

# Effects of Pharmaceutically Active Compounds on a Mixed Microbial Community Originating from a Municipal Wastewater Treatment Plant

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Received August 14, 2007. Revised manuscript received December 9, 2007. Accepted December 10, 2007.

The growth and composition of microorganisms found in a municipal wastewater treatment plant were investigated in the presence of four pharmaceutically active compounds (PhACs) [ketoprofen, naproxen, carbamazepine, and clofibrac acid] in batch reactors at varying organic loadings. Overall, the data suggest that microbial growth inhibition was correlated to organic loading rather than PhAC concentration. Significant inhibition ranging from 34 to 43% was observed under the lowest organic loading for all PhACs other than clofibrac acid. No inhibition was observed at the highest organic loading. Higher microbial inhibition was not observed with increased PhAC concentration for a given organic loading. These results indicate that the presence of PhAC may affect microbial growth especially under lower organic loading conditions. Further validation is required with additional PhACs, organic substrates, and a wider loading range. In addition, significant microbial shifts were observed in the presence of ketoprofen and naproxen. These data suggest that, in addition to their effect on overall microbial growth, PhACs may affect the microbial ecology and additional research should be carried out to identify PhACs that have the potential of affecting ecologically important microorganisms in wastewater treatment processes and aquatic environments in general.

## 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 (1, 2). Because many of the compounds are not fully metabolized prior to household discharge, the parent PhACs and their breakdown products commonly enter WWTPs (3). 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 (1, 4). Recently, more than 80 pharmaceuticals and drug metabolites

were detected in aquatic environments in the United States and even in drinking water samples (2, 5). 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 (5). Furthermore, pathogens may develop resistance to these compounds, ultimately leading to an increase risk of human diseases (2).

While there has been a recent interest in the development of technologies to remove PhACs from wastewater (6, 7), most treatment facilities are not designed to adequately remove them (8, 9). The persistent nature of PhACs is in large part due to their chemical characteristics as well as their slow biodegradation kinetics (10). 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 (10).

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. (11) 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. (12) 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 research was to determine the effect of PhAC and organic loading on the microbial growth and ecology of microorganisms found in a municipal WWTP. Four common PhACs were selected for this study and consisted of ketoprofen, naproxen, carbamazepine, and clofibrac acid (Table 1). These compounds were selected because they have been detected at fairly high concentrations (13). Ketoprofen and naproxen daily loads to a lake in Switzerland have been reported on the order of grams (10). Carbamazepine is among the 10 most frequently detected organic compounds according to the USGS survey (2), and clofibrac acid is known for its persistence (14).

## Materials and Methods

**Batch Reactors.** Batch reactors were prepared in 250 mL Erlenmeyer flasks containing 50 mL of basal medium prepared as described in Gunsch et al. (15) amended with 1 g/L (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>. Each reactor was inoculated with 1 mL of activated sludge obtained from the aeration basin from the North Durham WWTP (Durham, North Carolina). All flasks were heat-sterilized by autoclaving at 121 °C at 15 psi for 15 min. 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

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**TABLE 1. Target PhACs**

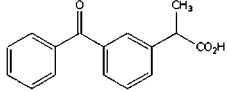
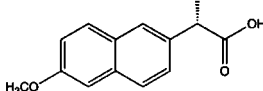
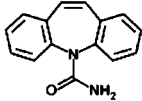
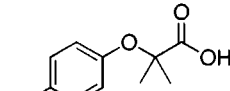
	Ketoprofen	Naproxen	Carbamazepine	Clofibric acid
CAS number	22071-15-4	22204-53-1	000298-46-4	882-09-7
Use	Non-steroidal anti-inflammatory drugs		Anti-epileptic drug	Lipid regulator
Chemical structure				

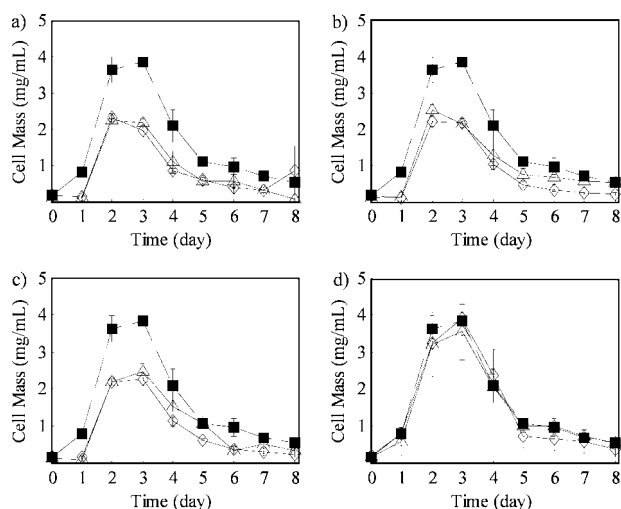
table at room temperature (approximately 20 °C). Oxygen level was measured periodically and remained in the range of 5–6 mg/L throughout the experimental phase. Ketoprofen was obtained from Sigma Aldrich (St. Louis, MO). Clofibric acid, naproxen, and carbamazepine were obtained from MP Biomedicals (Aurora, OH). All compounds were ACS grade.

**Microbial Growth Measurements.** Liquid samples were taken daily over a period of 7 days and monitored spectrophotometrically. All samples were collected under sterile conditions using a Labconco Purifier Class II biosafety cabinet (Kanasa, MO). Optical density was measured at 600 nm using a Hach DR/4000 U spectrophotometer (Loveland, CO). The spectrophotometric results were converted to dry cell mass based on a standard curve (see Supporting Information).

**Specific Oxygen Uptake Rate Measurements.** The specific oxygen uptake rate (SOUR) was calculated for each PhAC using standard methods as described in the Supporting Information (16).

**Nucleic Acid Analysis.** Cell samples were harvested from each reactor on days 0, 2, 4, 6, and 8. The details of sampling and extraction are presented in the Supporting Information. Denaturing gradient gel electrophoresis (DGGE) analyses were performed on the variable V3 region of the bacterial 16S rDNA using nested PCR products according to Muzer et al. (17); see also Table S1 in the Supporting Information. A D-code universal mutation detection system (Bio-Rad Laboratories, Hercules, CA) was used. A detailed protocol is presented in the Supporting Information.

**High-Pressure Liquid Chromatography.** PhAC concentrations were monitored by high-pressure liquid chromatography (HPLC). Samples totaling 500  $\mu$ L were collected from each reactor every two days. Prior to analysis, biomass was removed by filtering each sample using a VWR 0.2  $\mu$ m porosity polypropylene filter (Westchester, PA). A Prostar liquid chromatograph (Varian Inc., Palo Alto, CA) equipped with a 250 mm C-18 column (Alltech Inc., Newark, Delaware) was used for the analysis. All four PhACs 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  $\mu$ L were used. Wavelengths were optimized after performing a scan (Cary Bio100 UV spectrophotometer, Varian Inc., Palo Alto, CA). Absorbance wavelengths of 260, 230, 230, and 220 nm were used for ketoprofen, naproxen, clofibric acid, and carbamazepine, respectively. Using this protocol, the recovery rates for ketoprofen, naproxen, clofibric acid, and carbamaz-



**FIGURE 1.** Optical density at 600 nm ( $OD_{600}$ ) of bacteria in 0.2% (v/v) ethanol with 10  $\mu$ M PhAC (empty diamond), with 100  $\mu$ M PhAC (empty triangle), and without PhAC (solid square). (a) Ketoprofen. (b) Naproxen. (c) Carbamazepine. (d) Clofibric acid.

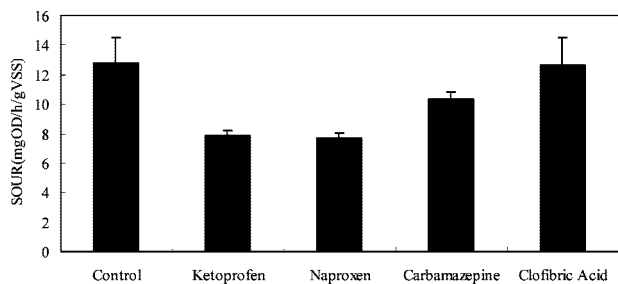
epine were 81%, 85%, 92%, and 99%, respectively. The HPLC method was a modification of previously published protocols (18, 19).

**Statistical Analysis.** Standard deviations were calculated and are shown in the figures. The student *t* test was used to assess the significance of the result with a 90% confidence interval.

## Results and Discussion

**Effects on Microbial Growth.** Microbial growth was lower in the presence of ketoprofen, naproxen, and carbamazepine as compared to the ethanol-only control at both the low (10  $\mu$ M) and high (100  $\mu$ M) PhAC concentrations in the presence of 0.2% (v/v) ethanol (Figure 1a–c). Overall cell concentration was statistically significant from days 3–6 with a 90% confidence interval. Maximum growth inhibition was observed on day 3 and resulted in a 42, 43, and 34% cell concentration decrease in the presence of ketoprofen, naproxen, and carbamazepine, respectively. No effect was observed in the presence of clofibric acid (Figure 1d). 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



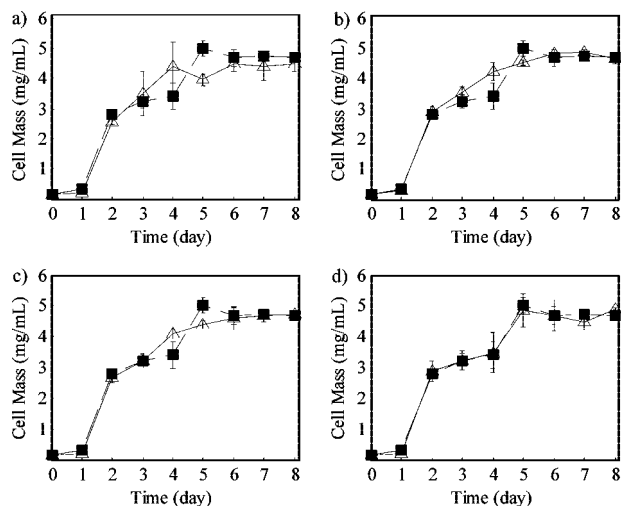
**FIGURE 2.** Specific oxygen uptake rate of activated sludge in the presence of 10  $\mu\text{M}$  PhAC.

0.2% (v/v) ethanol, microbial growth was independent of PhAC concentration in the 10 to 100  $\mu\text{M}$  range. The growth curves for the 10 and 100  $\mu\text{M}$  PhAC concentrations are virtually identical (Figure 1). Additional experiments should be carried out to determine if this observation holds true outside of this range. In addition to the decreased microbial growth, a 39, 39, and 19% decrease in SOUR was observed in the presence of 10  $\mu\text{M}$  naproxen, ketoprofen, and carbamazepine, respectively (Figure 2). No statistically significant difference was observed with clofibric acid (Figure 2). Similar results were obtained in the presence of 100  $\mu\text{M}$  PhAC concentrations (results not shown). 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.

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 different microbial culture and/or experimental platform. Kumagai et al. (20) showed that 983  $\mu\text{M}$  ketoprofen resulted in 50% 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. Nonetheless, this result suggests that microbial growth inhibition and PhAC concentration are not linearly correlated. Thus, it is possible that significant effects on overall microbial growth could still occur at even lower concentrations than those tested in the present study.

Kruszewska et al. (21) 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. (22). In that study, 10  $\mu\text{g/L}$  carbamazepine reduced the amount of bacterial biomass produced on riverine biofilms. Dokianakis et al. (23) reported that carbamazepine did not significantly inhibit nitrifiers. Thus, it is possible that carbamazepine may affect other microbial populations in the activated sludge process.

No inhibition was observed in the presence of clofibric acid at either the 10 or 100  $\mu\text{M}$  concentration. Dokianakis et al. (23) 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 (24, 25). Thus, the absence of inhibition in our tests may indicate that microorganisms in activated



**FIGURE 3.** OD<sub>600</sub> of bacteria in 2% (v/v) ethanol with 100  $\mu\text{M}$  PhAC (empty triangle) and without PhAC (solid square). (a) Ketoprofen. (b) Naproxen. (c) Carbamazepine. (d) Clofibric acid.

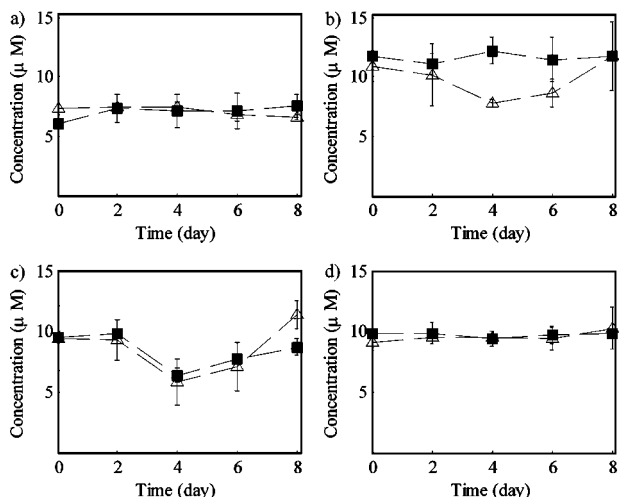
sludge are less sensitive to clofibric acid than the indicator organisms used in the toxicity tests.

**Organic Loading and Microbial Growth Inhibition.** In the presence of 2% (v/v) ethanol (Figure 3), the microbial growth curves have distinctively different shapes as compared to the 0.2% (v/v) ethanol case (Figure 1). In the presence of the higher ethanol concentration, no significant effect was observed even at the highest PhAC concentration (100  $\mu\text{M}$ ) for all four PhACs. There have been reports of more rapidly growing microorganisms outgrowing slower microorganisms in high organic loading environments (26). Rossello-Mora et al. (27) reported that high organic loading in sewage plants was linked to a high level of *Zoogloea ramigera*, a Gram negative bacillus. *Zoogloea* 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. Additional experiments are currently underway to verify this hypothesis.

Our data suggest that PhAC inhibition is correlated to the concentration of biodegradable carbon present in a given sample rather than the absolute PhAC concentration. This result has important implications as it suggests that PhACs might not inhibit microbial growth in treatment processes with organic loadings greater than some threshold values 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 used in this study. More extensive studies are needed to test additional organic substrates, PhACs, as well as the loading range to determine if the results obtained in this study can be extended to other compounds and to determine which microbial species are impacted by PhACs.

**PhAC Removal.** No significant PhAC degradation was observed throughout the experiment in either experimental or control reactors (Figure 4). 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 (28, 29). The exact mechanisms are not known for the four PhACs investigated in this research.

Quintana et al. (30) reported biodegradation for ketoprofen and naproxen in an aerobic membrane bioreactor. However the biodegradation was reported over a period of 28 days which is considerably longer than the experimental

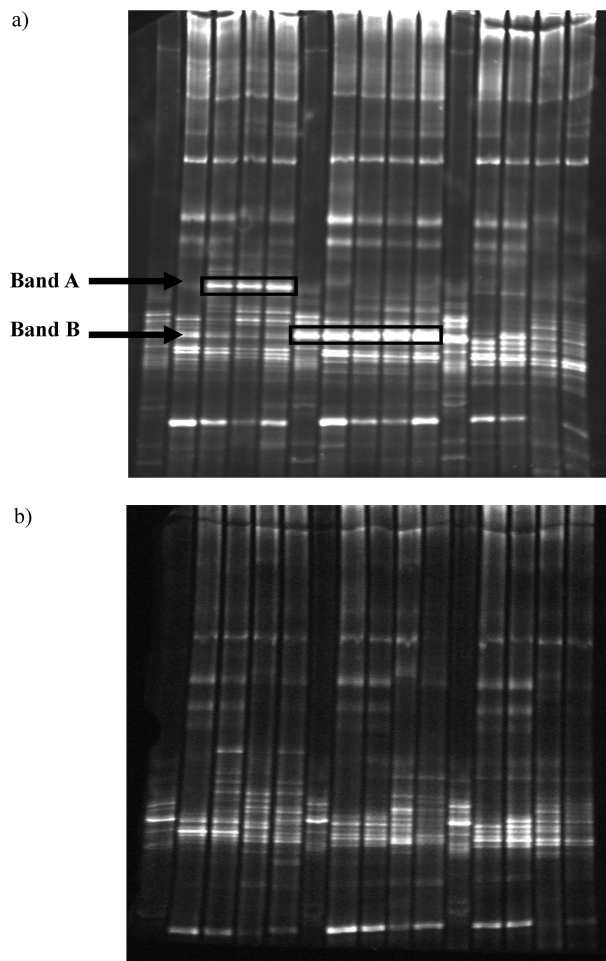


**FIGURE 4.** Concentrations of 10  $\mu\text{M}$  PhAC in 0.2% (v/v) ethanol in the presence of bacteria (empty triangle) and without bacteria (solid square). (a) Ketoprofen. (b) Naproxen. (c) Carbamazepine. (d) Clofibric acid.

time frame presented herein (i.e., 1 week). The experimental setup in our study was considerably different (batch reactors as compared to a continuous flow bioreactor), which makes a comparison between these two studies difficult. It is likely that the batch cultures in the present study suffered a certain amount of die-off, resulting in the decline of the activity at the later stages of incubation. In addition, nutrients were only introduced at the beginning of the experimental phase which may have contributed to a decline in microbial activity especially for the low organic concentration treatment.

**Microbial Community Fingerprinting.** Because a decrease in overall microbial growth was only observed under low organic loading (Figure 1), 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 5a). No significant microbial community changes were observed for either carbamazepine or clofibric acid (Figure 5b). The clofibric acid result was expected since no significant difference was observed in either microbial growth or SOUR experiments. However, microbial shifts were expected in the presence of carbamazepine, and thus this result is surprising. However, because of the inherent PCR biases which come with DGGE analysis (31) as well as its aforementioned qualitative nature, it is possible that the microbial communities affected by carbamazepine simply are not detected. It is anticipated that other methods, such as stable isotope probing, which overcome PCR biases (32) will be used in the follow up experiments.

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 bands A and B, respectively (Figure 5a). 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 microbial population and thus enrichment.



**FIGURE 5.** DGGE results of 10  $\mu\text{M}$  PhAC with 0.2% ethanol (v/v). (a) From left to right: ketoprofen on day 0, 2, 4, 6, 8; naproxen on day 0, 2, 4, 6, 8; ethanol control on day 0, 2, 4, 6, 8; (b) carbamazepine on day 0, 2, 4, 6, 8; clofibric acid on day 0, 2, 4, 6, 8; ethanol control on day 0, 2, 4, 6, 8.

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 (33). Their survival is thought to be linked to their increased levels of antibiotic resistance as compared to other bacterial species (33). In addition, some strains of *Acinetobacter* have been reported in wastewater treatment (34). Thus, it is quite probable that *Acinetobacter* spp. were present in the inoculum which was obtained from a municipal WWTP. Work is underway to purify the enriched strains and study their respective sensitivity to naproxen and ketoprofen.

### Acknowledgments

This research was funded by the Pratt School of Engineering at Duke University. We thank the National Science Foundation for supporting Ryan M. Holzem under Grant No. EEC-0139460. Any opinions, findings and conclusions, or recommendations expressed in this material are those of the authors and do not necessarily reflect the views of the National Science Foundation.

## Supporting Information Available

Detailed information showing the correlation between OD<sub>600</sub> and dry weight as well as SOUR, DNA extraction, and DGGE protocols. This material is available free of charge via the Internet at <http://pubs.acs.org>.

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ES072026X