pharmaceuticals and other organic compounds in chlorinated drinking water as a function of time. *Sci. Total Environ*., 373, 240-249.

Glassmeyer, S. T.; Shoemaker, J. A. (2005) Effects of chlorination on the persistence of pharmaceuticals in the environment. *Bull. Environ. Contam. Toxicol*., 74, 24-31.

Grenni, P.; Patrolecco, L.; Ademollo, N.; Tolomei, A.; Caracciolo, A. B. (2013) Degradation of gemfibrozil and naproxen in a river water ecosystem. *Mirochem. J.,* 107, 158-164.

Gros, M.; Petrović, M.; Barceló, D. (2006) Development of a multi-residue analytical methodology based on liquid chromatography-tandem mass spectrometry (LC-MS/MS) for screening and trace level determination of pharmaceuticals in surface and wastewaters. *Talanta*, 70, 678-690.

Gros, M.; Petrović, M.; Barceló, D. (2009) Tracing pharmaceutical residues of different therapeutic classes in environmental waters by using liquid chromatography/quadrupole-linear ion trap mass spectrometry and automated library searching. *Anal. Chem*., 81, 898-912.

Gross, B.; Montgomery-Brown, J.; Naumann, A.; Reinhard, M. (2004). Occurrence and fate of pharmaceuticals and alkylphenol ethoxylate metabolites in an effluent-dominated river and wetland. *Environ. Toxicol. Chem.,* 23(9), 2074-2083.

Grossert, J. S.; Cook, M. C.; White, R. L. (2006) The influence of structural features on facile McLafferty-type, even-electron rearrangements in tandem mass spectra of carboxylate anions. *Rapid Commun. Mass Spectrom*., 20, 1511-1516.

Halling-Sorensen, B.; Nors, N. S.; Lanzky, P. F.; Ingerslev, F.; Holten Lutzhoft, H. C.; Jorgensen, S. E. (1998) Occurrence, fate and effects of pharmaceutical substances in the environment-a review. *Chemosphere,* 36, 357-393.

Hawkins, C. L.; Shipitalo, M. J.; Rutledge, E. M.; Savin, M. C.; Brye, K. R. (2008) Earthworm populations in septic system filter fields and potential effects on wastewater renovation. *Appl. Soil Ecol.,* 40(1), 195-200.

Heberer, T. (2002). Occurrence, fate, and removal of pharmaceutical residues in the aquatic environment; a review of recent research data. *Toxicol Letters,* 131, 5-17.

Hernando, M. D.; Mexcua, M.; Fernandez-Alba; A. R.; Barcelo, D. (2006) Environmental risk assessment of pharmaceutical residues in wastewater effluents, surface waters and sediments. *Talanta,* 69, 334-342.

Hernando, M. D.; Petrovic, M.; Fernández-Alba, A. R.; Barceló, D. (2004) Analysis by liquid chromatography-electrospray ionization tandem mass spectrometry and acute toxicity evaluation for β-blockers and lipid-regulating agents in wastewater samples. *J. Chromatogr. A.,* 1046(1-2), 133-140.

Heydorn, A.; Neilsen, A. T.; Hentzer, M.; Sternberg, C.; Givskov, M.; Ersboll, C. K.; Molin, S. (2000) Quantification of biofilm structures by the novel computer program COM-STAT. *Microbiology,* 146, 2395-2407.

Hu, J. Y.; Aizawa, T.; Ookubo, S. (2002a) Products of aqueous chlorination of bisphenol A and their estrogenic activity. *Environ. Sci. Technol*., 36, 1980–1987.

Hu, J. Y.; Cheng, S.; Aizawa, T.; Terao, Y.; Kunikane, S. (2003) Products of aqueous chlorination of 17b-estradiol and their activities. *Environ. Sci. Technol*., 37, 5665–5670.

Hu, J. Y.; Xie, G. H.; Aizawa, T. (2002b) Products of aqueous chlorination of 4-nonylphenol and their estrogenic activity. *Environ. Toxicol. Chem*., 21, 2034–2039.

Hu, Z.; Gagnon, G. A. (2006a) Impact of filter media on the performance of full-scale recirculating biofilters for treating multi-residential wastewater. *Water Res*., 40, 1474 – 1480.

Hu, Z; Gagnon, G. A. (2006b) Factors affecting recirculating bio-filters (RBFs) for treating municipal wastewater. *J. Environ. Eng. Sci*., 5(4), 349-357.

Hyland, K. C.; Dickenson, E. R. V.; Drewes, J. E.; Higgins, C. P. (2012) Sorption of ionized and neutral emerging trace organic compounds onto activated sludge from different wastewater treatment configurations. *Water Res.,* 46, 1958-1968.

Inaba, K.; Doi, T.; Isobe, N.; Yamamoto, T. (2006) Formation of bromo-substituted triclosan during chlorination by chlorine in the presence of trace levels of bromide. *Water Res*., 40, 2931-2937.

Ings, J.S.; Servos, M.R.; Vijiyan, M.M.; Hepatic transcriptomics and protein expression in rainbow trout exposed to municipal wastewater effluent. *Environ. Sci. Technol.,* 45(6), 2367-2376.

Isidori, M.; Nardelli, M.; Pascarella, L.; Rubino, M.; Parrella, A. (2007) Toxic and genotoxic impacts of fibrates and their photoproducts on non-target organisms. *Environ. Int.,* 33, 635-641.

Jackevicius, C. A.; Tu, J. V.; Ross, J. S.; Ko, D. T.; Carreon, D.; Krumholz, H. M. (2011) Use of fibrates in the Unitest States and Canada. *J. Am. Med. Ass.,* 23(30), 1217 – 1224.

Jiao, S.; Zheng, S., Yin, D.; Wang, L.; Chen, L. (2008) Aqueous photolysis of tetracycline and toxicity of photolytic products to luminescent bacteria. *Chemosphere*, 73, 377-382.

Jones-Lepp, T. L.; Stevens, R. (2007) Pharmaceuticals and personal care products in biosolids/sewage sludge: the interface between analytical chemistry and regulation. *Anal. Bioanal. Chem.,* 387, 1173-1183.

Jos, A.; Repetto, G.; Rios, J. C.; Hazen, M. J.; Molero, M. L.; del Peso, A.; Salguero, M.; Fernandez-Freire, p.; Perez-Martin, J. M.; Camean, A. (2003) Ecotoxicological evaluation of Carbamazepine using six different model systems with eighteen endpoints. *Toxicol. in Vitro.,* 17, 525-532.

Joss, A.; Keller, E.; Alder, A. C.; Gobel, A.; McArdell, C. S.; Ternes, T.; Siegrist, H. (2005) Removal of pharmaceuticals and fragrances in biological wastewater treatment. *Water Res.,* 39, 3139-3152.

Joss, A.; Zabcynski, S.; Gobel, A.; Hoffmann, B.; Loffler, D.; McArdell, C. S.; Ternes, T. A.; Thomsen, A.; Siegrist, H. (2006) Biological degradation of pharmaceuticals in municipal wastewater treatment: Proposing a classification scheme. *Water Res.,* 40(8), 1686-1696.

Kanokkantapong, V.; Marhaba, T. F.; Panyapinyophol, B.; Pavasant, P. (2006) FTIR evaluation of functional groups involved in the formation of haloacetic acids during the chlorination of raw water. *J. Hazard. Mater*., 136, 188-196.

Kolpin, D. W.; Furlong, E. T.; Meyer, M. T.; Thurman, M. E.; Zaugg, S. D.; Barber, L. B. (2002) Pharmaceuticals, hormones, and other organic wastewater contaminants in US streams, 1999-2000: A national reconnaissance. *Environ. Sci. Technol.,* 36, 1202-1211.

Kosjek, T.; Heath, E.; Compare, B. (2007) Removal of pharmaceutical residues in a pilot wastewater treatment plant. *Anal. Bioanal. Chem.,* 387, 1379-1387.

Koziar, S. A. (2007) Chlorination of pharmaceuticals during water treatment. unpublished Master's Thesis. Dalhousie University, Halifax, Nova Scotia, Canada.

Krasner, S. W.; Weinberg, H. S.; Richardson, S. D.; Pastor, S. J.; Chinn, R.; Sclimenti, M. J.; Onstad, G. D.; Thruston, A. D. (2006) Occurrence of a new generation of disinfection byproducts. *Environ. Sci. Technol*., 40, 7175–7185.

Kreuzinger, N.; Clara, M.; Strenn, B.; Krolss, H. (2004) Relevance of the sludge retention time (SRT) as design criteria for wastewater treatment plants for the removal of endocrine disruptors and pharmaceuticals from wastewater. *Wat. Sci.Technol.,* 50(5), 149-156.

Krkošek, W. H.; Koziar, S. A.; White, R. L.; Gagnon, G. A. (2011) Identification of reaction products from reactions of free chlorine with the lipid-regulator gemfibrozil. *Water Res*., 45(3): 1414-1422.

Lawrence, J. R.; Swerhone, G. D. W.; Wassenaar, L. I.; Neu, T. R. (2005) Effects of selected pharmaceuticals on riverine biofilm communities. *Can. J. Microbiol.,* 51(8), 655-669.

Lin, W. C.; Chen, H. C.; Ding, W. H. (2005) Determination of pharmaceutical residues in waters by solid-phase extraction and large-volume on-line derivatization with gas chromatography-mass spectrometry. *J. Chromat. A.,* 1065, 279-285.

Lishman, L.; Smyth, S.; Sarafin, K.; Kleywegt, S.; Toito, J.; Peart, T.; Lee, B.; Servos, M.; Beland, M.; and Seto, P. (2006) Occurrence and reductions of pharmaceuticals and personal care products and estrogens by municipal wastewater treatment plants in Ontario, Canada. *Sci. Total Environ.,* 367, 544-558.

Liu, R. C.; Hahn, C.; Hurtt, M. E. (1996) The direct effect of hepatatic peroxisome proliferators on rat Leydig cell function *in vitro. Fund. Appl. Toxicol.,* 30, 102-108

Löffler, D.; Ternes, T. A.; 2003. Determination of acidic pharmaceuticals, antibiotics and ivermectin in river sediment using liquid chromatography-tandem mass spectrometry. *J. Chromatogr. A.,* 1021, 133-144.

Loupasaki, E.; Diamadopoulos, E. (2012) Attached growth systems for wastewater treatment in small and rural communities: A Review. *J Chem. Technol. Biot.,* 88(2), 190-204.

Maeng, S. K.; Choi, B. G.; Lee, K. T.; Song, K. G. (2013) Influences of solid retention time, nitrification and microbial activity on the attenuation of pharmaceuticals and estrogens in membrane bioreactors. *Water Res.,* 47, 3151-3162.

Matamoros, V,; Arias, C.; Brix, H.; Bayona, J. M. (2009) Preliminary screening of small-scale domestic wastewater treatment systems for removal of pharmaceutical and personal care products. *Water Res.,* 43, 55-62.

Medina, M. H.; Correa, J. A.; Barata, C. (2007) Micro-evolution due to pollution: Possible consequences for ecosystem responses to toxic stress. *Chemosphere,* 67, 2105-2114.

Metcalf & Eddy Inc. (2003) *Wastewater Engineering. Treatment and Reuse*. Fourth edition. McGraw-Hill. New York, pp. 1848.

Metcalfe, C.D.; Chu, S.G.; Judt, C.; Li, H.X.; Oakes, K.D.; Servos, M.R.; Andrews, D.M. (2010) Antidepressants and their metabolites in municipal wastewater, and downstream exposure in an urban watershed. *Environ. Tox. Chem.,* 29(1), 79-89.

Metcalfe, C. D.; Koenig, B. G.; Bennie, D. T.; Servos, M.; Ternes, T. A.; Hirsch, R. (2003a) Occurrence of neutral and acidic drugs in the effluents of Canadian sewage treatment plants. *Environ. Toxicol. Chem*., 22, 2872-2880.

Metcalfe, C. D.; Miao, X.; Koenig, B. G.; Struger, J. (2003b) Distribution of acidic and neutral drugs in surface waters near sewage treatment plants in the lower great lakes, Canada. *Environ. Toxicol. Chem*., 22, 2881-2889.

Meylan, W. M.; Howard P. H. (1995) Atom fragment contribution method for estimate octanol water partition coefficients. *J Pharm. Sci*., 84, 83-92.

Miao, X.-S.; Koenig, B. G.; Metcalfe, C. D. (2002) Analysis of acidic drugs in the effluents of sewage treatment plants using liquid chromatography-electrospray ionization tandem mass spectrometry. *J. Chromatogr. A.,* 952, 139-147.

Miège, J. M.; Choubert, L.; Ribeiro, M.; Eusebe, M.; Coquery, M. (2008) Removal efficiency of pharmaceuticals and personal care products with varying wastewater treatment processes and operating conditions – conception of a database and first results. *Water Sci. Technol.,* 57(1), 49-56.

Mimeault, C.; Woodhouse, A. J.; Miao, X. -S.; Metcalfe, C. D.; Mon, T. W.; Trudeau, V. L. (2005) The human lipid regulator, gemfibrozil bioconcentrates and reduces testosterone in the goldfish, Carassius auratus. *Aquat. Toxicol*., 73, 44-54.

Mompelat, S.; Le Bot, B.; Thomas, O. (2009) Occurrence and fate of pharmaceutical products and by-products, from resource to drinking water. *Environ. Int*., 35, 803-814.

Moriyama, K.; Matsufuji, H.; Chino, M.; Takeda, M. (2004) Identification and behaviour of reaction products formed by chlorination of ethynylestradiol. *Chemosphere*, 55, 839–847.

Morris, J. C. (1966) The acid ionization constant of HOCl from 5 to 351. *J. Phys. Chem*., 70, 3798–3805.

Murai, T.; Iwabuchi, H.; Ikeda, T. (2004) Identification of gemfibrozil metabolites, produced as positional isomers in human liver microsomes, by on-line analyses using liquid chromatography/mass spectrometry and liquid chromatography/nuclear magnetic resonance spectroscopy. *J. Mass Spectrom. Soc. Jpn*., 52, 277-283.

Namkung, E.; Rittmann, B. E. (1987) Estimating volatile organic compound emissions from publicly owned treatment works. *J. Water Pollut. Control Fed.,* 59(7), 670-678.

Ollers, S.; Singer, H. P.; Fassler, P.; Muller, S. R. (2001) Simultaneous quantification of neutral and acidic pharmaceuticals and pesticides at the low-ng/l level in surface and waste water. *J. Chromat. A,* 911, 225-234.

Onesios, K.M.; Yu, J.T.; Bouwer, E.J. (2009) Biodegradation and removal of pharmaceuticals and personal care products in treatment systems: a review. *Biodeg.,* 20, 441-466.

Organization for Economic Cooperation and Development. (2004) *The testing of chemicals – Sediment – water chironomid toxicity test using spiked water*. OECD guideline 218: Paris, France, pp. 21.

Ottmar, K. J.; Colosi, L. M.; Smith, J. A. (2010) Sorption of statin pharmaceuticals to wastewater treatment biosolids, terrestrial soils, and freshwater sediment. *J. Environ. Eng.,* 136(3), 256-264.

Palleroni, N. J. (2010) The *Pseudomonas* story. *Environ. Microbiol.,* 12, 1377-1383.

Paxeus, N. (2004) Removal of selected non-steroidal anti-inflammatory drugs (NSAIDs), gemfibrozil, carbamazepine, beta-blockers, trimethoprin, and triclosan in conventional wastewater treatment plants in five EU countries and their discharge to the aquatic environment. *Wat. Sci. Technol.,* 50(5), 253-260.

Petrovic, M.; Gros, M.; Barcelo, D. (2006) Multi-residue analysis of pharmaceuticals in wastewater by ultra-performance liquid chromatography-quadrupole-time-of-flight mass spectrometry. *J. Chromatogr. A.,* 1124, 68-81.

Pinkston, K. E.; Sedlak, D. L. (2004) Transformation of aromatic ether- and amine-containing pharmaceuticals during chlorine disinfection. *Environ. Sci. Technol*., 38, 4019-4025.

Plant, N. (2003) *Molecular Toxicology*. Bios Scientific Publishers: New York, pp. 150.

Pomiès, M.; Choubert, J.- M.; Wisniewski, C.; Coquery, M. (2013) Modelling of micropollutant removal in biological wastewater treatments: A review. *Sci. Total Environ.,* 443, 733-748.

Poseidon. (2004) *Assessment of Technologies for the Removal of Pharmaceuticals and Personal Care Products in Sewage and Drinking Water Facilities to Improve the Indirect Potable Water Reuse.* [Online]. Available: http://poseidon.bafg.de/servlet/is/2888/ [2008, July]

Pretsch, E.; Bühlmann, P.; Affolter, C. (2000) *Structure Determination of Organic Compounds*. Springer-Verlag: Berlin, pp. 182-183.

Quinn, B.; Schmidt, W.; O'Rourke, K.; Hernan, R. (2011) Effects of the pharmaceuticals gemfibrozil and diclofenac on biomarker expression in the zebra mussel (*Dreissena polymorpha*) and their comparison with standardized toxicity tests. *Chemosphere*, 84, 657-663.

Quintana, J. B.; Rodil, R.; Lopez-Mahia, P.; Muniategui-Lorenzo, S.; Prada-Rodriguez, D. (2010) Investigating the chlorination of acidic pharmaceuticals and by-product formation aided by an experimental design methodology. *Water Res*., 44, 243-255.

Radjenovic, J.; Petrovic, M.; Barcelo, D. (2009) Fate and distribution of pharmaceuticals in wastewater and sewage sludge of the conventional activated sludge (CAS) and advanced membrane bioreactor (MBR) treatment. *Water Res*., 43, 831-41.

Ramirez, A. J.; Mottaleb, M. A.; Brooks, B. W.; Chambliss, C. K. (2007) Analysis of pharmaceuticals in fish using liquid chromatography-tandem mass spectrometry. *Anal. Chem*., 79, 3155-3163.

Reddersen, K.; Heberer, T. (2003) Multi-compound methods for the detection of pharmaceutical residues in various waters applying solid phase extraction and gas chromatography with mass spectrometric detection. *J. Sep. Sci.,* 26, 1443-1450.

Regan, J. M.; Cho, A-Y; Kim, S.; Smith, C. D. (2007) *Monitoring Ammonia-Oxidizing bacteria in Chloraminated Distribution Systems*. AwwaRF and AWWA: Denver, CO, pp. 104.

Richardson, S. D. (2005) New disinfection by-product issues: emerging DBPs and alternative routes of exposure. *Global NEST J.,* 7, 43–60.

Rittmann, B. E.; McCarty, P. L (2001) *Environmental Biotechnology: Principles and Applications.* McGraw Hill: New York, pp. 768.

Rodriguez, I.; Carpinteiro, J.; Quintana, J. B.; Carro, A. M.; Lorenzo, R. A.; Cela, R. (2004) Solid-phase microextraction with on-fiber derivatization for the analysis of anti-inflammatory drugs in water samples. *J. Chromat. A,* 1024, 1-8.

Rosal, R.; Gonzalo, M. S.; Boltes, K.; Leton, P.; Vaquero, J. J.; Garcia-Calvo, E. (2009) Identification of intermediates and assessment of ecotoxicity in the oxidation products generated during the ozonation of clofibric acid. *J. Hazard. Mater.,* 172, 1061-1068.

Rosal, R.; Rodea-Palomares, I.; Boltes, K.; Fernandez-Pinas, F.; Leganes, F.; Gonzalo, S.; Petre, A. (2010) Ecotoxicity assessment of lipid regulators in water and biologically treated wastewater using three aquatic species. *Environ. Sci. Pollut. Res*., 17, 135-144.

Rosi-Marshall, E. J.; Kincaid, D. W.; Bechtold, H. A.; Royer, T. V.; Rojas, M.; Kelly, J. J. (2013) Pharmaceuticals suppress algal growth and microbial respiration and alter bacterial communities in stream biofilms. *Ecol. Appl.,* 23(3), 583-593.

Rule, K. L.; Ebbett, V. R.; Vikesland, P. J. (2005) Formation of chloroform and chlorinated organics by free-chlorine-mediated oxidation of triclosan. *Environ. Sci. Technol*., 39, 3176-3185.

Sabater, S.; Guasch, H.; Ricart, M.; Romani, A.; Vidal, G.; Klunder, C.; Schmitt-Jansen, M., (2007) Monitoring the effect of chemicals on biological communities. The biofilm as an interface. *Anal. Bioanal. Chem*., 387, 1425-1434.

Sanderson, H.; Johnson, D. J.; Reitsma, T.; Brain, R. A.; Wilson, C. J.; Solomon, K. R. (2004) Ranking and prioritization of environmental risks of pharmaceuticals in surface waters. *Regul. Toxicol. Pharm.,* 39, 158-183.

Santos, L. H. M. L. M.; Araujo, A.N.; Fachini, A.; Pena, A.; Delelrue-Matos, C.; Montenegro, M. C. B. S. M. (2010) Ecotoxicological aspects related to the presence of pharmaceuticals in the aquatic environment. *J. Hazard. Mater*., 175, 45-95.

Schmitt-Jansen, M..;Bartels, P.; Adler, N.; Altenburger, R. (2007) Phytotoxicity assessment of diclofenac and its phototransformation products. *Anal. Bioanal. Chem*., 387, 1389-1396.

Sebők, Á.; Vasanits-Zsigrai, A.; Helenkár, A.; Záray, G.; Molnár-Perl, I. (2009) Multiresidue analysis of pollutants as their trimethylsilyl derivatives, by gas chromatography-mass spectrometry. *J. Chromatogr. A*., 1216, 2288-2301.

Servos, M. R.; Bennie, D. T.; Burnisonb, B. K.; Jurkovic, A.; McInnis, R.; Nehelib, T.; Schnell, A.; Seto, P.; Smyth, S. A.; Ternes, T. A. (2005) Distribution of estrogens, 17h-estradiol and estrone, in Canadian municipal wastewater treatment plants. *Sci. Total Environ.,* 336, 155– 170.

Singer, P.C. (2006) DBPs in drinking water: additional scientific and policy considerations for public health protection. *J.–Am. Water Works Assoc*., 98, 73-82.

Sirivedhin, T.; Gray, K. A. (2005) Comparison of the disinfection by-product formation potentials between a wastewater effluent and surface waters. *Water Res*., 39, 1025-1036.

Skolness, S.Y.; Durham, E.J.; Jensen, K.M.; Kahl, M.D.; Makynen, E.a., Villeneuve, D.L., Ankley, G.T. (2012) Effects of gemfibrozil on lipid metabolism, steroidogenesis, and reproduction in the fathead minnow (*Pimephales promelas*). *Environ. Toxicol.Chem.,* 31, 2615-2624.

Slack, R. J.; Zerva, P.; Gronow, J. R.; Voulvoulis, N. (2005) Assessing quantities and disposal routes for household hazardous products in the United Kingdom. *Environ. Sci. Technol*., 39, 1912-1919.

Stevens-Garmon, J; Drewes, J. E.; Khan, S. J.; McDonald, J. A.; Dickenson, E. R. V. (2011) Sorption of emerging trace organic compounds onto wastewater sludge solids. *Water Res.,* 45, 3417-3426.

Stumpf, M.; Ternes, T. A.; Wilken, R. D.; Rodrigues, S. V.; Baumann, W. (1999) Polar drug residues in sewage and natural waters in the state of Rio de Janiero, Brazil. *Sci. Total Environ.,* 225, 135-141.

Su, J. L.; Ouyang, C. F. (1984) Advanced biological enhanced nutrient removal processes by the addition of rotating biological contactors. *Water Sci. Technol.,* 35(8), 153-160.

Suarez, S.; Lema, J. M.; Omil, F. (2010) Removal of pharmaceutical and personal pare products (PPCPs) under nitrifying and denitrifying conditions. *Water Res.,* 44, 3214-3224.

Ternes, T. A. (1998) Occurrence of drugs in German sewage treatment plants and rivers. *Water Res.,* 32(11), 3245-3260.

Ternes, T. A.; Hirsch, R.; Mueller, J.; Haberer, K. (1998) Methods for the determination of neutral drugs as well as betablockers and beta(2)-sympathomimetics in aqueous matrices using GC-MS and LC/MS/MS. *Fresen. J. Anal. Chem*., 362(3), 329-340.

Ternes, T.; Jos, A.; Siegrist, H. (2004) Scrutinizing pharmaceutical and personal care products in wastewater treatment. *Environ Sci Technol.,* 393-399.

Thibaut, R.; Schnell, S.; Porte, C. (2006) The interference of pharmaceuticals with endogenous and xenobiotic metabolizing enzymes in carp liver: an in-vitro study. *Environ. Sci. Technol.,* 40, 5154-5160.

Thomas, B. F.; Burgess, J. P.; Coleman, D. P.; Scheffler, N. M.; Jeffcoat, A. R.; Dix, K. J. (1999) Isolation and identification of novel metabolites of gemfibrozil in rat urine. *Drug Metab. Dispos*., 27, 147-157.

Thomas, P. M.; Foster, G. D. (2005) Tracking acidic pharmaceuticals, caffeine, and triclosan through the wastewater treatment process. *Environ. Toxicol. Chem*., 24, 25-30.

Tolls, J. (2001) Sorption of veterinary pharmaceuticals in soils: a review. *Environ. Sci Technol.,* 35(17), 3397-406.

Urase, T.; Kikuta, T. (2005) Separate estimation of adsorption and degradation of pharmaceutical substances and estrogens in the activated sludge process. *Water Res*., 39, 1289-1300

USEPA. (2008) *Onsite Wastewater Treatment System Technology Fact Sheet 11; Recirculating Sand/Media Filters. U.S Environmental Protection Agency*. [Online]. Available: http://www.epa.gov/nrmrl/pubs/625r00008/html/tfs11.htm [2008, June 26].

Verlicchi, P.; Aukidy, M. Al.; Zambello, W. (2012) Occurrence of pharmaceutical compounds in urban wastewater: Removal, mass load and environmental risk after a secondary treatment – a review. *Sci. Total Environ.,* 429, 123-155.

Vogna, D.; Marotta, R.; Andreozzi, R.; Napolitano, A.; d'Ischia, M. (2004) Kinetic and chemical assessment of the UV/H2O2 treatment of antiepileptic drug carbamazepine. *Chemosphere*, 54, 497-505.

Wang, S.; Holzem, R. M.; Gunsch, C. K. (2008) Effects of pharmaceutically active compounds on a mixed microbial community originating from a municipal wastewater treatment plant. *Environ. Sci. Technol.,* 42, 1091-1095.

Wang, T. X.; Margerum, D. W. (1994) Kinetics of reversible chlorine hydrolysis: temperature dependence and general-acid/ base-assisted mechanisms. *Inorg. Chem*., 33, 1050–1055.

Westerhoff, P.; Chao, P.; Mash, H. (2004) Reactivity of natural organic matter with aqueous chlorine and bromine. *Water Res*., 38, 1502-1513.

Westerhoff, P.; Yoon, Y.; Snyder, S.; Wert, E. (2005) Fate of endocrine-disruptor, pharmaceutical, and personal care product chemicals during simulated drinking water treatment processes. *Environ. Sci. Technol*., 39, 6649-6663.

Williamson, E. (2010) Cold climate performance analysis of on-site domestic wastewater treatment systems. *Water Environ. Res.,* 82(6), 512-518.

Writer, J. H.; Ferrer, I.; Barber, L. B.; Thurman, E. M. (2013) Widespread occurrence of neuro-active pharmaceuticals and metabolites in 24 Minnesota rivers and wastewaters. *Sci. Tot. Environ.,* 461, 519-527.

Wu, C.; Witter, J. D.; Spongberg, A. L.; Czajkowski, K. P. (2009) Occurrence of selected pharmaceuticals in an agricultural landscape, western Lake Erie basin. *Water Res*., 43, 3407-3416.

Xagoraraki, I.; Hullman, R.; Song, W.; Li, H.; Voice, T. (2008) Effect of pH on degradation of acetaminophen and production of 1,4-benzoquinone in water chlorination. *J. Water Supply Res. T*., 57, 381-390.

Xia, Y.-Q.; Miller, J. D.; Bakhtiar, R.; Franklin, R. B.; Liu, D. Q. (2003) Use of a quadrupole linear ion trap mass spectrometer in metabolite identification and bioanalysis. *Rapid Commun. Mass Spectrom*., 17, 1137-1145

Yergeau, E.; Sanschagrin, S.; Waiser, M. J.; Lawrence, J. R.; Greer, C. W. (2012) Sub-inhibitory concentrations of different pharmaceuticals products affect the meta-transcriptome of river biofilm communities cultivated in annular reactors. *Environ. Microbiol. Rep.,* 4(3), 350-359.

Yu, Z. R.; Peldszus, S.; Huck, P. M. (2007) Optimizing gas chromatographic-mass spectrometric analysis of selected pharmaceuticals and endocrine-disrupting substances in water using factorial experimental design. *J. Chromatogr. A*., 1148, 65-77.

Zimetbaum, P.; Frishman, W. H.; Kahn, S. (1991) Effects of gemfibrozil and other fibric acid derivates on blood lipids and lipoproteins. *J. Clin. Pharmacol*., 31, 25–37.

Zorita, S.; Martensson, L.; Mathiasson, L. (2009) Occurrence and removal of pharmaceuticals in a municipal sewage treatment system in the south of Sweden. *Sci. Total Environ.,* 407(8), 2760-2770.

Zupanc, M.; Kosjek, T.; Petkovšek, M.; Dular, M.; Kompare, B.; Širok, B.; Blažeka, Ž.; Heath, E. (2013) Removal of pharmaceuticals from wastewater by biological processes, hydrodynamic cavitation and UV treatment. *Ultrason. Sonochem.,* 20, 1104-1112.

Zurita, J. L., Repetto, G., Jos, A., Salguero, M., Lopez-Artiguez, M., Camean, A. M., (2007) Toxicological effects of the lipid regulator Gemfibrozil in four aquatic systems. *Aquat. Toxicol.,* 81, 106-115.

Zwiener, C. (2007) Occurrence and analysis of pharmaceuticals and their transformation products in drinking water treatment. *Anal. Bioanal. Chem.,* 387, 1159-1162.

Zwiener, C.; Frimmel, F. H. (2003) Short-term tests with a pilot sewage plant and biofilm reactors for the biological degradation of the pharmaceutical compounds clofibric acid, ibuprofen and diclofenac. *Sci. Total Environ.,* 309, 201-211.

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