Microbial Impacts of Selected Pharmaceutically Active Compounds Found in Domestic Wastewater Treatment Plants

by

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Heather Stapleton

Dissertation submitted in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Civil and Environmental Engineering in the Graduate School of Duke University

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ABSTRACT

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Abstract

Large amounts of human pharmaceutical products are consumed worldwide. Many drugs and their metabolites, referred to as pharmaceutically active compounds (PhACs), are not fully metabolized prior to household discharge resulting in their common occurrence in wastewater treatment plants (WWTPs). In most instances, WWTPs present the first treatment opportunity for removing PhACs and preventing significant environmental exposure. Because most municipal WWTPs rely on the microbial component of the activated sludge process, there is a need to estimate the influence of PhACs in wastewater influent on the activated sludge microbial communities and the treatment performance of WWTPs. The objective of this dissertation was to determine the impact of selected PhACs (i.e., ketoprofen, naproxen, clofibric acid, carbamazepine and gemfibrozil) on activated sludge microorganisms and key individual microbial species in domestic wastewater treatment. Analyses were performed in batch reactors initially and then in laboratory-scale sequencing batch reactors (SBR) which mimic WWTP operations. Ammonia oxidizing bacteria (AOB) were selected as indicator organisms because of their importance in wastewater treatment and demonstrated sensitiveness to toxic compounds.

The batch experiments results suggested that microbial growth inhibition was correlated to organic loadings. In the presence of 0.2% (v/v) ethanol, significant
inhibition, ranging from 34 to 43%, was observed for all PhACs other than clofibric acid.

Nitrification inhibition studies using *Nitrosomonas europaea*, a model AOB strain showed that ketoprofen, naproxen, carbamazepine and gemfibrozil inhibited nitrite production. The corresponding maximum nitrification inhibition rates were 25, 29, 22 and 26%, respectively. Inhibition was shown to increase with PhAC concentration for concentrations greater than 0.1 µM. Results from membrane integrity tests suggest that the inhibition may be due to the disturbance of the cell membrane by PhACs and such inhibition was shown to be irreversible.

Even though PhACs were shown to inhibit the nitrification rate in pure culture studies, the performance of SBRs exposed to individual PhACs was not adversely affected neither in terms of COD nor ammonia removal. Microbial fingerprinting for both total bacteria and AOB confirmed that no significant shifts occurred when microbial communities were exposed to PhACs. However, some PhACs introduced in binary mixture were found to both inhibit the nitrification of *N. europaea* as well as the performance of SBRs. The mixture composed of 0.5 µM ketoprofen and 0.5 µM naproxen showed significant inhibition (25%) on the nitrite production of *N. europaea* although neither 0.5 µM ketoprofen nor 0.5 µM naproxen had significant effect when presented alone. Similarly, both COD and ammonia removal were significantly impacted by binary
mixtures of PhACs. These results suggest that mixture effects can play an important role in an overall treatment’s nitrification potential and this phenomenon should be further investigated.
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Chapter 1  Introduction

Every year, large quantities of human pharmaceutical products are consumed worldwide. Because many of them are not designed to be totally metabolized by the human body, they are directly excreted to the domestic sewer system. In addition, a considerable amount of medicines are disposed of directly through household waste or flushed in the sink/toilet (Bound and Voulvoulis 2005). The net result of these actions is that a wide variety of metabolized and un-metabolized forms of these drugs enter domestic wastewater treatment plants.

Drugs and their metabolites, commonly referred to as pharmaceutically active compounds (PhACs) (Sedlak D.L. 2000), are of growing concern because of their endocrine disruption potential to humans and wildlife as well as their role in antibiotic resistance development (Daughton and Ternes 1999; Sumpter 2007). Because of their relatively complex chemical structures, PhACs are rarely entirely removed during wastewater treatment processes and are continuously released into the environment through wastewater discharge. In recent surveys, more than 80 PhACs and their metabolites were detected in various aquatic environments in the United States and even in drinking water samples (Halling-Sorensen et al. 1998b; Kinney et al. 2006a; Kolpin et al. 2002).
The extent and magnitude of the risks posed by the presence of PhACs is not yet known due to a lack of research data. However, there are many concerns that PhACs may threaten the physiological and reproductive processes of micro and macro aquatic organisms because of their endocrine disruption potential and high bioactivity to human and wildlife (Akarsubasi et al. 2005; Kinney et al. 2006a).

1.1 Problem Identification

In most instances, wastewater treatment plants (WWTPs) commonly present the first treatment opportunity for removing PhACs and preventing significant environmental exposure. Most treatment facilities, however, are not designed to adequately remove PhACs (Joss et al. 2006; Lindqvist et al. 2005). Thus, many PhACs are not transformed in the wastewater treatment process and are released into the environment through WWTP discharges. A number of PhACs have been detected both in WWTP effluents and in sludge/biosolids with levels ranging from below detection limits up to mg/L (Kinney et al. 2006b; Ternes 1998).

Because most municipal WWTPs rely on the microbial component of the activated sludge process, there is a need to determine if the presence of PhACs in wastewater has the potential of negatively impacting activated sludge microbial communities. To date, very few studies have focused on studying the impact of PhACs on microbial metabolism and growth in engineered treatment systems. Carrucci et al.
(2006) reported that some PhACs inhibited nitrification in a laboratory-scale sequencing batch reactor (SBR) but did not investigate the effect of PhACs on the non-nitrifying microbial fraction. Wittebolle et al. (2005) linked failure of ammonia oxidation in pharmaceutical wastewater treatment with shifts of bacterial communities. However, bacterial shifts linked to PhACs have not been studied in municipal wastewaters.

1.2 Objectives

The main objective of this dissertation is to determine the microbial impact of selected PhACs in municipal WWTPs. The selected PhACs for this study were ketoprofen, naproxen, clofibric acid, carbamazepine and gemfibrozil. These PhACs were expected to partition differently in WWTPs and their fate and effect on microbial communities should cover a broad range.

The specific research objectives of this dissertation were to:

- Estimate the effect of PhACs individually and in binary mixtures on the activity and composition of microbial communities in WWTPs;
- Ascertain the effect of PhACs on sensitive microorganisms (i.e. ammonia-oxidizing bacteria, AOB) in wastewater treatment; and
- Determine if PhACs could adversely impact treatment performance in WWTPs.
1.3 Research Hypothesis and Approach

The general hypothesis for this dissertation is that the presence of some PhACs will adversely impact microbial populations in domestic wastewater treatment. This research investigated the impact of selected PhACs on microbial community structure and the performance of key individual microbial species (i.e., AOB) in domestic wastewater treatment. Specifically, analyses were performed in batch reactors and sequencing batch reactors to determine the impact of PhACs on the microbial community both in terms of bioreactor performance and microbial community structure. AOB were selected as the target individual species because of their importance in wastewater treatment and sensitiveness to toxic compounds. Finally, because PhACs are commonly found in mixtures in the environment, it was of interest to determine if PhAC mixtures affected the microbial population differently than individual PhACs.

First, experiments were carried out in batch reactors. Using this reactor configuration, the effect of each PhAC on microorganisms originating from activated sludge was investigated. Microbial growth and activity as well as community structure were monitored under several PhAC concentrations and organic loading conditions. In addition, the reduction of PhACs in the batch systems was investigated. Next, batch tests were conducted to determine the effect of PhACs on the nitrification rates of AOB. Experiments were performed using *Nitrosomonas europaea* as the model strain.
Reversibility experiments and membrane integrity tests were also performed to understand the mechanism of the inhibition.

The second half of this research consisted of investigating the effects of PhACs in SBRs mimicking WWTP operations. The performance of SBRs exposed to PhACs was compared to control SBRs in terms of chemical oxygen demand and ammonia removal. The microbial communities under each treatment condition were also compared using denaturing gradient gel electrophoresis and terminal restriction fragment length polymorphism. Finally, the effect of binary PhAC mixtures was investigated using the same methods.
Chapter 2  Background and Literature Review

In WWTPs, most PhACs are not completely removed. As a result, PhACs are commonly present in wastewater effluent which is discharged into receiving waters as well as in biosolids originating from wastewater operation which are commonly land applied (Jones-Lepp and Stevens 2007; Ternes 1998). Because it is likely that PhAC concentrations will keep increasing in wastewater influent because of worldwide increasing drug consumption, it is essential to understand how PhACs impact activated sludge microbial communities.

This chapter presents an overview of the current state of knowledge surrounding PhACs in wastewater treatment operations. The literature review begins with a section introducing some background information about PhACs including the various types, their occurrence and toxicity. The second section presents a summary of published studies concerning the removal of PhAC in wastewater treatment plant (WWTP) operations. The third section specifically discusses the properties of the target PhACs which were studied in the context of this dissertation. A summary of published work concerning the target PhACs in WWTPs is also presented. Special focus is put on ammonia oxidizing bacteria (AOB) and their role in wastewater treatment.


2.1 Introduction

2.1.1 Sources and Categories

PhACs are introduced into the environment via community and on-site wastewater treatment facilities, through the overflow or leakage of storage facilities as well as through the land application of untreated animal wastes, and through manufacturing residues. Generally speaking, drugs for medical or veterinary use are not designed to be totally metabolized by the patient and a portion ends up being excreted. This portion generally is discharged in the sanitary sewer system. In addition to the excreted PhACs, some compounds (e.g., expired medicines) are directly discarded as household waste or flushed into the sink/toilet (Bound and Voulvoulis 2005).

Most of the PhACs originating from the sources described above as well as their breakdown products enter WWTPs following their discharge. If these compounds are not removed during the treatment process, they will ultimately be discharged as wastewater effluent and enter the aquatic environment. A schematic representation of possible PhAC sources as well as means of environmental distribution is shown in Figure 2.1. The input of PhACs into the environment is highly dependent upon their rates of consumption, human and animal metabolism as well as removal efficiency in WWTPs.
PhACs are a highly diverse group of compounds with different chemical functionalities as well as biological and physiochemical properties. In general, PhACs are classified according to their therapeutic purpose (e.g., antibiotic, beta-blockers anti-inflammatory drug) [Table 2.1]. Classification according to chemical structure is less common even though this type of classification might be more useful for studying their environmental fate. Such a classification system enables comparison between PhACs with closely related structures which are likely to behave similarly. In this way, PhACs can be treated as groups and surrogates to estimate the behavior of all compounds which fall in a specific group. For PhACs, several biological and physiochemical
properties including log $K_{ow}$, endocrine disruption potential and biodegradability have been used as standards for classification.

<table>
<thead>
<tr>
<th>Drug</th>
<th>Examples</th>
<th>Risk (Exposure and Toxicity)</th>
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<tr>
<td>Antibiotics</td>
<td>Penicillins, sulfamethoxazole</td>
<td>High volumes (About 23,000 tons of antibiotics/year in the U.S.\textsuperscript{a}); concerns over toxicity and antibacterial resistance.</td>
</tr>
<tr>
<td>Painkiller</td>
<td>Ibuprofen, naproxen, ketoprofen</td>
<td>Very high prescription and over-the-counter volumes (70 million prescriptions and 30 billion over-the-counter doses sold annually in the U.S.\textsuperscript{b}); toxicity has not been claimed.</td>
</tr>
<tr>
<td>Antiepileptics</td>
<td>Carbamazepine, Phenobarbital</td>
<td>Detected in the environment \textsuperscript{c}; persistent\textsuperscript{c,d}</td>
</tr>
<tr>
<td>Lipid regulators</td>
<td>Clofibrate acid, gemfibrozil</td>
<td>Long-term prescriptions; commonly detected \textsuperscript{a}</td>
</tr>
<tr>
<td>β-blockers</td>
<td>Propranolol, metoprolol</td>
<td>High volumes; detected in the environment \textsuperscript{c,e,f}</td>
</tr>
<tr>
<td>Antidepressants</td>
<td>Fluoxetins, risperidone</td>
<td>High volumes\textsuperscript{a}; Subject of toxicity testing</td>
</tr>
<tr>
<td>Antihistamines</td>
<td>Loratadine, cetrizine</td>
<td>Commonly held nonprescription medicine</td>
</tr>
<tr>
<td>Others</td>
<td>Contraceptive pills, estradiol</td>
<td>Associated with endocrine disruptions</td>
</tr>
</tbody>
</table>


### 2.1.2 PhAC Occurrence and Abundance

PhACs have been shown to persist following wastewater treatment (Heberer 2002; Quintana JB 2005; Ternes et al. 2002; Tixier et al. 2003). As a result, PhACs tend to enter to and accumulate in aquatic bodies. In a landmark survey of 139 streams across 30
states conducted by the U.S. Geological Survey (USGS), 95 contaminants including 45 pharmaceuticals and other organic wastewater contaminants were detected between the years 1999 and 2000 (Kolpin et al. 2002). The widespread presence of these compounds was further confirmed to be global based on additional investigations performed in Austria, Brazil, Canada, Croatia, England, Germany, Greece, Italy, Spain, Switzerland, the Netherlands, and the U.S. More than 80 pharmaceuticals and drug metabolites were detected in aquatic environments at concentrations up to µg/L-level (Heberer 2002). Their presence in such aquatic bodies impacts the sources of public drinking water supplies and thus careful attention should be taken to determine the effects of these chemicals on human and environmental health. It has been reported that diclofenac, ibuprofen, and phenazone residues, amongst others, have been found at very low concentrations in drinking water samples (Ternes et al. 2002). Researchers also detected 34 PhACs and Endocrine Disrupting Chemicals (EDCs) in source water, finished drinking water, and distribution system (tap) water from 19 U.S. water utilities (Benotti et al. 2008).

2.1.3 Effects and Risks

As discussed in the previous section, there is undeniable evidence that PhACs are present in aquatic environments. As a result, a large amount of research has focused on determining the toxicological impacts of these compounds in order to estimate their threat to the environment. The test species have ranged from micro-organisms (e.g.,
*Escherichia coli, Vibrio fischeri* to algae and higher plants (e.g. *Microcystis aeruginosa*) as well as invertebrate (e.g. *Daphnia*) to fish (*Oryzias latipes*). Studies have looked at both acute toxicity and chronic exposure (Crane et al. 2006; Jones et al. 2001).

Data relating to antibiotics are more prevalent than for other therapeutic classes in large part due to their targeted activity on specific organisms. Many antibiotics have been reported to have acute or chronic toxicity to the environment and present a potential danger to human health because of the development of antibiotic resistance in pathogens. For instance, levofloxacin and clarithromycin showed high toxicity to microalgae as well as chronic toxicity to crustaceans (Yamashita et al. 2006). In another study, sulfamethoxazole’s hazard quotient, derived from the acute toxicity concentration on *Daphnia magna* and its predicted environment concentration (PEC) was reported to be 6.3, which suggests potential environmental concerns (Kim et al. 2007).

Endocrine disruptors (e.g., diethylstilbestrol and 17-α ethinylestradiol) are another group of PhACs which have received much attention. This class of compounds has been shown to have profound ecological impacts as they mimic a natural hormone, fooling the body or blocking the effects of a hormone from certain receptors. It was reported that a single dose of 2 ng/L 17-α ethinylestradiol in water can retard testes growth and development by 50% in maturing male trout (Tyler et al. 1998). In addition, exposure to 30 ng/L of either 17-β ethinylestradiol or estrone in water for 21 days induced vitellogenin (an egg yolk precursor protein that is normally produced only by
adult females) synthesis and abnormal testicular growth in male fathead minnows
(Pimephales promelas) (Panter et al. 2000).

Besides antibiotics and endocrine disruptors, the toxicity of other therapeutic
classes have only been reported sporadically (Brain et al. 2004; Henry et al. 2004; Isidori
et al. 2006; Kim et al. 2007; Zurita et al. 2007). Some research indicated that PhACs have
cytotoxicity, which shows a correlation with their Log $D_{lipw}$ (Caminada et al. 2006; Escher
et al. 2005). In a test using membrane vesicles isolated from a photosynthetic bacterium,
Rhodobacter sphaeroides, seven PhACs (i.e. clofibrate, acetaminophen, propranolol,
diazepam, diclofenac, ethinylestradiol and ibuprofen) clearly exhibited baseline toxicity.
This finding is alarming especially if their toxicities become additive in the environment
where mixtures containing a large number of different PhACs are likely to be found
(Escher et al. 2002). However, compared with their concentrations in water, most PhACs
are not expected to present acute risks to the aquatic biota (Lienert et al. 2007).
Nevertheless, the chronic toxicity of PhACs at environmentally relevant concentrations
have not been well documented and thus it cannot be concluded that PhACs do not
have any ecological impacts (Crane et al. 2006).

2.1.4 Mixture Effect

PhACs never occur alone in the environment but, rather, are always found in
combination with other compounds. Human and wildlife are always exposed to mixture
of chemicals instead of single compounds. Therefore, the characterization of effects of single compounds is not sufficient to elucidate the potential hazard of PhACs in the environment.

The combination effects of chemicals can be grouped based on whether they interact and whether they act in a similar mode. For those chemicals that react with each other, their mixture effect may be either higher if more toxic products at the end point are derived or lower when products are less toxic. On the other hand, if the components in the mixture do not interact, two pharmacological concepts for the expected additive mixture effects have been generally accepted, concentration addition, and independent action (Bliss 1939; Glenn 1992).

Compounds that share a common mode of toxic action typically act in a concentration additive manner, which means that every mixture component contributes to the overall combination effect in proportion to its concentration, even below their non-observed effect concentration (NOEC) (Altenburger et al. 2000; Silva et al. 2002). Independent action, which is usually applied to chemicals with different modes of action, assumes that mixture effects are the result of interactions of individual mixture constituents with different subsystems of an organism (Bliss 1939; Glenn 1992). In such mode, chemicals that are below their NOEC are not expected to contribute to the total mixture effect.
Studies have revealed that the PhACs working in the mode of concentration addition resulted in dramatic mixture effects. For example, eight weak endocrine disrupting chemicals (EDCs) were shown to act together to produce significant effects in a recombinant yeast estrogen screen (YES) system even though their individual concentrations were below their respective NOECs (Silva et al. 2002). This phenomenon was further confirmed by a study using fathead minnow as target organisms. In that study, a mixture containing five estrogenic chemicals with concentrations below their NOECs affected the reproductive performance of fish (Brian et al. 2007). Besides EDCs, mixture toxicity of four NSAIDs at their NOEC concentration was also found to be significant in the Daphnia test. In that study, the toxicity level could be accurately predicted using the concept of concentration addition (Cleuvers 2004). In another study using membrane vesicles isolated from a photosynthetic bacterium, Rhodobacter sphaeroides, mixtures of four pharmaceuticals (i.e. diazepam, diclofenac, ethinylestradiol and inbuprofen) were concentration-additive with each other as well as with the pure baseline toxicants (Escher et al. 2002). These studies suggest that the risk assessment of pharmaceuticals is not only specific to a given compound (e.g., endocrine disruption) but that the cumulative risk assessment of a mixture must be considered. The nonspecific but additive baseline toxicity has to be taken into account.
2.2 PhACs in WWTP

The main pathway by which PhACs are introduced into the environment is through municipal WWTPs (Figure 2.1). Because the treatment process at most municipal WWTPs is largely biological, it is essential to understand the impact this class of contaminants might have on the microbial communities including their activity and structure in WWTPs. The following sections present an overview of the current state of knowledge concerning the fate and microbial impact of PhACs in WWTP.

2.2.1 Fate of PhACs in WWTPs

The removal of PhACs in WWTPs mainly results from sorption and degradation processes which are either chemically or biologically mediated (Kummerer et al. 2004). Most PhACs are large molecules with low Henry’s constants (Maurin and Taylor 2000; Poiger et al. 2003). Thus, volatilization is usually considered negligible as an elimination mechanism and is rarely considered as a removal mechanism.

The removal rates as well as typical measured influent concentrations for a variety of PhACs are shown in Table 2.2. Their fate depends on their physicochemical properties (e.g., chemical structure, aqueous solubility, octanol/water partition coefficient, and Henry’s law constant) as well as the operation of WWTPs.
Table 2.2 Concentration and Removal Rates of some PhACs in WWTP

<table>
<thead>
<tr>
<th>PhACs</th>
<th>Influent ($\mu$g/L)</th>
<th>Removal (%)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aspirin</td>
<td>0.3-3.1</td>
<td>81-88</td>
<td>Heberer (2002)</td>
</tr>
<tr>
<td>Bezafibrate</td>
<td>1.2-5.3</td>
<td>27-83</td>
<td>Ternes (1998)</td>
</tr>
<tr>
<td>Caffeine</td>
<td>230</td>
<td>99.9</td>
<td>Heberer (2002)</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>1.78-2.1</td>
<td>7-8</td>
<td>Heberer (2002)</td>
</tr>
<tr>
<td>Clofibric Acid</td>
<td>0.46-1.2</td>
<td>0-15</td>
<td>Heberer (2002)</td>
</tr>
<tr>
<td>Cyclophosphamide</td>
<td>0.007-0.143</td>
<td>0-94</td>
<td>StegerHartmann et al.(1997)</td>
</tr>
<tr>
<td>Fenofibric acid</td>
<td>0.5-1.03</td>
<td>6-64</td>
<td>Stumpf et al.(1999)</td>
</tr>
<tr>
<td>Gemfibrozil</td>
<td>0.35-0.9</td>
<td>16-69</td>
<td>Ternes (1998) Stumpf et al.(1999)</td>
</tr>
<tr>
<td>Ibuprofen</td>
<td>0.3-4.1</td>
<td>90</td>
<td>Ternes (1998) Stumpf et al.(1999)</td>
</tr>
<tr>
<td>Ketoprofen</td>
<td>0.6</td>
<td>48-69</td>
<td>Stumpf et al.(1999)</td>
</tr>
<tr>
<td>Naproxen</td>
<td>0.6-1.3</td>
<td>15-78</td>
<td>Ternes (1998)</td>
</tr>
<tr>
<td>Phenazone</td>
<td>0.3</td>
<td>33</td>
<td>Ternes (1998)</td>
</tr>
</tbody>
</table>

2.2.1.1 Sorption, Photolysis and Hydrolysis

Sorption processes result in the redistribution of PhACs after they enter WWTPs. For instance, in the case of ciprofloxacin (a common antibiotic), 20% sorbs onto primary sludge and 40% of the remainder partitions onto the secondary sludge (Gobel et al. 2005). Carbamazepine, an antiepileptic drug, has been shown to be effectively removed by granular activated carbon (Ternes et al. 2002). On the other hand, acidic pharmaceuticals, such as the non-steroidal anti-inflammatories (e.g., ibuprofen), do not significantly sorb onto sludge. PhACs are thought to be removed by sedimentation in surface waters (Tixier et al. 2003). PhACs which lack functional groups (e.g., –OH, –COOH, or –NH₂
functional groups) tend to not be charged at neutral pH; hence, the sorption for this class of PhACs is probably caused by nonspecific sorption interactions.

Besides $pK_a$, some characteristics including octanol/water distribution coefficient ($K_{ow}$), liposome/water distribution coefficient ($D_{lipw}$), solid-water distribution coefficient ($K_d$) and biosolids/water distribution coefficient ($K_p$) are also good indicators of a compound’s ability to sorb. The higher these coefficients are, the more likely the PhAC is to partition to the sludge/biosolids as well as the nonpolar fats and lipids, mineral oils, and greases generally present in wastewater (Krogmann et al. 1999; Maurer et al. 2007).

Photolysis has been demonstrated as another possible removal pathway for PhACs. Direct phototransformation has been demonstrated as an elimination process in surface waters for diclofenac (Buser et al. 1998b). For ketoprofen and naproxen, direct phototransformation was considered as possible elimination processes in surface waters (Tixier et al. 2003).

Several reports have shown that for drinking water treatment applications, ozonation and advanced oxygen processes (AOP) are very effective in oxidizing pharmaceuticals (Ternes et al. 2002). The application of AOP (e.g., $O_3/H_2O_2$) improved the degradation efficiency of clofibric acid, ibuprofen, and diclofenac significantly. Under optimal conditions, removal as high as 90% were obtained for ibuprofen and diclofenac (Zwiener and Frimmel 2000). However, the toxicity of the oxidized products of PhACs need to be studied before the applications.
Hydrolysis is another possible removal pathway for PhACs although there are limited reports on this topic. It has been demonstrated that in some cases PhACs undergoing hydrolysis generate more reactive and toxic products than the parent compounds (Halling-Sørensen et al. 1998a).

2.2.1.2 Biodegradation

Some PhACs are bioavailable and thus the biodegradation of these contaminants is possible. Generally, unbranched compounds with short side chains are more biodegradable than molecules with long, highly branched side chains (Schwarzenbach et al. 2003); unsaturated aliphatic compounds are more accessible than saturated analogues or aromatic compounds with complicated aromatic ring structures and sulfate or halogen groups (Rogers 1996). However, no quantitative relationship between structure and biodegradability can be derived from the limited number of published studies.

In a laboratory scale sequencing batch reactor (SBR), ranitidine, an H2 antagonist, showed removal efficiencies ranging from 17 to 26% while atenolol, a ß-blocker, showed removal efficiencies ranging from 36 (real wastewater) to 90% (synthetic wastewater) (Carucci et al. 2006). For polar compounds, such as acidic PhACs, microbial degradation tends to play a more important role as a removal process since they are less likely to be removed by sorption in wastewater treatment (Quintana et al. 2005). High removals
mainly resulting from biodegradation have been reported for ibuprofen (82%) in pilot nitrifying-denitrifying activated sludge system (Suarez et al. 2005).

Besides the properties of PhACs, the biota of WWTPs, which depends on treatment processes (i.e., aerobic or anaerobic, suspended sludge or biofilm), WWTP operational parameters (i.e., solid retention time, SRT) are another factor impacting the biodegradation of PhACs. A study demonstrated that, in an up-flow anaerobic stage reactor (UASB), an average of 95% removal of the antibiotic tylosin can be achieved (Chelliapan et al. 2006) while only 63% tylosin was removed in a activated sludge treatment process (Watkinson et al. 2007). Another study showed that a membrane bioreactor (MBR) with 65 day SRT has better biodegradation of ketoprofen and diclofenac than the one with 15 day SRT (Kimura et al. 2007). This phenomenon may due to the growth of slower growing bacteria with longer SRT.

Among the slow growing bacteria, ammonia oxidizing bacteria (AOB) are a group of bacteria that draw attention of researchers for their ability to co-metabolize organic pollutants. It was reported that the activity of AOB dominated the degradation of estrone, estradiol, estriol and ethinylestradio (Ren et al. 2007). Several studies also demonstrated some PhACs and EDCs exhibited higher removal in nitrifying activated sludge (Drewes et al. 2002; Kreuzinger et al. 2004; Vader et al. 2000). AOB have been demonstrated to be a very sensitive microbial population in WWTPs and for this reason are a good model organism to use for testing the toxicity of contaminants (Hooper et al.
1997; Iizumi et al. 1998; Wood et al. 1981). These microorganisms will be discussed in more detail later in this chapter.

### 2.2.2 Impact on Total Microbial Communities

#### 2.2.2.1 Microbial Activities

To date, very few studies have focused on studying the impact of PhACs on microbial metabolism and growth in engineered treatment systems. In a laboratory-scale completely mixed anaerobic reactor, the COD removal decreased from 90 to 71% and acetoclastic methanogenic activity decreased by half when the feed was switched from a municipal wastewater to a pharmaceutical wastewater (Akarsubasi et al. 2005). Another similar study also showed that mixing pharmaceutical and municipal wastewaters resulted in a decrease of COD removal efficiency, methane yield and acetoclastic methanogenic activity (Oz et al. 2003). With the addition of 1 mg/L erythromycin, a laboratory-scale anaerobic sequencing batch reactor was found to have a reduction of biogas production by 5%. Further tests showed the conversion of butyric acid was inhibited, which could lead to the accumulation of volatile fatty acids and thus could have potential impacts on system stability (Amin et al. 2006).

#### 2.2.2.2 Effect on Microbial Community Structure

Two polymerase chain reaction (PCR)-based techniques, Denaturing gradient gel electrophoresis (DGGE) and Terminal restriction fragment length polymorphism (T-
RFLP) are typically used to assess the impact of contaminants on bacterial community stability. In a laboratory-scale completely mixed anaerobic reactor treating pharmaceutical wastewaters, DGGE results showed the composition of eubacterial population changed whereas the overall archeal profile was not altered (Oz et al. 2003). With an increase in the proportion of pharmaceutical wastewater in the influent, the relative abundance of key methanogens was shown to decrease (Akarsubasi et al. 2005).

In a recent study, T-RFLP applied to an aerobic sludge bacterial community showed that a structural divergence and a reduced diversity of bacterial community occurred in the presence of 50 µg/L ibuprofen, naproxen, ketoprofen, diclofenac and clofibric acid. Furthermore, clone-library analysis identified the genus *Nitrospira* sp., key nitrite-oxidizing bacteria, were only present in the clone library for the reactor without pharmaceuticals (Kraigher et al. 2008). This result suggests that pharmaceuticals could have a potentially important influence on the treatment performance of WWTPs.

### 2.2.3 Impact on Nitrifiers

Nitrifiers or nitrifying bacteria, are chemoautotrophic bacteria that grow at the expense of inorganic nitrogen compounds (Mancinelli 1996). They are widespread in soil and water, and thrive in places with high amount of ammonia like WWTPs. They include two groups: ammonia-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). The functions for both of these bacterial groups are shown in Figure 2.2.
2.2.3.1 Ammonia-Oxidizing Bacteria (AOB)

AOB make up a key group of bacteria in WWTPs which remove ammonia. They oxidize ammonia to nitrite in a two-step process: the first step is the oxidization of ammonia to hydroxylamine (NH$_2$OH) by the membrane-bound enzyme ammonia monooxygenase (AMO); the subsequent oxidation of hydroxylamine to nitrite is catalyzed by periplasmic enzyme, hydroxylamine oxidoreductase (HAO) (Gunther et al. 2007; Hooper et al. 1997) [Figure 2.2].

![Figure 2.2 Nitrification process (Gunther et al. 2007).](image)

AOB are believed to be particularly susceptible to inhibition by certain chemical compounds at low concentrations compared to NOB and heterotrophs (Hooper et al. 1997; Iizumi et al. 1998; Wood et al. 1981). It is well accepted that inhibition of ammonia oxidation by AOB leads to a total failure of nitrogen removal (Jonsson et al. 2001; Moussa et al. 2006). The major reason for AOB inhibition is the inhibition of the AMO-mediated ammonia oxidation process. AMO is a membrane-bound enzyme containing...
copper in its active site. Therefore, metal binding compounds and chelating agents reversibly inhibit its activity at very low concentrations (Bedard and Knowles 1989). AMO has also been shown to oxidize substrates other than ammonia including sulfur, aromatic, and halogenated compounds, and these compounds competitively inhibit AMO activity (Hyman et al. 1988).

Due to their sensitivity to chemicals and importance in the environment, AOB are widely used as bioassay model strains or indicators in toxicity tests (Blum and Speece 1991; Gernaey et al. 1997; Iizumi et al. 1998; Ren and Frymier 2003). To protect this sensitive nitrifying activated sludge population in WWTPs, it is essential to understand the impact of PhACs on their nitrifying activity and livability. However, very few studies have focused on studying the impact of PhACs on AOB. Wittebolle et al. (2005) linked failure of ammonia oxidation in pharmaceutical wastewater treatment to shifts of bacterial communities. Carrucci et al. (2006) reported that some PhACs (ranitidine, atenolol and lincomycin) inhibited nitrification of activated sludge in both WWTP and lab-scale sequencing batch reactor by monitoring the acidity production. Halling-Sorensen (2001) showed that 11 antibacterial agents adversely affected the growth and nitrification rate of activated sludge microorganisms and _N. europaea_.

In a study comparing heterotrophs, methanogens and _Nitrosomonas_ sp., _Nitrosomonas_ was shown to be the most effective microorganism because of its higher sensitivity (Blum and Speece 1991). In the present dissertation, focus will be given to
Nitrosomonas europaea, a well studied AOB which utilizes only NH₃, CO₂ and mineral salts for growth with a double-time of 8-10 hours (Radniecki et al. 2008). Most of our understanding for biochemistry and molecular biology of AOB have been achieved from this strain (Arp et al. 2002). N. europaea is widely used as a model strain for fast and sensitive acute toxicity detection (Cui et al. 2005; Gernaey et al. 1997; Iizumi et al. 1998).

2.2.3.2 Nitrite-oxidizing Bacteria (NOB)

All isolated chemolithoautotrophic NOB belong to one of four different genera: *Nitrobacter* (alpha subclass of *Proteobacteria*), *Nitrococcus* (gamma subclass of *Proteobacteria*), *Nitrospina* (delta subclass of *Proteobacteria*), and *Nitrospira* (phylum *Nitrospira*) (Daims et al. 2001). *Nitrobacter* species can be isolated from many environmental samples, whereas *Nitrospira* are unculturable. *Nitrospira* have been shown to be the most important group of NOB in wastewater treatment plants (Burrell et al. 1998; Juretschko et al. 1998). Bacteria of both genera rely on nitrite oxidoreductase (NOR or NXR) to oxidize nitrite to nitrate (Figure 2.2). This enzyme is bound to the inner cytoplasmic surface of the bacterial membrane and contains multiple subunits, iron-sulphur centers and a molybdenum cofactor (Meincke et al. 2004).

A research study focused on nitrite reduction rates indicated that some PhACs (i.e., triclosan, ofloxacin and sulfamethoxazole) inhibited the activity of NOB (Dokianakis et al. 2004). Analyses of T-RFLP profiles and two clone libraries
indicated that a mixture of pharmaceuticals containing four NSAIDs and clofibric acid at concentration of 50 µg/L caused the inability to detect *Nitrospira* in the reactor with addition of pharmaceuticals further suggesting that PhACs could adversely affect WWTP performance.

### 2.3 Target PhACs Characteristics

In the context of this dissertation work, research experiments focused on 5 common PhACs, which have been detected at fairly high concentrations in the environment and in WWTPs. The five compounds selected consist of naproxen, ketoprofen, clofibric acid, carbamazepine and gemfibrozil.

#### 2.3.1 Occurrence in the Environment

The daily loads of naproxen and ketoprofen to a lake in Switzerland have been reported on the order of grams (Tixier et al. 2003). Carbamazepine is among the 10 most frequently detected organic compounds in the surface water of United States according to the USGS survey (Kolpin et al. 2002), and clofibric acid is known for its persistence (Buser et al. 1998a; Khetan and Collins 2007). The concentration of gemfibrozil in surface waters in North America and Europe was found to be approximately 3 and 6 nM, respectively (Sanderson et al. 2003).
In three WWTPs in Switzerland, the concentrations of naproxen and ketoprofen in the effluents reach 2.6 µg/L and 0.18 µg/L, resulting in their daily load to surface water as high as 11.7 and 0.41 g/day, respectively (Tixier et al. 2003). The average concentration of carbamazepine in the WWTP influent ranges from 1.78~2.1 µg/L and the removal rate was reported to be in the range of 7 to 8 % (Heberer 2002). For clofibric acid, the daily load to a WWTP in Germany was around 60 g/day, and the daily discharge of gemfibrozil from WWTPs in France was 0.8 µg/L.

These five compounds are also present in source water of drinking water, the treated water and distribution water from water treatment plants. In a 1-year study based on the examination from 19 drinking-water treatment facilities, naproxen, carbamazepine and gemfibrozil are among the 11 most frequently detected compounds. Their concentrations in source water reach 51, 24 and 32 ng/L (Benotti et al. 2008).

2.3.2 Physical and Chemical Properties

The physicochemical properties of PhACs (e.g., chemical structure, aqueous solubility, octanol/water partition coefficient, and Henry’s law constant) decide their fate including their bioavailability and microbial impact. Naproxen, ketoprofen and clofibric acid are organic acids with $pK_a$ values ranging from 4.9 to 2.9 and consequently belong to the group of acidic pharmaceuticals (Table 2.3). Although they have moderately high $\text{Log}K_{ow}$ (2.57-3.2), sorption has not been shown to be the main mechanism of removal
due to their ionic form in the environment. The contribution of sorption to elimination of naproxen, ketoprofen and clofibric acid was less than 0.7, 0.5 and 2%, respectively (Kimura et al. 2007). In this case, Log$D_{ow}$ would be a better indicator of sorption potential. Based on these data, carbamazepine and gemfibrozil are likely to have only moderate potential to accumulate in biosolids and sludge. In all cases, due to their very low Henry’s law constant, these compounds are not expected to partition into the gase phase. Gas exchange can be neglected for all five compounds.
Table 2.3 Properties of Target PhACs

<table>
<thead>
<tr>
<th>CAS number</th>
<th>Medical Usage</th>
<th>Structure</th>
<th>Molecular Weight</th>
<th>Log $K_{ow}$</th>
<th>Log $D_a$ (pH=7)</th>
<th>pKa</th>
<th>Water Solubility (mg/L)</th>
<th>Henry's Law Constant: (atm-m$^3$/mole, 25°C)</th>
<th>Vapor pressure (mm Hg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>22204-53-1</td>
<td>Nonsteroidal anti-inflammatory (NSAID)</td>
<td><img src="image1" alt="Structure of Naproxen" /></td>
<td>230.26</td>
<td>3.18</td>
<td>0.85</td>
<td>4.15 or 4.84</td>
<td>15.9</td>
<td>3.39E-010</td>
<td>1.89E-006</td>
</tr>
<tr>
<td>22071-15-4</td>
<td>Anti-epileptic drug</td>
<td><img src="image2" alt="Structure of Ketoprofen" /></td>
<td>254.29</td>
<td>3.12</td>
<td>Na</td>
<td>4.45</td>
<td>51</td>
<td>2.12E-011</td>
<td>3.72E-007</td>
</tr>
<tr>
<td>000298-46-4</td>
<td>Lipid regulator</td>
<td><img src="image3" alt="Structure of Carbamazepine" /></td>
<td>236.28</td>
<td>2.45/2.67</td>
<td>2.67</td>
<td>NA**</td>
<td>17.7</td>
<td>1.08E-010</td>
<td>1.84E-007</td>
</tr>
<tr>
<td>882-09-7</td>
<td>Antihyperlipidemic agent</td>
<td><img src="image4" alt="Structure of Clofibric Acid" /></td>
<td>214.65</td>
<td>2.57</td>
<td>-0.76</td>
<td>2.85</td>
<td>583</td>
<td>2.19E-008</td>
<td>1.13E-004</td>
</tr>
<tr>
<td>25812-30-0</td>
<td></td>
<td><img src="image5" alt="Structure of Gemfibrozil" /></td>
<td>250.33</td>
<td>4.77/4.39</td>
<td>2.15</td>
<td>4.75</td>
<td>10.9</td>
<td>1.19E-008</td>
<td>3.05E-005</td>
</tr>
</tbody>
</table>

* Most data are from Interactive PhysProp Database of Syracuse Research Corporation. ([http://www.syrres.com/esc/physdemo.htm](http://www.syrres.com/esc/physdemo.htm))
** Data not available

2.3.3 Transformation and Degradation

For naproxen and ketoprofen, direct phototransformation and biodegradation are considered as possible elimination processes (Tixier et al. 2003). In surface water,
naproxen was rapidly transformed via direct photolysis ($t_{1/2} = 42$ min) under summer sunlight (Packer et al. 2003). However, its photoproducts were more toxic for both acute and chronic effects on aquatic organisms. In WWTPs, it has been reported that 68% of naproxen was removed in a pilot nitrifying-denitrifying activated sludge system (Suarez et al. 2005) and 50 to 65% of ketoprofen in activated sludge municipal wastewater treatment (Quintana J.B 2005). Because the removal is unlikely from sorption (low sorption potential) and phototransformation (turbid environment in WWTPs), naproxen and ketoprofen removal is mostly attributed to biodegradation (Carballa et al. 2007).

The biodegradation for naproxen was considered to be co-metabolic (Quintana J.B 2005). In the same study, ketoprofen was demonstrated to be transformed as a sole source of carbon and energy to biphenyls and related compounds based on LC-MS analysis. However, the metabolites of ketoprofen seemed more stable and resulted in partial mineralization of ketoprofen.

Removal of carbamazepine is generally very low (<10%) as reported in several studies (Joss 2005; Metcalfe et al. 2003; Radjenovic et al. 2007). The poor biodegradability of carbamazepine can be hypothesized based on the low removal rate in WWTPs where microorganisms have much higher activity and diversity than in the natural environment. It has been shown to be recalcitrant in both membrane bioreactor and activated sludge systems (Radjenovic et al. 2007). Furthermore, the increase of sludge retention time did not improve the removal (Bernhard et al. 2006).
concentration of effluent sometimes was higher than influent which may have resulted from the cleavage of glucuronide-conjugated carbamazepine in influent (Radjenovic et al. 2007). However, carbamazepine may undergo photolysis during treatment processes. Using solar irradiation, Andreozzi et al. (2002) showed that carbamazepine degradation follows a first order reaction with a kinetic constant value of $5.7 \times 10^{-3}$/h, which corresponded to a half-life of 121.6 h.

Clofibric acid is known for its persistence (Buser et al. 1998a; Zwiener et al. 2000). Henschel et al. (1997) suggested that clofibric acid is not biodegradable. However, slow direct photolysis of clofibric acid has been observed in both Milli-Q water and surface water. In the natural water, indirect photolysis is evolved, resulting in higher degradation rate ($t_{1/2}=50$ hr) because of the presence of nitrate and humic acids (Packer et al. 2003).

The removal of gemfibrozil in WWTPs have been reported in the range of 16 to 69% (Ternes 1998). To date, there are few reports concerning the fate and degradation of gemfibrozil in domestic WWTPs and the aquatic environment.

### 2.3.4 Toxicity and Inhibition

A study based on thiazolyl blue tetrazolium bromide (MTT) and neutral red (NR) assays for fish cells (PLHC-1 and RTG-2 cells) indicates that the cytotoxicity of naproxen, carbamazepine, clofibric acid and gemfibrozil are low (Caminada et al. 2006). The half
maximal effective concentration (EC50) values, which represent the concentration of agonist required to induce a response halfway between the baseline and maximum toxicity, are shown in Table 2.4.

<table>
<thead>
<tr>
<th>PhACs</th>
<th>MTT (PLHC-1) EC50 [mM]</th>
<th>NR (PLHC-1) EC50 [mM]</th>
<th>MTT (RTG-2) EC50 [mM]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naproxen</td>
<td>2.54</td>
<td>1.49</td>
<td>4.48</td>
</tr>
<tr>
<td>Carbamazepine</td>
<td>&gt;2.5</td>
<td>&gt;2.5</td>
<td>&gt;2.5</td>
</tr>
<tr>
<td>Clofibric acid</td>
<td>3.77</td>
<td>3.15</td>
<td>5</td>
</tr>
<tr>
<td>Gemfibrozil</td>
<td>0.873</td>
<td>0.843</td>
<td>3.94</td>
</tr>
</tbody>
</table>

Ketoprofen was reported to be associated with peroxidation of membrane lipids, leading to cell lysis (Bosca et al. 1995). Lipid peroxidation is an oxidative degradation of lipids, whereby unsaturated lipids are initiated by reactive oxygen species (ROS) to fatty acid radicals and finally to polar lipid hydroperoxides. Such effects can profoundly influence membrane functions (Laskin et al. 2006). In the presence of 10 µM ketoprofen, a significant amount of lipid-derived hydroperoxides were detected, which indicated the lipid peroxidation. The decrease in intracellular lactate dehydrogenase activity also confirmed the leakage of the cells. The cytotoxic effect was explained by the generation of radical intermediates derived by ketoprofen.

Naproxen with comparable properties to ketoprofen on the basis of a structural criterion (De Guidi et al. 2005), was also reported to induce photosensitization, which
resulted in lipid peroxidation and cell lysis, on red blood cells causing by drug-derived free radicals (Condorelli et al. 1996). In a study about the impact of NSAIDs on aquatic life, the EC50 of naproxen was reported to be 625 mg/L in an algal growth test and 166 mg/L for *Daphnia* immobilization tests (Cleuvers 2004).

Although carbamazepine did not exert significant effect on the microalgae *Aplanes braunii* within 96 hours, the 60 day carbamazepine exposure experiment showed this algal strain accumulated the substrate by adopting high concentrations in comparison with the culture medium. The uptake and concentration into algal cells of carbamazepine could cause bioaccumulation through food net to consumer organisms in the upper trophic level (Andreozzi et al. 2002). In prokaryotes, it was reported that 10 µg/L carbamazepine reduced the amount of bacterial biomass produced on riverine biofilms (Lawrence et al. 2005).

The toxic effects of gemfibrozil have been investigated in four aquatic systems. The most sensitive system, *Daphnia magna* immobilization, showed the non-observed adverse effect level of gemfibrozil was 30 µM. The possible mechanism was the binding to sulphydryl groups (Zurita et al. 2007).

To date, very few studies have focused on studying the impact of the five PhACs on microbial metabolism and growth in engineered treatment systems. Kumagai et al. (2006) showed that 983 µM ketoprofen resulted in 50% OUR inhibition of activated
sludge. Kruszewska et al. (2002) showed that naproxen in the form of Nalgesin tablets significantly inhibited microbial growth of select individual microorganisms.

2.4 Microbial Community Analysis

To understand impact on microbial communities, it is important to examine microbial community structure and community dynamics in response to changes in different environmental parameters (Torsvik et al. 1996). To identify microbial community structural changes, molecular techniques are critical because traditional cultivation-based techniques typically include only a small percentage of the total cells present in a given sample (Amann et al. 1995). Two commonly used polymerase chain reaction (PCR)-based microbial community analysis tools are introduced in this section.

2.4.1 Denaturing Gradient Gel Elecrophoresis (DGGE)

DGGE is based on the separation of PCR-amplifed fragments of genes coding for the 16S rRNA gene by electrophoresis using a denaturing gradient gel (Amann et al. 1995; Muyzer et al. 1993; Torsvik et al. 1996). This technique allows for the side-by-side comparison of microbial profiles. Each gel is composed of DNA bands and their patterns can be used to diagnose for the presence and absence of microorganisms. The relative intensity of each band and its position roughly represented the relative abundance of a particular species in the population. To further identify microorganisms of interest, DNA bands can be excised from the DGGE gel, reamplified and sequenced.
To obtain information about the presence of a particular species or a group of bacteria, DGGE gels can be transferred to hybridization membranes and probed with specific oligonucleotides probes. A more quantitative analysis can also be applied to DGGE gels by calculating the Sorenson similarity index: \( S = \frac{2c}{a+b} \), where \( a \) and \( b \) are the number of bands in any two samples and \( c \) is the number of bands shared between those samples (Turpeinen et al. 2004).

Because DGGE can provide the constituent of a population in both qualitative and semi-quantitative ways, it has been successfully applied to the identification of a number of genes from several different organisms including total bacteria and AOB (Ceccherini et al. 2007; Ferris et al. 1996; Kowalchuk et al. 1997). However, DGGE has been shown to not always provide a completely accurate and unbiased fingerprint of the microbial community. This PCR-based technique inevitably has a built-in PCR bias, i.e. differential amplification of different rRNA genes, as well as different lysis rate of cells (Reysenbach et al. 1992; Suzuki and Giovannoni 1996). It is also limited in detecting all microbial strains. It has been reported that it is capable of detecting only 95–99% of the bacterial community (Gelsomino et al. 1999).

### 2.4.2 Terminal Restriction Fragment Length Polymorphism (T-RFLP)

Similar to DGGE, T-RFLP analysis is a PCR-based technique used to study complex microbial communities based on variation in the 16S rRNA gene. T-RFLP
analysis consists of 3 steps: 1) 16S rRNA genes in a sample are amplified using a fluorescently-labeled primer to yield a mixture of labelled 16S rRNA genes; 2) These amplification products are digested with restriction enzymes to produce labelled terminal restriction enzyme fragments (TRFs); 3) T-RFs are separated by electrophoresis and transformed to a series of peaks (fragments) of various sizes and heights that represents the profile of that sample. T-RFLP is generally accepted as a culture-independent, rapid, sensitive and reproducible method of assessing diversity of complex communities without the need for any genomic sequence information (Osborn et al. 2000). T-RFLP has been applied to the study of complex microbial communities in diverse environments such as soil and activated sludge systems (Fierer and Jackson 2006; Kraigher et al. 2008).

T-RFLP profiles are generally analyzed to reveal the diversity indices of communities: richness (S), Shannon diversity index (H) and evenness (E). S is obtained from distinct T-RFs of each derivate profile, indicating the species of the communities. H can be calculated as described by Briones et al. (2007) using Equation 2-1

\[ H = -\sum p_i \ln p_i \]  

Equation 2-1

where \( p_i \) is the ratio of an individual T-RF peak area relative to the cumulative peak area (Briones et al. 2007). E can be calculated using Equation 2-2

\[ E = \frac{H}{H_{\text{max}}} \]  

Equation 2-2
where $H_{\text{max}} = \ln S$.

In addition to estimating the diversity indices of communities, the T-RFLP pattern of a community can be viewed as a community fingerprint and used to assess the similarity of different communities. Each T-RFLP pattern constitutes a distinct phylogenetic fingerprint of a community based on the number of T-RFs and their intensities. As a PCR based technique, T-RFLP has the same bias problem as DGGE which was discussed above. In addition, some organisms may produce more than one T-RF because of rrn operon copy number heterogeneity (Crosby and Criddle 2003).
Chapter 3  Effect of PhACs on Activated Sludge Microorganisms in Batch Reactor Systems

The main objective of this dissertation is to determine the elimination of select PhACs in WWTPs as well as their effect on the microbial communities. To meet this objective, the effect of PhAC on the microbial growth and ecology of microorganisms originated from a municipal WWTP was first characterized in batch reactor systems. The results from this portion of the study are reported in this chapter.

3.1 Introduction

Pharmaceutically active compounds (PhACs) are being introduced into the environment via community and full-scale wastewater treatment plants (WWTPs), through the overflow or leakage of storage facilities and land application of untreated animal wastes, as well as through manufacturing residues (Daughton and Ternes 1999; Kolpin et al. 2002). Because many of the compounds are not fully metabolized prior to household discharge, the parent PhACs and their breakdown products commonly enter WWTPs (Bound and Voulvoulis 2005). The prevalence of pharmaceutical residuals and antibacterial chemicals in municipal wastewaters and other aquatic environments is of growing concern because of their endocrine disruption potential to humans and wildlife as well as their role in antibiotic resistance development (Daughton and Ternes 1999;
Recently, more than 80 pharmaceuticals and drug metabolites were detected in aquatic environments in the United States and even in drinking water samples (Kinney et al. 2006a; Kolpin et al. 2002). The extent and magnitude of the risks posed by PhACs is not yet known due to a lack of research data. However, there are many concerns that PhACs may threaten the physiological and reproductive processes of micro and macro aquatic organisms (Kinney et al. 2006a). Furthermore, pathogens may develop resistance to these compounds, ultimately leading to an increase risk of human diseases (Kolpin et al. 2002).

While there has been a recent interest in the development of technologies to remove PhACs from wastewater (Kimura et al. 2005; Rooklidge et al. 2005), most treatment facilities are not designed to adequately remove them (Joss et al. 2006; Lindqvist et al. 2005). The persistent nature of PhACs is in large part due to their chemical characteristics as well as their slow biodegradation kinetics (Tixier et al. 2003). PhACs are not transformed in the wastewater treatment process and are released into the environment through WWTP discharges. As a result, PhACs tend to accumulate in aquatic bodies, thereby increasing the possibility of human exposures (Tixier et al. 2003).

Because most municipal WWTPs rely on the microbial component of the activated sludge process, there is a need to determine if the presence of PhACs in wastewater has the potential of negatively impacting activated sludge microbial communities. To date, very few studies have focused on studying the impact of PhACs
on microbial metabolism and growth in engineered treatment systems. Carrucci et al. (2006) reported that some PhACs inhibited nitrification in a laboratory-scale sequencing batch reactor but did not investigate the effect of PhACs on the non-nitrifying microbial fraction. Wittebolle et al. (2005) linked failure of ammonia oxidation in pharmaceutical wastewater treatment with shifts of bacterial communities. However, bacterial shifts linked to PhACs have not been studied in municipal wastewaters. The primary objective of this chapter was to determine the effect of PhAC and organic loading on the microbial growth and ecology of microorganisms found in a municipal WWTP. These experiments were carried out in batch reactors.

### 3.2 Materials and Methods

#### 3.2.1 Batch Reactors Description

Batch reactors were prepared in 250 mL Erlenmeyer flasks containing 50 mL of basal medium prepared as described in Gunsch et al. (2005) amended with 1 g/L (NH₄)₂SO₄. Each reactor was inoculated with 1 mL of activated sludge obtained from the aeration basin at the North Durham WWTP (Durham, North Carolina). All flasks were heat-sterilized by autoclaving at 121°C at 15 psi for 15 minutes. Stock solutions were prepared by dissolving each PhAC in pure ethanol. The final PhAC concentrations were 10 and 100 µM corresponding to 0.2 and 2% (v/v) ethanol concentrations, respectively.
Triplicates of each treatment condition were prepared. An additional set of bottles with PhACs and media but without any bacteria was used as an abiotic control. The purpose of this control was to verify that no hydrolysis or photolysis was occurring. In addition, either 0.2 or 2% (v/v) ethanol controls were prepared without PhAC and monitored throughout each experiment for comparison purposes. To ensure uniform oxygen and nutrient distribution, all batch reactors were incubated at 150 rpm on a shaker table at room temperature (approximately 20°C). Oxygen level was measured periodically and remained in the range of 5-6 mg/L throughout the experimental phase. Ketoprofen, gemfibrozil were obtained from Sigma Aldrich (St. Louis, Missouri). Clofibric acid, naproxen and carbamazepine were obtained from MP Biomedicals (Aurora, Ohio). All compounds were ACS grade.

3.2.2 Microbial Growth Measurements

Liquid samples were taken daily over a period of 8 days and monitored spectrophotometrically. All samples were collected under sterile conditions using a Labconco Purifier Class II biosafety cabinet (Kanasa, Missouri). Optical density was measured at 600 nm (OD600) using a Hach DR/4000 U spectrophotometer (Loveland, Colorado). The spectrophotometric results were converted to dry cell mass based on a standard curve (Figure 3.1). The standard curve was prepared by vacuum filtering 10 mL cell cultures with known OD600 values through dry Whatman filter membranes (0.2
µM). Membranes were placed in aluminum planchettes, dried at 105°C for 4 hours, cooled to room temperature in a desiccator and weighed on a four-place balance. All measurements were performed in triplicates.

\[ y = 1.2484x - 0.1555 \]
\[ R^2 = 0.9926 \]

Figure 3.1 Standard curve relating OD$_{600}$ and cell mass.

3.2.3 Specific Oxygen Uptake Rate Measurements

The specific oxygen uptake rate (SOUR) was calculated for each PhAC using standard methods (Eaton A.D. et al. 2005). Briefly, 10 mL of Day 0 sludge were added to a 300-mL bottles filled with basal medium and containing 10 µM PhAC. The DO levels were measured using a pre-inserted DO probe (YSI model 50B, Yellow Springs, Ohio) over a 20 minute period. VSS was measured using standard methods (Eaton A.D. et al.)
SOUR measurements were performed at a final PhAC concentration of 10 µM in 0.2% (v/v) ethanol. Triplicate tests were performed for each PhAC.

3.2.4 Nucleic Acid Analysis

Cell samples were harvested from each reactor on days 0, 2, 4, 6, and 8. One hundred µL biomass samples were obtained by centrifuging at 16,300 × g RCF for 1 minute at room temperature using a Spectrafuge 24D microcentrifuge (Eppendorf, Inc., Woodbridge, New Jersey). If DNA extraction was not performed immediately, the cell pellets were stored at -80°C in a Revco Ultima Ultra-Cold Freezer (Thermo Electron Corporation, Marietta, Ohio). DNA was extracted using Mo Bio Ultra Clean™ microbial DNA kits (Carlsbad, California). The extraction was performed following the manufacturer’s instructions without any modifications. The purified DNA was eluted in sterile water and stored at -20°C (GE® Upright Freezer, Louisville, Kentucky) for PCR amplification. DNA quality was assessed by measuring the A260/A280 ratio using a ND-1000 Spectrophotometer (Nanodrop®, Wilmington, Delaware). Only samples with ratios between 1.8 and 1.9 were used (Glasel 1995).
3.2.5 PCR-DGGE

The first PCR product was amplified using the bacterial forward primer 8F and the universal reverse primer 1492R (Weisburg et al. 1991). The final PCR amplicon was obtained using the I-341-fGC and I-533r primers (Watanabe et al. 2001). A summary of the primers sequences is shown in Table 3.1. All PCR amplifications were performed using a GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, CA). All reagents were obtained from the Eppendorf MasterTaq® kit (Hamburg, Germany) and used following the manufacturer’s instructions. The program for the first PCR amplification consisted of a 30-second denaturation step at 94°C, followed by a 30-second annealing step at 52°C and a 30-second extension step at 72°C (30 cycles). Touch-down PCR was used for the second PCR amplification. The first two cycles consisted of a 30-second denaturation step at 94°C, a 30-second annealing step at 52°C and a 30-second extension step at 72°C. The next 30 cycles consisted of a 30-second denaturation step at 94°C, a 30-second annealing step at 47°C and a 30-second extension step at 72°C.
Table 3.1 Primer sequences for DGGE analysis

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5'-3')&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>8F</td>
<td>AGAGTTTGATCCTGGCTCAG</td>
</tr>
<tr>
<td>1492R</td>
<td>GGTACCTTGTTACGACCTT</td>
</tr>
<tr>
<td>I-341-fGC</td>
<td>CGCCCGCCCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGCGACCGGGGGCCCTAC</td>
</tr>
<tr>
<td>I-533r</td>
<td>TIACCGIIICTICTGGCAC</td>
</tr>
</tbody>
</table>

<sup>a</sup> Numbers correspond to the nucleotide positions in *Escherichia coli*; F or f, forward primer; R or r, reverse primer. <sup>b</sup> I, inosine.

Denaturing Gradient Gel Electrophoresis (DGGE) analyses were performed on the variable V3 region of the bacterial 16S rDNA using nested PCR products according to Muyzer et al. (1993). A D-Code Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, California) was used. All PCR amplifications were loaded on an 8% (w/v) polyacrylamide gel with a denaturing gradient ranging from 20 to 55% urea-formamide (100% denaturing solution corresponded to 40% (v/v) deionized formamide and 7 M urea). The gel was run at 57°C under a voltage of 50 V for 18 h and then stained with 1X SYBR Gold (Molecular Probes, Eugene, Oregon). Sequencing was performed on excised bands using the I-533r primer. DNA sequence analysis was performed using the BLAST server of the National Centre for Biotechnology Information ([http://www.ncbi.nlm.nih.gov](http://www.ncbi.nlm.nih.gov)).
3.2.6 High-Pressure Liquid Chromatography (HPLC)

Prior to HPLC analysis, biomass was removed by filtering each sample using a VWR 0.2 µm porosity polypropylene filter (Westchester, Pennsylvania). A Prostar Liquid Chromatograph (Varian Inc., Palo Alto, California) equipped with a 250 mm C-18 column (Alltech Inc., Newark, Delaware) was used for the analysis. Ketoprofen, naproxen, clofibric acid and carbamazepine were resolved using a mobile phase composed of 29% acetonitrile, 19% methanol, and 52% formic acid by volume, adjusted to pH 3.4. A flow rate of 1.0 mL/min and an injection volume of 20 µL were used. Wavelengths were optimized after performing a scan (Cary Bio100 UV spectrophotometer, Varian Inc, Palo Alto, California). Absorbance wavelengths of 260, 230, 230 and 220 nm were used for ketoprofen, naproxen, clofibric acid and carbamazepine, respectively. The HPLC method was a modification of previously published protocols (Du et al. 2003; Zakeri-Milani et al. 2005). Gemfibrozil was separated using a mobile phase consisted of 0.4% phosphoric acid solution–acetonitrile (47:53, v/v) in a flow rate of 1.2 mL/min. The fluorescence detection was performed at excitation and emission wavelengths of 242 nm and 300 nm to detect gemfibrozil (Kim et al. 2006).
3.3 Results and Discussion

3.3.1 PhAC Concentrations and Microbial Growth

Microbial growth was lower in the presence of ketoprofen, naproxen, carbamazepine and gemfibrozil as compared to the ethanol only control at both the low (10 µM) and high (100 µM) PhAC concentrations in the presence of 0.2 % (v/v) ethanol (Figure 3.2 a-c, e). Overall cell concentration was significantly different as compared to the control from day 3 to 6 with a 90% confidence interval. Maximum growth inhibition was observed on day 3 and resulted in a 42, 43, 34 and 37% cell concentration decrease in the presence of ketoprofen, naproxen, carbamazepine and gemfibrozil respectively. No effect was observed in the presence of clofibric acid (Figure 3.2 d). As expected, no growth was detected in the abiotic controls.

Microbial growth inhibition was especially apparent during the first three days. Interestingly, in the presence of 0.2% (v/v) ethanol, microbial growth was independent of PhAC concentration in the 10 to 100 µM range. The growth curves for the 10 and 100 µM PhAC concentrations are virtually identical (Figure 3.2). In addition to the decreased microbial growth, a 39, 39, 19 and 33% decrease in SOUR was observed in the presence of 10 µM naproxen, ketoprofen, carbamazepine and gemfibrozil respectively (Figure 3.3). No statistically significant difference was observed with clofibric acid (Figure 3.3). Similar results were obtained in the presence of 100 µM PhAC.
concentrations (Figure 3.4). This finding is consistent with the lower microbial growth rate and further indicates that the presence of some PhACs may inhibit microbial growth as well as respiratory activity of some activated sludge microorganisms under certain growth conditions.
Figure 3.2 Bacterial growth in 0.2% (v/v) ethanol with 10 μM PhAC (◊), 100μM PhAC (△) and without PhAC (■). a) Ketoprofen, b) Naproxen, c) Carbamazepine, d) Gemfibrozil, e) Clofibric acid.
Figure 3.3 Specific oxygen uptake rate of active sludge in the presence of 10 \( \mu \text{M} \) PhAC. Star (*) indicates statistically significant difference from control samples with 95\% confidence interval.

Figure 3.4 Specific oxygen uptake rate of active sludge in the presence of 100 \( \mu \text{M} \) of PhAC. Star (*) indicates statistically significant difference from control samples with 95\% confidence interval.
Other studies have reported similar effects although most experiments presented in the literature were conducted either at significantly higher concentrations than those tested herein or with a different microbial culture and/or experimental platform. Kumagai et al. (2006) showed that 983 µM ketoprofen resulted in 50% oxygen uptake rate (OUR) inhibition. The OUR inhibition is approximately 22% higher than the rate reported in the present study even though the ketoprofen concentration used in that study was approximately 10 times higher. This variation may be due to differences in either activated sludge sources (e.g., community structure and/or metabolic activity deviations) or carbon substrates.

Kruszewska et al. (2002) showed that naproxen in the form of Nalgesin tablets significantly inhibited microbial growth of select individual microorganisms. This result is consistent with our findings which indicate a 36% SOUR decrease with microbes originating from an activated sludge mixed community. The final PhAC which exhibited significant microbial inhibition is carbamazepine. Our result is consistent with that reported by Lawrence et al. (2005). In that study, 10 µg/L carbamazepine reduced the amount of bacterial biomass produced on riverine biofilms. Dokianakis et al. (2004) reported that carbamazepine did not significantly inhibit nitrifiers. Thus, it is possible that carbamazepine may affect other microbial populations in the activated sludge process. Gemfibrozil was reported to impact the immobilization of *Daphnia magna* at the
mean concentration of 120 µM after 72 hours and other three aquatic toxicological models (Zurita et al. 2007). This result and our finding indicated gemfibrozil may impact the microorganisms in either wastewater treatment plant or aquatic environment.

No inhibition was observed in the presence of clofibric acid at either the 10 or 100 µM concentration. Dokianakis et al. (2004) reported a similar result for this PhAC with respect to nitrifiers. The only other published reports on clofibric acid concern the aquatic toxicology of this PhAC. There are reports which suggest that clofibric acid is toxic to several indicator organisms (Ferrari et al. 2003; Henschel et al. 1997). Thus, the absence of inhibition in our tests may indicate that microorganisms in activated sludge are less sensitive to clofibric acid than the indicator organisms used in the toxicity tests.

3.3.2 Organic Loadings and Microbial Growth

In the presence of 2% (v/v) ethanol (Figure 3.5), the microbial growth curves have distinctively different shapes as compared to the 0.2% (v/v) ethanol case (Figure 3.2). In the presence of the higher ethanol concentration, no significant effect was observed even at the highest PhAC concentration (100 µM) for all four PhACs. There have been reports of more rapidly growing microorganisms outgrowing slower microorganisms in high organic loading environments (Zheng et al. 2006). Rossello-Mora et al (1995) reported that high organic loading in sewage plants was linked to high level of Zoogloea ramigera, a Gram negative bacillus. Zoogloea spp. organisms are known to have the ability to block
toxic compounds using their exocellular matrix (slime layer). Thus, it is possible that the increased ethanol concentration resulted in additional microbial growth of some strains which concealed the inhibition of other strains.
Figure 3.4 Bacterial growth in 2% (v/v) ethanol with 100 μM PhAC (◊), 100μM PhAC (△) and without PhAC (■). a) Ketoprofen, b) Naproxen, c) Carbamazepine, d) Gemfibrozil.

The data suggest that PhAC inhibition can vary depending on the concentration of biodegradable carbon present in a given sample. This result has important implications as it suggests that PhACs might not inhibit the overall microbial growth in treatment processes with organic loadings greater or than some threshold value even at fairly high PhAC concentrations. Care should be taken however in extrapolating this
result to a full scale WWTP since the operational setup is very different than the batch reactor model.

3.3.3 Microbial Community Shifts

Because a decrease in overall microbial growth was only observed under 0.2% (v/v) ethanol (Figure 3.2), DGGE experiments were carried out only under that treatment condition. Since DGGE is a largely qualitative method, analysis was only done on bands which either clearly appeared, disappeared or changed in intensity relative to the control treatment (i.e., ethanol only). Using these parameters, the DGGE analysis suggests that shifts in microbial community structure may have occurred in the presence of ketoprofen and naproxen as compared to the ethanol only control (Figure 3.6a). No significant microbial community changes were observed for either carbamazepine, clofibric acid or gemfibrozil (Figure 3.6b, c). The clofibric acid result was expected since no significant difference was observed in either microbial growth or SOUR experiments. Microbial shifts were expected in the presence of carbamazepine and gemfibrozil, and thus this result is surprising. However, because of the inherent PCR biases which comes with DGGE analysis (Ishii and Fukui 2001) as well as its aforementioned qualitative nature, it is possible that the microbial communities affected by carbamazepine and gemfibrozil simply are not detected. It is possible that other methods such as stable isotope probing (SIP) could overcome PCR biases (Radajewski et al. 2000).
No bands were detected which clearly either appeared or disappeared when comparing treatments with and without PhACs at a specific time point. However, a single band was identified which showed increasing intensity in the ketoprofen and naproxen grown reactors. They were designated as Band A and B, respectively (Figure 3.6a). Because the only difference between these treatment and control reactors is the presence of ketoprofen and naproxen, the PhAC presence was attributed to having caused that effect. Furthermore, since other bands remain at similar intensities when comparing treatments and controls, an increase in a specific band’s intensity could possibly be linked to an increase in that species population and thus enrichment.

Sequencing results showed that band A was 97% homologous to *Acinetobacter* sp. (EF103571) and band B had 98% homology to *Acinetobacter* sp. (EF103567). Because of the previously mentioned PCR bias inherent to DGGE analysis, further experiments need to be carried out to confirm that these species were enriched as suggested by these results.

The possible enrichment of *Acinetobacter* spp. is consistent with other published studies. *Acinetobacter* spp. are able to survive in water with high levels of PhACs such as hospital and pharmaceutical plant effluents. Their survival is thought to be linked to their increased levels of antibiotic resistance as compared to other bacterial species (Guardabassi et al. 1998). In addition, some strains of *Acinetobacter* have been reported in wastewater treatment (Ghigliazza et al. 1998). Thus, it is quite probable that *Acinetobacter* spp. were present in the inoculum which was obtained from a WWTP.
Figure 3.5 DGGE results a) from left to right: ketoprofen-day 0, 2, 4, 6, 8, naproxen-day 0, 2, 4, 6, 8, ethanol control-day 0, 2, 4, 6, 8; b) carbamazepine -day 0, 2, 4, 6, 8, clofibric acid-day 0, 2, 4, 6, 8, ethanol control-day 0, 2, 4, 6, 8; c) ethanol control-day 0, 2, 4, 6, 8, gemfibrozil-day 0, 2, 4, 6, 8.
3.3.4 PhAC Removal

No significant PhAC degradation was observed throughout the experiment in either experimental or control reactors (Figure 3.6). This result indicates that the parent PhAC rather than its metabolites were responsible for microbial growth inhibition. It is likely that the PhACs are toxic to certain microbial strains and/or block key microbial activities. These mechanisms have been shown to occur with other PhACs (Quintana J.B 2005; Tixier et al. 2003). The exact mechanisms are not known for the four PhACs investigated in this research.
Figure 3.6 Concentrations of 10 μM PhAC in 0.2% (v/v) ethanol in the presence of bacteria (Δ) and without bacteria (■). (a) Ketoprofen. (b) Naproxen. (c) Carbamazepine. (d) Gemfibrozil. (e) Clofibrac acid.
3.4 Conclusions

Batch reactors inoculated with activated sludge were incubated for 7 days in the presence of ethanol (primary carbon source) and one PhAC at either 10 or 100 µM. Significant microbial inhibition was observed by spectrometry at 10 µM and 100 µM for ketoprofen, naproxen and carbamazepine in the presence of 0.2% (v/v) ethanol. Meanwhile, specific oxygen uptake rate revealed the inhibition of these three PhACs on microbial activity. Molecular fingerprinting depicted *Acinetobacter* spp. were in enriched in 100 µM ketoprofen and naproxen. No degradation of any of the four PhACs was observed. These results show that though some strains of microorganism might be impacted by certain PhACs and that these PhACs may inhibit their growth.
Chapter 4  Effects of PhACs on Ammonia Oxidizing Bacteria (AOB)

4.1 Introduction

While there has been recent interest in the development of technologies to remove PhACs from wastewater (Kimura et al. 2005; Rooklidge et al. 2005), there have been very few studies focusing on determining the impact of PhACs on microbial activity in WWTPs. It is especially important to determine if PhACs have an adverse impact on municipal WWTP performance since WWTPs rely heavily on the microbial component of the activated sludge process.

As discussed in Chapter 2, ammonia-oxidizing bacteria (AOB) are a key group of bacteria for the removal of ammonia. They oxidize ammonia to nitrite in a two-step process: the first step consists of the oxidization of ammonia to hydroxylamine (NH$_2$OH) by the membrane-bound enzyme ammonia monooxygenase (AMO); the subsequent oxidation of hydroxylamine to nitrite is catalyzed by the periplasmic enzyme, hydroxylamine oxidoreductase (HAO) (Hooper et al. 1997). AOBs are believed to be particularly susceptible to inhibition by certain chemical compounds at low concentrations (Hooper et al. 1997; Wood et al. 1981), and for this reason are a well accepted target microorganism used for the study of microbial inhibition caused by chemicals in wastewater. It is generally accepted that inhibition of ammonia oxidation
by AOB results in a total failure of nitrogen removal (Jonsson et al. 2001; Moussa et al. 2006), one of the key wastewater treatment goals. Due to their sensitivity to chemicals and importance in the environment, AOB are hence widely used as the bioassay model strains or indicators in toxicity tests (Blum and Speece 1991; Ren and Frymier 2003).

Very few studies have focused on studying the impact of PhACs on AOBs even though, as discussed above, AOBs are arguably the most sensitive microorganisms in the municipal wastewater treatment process and PhACs are widely distributed in wastewater. In the present study, the impacts of PhACs on AOBs were investigated using Nitrosomonas europaea as the model strain. Several possible inhibition mechanisms are proposed and discussed. Microbial inhibition was monitored by measuring the nitrite production of N. europaea in the presence of PhACs. To further understand the mechanism of their impact, reversibility experiments and membrane integrity tests were also performed.
4.2 Material and Methods

4.2.1 Cell Growth and Preparation

*N. europaea* (ATCC 19718) was cultured in a minimal growth media (ATCC medium 2265) [25 mM (NH₄)₂SO₄, 43 mM KH₂PO₄, 3.92 mM NaH₂PO₄, 3.77 mM Na₂CO₃, 750 µM MgSO₄, 270 µM CaCl₂, 18 µM FeSO₄, 17 µM EDTA Free Acid, and 1 µM CuSO₄] in 250 mL Erlenmeyer flasks. The media was maintained in suspension by constant stirring at 600 rpm using a magnetic stir bar. The cultures were maintained in the dark at room temperature (~22°C). A portion of media was periodically transferred to R2A agar plates to check for contamination. After one week, 500 mL of AOB cells in the late-exponential growth (OD₆₀₀ approximately reached 0.06) were harvested by centrifugation at 9000 rpm for 30 minutes using an Eppendorf Centrifuge 5804 (Westbury, New York). Cells were then washed with 40 mM KH₂PO₄ buffer (pH 7.8) and spun down again at 9000 rpm for 30 min. The cell pellet was resuspended in 10 mL of 40 mM KH₂PO₄ buffer for subsequent experiments. The cellular protein content was determined using a Micro BCA™ Protein Assay Kit (Rockford, Illinois) after the cells were digested in 3 N NaOH at 65°C for 30 minutes.

4.2.2 Nitrite production

To determine the effect of PhACs on AOB nitrification, nitrite production inhibition tests were performed. The nitrite production experiments were carried out in
batch reactors consisting of 250 mL Erlenmeyer flasks each containing 200 mL of minimal growth media (ATCC 2265 – described in the previous section) amended with 2.5 mM (NH₄)₂SO₄. All flasks were heat-sterilized by autoclaving at 121°C at 15 psi for 15 minutes prior to the beginning of each experiment. PhACs were added as stock solutions of PhAC dissolved in pure ethanol. The stock solution was added to the empty flasks, the ethanol was then blown off using a gentle stream of nitrogen gas. Finally, the minimal growth media was added to dissolve the PhAC and stirred using magnetic stir bars. The recovery rates using this method were found to be over 95% for the four PhACs in the concentration of 0.1, 1 and 10 µM (Table 4.1). Triplicates of each treatment condition were prepared. Batch reactors without PhACs were used and monitored in parallel as negative controls. Prior to the beginning of each experiment, the medium was inoculated with 1 mL of cells obtained as described above and the reactors were continuously stirred at 700 rpm in the dark at room temperature (21-22°C) for 4 hours. Nitrite was measured every 60 min throughout the experimental period using NitriVer® 3 Reagents (Hach, Loveland, Colorado). A 4 hour exposure time was chosen for these experiments because the doubling time for *N. europaea* ranges from 8 to 12 hours and is longer than that used in most studies (Cui et al. 2005; Iizumi et al. 1998; Radniecki et al. 2008). Nitrite production inhibition rate was calculated using Equation (4-1).
% Nitrite production inhibition = \left(1 - \frac{NO_2^-_{\text{sample}}}{NO_2^-_{\text{control}}}\right) \times 100\% \quad \text{Equation (4-1)}

Table 4.1 Recovery rate (%) of PhACs in aqueous phase from stock solutions

<table>
<thead>
<tr>
<th></th>
<th>ketoprofen</th>
<th>naproxen</th>
<th>carbamazepine</th>
<th>gemfibrozil</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 μM</td>
<td>96.1</td>
<td>94.3</td>
<td>97.1</td>
<td>96.1</td>
</tr>
<tr>
<td>1 μM</td>
<td>90.2</td>
<td>99.0</td>
<td>94.3</td>
<td>95.0</td>
</tr>
<tr>
<td>10 μM</td>
<td>91.8</td>
<td>98.8</td>
<td>99.4</td>
<td>98.9</td>
</tr>
</tbody>
</table>

4.2.3 Reversibility Experiments

Reversibility experiments were conducted to see if *N. europae* regained their nitrification potential following the removal of PhAC. To this end, nitrite production was monitored in *N. europae* cells following a 2 hour exposure to PhACs. These experiments were performed by placing *N. europae* cells into 250 mL Erlenmeyer flasks containing 50 mL minimal growth media with 2.5 mM (NH₄)₂SO₄ and PhACs. Batch reactors without PhACs served as the negative controls. Cells were continuously stirred at 700 rpm in the dark at room temperature (21-22°C) for 2 hours. Following the initial 2 hour incubation, nitrite production was measured using NitriVer® 3 Reagents (Hach, Loveland, Colorado). Cells were then centrifuged and washed 4 times in 40 mM KH₂PO₄ buffer (pH 7.8). The washed cells were then placed into fresh medium without any PhACs. The NO₂⁻ production was re-measured after a 2 hour incubation period to
determine if the inhibition was reversible. Protein concentrations of cells were measured before and after wash procedure.

### 4.2.4 Membrane Integrity Testing

Membrane integrity was compared in the presence and absence of PhACs to determine the effect of PhACs on the cell viability of *N. europaea* cells using a LIVE/DEAD Baclight™ kit (Molecular Probes, Eugene, Oregon). A range of PhAC concentrations were tested including 0.1, 1 and 10 µM. Viable and dead cells were detected by differential staining with a mixture of a green fluorochrome, SYTO 9 (which stains all cells, live or dead), and a red fluorochrome, propidium iodide (which stains only bacteria with damaged membranes). After a 4 hour PhAC exposure time, cells were harvested via centrifugation at 9000 rpm for 30 minutes using an Eppendorf 5804 (Westbury, New York). The cell pellet was resuspended in 50mL of 0.8% NaCl solution to reduce background fluorescence and inhibitor. Cells were spun again as described above and finally resuspended in 0.5 mL 0.8% NaCl solution. The cell suspension (0.2 mL) was mixed with the Live/Dead® Baclight™ reagents following the manufacturer’s instructions. After a 10 min incubation time, 5 µL of cells were placed on glass slides and observed using a Nikon Eclipse E600 microscope (Melville, New York) equipped with a Y-FL EPI-fluorescence attachment and a Nikon HB-10104AF 100 W Hg arc lamp (Marietta, Georgia). The fluorescence intensities of the stained microbial cells at 535 nm
(green) and 642 nm (red) represent the relative distribution of live and dead cells, respectively. A filter unit of EX465-495, DM505, BA515-555 nm and EX540/25, DM565, BA605/55 nm was used to detect green and red signal, respectively. Ten spots were randomly chosen from each slide and documented by SPOT RT Slider microscope digital camera (Diagnostic Instruments, Inc, Sterling Heights, Michigan). Two images under either 535 nm (green) or 642 nm (red) filters were taken for each spot and each treatment was run in triplicate. Thus for each experimental condition, a total of 60 images were analyzed. The ratios of live/total cells for each spot were calculated and compared using Image J software (version 1.41, National Institute of Health, Bethesda, Maryland). Membrane integrity inhibition rate was calculated using Equation (4-2).

\[
\text{% Membrane integrity inhibition} = (1 - \frac{\text{Live/total}_{\text{sample}}}{\text{Live/total}_{\text{control}}}) \times 100\% \quad \text{Equation (4-2)}
\]

4.2.5 Analytical Methods for PhAC Detection

PhAC analysis for ketoprofen, naproxen, carbamazepine and gemfibrozil was performed using high pressure liquid chromatography as described in Chapter 3.

4.2.6 Statistical Analysis

Standard deviations were calculated and are shown in the figures. Standard error was applied to the membrane integrity results. For all experiments, the student t-
test was used to assess the statistical significance of the results with a 95% confidence interval.

4.3 Results and Discussion

4.3.1 Inhibitory effects of PhACs on nitrification

The production rate of nitrite by *N. europaea* was significantly inhibited in the presence of 1 and 10 µM ketoprofen, naproxen, carbamazepine and gemfibrozil (Figure 4.1 b and c, Figure 4.2 b and c, respectively), but not in the presence of 0.1 µM for any of the PhACs. Nitrite concentration was significantly different (95% confidence interval) in the presence of the PhACs as compared to the control treatment after a 2 hour exposure and remained significantly different through the remainder of the experimental period. The inhibition rates for each treatment condition were calculated at the endpoint (i.e., 4 hours) and are shown in Table 4.2. Naproxen showed the highest inhibition of nitrification (28.9%) whereas carbamazepine had the lowest inhibition (18.1%). The inhibition rate generally increased with an increase of PhAC concentrations except for gemfibrozil.
Figure 4.1 Nitrite production comparison between *N. europaea* control cells and cells exposed to (a) 0.1 μM, (b) 1 μM and (c) 10 μM of ketoprofen and naproxen. Error bars represent ± 1 standard deviation.
Figure 4.2 Nitrite production comparison between *N.europaea* control cells and cells exposed to (a) 0.1 μM, (b) 1 μM and (c) 10 μM of carbamazepine and gemfibrozil. Error bars represent ± 1 standard deviation.
Table 4.2 Inhibition rate (%) of nitrite production as compared to controls at endpoint

<table>
<thead>
<tr>
<th></th>
<th>Ketoprofen</th>
<th>Naproxen</th>
<th>Carbamazepine</th>
<th>Gemfibrozil</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 µM</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1 µM</td>
<td>20.1±5.6</td>
<td>21.3±6.2</td>
<td>18.1±2.8</td>
<td>26.1±0.9</td>
</tr>
<tr>
<td>10 µM</td>
<td>25.2±2.7</td>
<td>28.9±3.8</td>
<td>21.9±9.7</td>
<td>22.1±7.1</td>
</tr>
</tbody>
</table>

These results suggest that some PhACs inhibit AOB activity. These findings are consistent with the limited number of previously published studies investigating the effect of other PhACs on nitrification. Carucci et al. (2006) reported that ranitidine in concentrations ranging from 31.8 to 127.2 µM and lincomycin (1.2 to 26.1 µM) inhibited ammonia consumption of activated sludge from both WWTP and lab-scale sequencing batch reactor. Furthermore, some antibacterial agents have been reported to inhibit the growth and nitrification of *N. europaea* (Halling-Sorensen 2001).

The data also suggest that the inhibition on *N. europaea* is directly correlated to the concentration of PhACs (Table 4.2). As mentioned above, no effect was observed at the lowest concentration (0.1 µM) whereas significant inhibition was observed at the higher concentrations with the maximum inhibition observed at the highest concentration tested in this study. Additional experiments should be carried out to determine if a mathematical correlation exists between inhibition level and PhAC concentration. Furthermore, additional experiments should be carried out with other
AOB strains as it is possible that other AOB strains would behave differently when exposed to the same PhACs.

There are some possible mechanisms which may explain the inhibition observed in this study. First, PhACs may directly compete with ammonia on the available binding site of AMO, leading to a decreased rate of nitrite production. Alternative substrates for AMO have been demonstrated to have effects on the ammonia oxidizing activity of *N. europaea*. Chang et al. (2002) reported that naphthalene had a strong inhibitory effect on ammonia oxidation in *N. europaea*. This study also showed that the oxidation between ammonia and naphthalene had a partially competitive inhibition. However, the competitive inhibition model described in that study does not explain such effect especially when the concentrations of organic compounds are high. Inhibition could also be linked to substrate concentration. For instance, phenol has been shown to act as a competitive inhibitor of AMO at low concentrations (Keener and Arp 1994). The inhibition of *N. europaea* in the presence of 10 µM phenol was shown to be completely reversible (Radniecki et al. 2008). However, at 50 µM, phenol behaves not only as an AMO inhibitor but also as a toxic compound which irreversibly inhibits certain cellular metabolic pathways (Iizumi et al. 1998). Such phenomenon was explained by the membrane toxicity of some organic chemicals such as phenol which can denature proteins and membranes at high concentrations (Sikkema et al. 1995). The impact of organic compounds on cell membranes is believed to be associated with the
octanol-water partition coefficient ($K_{ow}$) or distribution coefficient ($D_{ow}$) (Sikkema et al. 1995). The reported log $D_{ow}$ for phenol at pH 7 is 1.48 (Onuska and Terry 1995) whereas log $D_{ow}$ of PhACs in the present studies range from 0.85 to 2.67 (Chapter 2). Although no quantitative structure-activity relationships (QSARS) about $K_{ow}$ for N. europaea is available in the literature, the similarity of the distribution properties between phenol and selected PhACs indicates that PhACs may act in a similar fashion.

### 4.3.2 PhAC removal

Throughout the experimental period, no significant PhAC degradation was observed (Figure 4.3). AMO in N. europaea has been shown to rapidly (i.e., within 1 hour) cometabolize various hydrocarbon compounds including alkanes, alkenes, and alkynes within 1 hour (Hyman et al. 1988) as well as estrogens (Shi et al. 2004), however no significant degradation was observed in this study. This result suggests that AMO is unable to cometabolize the PhACs in this study. The PhAC removal data suggest that PhACs are likely to either be toxic to cells or act as non-competitive inhibitors rather than competitive inhibitors since these PhACs most likely do not bind to the AMO binding site as they are not cometabolized by AMO. This result also indicates that the parent PhAC rather than its metabolites are most likely responsible for nitrite inhibition.
4.3.3 Reversibility of PhAC inhibition

Reversibility experiments were performed to investigate if the inhibition of PhAC on *N. europaea* is reversible. The reversibility of PhAC inhibition on nitrite production was evaluated by comparing the nitrite production rate with PhACs and the rate after washing off PhACs. As shown in Figure 4.4, the data suggest that all of the

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Figure 4.3 PhAC concentration profiles in the presence of *Nitrosomonas europaea*
PhACs led to a significant irreversible inhibitory effect on nitrite production at a 95% confidence level. After extensive washing, the inhibition rates of nitrite production in *N. europae* cells having been exposed to PhACs were 25.5, 19.8, 35.6 and 19.3% for ketoprofen, naproxen, carbamazepine and gemfibrozil, respectively, as compared to the control cells. This result suggests that the inhibitory effect of selected PhACs was irreversible.
Figure 4.4 Nitrite production before and after washing in the presence of ketoprofen and naproxen (a) and carbamazepine and gemfibrozil (b).
4.3.4 Membrane Integrity

Lipids are important membrane constituents and their integrity provides a general indication as to whether or not a cell has been lysed. The LIVE/DEAD BacLight Bacterial Viability kit can be used to provide a measure of the relative amount of cells with intact and damaged cytoplasmic membranes using staining. As mentioned in the methods section, the fluorescence intensities of the stained microbial cells at 535 nm (green) and 642 nm (red) represent the relative distribution of live and dead cells, respectively.

In the presence of 0.1 µM PhACs (Figure 4.5a and 4.6a), intact/total cell ratios obtained from the cells treated with PhACs did not show significant difference when compared to controls (P>0.05). However, when the concentration of PhACs increased to 1 and 10 µM (Figure 4.5 b and c, Figure 4.6d and e, respectively), significant leakage of cell membranes was observed for all PhACs suggesting membrane damage. The rate of membrane damage ranged from 5.4% for carbamazepine to 16.3% for ketoprofen when compared to the control samples (Table 4.3). The results also indicate that the membrane damage caused by carbamazepine and gemfibrozil was dose-dependent. The live/dead ratios for carbamazepine and gemfibrozil at 1 µM are 5.4 and 6.5% lower than the control, respectively. This ratio is 13.0 and 9.5% higher, respectively, when the concentration is increased to 10 µM. The concentration of ketoprofen and naproxen did
not have an effect on membrane integrity. At 1 µM ketoprofen, the ratio of intact cells was 16.3% lower than the control cells whereas a concentration increase to 10 µM only resulted in 8.8% inhibition.

**Table 4.3 Damage rate (%) of live/dead ratio related to controls at endpoint**

<table>
<thead>
<tr>
<th></th>
<th>Ketoprofen</th>
<th>Naproxen</th>
<th>Carbamazepine</th>
<th>Gemfibrozil</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1 µM</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>1 µM</td>
<td>16.3 ± 5.0</td>
<td>13.2 ± 4.0</td>
<td>5.4 ± 1.9</td>
<td>6.6 ± 1.7</td>
</tr>
<tr>
<td>10 µM</td>
<td>8.8 ± 2.6</td>
<td>11.5 ± 3.4</td>
<td>13.0 ± 3.7</td>
<td>9.5 ± 4.1</td>
</tr>
</tbody>
</table>

Other studies have reported similar effects although most experiments presented in the literature were conducted in red blood cells and in mammalian hepatocytes. Ketoprofen has been reported to be associated with the peroxidation of membrane lipids, leading to cell lysis (Bosca et al. 1995). In the presence of 10 µM ketroprofen, a significant amount of lipid-derived hydroperoxides were detected which is an indicator of lipid peroxidation. In the same study, approximately a 20 and 10% decrease in intracellular lactate dehydrogenase activity was observed for hepatocytes and fibroblasts, respectively, when these cells were exposed to 100 µM ketroprofen. This result further confirms cell leakage, which are in the same range of our inhibition rates albeit in different cells. Bosca et al. (1995) suggest that the cytotoxic effect is related to the generation of radical intermediates derived from ketoprofen. Naproxen, which also belongs to the class of nonsteroidal anti-inflammatory drugs, has been reported to have
comparable properties to ketoprofen on the basis of a structural criterion (De Guidi et al. 2005). Thus, it is likely that naproxen will cause the same effects as ketoprofen.

Condorelli et al. (1996) showed that naproxen-derived free radicals also caused the lipid peroxidation and cell lysis of red blood cells.
Figure 4.5 Live/dead ratio of *N.europaea* control cells and cells exposed to (a) 0.1 μM (b) 1 μM and (c) 10 μM ketoprofen and naproxen. Error bars represent +/- standard error. Star (*) indicates statistically significant difference from control samples with 95% confidence interval.
Figure 4.6 Live/dead ratio of *N. europaea* control cells and cells exposed to (a) 0.1μM (b)1μM and (c) 10μM carbamazepine and gemfibrozil. Error bars represent+/-standard error. Star (*) indicates statistically significant difference from control samples with 95% confidence interval.
A study using a membrane-impermeant fluorescent nucleic acid dye to elucidate the cellular toxicity of carbamazepine found that 62.5 µM carbamazepine increased the death of human lymphocytes by 11% (Tabatabaei et al. 1997). Suwalsky et al. (2006) also confirmed that carbamazepine interacts with red blood cell membranes. In that study, 50 µM carbamazepine was shown to disturb a class of lipids found in red blood cell membranes and disrupts the polar head groups and acyl chains of the membrane lipid bilayer. These membrane integrity results are consistent with our observations although in different cells.

Only one study has reported the effects of gemfibrozil on cell membrane integrity. Zurita et al. (2007) studied the effects of gemfibrozil on fish cells (PLHC-1) looking for morphological and basal cytotoxicity (Zurita et al. 2007). In that study, gemfibrozil (860 µM) resulted in 50% leakage of glucose-6-phosphate dehydrogenase, which served as a marker of cell death while 100 µM gemfibrozil caused cellular swelling and cell loss.

4.3.5 Nitrite production adjusted to the viable fraction of *N. europaea* cells

The nitrite production rate for the viable fraction of the cells was calculated and compared to the control cells. Assuming that all cells have the same protein content, a modified nitrite production rate was calculated at the endpoint adjusted to the relative amount of viable cells measured using the membrane integrity test. As shown in Figure
4.7, the viable cell nitrite productions for cells exposed to ketoprofen and naproxen are significantly lower than the rate for the control cells. The nitrite production rates for cells incubated in the presence of 10 µM ketoprofen and naproxen were 1.64 and 1.62 mg NO₂/mg protein, respectively, as compared to 2.01 mg NO₂/mg protein for the control (Figure 4.8). Similar results were also observed for carbamazepine and gemfibrozil. The nitrite production rates for these two PhACs were 17.3% and 13.8% lower than the control. This result indicates that, after excluding the membrane integrity factor, the surviving cells were still inhibited by PhACs. These results suggest that loss of membrane integrity is a possible explanation for the nitrification inhibition. Furthermore, it is also possible that PhACs impact the cells at the level of the membrane-embedded electron transfer enzymes (e.g., AMO). Toxic effects on membrane-bound enzymes caused by the accumulation of lipophilic compounds, such as the PhACs used in this study, in lipid membranes have previously been demonstrated (Sikkema et al. 1995). Diphenyliodonium was shown to irreversibly inhibit AMO in *N. europaea* in addition to modifying its membrane integrity (Shiemke et al. 2004). Thus, it is possible that PhACs impact the surviving cells in a similar manner.
Figure 4.7 Nitrite production rate of live *N.europaea* cells at the endpoint (a) 1 μM (b) 10 μM ketoprofen and naproxen.

Figure 4.8 Nitrite production rate of live *N.europaea* cells at the endpoint (a) 1 μM (b) 10 μM carbamazepine and gemfibrozil.
4.4 Conclusions

This study demonstrated that ketoprofen, naproxen, carbamazepine and gemfibrozil inhibited the nitrite production of *N. europaea*. The maximum inhibition rates were 25.2, 28.9, 21.9 and 26.1% respectively. The degree of inhibition appears to be correlated to the concentration of PhACs. Inhibition increased with increasing PhAC concentration (1 and 10 µM) while no inhibition was observed at the lowest PhAC concentration tested (0.1µM). The membrane integrity data suggest that the inhibition may be due to a disturbance of the cell membrane. For ketoprofen, naproxen, carbamazepine and gemfibrozil, a 16.3, 13.2, 12.9 and 9.5 % decrease of the live/dead ratio was observed respectively.

The reversibility experiments showed that after PhACs were removed, the difference in nitrite production between the PhAC treatments and controls were still significant suggesting that inhibition is irreversible. The inhibition rates were 25.5, 19.8, 35.6 and 19.3% for ketoprofen, naproxen, carbamazepine and gemfibrozil, respectively. The calculation of live cell inhibition rate suggested that PhACs not only resulted in the death of cells but also inhibited their activity.
Chapter 5  Effects of PhACs on Treatment Performance in Sequencing Batch Reactors Mimicking WWTP Operations

5.1 Introduction

WWTPs generally provide the first treatment opportunity for removing pollutants and preventing significant environmental exposure. Because most municipal WWTPs rely on the microbial component of the activated sludge process, it is important to study the impact of anthropogenic compounds in wastewater influent on the treatment performance of WWTPs. The importance of microbial activity and community composition has been reported in studies investigating the impact of mercury as well as other anthropogenic contaminants (e.g., nanomaterial and antibiotics) on bioreactor performance (Carucci et al. 2006; Nyberg et al. 2008; von Canstein et al. 2002). However, very few studies have focused on studying the ecological impacts of PhACs on WWTP microorganisms as well as to determine their effects on treatment performance. Thus, there is a need to determine if the presence of PhACs in wastewater has the potential of negatively impacting activated sludge microbial communities. The present study served to fill this research gap.

One of the primary functions of WWTPs is the removal of nitrogen which is mainly present in the form of ammonia in wastewater influent. As previously discussed in Chapters 2 and 4, ammonia-oxidizing bacteria (AOB) are a key group of bacteria for
the transformation and removal of ammonia. Due to their sensitivity to chemicals and importance in the wastewater treatment process, AOB have been widely used as a bioassay model or indicator in toxicity tests (Blum and Speece 1991; Ren and Frymier 2003).

In the present study, the impacts of four commonly used PhACs were investigated on the composition of activated sludge communities. The selected PhACs consisted of ketoprofen, naproxen, carbamazepine, and gemfibrozil. These compounds were selected because they have been detected at fairly high concentrations in the aquatic environment and in WWTPs (Ternes 1998). Following up on the findings in batch reactor (Chapter 3) and in pure culture with *Nitrosomonas europaea* (Chapter 4), bench scale experiments were carried out in reactors mimicking WWTP operations to determine their impact in such a setting. The sequencing batch reactor (SBR) configuration was selected for these experiments because they are widely used in many small scale WWTPs and have been demonstrated to have similar performance as continuous stirred-tank reactors (CSTRs) (Grady et al. 1999). The impacts were measured in terms of SBR performance (total chemical oxygen demand (COD) and ammonia removal) and microbial community structure shifts for both total bacteria and AOB. The removal of individual PhACs was also examined.
5.2 Materials and Methods

5.2.1 Bioreactors description and sampling

Three 2-L laboratory-scale reactors (Figure 5.1) with final liquid volumes of 1.5 L were constructed for the bioreactor study. The reactors were operated with an 8 hour cycle consisting of a 30 minute feeding, a 5 hour aerobic phase, a 2 hour settling phase and a 30 minute drawing phase (Figure 5.1). A 12 hour hydraulic residence time (HRT) was selected to provide enough reaction time for PhACs. This HRT was selected because it has been reported to be an efficient amount of time to remove pollutants in industrial wastewater (Franta and Wilderer 1997; Ganesh et al. 2006). Solid retention time (SRT) was maintained at 10 days to maximize the growth of slow-growing nitrifiers (Mace and Mata-Alvarez 2002). The bioreactor received synthetic wastewater as influent (Table 5.1). Air, feed and effluent pumps (Cole Parmer, Masterflex L/S™, Vernon Hills, Illinois) were operated by time-controllers (Model TN311C, Grove, Illinois). Throughout the operation, Mixed Liquor Suspended Solids (MLSS) levels were maintained between 2,000 and 2,500 mg/L, dissolved oxygen was maintained between 5.8 and 6.5 mg/L and pH between 6.8 and 7.5.
During each experimental phase, three bioreactors were operated in parallel.

Each bioreactor was inoculated with 500 mL activated sludge obtained from the aeration basin of the North Durham WWTP (Durham, North Carolina). The control reactor was operated without PhAC while the other two reactors received PhAC in their influent.
During the first 30 days of operation, all three reactors were operated with synthetic wastewater without PhAC to ensure steady state conditions had been reached prior to PhAC addition. Steady state operation was verified by checking microbial community structure as well as monitoring COD and ammonia removal rates. Once steady state was achieved, two of the bioreactors were spiked with PhACs while the control reactor was operated without PhACs. All reactors were covered in aluminum foil to prevent photolysis. New bioreactors were prepared for each PhAC tested. The treatment conditions as well as the order in which each condition was tested are shown in Table 2. Each experimental condition was tested for 30 days.

Table 5.2 Treatment conditions of SBRs

<table>
<thead>
<tr>
<th>Run</th>
<th>SBR 1</th>
<th>SBR 2</th>
<th>SBR 3 (Control Reactor)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Startup (No PhAC)</td>
<td>Startup (No PhAC)</td>
<td>Startup (No PhAC)</td>
</tr>
<tr>
<td>1</td>
<td>1 µM Ketoprofen</td>
<td>1 µM Naproxen</td>
<td>No PhAC</td>
</tr>
<tr>
<td>2</td>
<td>10 µM Ketoprofen</td>
<td>10 µM Naproxen</td>
<td>No PhAC</td>
</tr>
<tr>
<td>3</td>
<td>100 µM Ketoprofen</td>
<td>100 µM Naproxen</td>
<td>No PhAC</td>
</tr>
<tr>
<td>4</td>
<td>Startup (No PhAC)</td>
<td>Startup (No PhAC)</td>
<td>Startup (No PhAC)</td>
</tr>
<tr>
<td>5</td>
<td>0.1 µM Carbamazepine</td>
<td>0.1 µM Gemfibrozil</td>
<td>No PhAC</td>
</tr>
<tr>
<td>6</td>
<td>1 µM Carbamazepine</td>
<td>1 µM Gemfibrozil</td>
<td>No PhAC</td>
</tr>
<tr>
<td>7</td>
<td>10 µM Carbamazepine</td>
<td>10 µM Gemfibrozil</td>
<td>No PhAC</td>
</tr>
<tr>
<td>8</td>
<td>Startup (No PhAC)</td>
<td>Startup (No PhAC)</td>
<td>Startup (No PhAC)</td>
</tr>
<tr>
<td>9</td>
<td>0.1 µM Ketoprofen</td>
<td>0.1 µM Naproxen</td>
<td>No PhAC</td>
</tr>
</tbody>
</table>
5.2.2 Analytical Methods

Reactor performance was measured by monitoring total COD, NH$_4^+$-N, NO$_2^-$-N and NO$_3^-$-N using Hach reagents (catalog numbers 21258-25, 26069-45, 21071-69, and 2106169, respectively). PhAC concentrations were monitored by high pressure liquid chromatography (HPLC). Performance was measured every two days using 1 mL samples collected from each reactor. Prior to analysis, biomass was removed by filtering each sample using a VWR 0.2 µm porosity polypropylene filter (Westchester, Pennsylvania).

A Prostar Liquid Chromatograph (Varian Inc., Palo Alto, California) equipped with a 250 mm C-18 column (Alltech Inc., Newark, Delaware) was used for the HPLC analysis. Naproxen, ketoprofen, and carbamazepine were resolved using a mobile phase composed of 29% acetonitrile, 19% methanol, and 52% water by volume, adjusted to pH 3.4 using formic acid using a method adapted from Du et al.(2003) and Zakeri-Milani et al. (2005) A flow rate of 1.0 mL/minute and an injection volume of 20 µL were used. Wavelengths were optimized after performing a scan using a Cary Bio100 UV spectrophotometer (Varian Inc., Palo Alto, California). Absorbance wavelengths of 260, 230 and 220 nm were used for ketoprofen, naproxen and carbamazepine, respectively. Using this protocol, the recovery rate for ketoprofen, naproxen, and carbamazepine was 81%, 85% and 99%, respectively while the limit of quantification (LOQ) was 0.5, 0.1 and 1 µM, respectively. Gemfibrozil was separated using a mobile phase consisting of
methanol and water (80:20, v/v) at a flow rate of 1.1 mL/minute with the UV detection set at 280 nm (Ulu 2006). The recovery rate was found to be 95% and the LOQ was 1 µM.

To detect PhAC concentrations lower than the LOQ, solid phase extraction (SPE) method was applied to enrich PhACs adapting a method developed by Pedrouzo et al. (2007). Briefly, 60 mg Oasis HLB extraction cartridges (Waters, Milford, Massachusetts) were conditioned with 3 mL methanol and 3 mL water. After the cartridges were vacuum dried for 10 minutes, either 10 mL (ketoprofen and carbamazepine) or 25 mL (gemfibrizil) of water was passed through the cartridge. Next, 1 mL of 5% methanol solution was used to wash the cartridge. An elution step was then carried out following 15 minutes of vacuuming using 0.5 mL methanol three times. Finally, the extract was blown out to dryness using a gentle nitrogen stream and dissolved in 1 mL of water. Using this method, the detection limits were 0.05, 0.1 and 0.1 for ketoprofen, carbamazepine and gemfibrizil, respectively.

5.2.3 Denaturing Gradient Gel Electrophoresis (DGGE)

To determine the effect of PhAC on microbial community structure, two different bacterial communities were followed: total bacterial and AOB. In both cases, a nested Polymerase Chain Reaction (PCR) approach was used. A summary of the primers sequences and thermal cycling conditions used are shown in Table 5.3. For all PCR runs, a positive control (1 µl of purified DNA from strain *N. europaea* ATCC 19718) and a
negative control (1 µl of DNase/RNase-free sterilized water) were used. All PCR amplifications were performed using a GeneAmp® PCR System 9700 (Applied Biosystems, Foster City, California). All reagents were obtained from the Eppendorf MasterTaq® kit (Hamburg, Germany) and used following the manufacturer’s instructions.

A D-Code Universal Mutation Detection System (Bio-Rad Laboratories, Hercules, California) was used for DGGE analysis. All PCR amplifications were loaded on an 8% (w/v) polyacrylamide gel with a denaturing gradient ranging from 20 to 55% urea-formamide (100% denaturing solution corresponded to 40% (v/v) deionized formamide and 7 M urea). The gel was run at 57°C under a voltage of 50 V for 18 hours and then stained with 1X SYBR Gold (Molecular Probes, Eugene, Oregon).
### Table 5.3 Primers for DGGE analysis

<table>
<thead>
<tr>
<th>Primers</th>
<th>Thermal cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Bacterial 16S rDNA</strong></td>
<td></td>
</tr>
<tr>
<td>1st round&lt;sup&gt;a&lt;/sup&gt;</td>
<td>8f: AGAGTTTGTACCTGCTAG&lt;br&gt;1492r: GGTACCTTGGTACGACTT&lt;br&gt;5 minutes at 94°C, 30 cycles at 94°C for 30 seconds, 52°C for 30 seconds and 72°C for 30 seconds, and 10 minutes final extension at 72°C.</td>
</tr>
<tr>
<td>2nd round&lt;sup&gt;b&lt;/sup&gt;</td>
<td>I-341-fGC: GCclamps-GCACGGGGGGCCTACGGGGGCGC&lt;br&gt;A&lt;br&gt;I-533r-TIACCIIICTICTGGCAC&lt;br&gt;95°C for 10 minutes, 4 cycles at 94°C for 30 seconds, 55°C for 30 seconds and 72°C for 30 seconds. The annealing temperature was subsequently lowered by 2°C every 2 cycles until a touchdown at 47°C, at which an additional 25 cycles were carried out.</td>
</tr>
<tr>
<td><strong>Ammonia-oxidizing bacterial 16S rDNA</strong></td>
<td></td>
</tr>
<tr>
<td>1st round&lt;sup&gt;c&lt;/sup&gt;</td>
<td>CTO189fA/B:&lt;br&gt;GGAGRAAGCAGGGGATCG&lt;br&gt;CTO189fC:&lt;br&gt;GGAGGAAAGTAGGGGATCG&lt;br&gt;CTO654r:&lt;br&gt;CTAGCYTTGTAGTTTCAAACGC&lt;br&gt;2 minutes at 94°C, 30 cycles at 94°C for 30 seconds, 57°C for 60 seconds and 68°C for 60 seconds, and 10 minutes final extension at 68°C.</td>
</tr>
<tr>
<td>2nd round&lt;sup&gt;d&lt;/sup&gt;</td>
<td>357F-GC: GCclamps-GCACGGGGGGCCTACGGGGCAGC&lt;br&gt;CAG&lt;br&gt;518R: ATTACCCGGGCTGCTGG&lt;br&gt;95°C for 10 minutes, 4 cycles at 94°C for 1 minutes, 65°C for 1 minutes and 72°C for 2 minutes. The annealing temperature was subsequently lowered by 2°C every four cycles until a touchdown at 55°C, at which an additional ten cycles were carried out.</td>
</tr>
</tbody>
</table>

<sup>a</sup> (Weisburg et al. 1991); <sup>b</sup> (Watanabe et al. 2001); <sup>c</sup>(Kowalchuk et al. 1997); <sup>d</sup>(Muyzer et al. 1993)
DNA bands were excised from the DGGE polyacrylamide gels using a sterile razor and resuspended in water for identification. Each PCR product was then reamplified using the same primer pair without GC clamps. PCR products were purified using the QIAquick® PCR Purification Kit (Qiagen, Valencia, CA). Sequencing was performed by Duke University DNA Sequencing Facility using an Applied Biosystems 3730 DNA Analyzer (Foster City, California). DNA sequence analysis was performed using the BLAST server of the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov).

Pairwise comparisons of DGGE bands was performed using the Sorenson similarity index (SI): \( SI = \frac{2c}{a+b} \), where \( a \) and \( b \) are the number of bands in any two samples and \( c \) is the number of bands shared between those samples (Turpeinen et al. 2004). This index ranges from 0 (no common bands) to 1 (identical band patterns). Thus, the lower the SI, the less similarity exists between two microbial communities.

5.2.4 Terminal restriction fragment length polymorphism (T-RFLP)

T-RFLP analysis was applied to quantitatively compare the microbial community structure variability following exposure to PhACs as compared to the control communities. The primers and PCR thermal cycles were performed as described by Blackwood et al. (2003). Briefly, an initial denaturation step of 95°C for 3 minutes followed by 22 cycles of a program consisting of denaturation at 94°C for 30 seconds,
annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. The forward and reverse primers used in this study were hexachlorofluorescein (hex)-labeled 8-27F (AGAGTTTGATCCTGGCTCAG) and 1392R (ACGGCGGTGTGTACA) and were manufactured by Integrated DNA Technologies (Skokie, Illinois). Fifty µL PCR products were purified using QIAquick® PCR Purification Kit (Qiagen, Valencia, California) following the manufacturer’s instructions except that the elution was performed using 30 µL sterile water. Thirty µL of the PCR purified product was mixed with 3.5 µL 10× reaction buffer and 1.5µL Rsal enzyme (Promega, Madison, Wisconsin) and incubated at 37°C for 3 hours, followed by 15 minutes incubation at 65°C for enzymatic inactivation. Fragment analysis was performed by the Duke University DNA Analysis Facility using an Applied Biosystems 3100 capillary sequencer (Foster City, California) using POP6 polymer and ROX-labeled MapMarker 1000 size standards (BioVentures, Inc., Murfreesboro, Tennessee). Data analysis was performed using Applied Biosystems GeneScan v3.7.1 software (Foster City, California).

5.2.5 Data Analysis

The analysis and standardization of the T-RFLP profiles was conducted as described in Dunbar et al.(2000). For the purposes of this study, an operational taxonomic unit (OTU) was considered to be a T-RF with a minimum peak height of 50 fluorescent units. An OTU represents a species identified by 16S rRNA gene sequence.
Only profiles with a cumulative peak height ≥ 5,000 fluorescence units were used in the analysis. Peaks differing by 0.7 bp or less were considered as a consensus peak (Kraigher et al. 2008). A subset of T-RF that appeared in all three replicates of the same sampling event was derived by comparing peak profiles under each experimental condition. The intensity of each T-RF was normalized by dividing peak areas of each T-RF by the cumulative peak area.

The richness (S), which represents the number of species in a given sample, was obtained from distinct T-RFs of each derivate profile. The Shannon diversity index (H), which is a measure of biodiversity in the sample which takes into account the number of species in a given sample, was calculated as described by Shannon (1948) using Equation 5-1.

\[ H = -\sum p_i \ln p_i \]  

(Equation 5-1)

where \( p_i \) is the ratio of an individual T-RF peak area relative to the cumulative peak area.

Evenness (E), which quantifies how equal the community is numerically, was calculated using Equation 5-2.

\[ E = \frac{H}{H_{\text{max}}} \]  

(Equation 5-2)

where \( H_{\text{max}} = \ln S \).
5.3 Results and Discussion

5.3.1 PhAC removal

PhAC degradation ranging from 3.7 to 81.9% was observed in the SBRs (Table 5.4). Naproxen and ketoprofen showed the highest removal rates. Their maximum removal rates were 81.9 and 48.1%, respectively. Carbamazepine removal was much lower with a rate ranging from 3.7 to 10.9%. The removal rate of gemfibrozil changed in function of its concentration. At the lowest concentration (0.1 µM), the highest removal rate (48.5%) was obtained while only 13.8% of gemfibrozil was removed at the concentration of 10 µM. These results are consistent with previous studies reporting on PhAC removal in WWTPs (Quintana and Reemtsma 2004; Stumpf et al. 1999; Suarez et al. 2005; Ternes 1998). Removal rates on the order 50 to 82% for naproxen and 23 to 78% for ketoprofen have been reported in municipal WWTPs (Heberer 2002; Quintana and Reemtsma 2004; Stumpf et al. 1999; Suarez et al. 2005; Ternes 1998). Several studies have reported that carbamazepine removal rates are very low (<10%) (Joss 2005; Metcalfe et al. 2003; Radjenovic et al. 2007). These removal rates have been shown to remain low even with an increase in SRT further suggesting that carbamazepine is recalcitrant and unlikely to be biodegraded (Bernhard et al. 2006). The removal of gemfibrozil in WWTPs has been reported in the range of 16 to 69% depending on the WWTPs operation (Stumpf et al.)
Our results showed that 13.8 to 48.5% of gemfibrozil was removed depending on the influent concentration.

<table>
<thead>
<tr>
<th></th>
<th>Ketoprofen</th>
<th>Naproxen</th>
<th>Carbamazepine</th>
<th>Gemfibrozil</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>0.1 μM</strong></td>
<td>25.2</td>
<td>16.3</td>
<td>3.7</td>
<td>48.5</td>
</tr>
<tr>
<td><strong>1 μM</strong></td>
<td>40.8</td>
<td>46.4</td>
<td>10.1</td>
<td>27.2</td>
</tr>
<tr>
<td><strong>10 μM</strong></td>
<td>48.1</td>
<td>81.9</td>
<td>10.9</td>
<td>13.8</td>
</tr>
</tbody>
</table>

### 5.3.2 Reactor performance

**COD Removal.** COD removal rates ranged from 91.2 to 99.6% (Tables 5.5 and 5.6). None of the removal rates in the presence of PhACs were statistically significantly different (p<0.05) from the removal rate in the control reactors. These data suggest that these PhACs do not notably impact the performance of COD removal in this reactor configuration. This result is consistent with other published reports. Suarez et al. (2005) showed that in a nitrifying and denitrifying process of CSTR system, the mixture of carbamazepine, diazepam, fluoxetine, citalopram, ibuprofen, naproxen and diclofenac at the concentration of 10-20 ppb (approximately 0.1 nM) did not affect the COD and nitrogen removal of activated sludge process.

These results are, however, inconsistent with those obtained in the batch reactor study reported in Chapter 3 in which microbial inhibition was observed. Those results
suggested that microbial inhibition might be correlated with a decrease in reactor
performance. However, it is possible that this result is due to differences in reactor
operation as well as carbon substrates. For the SBR portion of this work, bioreactors
received fresh synthetic wastewater every 8 hours whereas in the batch reactor study,
substrate was only provided at the beginning of the experiment. It is likely that the
limited nutrient conditions in the batch reactors may have caused some strains to die out
due to competition (Rittmann and McCarty 2000). Because microbial diversity has been
reported to be related to ecosystem stability when exposed to environmental
perturbations (Naeem and Li 1997; Tilman 1999), it is possible that the greater microbial
diversity in the SBRs allowed for a better performance to be maintained even in the
presence of high concentrations of PhACs. As will be discussed later in this chapter, this
explanation can be preliminarily supported by the comparison of DGGE results between
these two systems. Because both DGGE were performed using the same conditions (e.g.
primers, gel components), the microbial profiles of the two systems are comparable. The
number of bands for the batch reactors (cf. Figure 3.5) ranges from 15 to 20, whereas it
ranges from 20 to 30 for the SBRs (Figure 5.4). If each band represents a single
microorganism, this comparison suggests higher diversity in the SBRs.
Table 5.5 SBR performance summary in removal percentages

<table>
<thead>
<tr>
<th>Group 1</th>
<th>PhACs</th>
<th>COD</th>
<th>NH₃-N</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1μM</td>
<td>Control</td>
<td>99.5±1.2</td>
<td>90.4±8.2</td>
</tr>
<tr>
<td></td>
<td>Ketoprofen</td>
<td>99.6±1.1</td>
<td>89.0±10.2</td>
</tr>
<tr>
<td></td>
<td>Naproxen</td>
<td>99.6±2.4</td>
<td>75.1±19.6</td>
</tr>
<tr>
<td>1μM</td>
<td>Control</td>
<td>99.2±1.9</td>
<td>94.2±6.6</td>
</tr>
<tr>
<td></td>
<td>Ketoprofen</td>
<td>96.8±4.0</td>
<td>89.6±9.6</td>
</tr>
<tr>
<td></td>
<td>Naproxen</td>
<td>95.4±6.1</td>
<td>89.6±9.0</td>
</tr>
<tr>
<td>10μM</td>
<td>Control</td>
<td>97.0±3.6</td>
<td>90.6±4.9</td>
</tr>
<tr>
<td></td>
<td>Ketoprofen</td>
<td>93.3±4.9</td>
<td>90.2±5.8</td>
</tr>
<tr>
<td></td>
<td>Naproxen</td>
<td>94.9±5.2</td>
<td>90.6±4.5</td>
</tr>
</tbody>
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Table 5.6 SBR performance summary in removal percentages

<table>
<thead>
<tr>
<th>Group 2</th>
<th>PhACs</th>
<th>COD</th>
<th>NH₃-N</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.1μM</td>
<td>Control</td>
<td>96.3±4.6</td>
<td>67.0±27.8</td>
</tr>
<tr>
<td></td>
<td>Carbamazepine</td>
<td>94.7±3.7</td>
<td>62.0±27.8</td>
</tr>
<tr>
<td></td>
<td>Gemfibrozil</td>
<td>91.2±6.9</td>
<td>74.0±19.1</td>
</tr>
<tr>
<td>1μM</td>
<td>Control</td>
<td>94.2±1.8</td>
<td>64.4±19.7</td>
</tr>
<tr>
<td></td>
<td>Carbamazepine</td>
<td>97.1±2.1</td>
<td>68.0±29.4</td>
</tr>
<tr>
<td></td>
<td>Gemfibrozil</td>
<td>96.0±3.5</td>
<td>70.4±30.9</td>
</tr>
<tr>
<td>10μM</td>
<td>Control</td>
<td>94.2±4.2</td>
<td>64.4±6.7</td>
</tr>
<tr>
<td></td>
<td>Carbamazepine</td>
<td>97.1±3.2</td>
<td>68.0±13.2</td>
</tr>
<tr>
<td></td>
<td>Gemfibrozil</td>
<td>96.0±4.9</td>
<td>70.4±15.2</td>
</tr>
</tbody>
</table>

Ammonia Removal. Similar to the COD removal data, the ammonia removal in reactors receiving PhACs in their influent was similar to that in the control reactors. There was no significant effect at any PhAC concentration. As shown in Tables 5.5 and 5.6, there was, however, a difference in ammonia removal rates between the first and
second batch of experiments. A possible reason for this difference is the use of activated sludge collected at different time points from the North Durham WWTP (Durham, North Carolina). As will be described in a later section, the AOB microbial fingerprints for these two experiments are significantly different and thus confirm this hypothesis. Vazquez-Rodriguez et al (2003) reported that a variability of activated sludge used as inocula can result in very different biodegradation rates for the compound dodecyl benzene sulfonate, and this same finding can likely be extrapolated to other compounds, including ammonia.

5.3.3 Comparison of bacterial community structure using DGGE

DGGE was used to compare microbial community structure between the reactors receiving PhACs and the control reactors. DGGE has long been considered to be a powerful technique by which the diversity of PCR-amplified genes from a large number of samples can be compared in one gel to reveal changes in community structure over time (Muyzer et al. 1993). Because DGGE is a largely qualitative tool, the analysis in this research initially focused on locating bands which either clearly appeared or disappeared or changed in intensity relative to the control treatment. These results could serve as an indicator for microorganisms which disappeared or became enriched in the presence of each PhAC.
16S rDNA. Microbial community structure was first observed using the 16S rDNA genetic target. Several shifts can be seen when comparing the various treatments as well as the different time points during a given experimental period (Figures 5.2-5.4). These microbial shifts are characteristic of microbial adaptation to each treatment condition. As shown in Figure 5.2, in the presence of 0.1 µM ketoprofen and naproxen, Bands A and C faded away while Band B intensified in the presence of 0.1 µM naproxen. However, there were no consistent shifts in the control as well as in the ketoprofen reactors. Thus, it is likely that any changes in community structure are not significant.

In the presence of 0.1 µM carbamazepine and gemfibrozil, the conclusion was also reached that no notable shifts occurred. Although changes were observed for Bands A, B and C in the presence of 0.1 µM naproxen relative to the control reactors, such changes did not occur in the bioreactors treated with 1 µM and 10 µM naproxen (Figure 5.3a and Figure 5.4a). This result suggests that the change of Bands A, B and C may be random and not a result of either enrichment or inhibition. Similar results were observed in the presence of 1 and 10 µM for the other PhACs (Figures 5.3a and 5.3b). The lack of consistent microbial community shifts in the SBRs is consistent with the relatively constant COD removal rate which was observed in the presence of PhACs. These results suggest that carbamazepine, ketoprofen, naproxen and gemfibrozil at concentrations ranging from 0.1 to 10 µM do not affect the stability of microorganisms. These data are also reinforced by the earlier finding that COD removal treatment performance was
comparable in the presence and absence of PhACs and further suggests that microorganisms involved in COD removal are not affected. It is possible that the relative abundance of each microbial strain varies with treatment conditions, however DGGE cannot reveal such changes.

Figure 5.2 DGGE of bacteria communities exposed to: a) 0.1 μM ketoprofen (K) and naproxen (N); b) 0.1 μM carbamazepine (B) and gemfibrozil (G).

C     K    N     C     K    N    C     K    N  C     B    G     C     B    G      C      B     G

Day 0            Day 15         Day 30                   Day 0            Day 15         Day 30
Figure 5.3 DGGE of bacteria communities exposed to: a) 1 μM ketoprofen (K) and naproxen (N); b) 1 μM carbamazepine (B) and gemfibrozil (G).
The microbial community structure analysis based on SI calculations show that the difference between the microbial communities exposed to PhACs generally decreased over time (Table 5.7). For example, the fingerprints of day 0 were identical for 0.1 µM carbamazepine and gemfibrozil (SI=0.983). However, on Day 30, the SI value decreased to 0.963 and 0.868 for these two conditions, respectively. In general, SI values reveal a change of band pattern over time, which is informative to a more quantitative understanding of the microbial community structure differences. However, even though
a slight change in band pattern is observed, because no effects are observed on the treatment performance measurements, this change is likely not attributable to the PhACs used in the present study. Rather, this slight difference in the microbial fingerprints revealed by SI analysis is likely due to the chaotic dynamics of the bacterial community. Chaotic dynamics are based on the assumption that competition between taxa is finely poised. It is generally recognized in ecology that competition for three or more growth-limiting resources may generate oscillations and chaotic fluctuations in species abundances (Huisman and Weissing 1999). This theory has been applied in several studies to explain the difference in the bacterial community structure of replicate reactors (Curtis and Sloan 2004; Kaewpipat and Grady 2002; Saikaly et al. 2005). These dynamics in the bacterial community are especially likely in small-scale biological treatment plants (Curtis and Sloan 2004). The SBRs used in the present study were lab-scale reactors and fit within this description and thus, it is likely that the bacterial community in these reactors underwent oscillations and fluctuations during the experimental period. This result is consistent with the slight change in community structure observed in Figures 5.2, 5.3 and 5.4 which was previously discussed.
Table 5.7 Similarity index (SI) values for the total microbial profile under each treatment condition.

<table>
<thead>
<tr>
<th></th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>0</th>
<th>15</th>
<th>30</th>
<th>0</th>
<th>15</th>
<th>30</th>
</tr>
</thead>
<tbody>
<tr>
<td>ketoprofen</td>
<td>0.984</td>
<td>0.984</td>
<td>0.984</td>
<td>1.000</td>
<td>0.905</td>
<td>0.927</td>
<td>0.927</td>
<td>0.909</td>
<td>0.930</td>
</tr>
<tr>
<td>naproxen</td>
<td>0.984</td>
<td>0.984</td>
<td>0.968</td>
<td>1.000</td>
<td>0.864</td>
<td>0.933</td>
<td>0.930</td>
<td>0.889</td>
<td>0.889</td>
</tr>
<tr>
<td>carbamazepine</td>
<td>0.983</td>
<td>0.981</td>
<td>0.963</td>
<td>0.963</td>
<td>0.870</td>
<td>0.896</td>
<td>0.896</td>
<td>0.847</td>
<td>0.828</td>
</tr>
<tr>
<td>gemfibrozil</td>
<td>0.983</td>
<td>0.830</td>
<td>0.868</td>
<td>0.868</td>
<td>0.857</td>
<td>0.812</td>
<td>0.812</td>
<td>0.800</td>
<td>0.793</td>
</tr>
</tbody>
</table>

**AOB Analysis.** Figures 5.5, 5.6 and 5.7 show the DGGE results for the AOB community analysis. Comparison between group 1 treatments (i.e. ketoprofen and naproxen) [Figure 5.5a, 5.6a and 5.7a] and group 2 (i.e. carbamazepine and gemfibrozil) [Figure 5.5b, 5.6b and 5.7b] suggests that these two groups showed very different band patterns. This result indicates that the AOB composition of these two groups are very different and could help explain the differences in ammonia removal rates between these two groups (Table 5.5 and Table 5.6).
Figure 5.5 DGGE of ammonia-oxidizing bacteria (AOB) communities exposed to: a) 0.1 μM ketoprofen (K) and naproxen (N); b) 0.1 μM carbamazepine (B) and gemfibrozil (G).
Figure 5.6 DGGE of ammonia-oxidizing bacteria (AOB) communities exposed to: a) 1 μM ketoprofen (K) and naproxen (N); b) 1 μM carbamazepine (B) and gemfibrozil (G).
Figure 5.7 DGGE of ammonia-oxidizing bacteria (AOB) communities exposed to: a) 10 μM ketoprofen (K) and naproxen (N); b) 10 μM carbamazepine (B) and gemfibrozil (G).

The DGGE analysis for PhAC concentrations of 0.1, 1 and 10 μM shows that, in most cases, the AOB community structure did not vary significantly between the PhAC treatments and the control within each experimental group. Although the pure culture study performed in Chapter 4 showed that all four PhACs significantly inhibited the activity of *N. europaea*, this result is not totally unexpected because the ammonia removal rate was not significantly impacted. The stability of the SBR performance may be due to
the functional redundancy. Selected PhACs may impact some AOB strains (e.g. *N. europaea*) but not other AOB which perform the same ecological function. However, the 16S rDNA CTO primers used in the present study do not target all AOB strains but only the β-subclass AOB (Nicolaisen and Ramsing 2002). Thus, DGGE results might not provide a complete picture of the actual AOB diversity found in the samples. Thus, it is still possible that the AOB strains impacted by PhACs were not visualized by DGGE methods.

The only band which was shown to decrease in intensity is Band A (Figure 5.7) which is for the 10 µM naproxen treatment. Because the other bands in that lane appear to be of similar intensity, the decrease in intensity for Band A could be linked to a decrease in that species microbial population and thus inhibition. Sequencing results showed that Band A was 88% homologous to *Nitrosomonas* sp. No information on the specific strain was available from NCBI. This result suggests that 10 µM naproxen may inhibit the growth of *Nitrosomonas* strains. While these microorganisms have been shown to be linked to ammonia oxidation, this result was only observed at a concentration unlikely to be encountered in an actual WWTP and thus is not likely a source of concern.
5.3.4 Comparison of bacterial community structure by T-RFLP

DGGE is a qualitative method, which can be used to visually assess but not quantify the change of microbial communities. Because of this, the impact of PhACs on microbial community structure in the SBRs was investigated using T-RFLP to further compare differences in community richness and diversity. It has been widely used in microbial ecology for various purposes including determining the correlation between operational parameters of wastewater treatment plants and the effects of pollutant shocks (Eichner et al. 1999; Saikaly et al. 2005).

Diversity indices values for each of the experimental conditions tested are presented in Table 5.8. No trend was observed for richness values ($S$) for any of the treatments. For example, the richness of the control reactor is higher than the 0.1 µM ketoprofen and naproxen treatments on Day 30 but lower than 1 and 10 µM ketoprofen and naproxen. Similarly, although the richness values of 1 µM carbamazepine and gemfibrozil are lower than the control, this trend was not observed in the case of 0.1 and 10 µM. The same fluctuations were observed for evenness ($E$).

Because of the variability for both $S$ and $E$, a conclusion cannot be drawn that PhACs affect bacterial populations in the SBRs. These inconsistencies are not unique to the present study. Dunbar et al. (2000) showed that T-RF profiles were ineffective for describing community richness or evenness values in soil environments (Dunbar et al. 2000). Thus, it is possible that even though the analysis does not reveal any differences
that some do exist for these parameters. However, this finding would need to be reinforced by other molecular methods and because no effect was observed on treatment performance, no attempts were made to look any further.

A trend was however observed for the Shannon-Weaver index ($H$). The $H$ values for the control reactors are consistently higher than those for the reactors receiving ketoprofen and naproxen in all but one data point (Day 30 for the 10 µM treatment). This result is consistent with a previous study showing that some PhACs affected microbial diversity in the presence of 50 µg/L ibuprofen, naproxen, ketoprofen, diclofenac and clofibric acid (Kraigher et al. 2008). Because the diversity of microorganisms is related to ecosystem stability, this result may have important implications in that PhACs could possibly impact wastewater treatment plant community stability and hence performance.
Table 5.8 Comparison of richness, evenness, and diversity values for microbial communities exposure to PhACs

<table>
<thead>
<tr>
<th></th>
<th>S</th>
<th>H</th>
<th>E</th>
<th>S</th>
<th>H</th>
<th>E</th>
<th>S</th>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>54</td>
<td>2.977</td>
<td>0.746</td>
<td>39</td>
<td>3.04</td>
<td>0.828</td>
<td>46</td>
<td>3.083</td>
<td>0.805</td>
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<td>Ketoprofen</td>
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<td>2.964</td>
<td>0.741</td>
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<td>2.886</td>
<td>0.747</td>
<td>22</td>
<td>2.473</td>
<td>0.804</td>
</tr>
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<td>Naproxen</td>
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<td>2.923</td>
<td>0.742</td>
<td>44</td>
<td>3.030</td>
<td>0.801</td>
<td>37</td>
<td>2.770</td>
<td>0.767</td>
</tr>
<tr>
<td>1 μM</td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>52</td>
<td>3.184</td>
<td>0.808</td>
<td>38</td>
<td>3.140</td>
<td>0.867</td>
<td>19</td>
<td>2.599</td>
<td>0.781</td>
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<tr>
<td>Ketoprofen</td>
<td>46</td>
<td>3.156</td>
<td>0.827</td>
<td>17</td>
<td>2.559</td>
<td>0.914</td>
<td>23</td>
<td>2.574</td>
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<tr>
<td>Naproxen</td>
<td>30</td>
<td>2.723</td>
<td>0.805</td>
<td>15</td>
<td>2.452</td>
<td>0.906</td>
<td>20</td>
<td>2.585</td>
<td>0.87</td>
</tr>
<tr>
<td>10 μM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>54</td>
<td>3.304</td>
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<td>42</td>
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<td>0.798</td>
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<td>2.814</td>
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<td>Ketoprofen</td>
<td>45</td>
<td>3.234</td>
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<td>24</td>
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<td>54</td>
<td>3.323</td>
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<tr>
<td>Naproxen</td>
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<td>3.176</td>
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<td>3.183</td>
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<td>0.1 μM</td>
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<td></td>
<td></td>
<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>61</td>
<td>3.219</td>
<td>0.799</td>
<td>54</td>
<td>2.888</td>
<td>0.725</td>
<td>47</td>
<td>2.762</td>
<td>0.723</td>
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<tr>
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<td>3.175</td>
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<td>45</td>
<td>2.830</td>
<td>0.752</td>
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<td>0.808</td>
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<tr>
<td>Gemfibrozil</td>
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<td>0.765</td>
<td>55</td>
<td>3.024</td>
<td>0.767</td>
<td>54</td>
<td>2.993</td>
<td>0.752</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>66</td>
<td>3.159</td>
<td>0.755</td>
<td>58</td>
<td>3.301</td>
<td>0.813</td>
<td>75</td>
<td>3.578</td>
<td>0.829</td>
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<td>Carbamazepine</td>
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<td>3.206</td>
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<td>3.321</td>
<td>0.858</td>
<td>72</td>
<td>3.546</td>
<td>0.831</td>
</tr>
<tr>
<td>Gemfibrozil</td>
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<td>3.204</td>
<td>0.780</td>
<td>56</td>
<td>3.306</td>
<td>0.824</td>
<td>48</td>
<td>3.346</td>
<td>0.865</td>
</tr>
<tr>
<td>10 μM</td>
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<td></td>
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<td></td>
<td></td>
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<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>47</td>
<td>3.173</td>
<td>0.828</td>
<td>46</td>
<td>3.204</td>
<td>0.842</td>
<td>47</td>
<td>3.103</td>
<td>0.837</td>
</tr>
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<td>Carbamazepine</td>
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<td>0.888</td>
<td>27</td>
<td>2.661</td>
<td>0.808</td>
<td>44</td>
<td>2.813</td>
<td>0.832</td>
</tr>
<tr>
<td>Gemfibrozil</td>
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<td>3.086</td>
<td>0.828</td>
<td>42</td>
<td>2.971</td>
<td>0.796</td>
<td>48</td>
<td>3.099</td>
<td>0.849</td>
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</tbody>
</table>

Although some variability was observed for H, because no consistent trends were observed between T-RFLP and DGGE analyses as well as treatment performance criteria, these results should be careful interpreted. A previously published study investigating the relationships between T-RF diversity indices and true indices found that the T-RFLP results are sensitive to the relative abundance threshold and thus can be inaccurate to estimate the true diversity of microbial communities (Blackwood et al. 2007). The author also recommended that the T-RFLP results should be reinterpreted as
a reflection of differences in community composition rather than a true difference in community diversity. Kraigher et al (2008) suggested that indices calculated based on clone library and sequence data were more accurate than those based T-RFLP data for complex bacterial communities and this would be the logical next step to undertake. However, because no effects were observed at the lower concentrations which are more environmentally relevant, no attempts were undertaken to perform such in depth analysis.

5.4 Conclusions

Laboratory-scale SBRs were shown to efficiently transform ketoprofen and naproxen as well as remove gemfibrozil and carbamazepine to a smaller extent. The highest removal rates for each compound were 48.1, 81.9, 48.5 and 13.8%, respectively. No significant differences were observed in the performance of bioreactors exposed to PhACs with respect to COD and ammonia removal as compared to the control bioreactors. The DGGE analysis revealed that although SI values generally decreased over time with an increase in PhAC concentrations as compared to the controls, no major microbial community shifts were observed for total bacteria and AOB communities. Furthermore, the T-RFLP data suggest that the presence of selected PhAC in wastewater only slightly affected the diversity of bacterial community. The minor changes in community structure observed in this study further strengthen that PhACs at
environmental conditions may not significantly impact the performance of the treatment itself. Rather, PhACs are likely impacting some sensitive species and slightly change the profile of bacterial communities but under the conditions tested herein should not present any problems in a WWTP.
Chapter 6 PhAC Mixture Effects

6.1 Introduction

PhACs are detected in the environment at very low concentrations from ng/L to µg/L, which are far below the therapeutic doses applied in medical practice. However, PhACs may have chronic effects on a variety of organisms especially sensitive species in unknown modes different from their pharmacological action. Furthermore, PhACs never occur alone in the environment but, rather, are always found in combination with other compounds. Therefore, the ecological characterization of single compounds is not sufficient to elucidate the potential hazard of PhACs in the environment. Thus, the ecological effects of PhAC mixtures should be investigated.

The combination effects of chemicals can be grouped based on whether they interact with each other and whether they act in a similar toxicity mode. For those chemicals which react with each other, compounds in mixture may demonstrate either higher inhibition levels if more toxic products are derived (synergistic) or lower when products are less toxic (antagonistic). On the other hand, if the components in the mixture do not interact, two pharmacological concepts for the expected additive mixture effects have been generally accepted, concentration addition, and independent action (Bliss 1939; Glenn 1992).
Compounds that share a common mode of toxic action typically act in a concentration additive manner, which means that every mixture component contributes to the overall combination effect in proportion to its concentration, even below their non-observed effect concentration (NOEC) (Altenburger et al. 2000; Silva et al. 2002). Independent action, which is usually applied to chemicals with different modes of action, assumes that mixture effects are the result of interactions of individual mixture constituents with different subsystems of an organism (Bliss 1939; Glenn 1992). In such mode, chemicals that are below NOEC are not expected to contribute to the total mixture effect.

Studies have revealed that PhACs which behave in the concentration addition toxicity mode resulted in dramatic mixture effects. For example, eight weak endocrine disrupting chemicals (EDCs) are able to act together to produce significant effects in a recombinant yeast estrogen screen (YES) system even though their concentrations were below NOECs (Silva et al. 2002). This phenomenon was further confirmed by a study using fathead minnow as target organisms. In that study, the mixture of five estrogenic chemicals with concentrations below their NOECs affected the reproductive performance of fish (Brian et al. 2007). In addition to EDCs, the toxicity of NSAIDs have also been shown to be altered in mixtures. Cleuvers (2004) showed that the mixture toxicity of four NSAIDs (i.e., diclofenac, ibuprofen, naproxen, and acetylsalicylic acid) at the NOEC concentration was shown to be significant. In this study, the toxicity level
could be accurately predicted using the concept of concentration addition (Cleuvers 2004).

In the present study, a preliminary investigation of the effects of PhACs was performed to determine if their inhibition is synergistic, antagonistic or entirely independent. The specific aim of this chapter was to investigate the effect of binary mixtures of selected PhACs (i.e. ketoprofen, naproxen, carbamazepine and gemfibrozil) on a well-studied strain of ammonia-oxidizing bacteria (AOB), *Nitrosomonas europaea*. Nitrite production was used as a measure of microbial activity. The toxicity results of PhAC mixtures were compared to individual PhACs. Similarly to previous studies in this dissertation, experiments were first carried out in batch reactors with pure culture (*N. europae*) and followed up with experiments in sequencing batch reactors. The follow up experiments were performed only with those PhACs having shown the highest degree of inhibition on their own (i.e., ketoprofen, naproxen and gemfibrozil) in Chapter 4.

### 6.2 Material and Methods

#### 6.2.1 Nitrite production

Ketoprofen, naproxen and gemfibrozil were selected for the binary mixture tests because they were previously shown to have the highest inhibition rate among the
PhACs used in this research (cf. Table 4.2). Because naproxen had the highest inhibition level of all the PhACs, this compound was used as the primary PhAC in mixture with the other PhACs. Two PhAC combinations were tested: 1) naproxen and ketoprofen and; 2) naproxen and gemfibrozil.

Nitrite production experiments were performed as described in Chapter 4. Briefly, *N. europaea* cells were exposed to various combinations of PhACs including 0.25 μM ketoprofen and 0.75 μM naproxen, 0.5 μM ketoprofen and 0.5 μM naproxen, 0.75 μM ketoprofen and 0.25 μM naproxen, 0.25 μM gemfibrozil and 0.75 μM naproxen, 0.5 μM gemfibrozil and 0.5 μM naproxen, 0.75 μM gemfibrozil and 0.25 μM naproxen. Cells without PhACs were served as controls. Nitrite was measured every 60 minutes over 4 hour experiment using NitriVer® 3 Reagents (Hach, Loveland, Colorado).

### 6.2.2 Microbial community structure analysis in SBRs

The effect of PhAC mixture was tested in SBRs mimicking wastewater treatment plant (WWTP) operation. SBR configuration was previously described in Chapter 5. Microbial community variability was compared between reactors exposed to PhAC mixtures (i.e. 0.25μM ketoprofen and 0.75μM naproxen, 0.5μM ketoprofen and 0.5μM naproxen, 0.25μM gemfibrozil and 0.75μM naproxen, 0.5μM gemfibrozil and 0.5μM naproxen) and the control which did not receive PhAC in their influent. The comparison
was performed using Denaturing Gradient Gel Electrophoresis (DGGE). This method was previously described in Chapter 5.

6.2.3 Data analysis

Standard deviations were calculated and are shown in all figures. The student t-test was used to assess the significance of the result with a 95% confidence interval.

Nitrite production inhibition rate was calculated using Equation (6-1).

\[
\% \text{ Nitrite production inhibition} = (1 - \frac{NO_2^{-}_{\text{sample}}}{NO_2^{-}_\text{control}}) \times 100\%
\]  

Equation (6-1)

6.3 Results and Discussion

6.3.1 Nitrite production

The effects of 0.5 µM naproxen, ketoprofen, gemfibrizil and carbamazepine on nitrite production are shown in Figures 6.1 and 6.2. The data show that the individual PhACs at 0.5 µM did not show significant difference from control at a 95% confidence level. However, in mixture, when ketoprofen and naproxen were added, significant inhibition of nitrite production was observed in \textit{N. europaea}. This result indicates that individual PhACs applied at less than their individual NOECs (i.e., 0.5 µM) do contribute to the total mixture inhibition. These data suggest that considerable combination effects could occur if some PhACs were applied even in concentrations...
below their NOEC. The inhibition rate of each combination is summarized in Table 6.1. The mixture of ketoprofen and naproxen in the ratio of 1:1 generated the highest inhibition rate (25.2%) compared to the mixture with the ratio of 1:3 or 3:1. However, the statistical analysis showed that the differences among various ratio combinations of ketoprofen and naproxen are not significant. The other combination, mixture of gemfibrozil and naproxen had lower inhibition rates, ranging from 3.7 to 16.6%. Again, no significant difference of inhibition rates was found between the different mixtures.

![Figure 6.1 Nitrite production comparison between N.europaea control cells and cells exposed to ketoprofen, naproxen and their mixture. K stands for ketoprofen and N stands for naproxen. Error bars represent +/- standard derivation. Star (*) indicates statistically significant difference from control samples with 95% confidence interval.](image-url)
Figure 6.2 Nitrite production comparison between N.europaea control cells and cells exposed to gemfibrozil, naproxen and their mixture. G stands for gemfibrozil and N stands for naproxen. Error bars represent +/- standard derivation. Star (*) indicates statistically significant difference from control samples with 95% confidence interval.

Table 6.1 Nitrite production inhibition (%) after 4 hour exposure to PhAC mixture

<table>
<thead>
<tr>
<th>Mixture</th>
<th>0.5μM K+0.5μM N</th>
<th>0.25μM K+0.75μM N</th>
<th>0.75μM K+0.25μM N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition rate</td>
<td>25.2±8.2</td>
<td>19.7±5.3</td>
<td>18.6±6.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Mixture</th>
<th>0.5μM G+0.5μM N</th>
<th>0.25μM G+0.75μM N</th>
<th>0.75μM G+0.25μM N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Inhibition rate</td>
<td>5.39±0.8</td>
<td>16.2±8.9</td>
<td>3.7±1.4</td>
</tr>
</tbody>
</table>

These results provide evidence that PhACs present in a mixture can have inhibitory effects on nitrification even at concentrations lower than their individual NOEC. This result has important implications for WWTPs as their influent contains hundreds of different PhACs. Previous studies have reported similar phenomena using
different model systems. For example, Cleuvers (2004) reported that naproxen mixed with NSAIDs below the NOEC concentration showed significant effect in both the *Daphnia* test and algal test. Quinn et al. (2009) also observed an additive effect in *Hydra* following exposure to PhAC mixtures from various therapeutic classes including gemfibrozil, naproxen and carbamazepine. In their study, the effective concentration which showed a toxic effect for each PhAC in the mixture was 2 to 3 times lower than that for the same compound when exposed individually to *Hydra* (Quinn et al. 2009).

### 6.3.2 SBR experiments

SBR experiments were performed using the combinations which showed the highest degree of inhibition in the nitrite production tests on *N. europae*. The conditions tested included: 1) 0.5 µM ketoprofen and 0.5 µM naproxen and; 2) 0.25 µM ketoprofen and 0.75 µM naproxen. Microbial community structure changes as well as SBR performance were monitored for both of these conditions.

#### 6.3.2.1 SBR Performance

The COD removal rates were similar for both PhAC mixture and control reactors. The COD removal rates ranged from 97.6% to 99.2%. The highest removal rate was observed for the mixture composed of 0.25 µM ketoprofen and 0.75 µM naproxen, however it was not statistically significantly different from the control reactor. The
ammonia removal rate (66%) was significantly lower in the presence of 0.25 µM ketoprofen and 0.75 µM naproxen as compared to the control bioreactors. This result suggests that the combination of 0.25 µM ketoprofen and 0.75 µM naproxen had considerable inhibition on AOBs. No significant inhibition was observed in the presence of 0.5 µM ketoprofen and 0.5 µM naproxen suggesting that the relative concentrations of PhACs plays an important role in determining nitrification inhibition potential.

![Figure 6.3 SBR performance in the present of PhAC mixtures. Error bars represent ± 1 standard deviation.](image)

6.3.2.2 Microbial Community Structure

DGGE was used to compare microbial community structure between the reactors receiving PhACs in their effluent and the control reactors. Microbial community structures were compared for two different bacterial communities, total bacterial and
AOB. The DGGE results are shown in Figures 6.4a and 6.4b for total bacteria and AOB, respectively.

The total bacterial community profiles show no significant shifts for mixture 1 (0.5 μM ketoprofen and 0.5 μM naproxen). However, significant shifts were observed for mixture 2 (0.25 μM ketoprofen and 0.75 μM naproxen). These microbial shifts were observed starting on Day 15 and became stable by Day 30. As noted on Figure 6.4a,
several bands either appeared and/or disappeared in mixture 2 as compared to the control. Interestingly however, the shifts observed are directly correlated to the PhAC mixture composition. This result indicates that, not surprisingly, different microorganisms are susceptible to various concentrations of naproxen and ketoprofen. If such species play important roles in contaminants degradation, the presence of PhACs in WWTPs may impact the performance of pollutant removal. However, because no significant effect was observed on COD removal, this result suggests that the affected microorganisms are either not involved in COD removal or that the remaining organisms are more efficient at removing compounds contributing to the COD. It is however possible that the shift affects performance criteria other than COD.

AOB community shifts were observed for mixture 2 as compared to the control reactor. The numbers of bands dramatically decreased throughout the 30 day experimental period suggesting a decrease in the overall number of AOB species. This result is consistent with the SBR performance results where the SBR exposed to mixture 2 was found to have the lowest ammonia removal rate. Because DGGE data does not provide information about the total number of microorganisms, no conclusions can be drawn about the relative amounts of each AOB. However, the observed decrease in nitrification in conjunction with the decrease in AOB diversity suggests that the more efficient nitrifiers have been removed following exposure to mixture 2.
6.4 Conclusions

The effects of PhAC mixtures are rarely reported in the literature, despite these compounds being found in both WWTPs and aquatic environments as complex mixtures. In this chapter these effects are discussed for selected PhACs which have been demonstrated to affect nitrification in *N. europaea*. Overall, both a pure culture of *N. europaea* and mixed community in the SBRs were impacted by some binary mixtures of PhACs. For instance, 0.5 µM ketoprofen and 0.5 µM naproxen showed significant inhibition (25.18%) on the nitrite production of *N. europaea* although neither 0.5 µM ketoprofen or 0.5 µM naproxen had significant effect when present alone. There was no significant difference between the different mixture treatments. This result suggests that mixture effects can play an important role in an overall treatment’s nitrification potential. Furthermore, significant ammonia removal inhibition was observed in an SBR exposed to 0.25 µM ketoprofen and 0.75 µM naproxen. The microbial profile of total bacteria with the treatment of such mixture showed shifts compared to the control bioreactor although the COD removal remained high in all reactors. AOB community profile analysis suggests that the low ammonia removal rate may be due to a decrease in AOB diversity.
Chapter 7  Conclusions

The major objective of this dissertation was to determine the impact of selected PhACs (i.e., ketoprofen, naproxen, clofibric acid, carbamazepine and gemfibrozil) on activated sludge microorganisms and individual microbial species performing key activities (i.e., nitrification) in domestic wastewater treatment. The results show that some PhACs, both individually and in mixture, can impact the microbial activity of select microorganisms as well as the bacterial community structure found in WWTPs. The specific objectives and a summary of key findings are listed below.

- **Estimate the effect of PhACs individually and in binary mixtures on the activity and composition of microbial communities in WWTPs;**

  In batch reactors, microbial growth inhibition of PhACs was found to be correlated to both organic loading and PhAC concentrations. In the presence of 0.2% (v/v) ethanol, significant inhibition ranging from 34 to 43% was observed for 10 and 100 µM ketoprofen, naproxen, carbamazepine and gemfibrozil. However, no inhibition was observed at the organic loading of 2% (v/v) ethanol. Furthermore, ketoprofen and naproxen was found to significantly impact the microbial structure by enriching *Acinetobacter* spp. However, in a sequencing batch reactor system, PhAC treatment did not show significant impact on either total bacteria community or ammonia-oxidizing
bacteria (AOB) community based on DGGE analysis. T-RFLP data suggest a slight microbial community shift but because no effect was seen on COD or ammonia removal performance, this change is likely insignificant.

In contrast to individual PhACs, the binary mixtures of PhACs were found to impact mixed community in both batch and SBR experiments. Both the microbial fingerprinting of total bacteria and AOB community exposed to PhAC mixture (i.e. 0.25 µM ketoprofen and 0.75 µM naproxen) showed significant shifts compared to the control bioractors.

- **Ascertain the effect of PhACs on sensitive microorganisms (i.e. ammonia-oxidizing bacteria, AOB) in wastewater treatment;**

  Ketoprofen, naproxen, carbamazepine and gemfibrozil were shown to inhibit the nitrite production of *N. europaea*. The maximum inhibition rates were 25.2, 28.9, 21.9 and 26.1% respectively. Inhibition increased with concentration for all PhACs other than for gemfibrozil. Membrane integrity data suggest that the inhibition may be due to a disturbance of the cell membrane. The inhibition was shown to be irreversible. Because no removal of PhACs occurred, the inhibition is unlikely to be caused by the competition of PhACs with ammonia. The calculation of live cell inhibition rate suggested that PhACs not only resulted in the death of cells but also inhibited their activity.
PhAC mixtures were also demonstrated to affect nitrification rates in *N. europaea*. For instance, 0.5 µM ketoprofen and 0.5 µM naproxen showed significant inhibition (25.18%) on the nitrite production of *N. europaea* although neither 0.5 µM ketoprofen nor 0.5 µM naproxen showed any significant effect when present alone. These results provide evidence that PhACs present in a mixture can have inhibitory effects on nitrification even at concentrations lower than their individual NOEC.

- **Determine if PhACs could adversely impact treatment performance in WWTPs.**

  The performance of bioreactors exposed to PhACs (i.e. COD and ammonia removal) did not have significant difference with control bioreactors. However, significant ammonia removal decrease was observed in an SBR exposed to 0.25 µM ketoprofen and 0.75 µM naproxen. This result suggests that mixture effects can play an important role in an overall treatment’s performance.

  These findings could have important engineering significance for the design and operation of WWTPs especially with respect to nitrogen removal. Because some PhACs were shown to adversely affect a model AOB strain (i.e. *N. europaea*) and because some PhAC mixtures resulted in a decrease in ammonia removal, follow up experiments should be performed to confirm the results obtained in this dissertation. Specifically, further studies should be conducted to confirm the negative impacts found in this study.
using a range of PhACs as well as different model AOB strains. Finally, the experiments in this dissertation should be expanded upon using lower PhAC concentrations and more complex PhAC mixtures which are more representative of realistic WWTP conditions.
Appendix

List of abbreviations

AOB - Ammonia-oxidizing bacteria
DGGE - Denaturing gradient gel electrophoresis
EDC - Endocrine disrupting chemicals
NOEC - Non-observed effect concentration
NSAID - Nonsteroidal anti-inflammatory drugs
PCR - Polymerase chain reaction
PEC - Predicted environment concentration
PhAC – Pharmaceutically active compound
T-RFLP - Terminal restriction fragment length polymorphism
WWTP – Wastewater treatment plant
Sorption batch tests

Sorption tests performed in 250 mL Erlenmeyer flasks were performed to determine if sorption played an important role in the overall removal of the selected PhACs in the activated sludge from WWTPs. For these experiments, each reactor was seeded with activated sludge obtained from the aeration basin of the North Durham WWTP (Durham, North Carolina). The experiments were carried out by mixing activated sludge, PhACs and synthetic wastewater. Before mixing with synthetic wastewater (Table 1), 100 mL of activated sludge were centrifuged at 5,000 rpm for 5 minutes to obtain a bacterial pellet using an Eppendorf Centrifuge 5804 (Westbury, New York). The pellet was re-suspended in synthetic wastewater to a final volume of 100 mL. The final suspended solids concentration was approximately 2000 mg/L ± 10% (Beltran et al. 2000). For inactivated sludge control, sodium azide was added to a final concentration of 1% (v/v) to determine the relative contribution of biodegradation to the overall removal. An additional set of bottles with PhACs and sterile synthetic wastewater media but without any activated sludge inoculum was used as an abiotic control to determine if any hydrolysis or photolysis was occurring. All sorption samples were spiked with PhACs stocks to a final concentration of 10 µM. Triplicates of each treatment condition were prepared. All experiments were performed at ~20 ºC. All reactors were incubated in the dark on a VWR DS2-500E-1 orbital shaker table (Batavia,
Illinois) at 150 rpm. One mL samples were collected for chemical analysis every 4 hour over a 24-hour period.

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Concentration (mg/L)</th>
<th>Constituent</th>
<th>Concentration (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Glucose</td>
<td>400</td>
<td>MgSO₄.7H₂O</td>
<td>26</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>100</td>
<td>CaCl₂</td>
<td>3.5</td>
</tr>
<tr>
<td>K₂HPO₄</td>
<td>138</td>
<td>NaHCO₃</td>
<td>80</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>69</td>
<td>FeSO₄.7H₂O</td>
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</tr>
<tr>
<td>NH₄Cl</td>
<td>65</td>
<td>ZnSO₄.7H₂O</td>
<td>0.75</td>
</tr>
<tr>
<td>Yeast</td>
<td>9</td>
<td>MnSO₄.7H₂O</td>
<td>0.75</td>
</tr>
</tbody>
</table>

*Based on the recipe developed by Beltran et al. (2000)

Sorption tests were performed to measure the proportion of PhAC which could be expected to be removed by sorption as opposed to biodegradation. As shown in Figure A1, the amount of ketoprofen and naproxen was statistically significantly different in the activated sludge as compared to the inactivated sludge indicating that both of these compounds sorb onto sludge and are also partially biodegradable (Figures A1a and b). Approximately 19% of ketoprofen was removed from the aquatic phase over the 24 hour experimental period. The data show that 64% was biodegraded while the remaining 36% sorbed onto the activated sludge. The overall naproxen removal was approximately 93% consisting of 69% biodegradation and 31% sorption. These results are consistent with previously published studies which show that these two PhACs can be removed by biodegradation (Daughton and Ternes 1999; Kolpin et al. 2002) No
significant change was observed for carbamazepine, clofibrin acid and gemfibrozil (Figure A1c, d and e).

Naproxen and ketoprofen were degraded in this sorption study even though they were not degraded during the eight day batch experiments previously described (section 3.3.4). Two explanations are possible for these differences. First, the amount of inoculants was different. In the batch experiment, bioreactors were inoculated by 1 mL activated sludge whereas 50 mL of activated sludge were used in sorption tests. The abundance of microorganisms in sorption tests was much higher, which was more likely to remove PhACs by degradation and sorption. Second, the feeding was different. Synthetic wastewater, which was rich in nutrient, was applied in sorption testes whereas batch experiment used minimal medium with ethanol as the carbon source.
Figure A1 Concentration profile for a) ketoprofen, b) naproxen, c) carbamazepine, d) clofibric acid and e) gemfibrozil in mixture with activated sludge (solid square), with autoclaved sludge (empty triangle). The abiotic control is shown by the (star).
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