

The influence of six pharmaceuticals on freshwater sediment microbial growth incubated at different temperatures and UV exposures

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Abstract Pharmaceutical compounds have been detected in freshwater for several decades. Once they enter the aquatic ecosystem, they may be transformed abiotically (i.e., photolysis) or biotically (i.e., microbial activity). To assess the influence of pharmaceuticals on microbial growth, basal salt media amended with seven pharmaceutical treatments (acetaminophen, caffeine, carbamazepine, cotinine, ibuprofen, sulfamethoxazole, and a no pharmaceutical control) were inoculated with stream sediment. The seven pharmaceutical treatments were then placed in five different culture environments that included both temperature treatments of 4, 25, 37°C and light treatments of continuous UV-A or UV-B exposure. Microbial growth in the basal salt media was quantified as absorbance (OD₅₅₀) at 7, 14, 21, 31, and 48d following inoculation. Microbial growth was significantly influenced by pharmaceutical treatments ($P < 0.01$) and incubation treatments ($P < 0.01$). Colonial morphology of the microbial communities post-incubation identified selection of microbial and fungal species with exposure to caffeine, cotinine, and ibuprofen at 37°C; acetaminophen, caffeine, and cotinine at 25°C; and carbamazepine exposed to continuous UV-A. Bacillus and coccus cellular arrangements (1000X magnification) were consistently observed across incubation treatments for each pharmaceutical

treatment although carbamazepine and ibuprofen exposures incubated at 25°C also selected spiral-shaped bacteria. These data indicate stream sediment microbial communities are influenced by pharmaceuticals though physiochemical characteristics of the environment may dictate microbial response.

Keywords Pharmaceutical · Microbial · UV · Temperature

Introduction

Pharmaceuticals and personal care products have become ubiquitous contaminants in the aquatic environment (Barnes et al. 2008; Focazio et al. 2008; Glassmeyer et al. 2005; Kolpin et al. 2002; Kolpin et al. 2004). Primary sources of pharmaceutical compounds in freshwaters include human excretion in sewage, drug disposal, runoff associated with animal agriculture, and releases from medicated feeds in aquaculture (Ellis 2006; Jorgensen 2000). A multitude of drug classes are represented in these contaminants and consist of analgesics, veterinary and human antibiotics, stimulants, lipid regulators, and insect repellants (Jorgensen 2000). Although these compounds were only recognized as freshwater contaminants in the 1970s (Tabak and Bunch 1970), they have been used by humans for several decades and

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likely have been present in wastewater since their initial widespread development (Ellis 2006).

Pharmaceuticals have been detected in freshwaters throughout the United States (Barnes et al. 2008; Focazio et al. 2008; Glassmeyer et al. 2005; Kolpin et al. 2002; Kolpin et al. 2004) and in numerous other regions of the world (e.g., Camacho-Muñoz et al. 2010; Daneshvar et al. 2010; Sim et al. 2010; Vieno et al. 2005). Although the primary source of these contaminants is thought to be from wastewater effluent, pharmaceuticals are also found in freshwaters not influenced by wastewater point sources (i.e., industrial and agricultural areas) (Kolpin et al. 2002; Bunch and Bernot 2011). Pharmaceutical compounds most frequently detected in freshwater include acetaminophen, caffeine, carbamazepine, cotinine, ibuprofen, and sulfamethoxazole (Camacho-Muñoz et al. 2010; Daneshvar et al. 2010; Glassmeyer et al. 2005; Kolpin et al. 2002; Kolpin et al. 2004). Of these frequently detected compounds, acetaminophen and caffeine are the least recalcitrant; whereas, cotinine, carbamazepine, and sulfamethoxazole are more recalcitrant (Benotti and Brownawell 2009). Once these compounds enter the aquatic ecosystem, they may be transformed via sediment sorption, organismal assimilation, photolysis, or microbial activity (Jorgensen 2000).

Abiotic photodegradation has been documented as a degradation pathway for several contaminants (Andreozzi et al. 2003; Kim and Tanaka 2009; Lai and Hou 2008) though the effectiveness of photodegradation varies with specific compounds. Under UV irradiation (254 nm), acetaminophen and carbamazepine are more recalcitrant relative to sulfamethoxazole (Kim and Tanaka 2009). Andreozzi et al. (2003) conducted abiotic sunlight irradiation and UV-lamp experiments and found carbamazepine half-life is nearly 100 days during winter at latitudes of 50°N whereas sulfamethoxazole has a shorter half-life of 2.4 days under the same conditions. Further, the half-life of both carbamazepine and sulfamethoxazole can be reduced in the presence of nitrate (Andreozzi et al. 2003).

Biodegradation can also be a significant pathway for transforming pharmaceutical compounds in both wastewater treatment plants (Jones et al. 2005; Quintana et al. 2005; Zwiener et al. 2002) and in aquatic ecosystems (Benotti and Brownawell 2009; Bradley et al. 2007; Lawrence et al. 2005; Winkler

et al. 2001; Yamamoto et al. 2009). Although surface water communities of bacteria are less diverse and lower in number than in sewage treatment plants (Kummerer 2009), certain compounds can serve as a carbon source for microbial assimilation in any environment (Lawrence et al. 2005; Winkler et al. 2001). Multiple physiochemical factors likely influence the rate and degradation potential of pharmaceuticals in natural ecosystems. For example, Zwiener et al. (2002) found ibuprofen degradation and production of metabolites was dependent on oxygen conditions in microbial bioreactors. Further, pharmaceutical degradation rates are higher in more eutrophic waters, perhaps due to a higher number of bacteria or differences in microbial communities (Benotti and Brownawell 2009). Temperature also may influence microbial degradation of pollutants. For example, Manzano et al. (1999) measured microbial degradation of surfactants in river water and found lower temperatures (21°C) reduced degradation rates.

A better understanding of microbial responses to the presence of pharmaceutical contaminants in the aquatic environment is needed to describe which compounds influence microbial communities through toxicity, stimulation, or assimilation of pharmaceuticals.

The objective of this study was to assess microbial growth in response to exposure to frequently detected pharmaceuticals in freshwater ecosystems under different temperature and light conditions using a nutrient-minimal media amended with pharmaceuticals and inoculated with stream sediment microbial communities. We hypothesized that 1) moderate temperature treatment (e.g., 25°C) would yield the highest microbial growth rates in comparison to low (4°C) and high (37°C) temperature treatments; and, 2) UV exposure would yield higher microbial growth rates relative to temperature treatments due to photolytic degradation of pharmaceutical compounds yielding more labile compounds.

Materials and methods

Media preparation, inoculation, and incubation

A defined basal salts broth media (BSM), amended with pharmaceutical treatments, was prepared to act as a nutrient-minimal media. BSM promotes the growth of organisms that can utilize amended pharmaceuticals as

a potential carbon, nitrogen, or sulfur source. The broth BSM consisted of 1.6 g/l K_2HPO_4 , 0.4 g/l KH_2PO_4 , 0.1 g/l NaCl, 1 g/l sucrose, 1 g/l glucose, and 1 g/l Na-citrate added to 986 ml of deionized water and autoclaved. Autoclaved broth was then separated into individual 100 ml aliquots in autoclaved glass containers for subsequent aseptic addition of five additional stock solutions via a sterile pipette. One ml/l of a 10 mg/l $ZnSO_4 \times 7H_2O$, 0.01 mg/l $CuSO_4 \times 5H_2O$, 30 mg/l $CoCl_2$, 10 mg/l $MnSO_4 \times H_2O$ stock solution was added. Subsequently, 10 ml/l of a 0.5 g/l $MgSO_4 \times 7H_2O$ stock solution was then added. One ml/l of a 0.1 g/l yeast extract solution, one ml/l of a 0.1 g/l $CaCl_2$, and 1 ml/l of a 10 mg/l $FeSO_4 \times 7H_2O$ stock solution was added. At the termination of the experiment, solid BSM was prepared as above with the inclusion of 15 g/l of agar for inspection of the established microbial communities.

Pharmaceuticals were subsequently added to prepared broth BSM aseptically. A total of seven pharmaceutical-amended broth BSM treatments were prepared including acetaminophen (500 ng/l), caffeine (500 ng/l), carbamazepine (10 ng/l), cotinine (50 ng/l), ibuprofen (50 ng/l), sulfamethoxazole (10 ng/l), and a control treatment (no added pharmaceutical). Pharmaceutical amendment concentrations were selected to represent the highest environmentally-relevant concentrations (Bunch and Bernot 2011, Focazio et al. 2008; Glassmeyer et al. 2005; Kolpin et al. 2002; Kolpin et al. 2004). Ten ml of prepared pharmaceutical-amended basal salt broth was aseptically transferred to sterile glass test tubes ($N = 7$ per pharmaceutical treatment) under a laminar flow hood prior to inoculation. To obtain a natural community of microbes present in freshwater streams of central Indiana, sediment was collected from the top 10 cm of the stream benthos at Killbuck Creek in east-central Indiana. Killbuck Creek is a third-order stream influenced predominantly by agricultural input and septic systems and has a mean temperature of 12°C and temperature ranges of 0–27°C (see Veach and Bernot 2011). After sediment collection, the sediment was homogenized and debris and macroinvertebrates were removed using a USGS no. 6 sieve (2.35 mm pore size) followed by equilibration at room temperature for ~48 h prior to inoculation. A flamed inoculating loop was submerged into the homogenized sediment and aseptically transferred to a sterile test tube containing pharmaceutical-amended basal

broth under a laminar flow hood with repeated submerging and transfer for each sterile test tube. Once all media were inoculated with sediment, test tubes were randomly assigned incubation treatments. Each pharmaceutical treatment was exposed to five different stationary incubation treatments including incubation at 4, 25, and 37°C. In order to understand effects of UV light on microbial growth rates when exposed to different pharmaceuticals, there were two additional incubation treatments consisting of a continuous UV-A exposure under a 150 wattage [®]Exo terra Sun glo bulb at 38°C and a UV-B exposure under a 160 wattage [®]Solar Brite Hg vapor bulb at 31°C resulting in a total of 210 test tubes ($N = 7$ pharmaceutical treatments; $N = 5$ incubation treatments). Six replicate test tubes were prepared for each pharmaceutical treatment and each incubation treatment using a factorial design ($N = 210$). All tubes incubated under different temperature treatments were wrapped in aluminum foil to prevent any light from reaching the medium.

Absorbance measurements

Turbidity measured via absorbance was used as a proxy for microbial growth (Talaro 2008) and has been used at wavelengths of 550 nm to identify microbial cell density (Ogunsetian 1996). Absorbance (550 nm) was measured using a Shimadzu dual-beam spectrophotometer (UV-1700 Pharmaspec) at 7, 14, 21, 31, and 48 d after inoculation. The spectrophotometer was zeroed with control basal salt media to quantify any changes in turbidity. At every measurement, 5 ml of media was aseptically transferred from each individual test tube under a laminar flow hood to cuvettes for measurement on the spectrophotometer. After media transfer to cuvettes, 5 ml of pharmaceutical-amended fresh sterile medium was replaced for continued incubation.

Colony and cellular measurements

At the last turbidity measurement (48 d), broth BSM was aseptically transferred from test tubes and streaked to prepared solid basal salt media using a flamed inoculating loop under a laminar flow hood. Three plates were prepared for each pharmaceutical treatment ($N = 7$) at each incubation treatment ($N = 5$) for a total of 105 agar plates. Once all plates were inoculated, they were placed at the original

incubation regimes. Microbial colony morphology was evaluated for both UV-A and UV-B treatments at 4 d after plating, 37°C treatments at 5 d after plating, 25°C treatments at 6 d after plating, and 4°C treatments at 36 d after plating to ensure substantial growth had occurred.

Also following the final turbidity measurement, Gram stains were prepared to determine cellular arrangements of bacteria colonies. One Gram stain was prepared for each pharmaceutical treatment and each incubation treatment yielding a total of 34 slides prepared. The slide prepared for caffeine at 25°C was broken therefore no cellular arrangements are provided.

Statistical analyses

Microbial growth rates were calculated for each replicate as the linear change in absorbance over time (absorbance/d). Repeated measures analysis of variance (ANOVA) was used to evaluate differences in absorbance among incubation treatments within a pharmaceutical treatment. One-way ANOVA was used to evaluate differences in microbial growth rates among incubation treatments independent of pharmaceutical treatment. Also, one-way ANOVA was used to evaluate differences in microbial growth rates among pharmaceutical treatments across temperature incubation treatments and across UV incubation treatments. Repeated measures ANOVA was conducted using SPSS (®SPSS 17.2); One-way ANOVA was conducted using Minitab 16 (Minitab® Inc. 2010, USA).

Results

Turbidity measurements

Overall, absorbance increased throughout the experiment for all treatments. An increase in absorbance for the duration of the experiment suggests that microbial communities in all treatments were able to sustain

growth on the BSM. However, significant differences in relative rates of growth were observed among both pharmaceutical treatments and incubation treatments.

Pharmaceutical treatment ($P < 0.01$) and incubation treatment ($P < 0.01$) significantly influenced microbial growth as increased turbidity (Table 1). However, there was a significant interaction between pharmaceutical and incubation treatment ($P < 0.01$) (Table 1). Thus, pharmaceuticals differentially influenced microbial growth depending on the incubation treatment.

Overall, incubation treatments significantly influenced microbial growth rates (Fig. 1). Specifically, UV-A exposure yielded higher microbial growth rates (mean = 0.026 abs/d) than all temperature incubation treatments ($P < 0.01$). UV-B exposure (0.019 abs/d) resulted in higher microbial growth rates than 4°C (0.01 abs/d) and 37°C (0.007 abs/d) incubation treatments; whereas, 25°C (0.015 abs/d) treatments had higher growth rates than 37°C treatments. All incubation treatments, with the exception of 4°C incubation, had higher microbial growth rates than the 37°C incubation treatments (Fig. 1).

Across temperature incubation treatments (4, 25, 37°C), only incubation at 4°C resulted in significant differences in microbial growth among pharmaceutical treatments ($P < 0.01$; Fig. 2). Under 4°C incubation, both the control (0.012 abs/d) and cotinine treatment (0.014 abs/d) had ~2-fold increase in microbial growth compared to acetaminophen (0.007 abs/d) and caffeine (0.007 abs/d) ($P < 0.01$).

Across UV incubation treatments (UV-A, UV-B), UV-B exposure resulted in significant differences in microbial growth among pharmaceutical treatments (Fig. 3; $P < 0.01$). Specifically, ibuprofen treatments (0.042 abs/d) had higher microbial growth rates than control (0.006 abs/d), cotinine (0.013 abs/d), and sulfamethoxazole (0.007 abs/d) treatments ($P < 0.01$) with control (no pharmaceutical addition) treatments having the lowest microbial growth rate. No significant differences in microbial growth rates among pharmaceutical

Table 1 Repeated measures ANOVA ($N = 208$) comparing effects and interactions between pharmaceutical and incubation treatments for the duration of the experiment

Statistical test	Source	DF	Type III sum of squares	Mean square	F statistic	P value
Repeated Measures	Pharmaceutical	6	2.492	0.415	3.217	0.05
	Treatment	4	44.418	11.105	86.028	<0.01
	Pharmaceutical \times treatment	24	11.573	0.482	3.736	<0.01

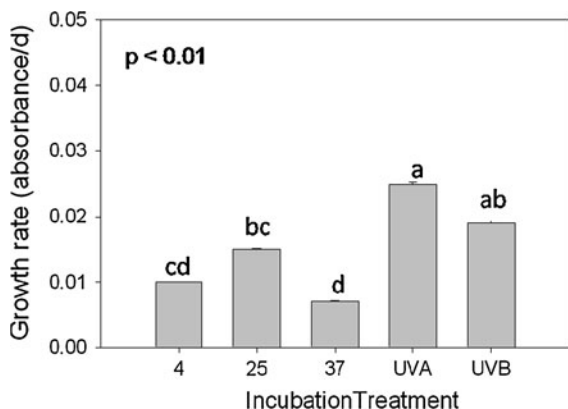


Fig. 1 Differences in microbial growth rates in response to incubation treatments of 4, 25, 37°C, UV-A exposure at 38°C, and UV-B exposure at 31°C. $N = 42 \pm$ S.E. for each bar

treatments were found with UV-A exposure ($P = 0.87$) (Fig. 3).

Colonial and cellular morphology

At the termination of the experiment, all broth BSM except those incubated at 4°C, contained a black precipitate and produced hydrogen sulfide as evidenced by the sulfide smell. Colonial morphology of the microbial communities varied among pharmaceutical and incubation treatments. Cellular shapes of bacillus and coccus were consistently identified across incubation treatments for each pharmaceutical treatment (Table 2). For example, caffeine treatments yielded single coccus, diplococci, streptococci, single bacillus, diplobacilli, and streptobacilli shapes whereas cotinine treatments also contained cocci tetrad configurations. Colony surface configuration for all pharmaceutical and incubation treatments had smooth configuration although only 25, 37°C, and UV-A incubation treatments exhibited filamentous or rhizoid margin configurations across pharmaceutical treatments (Table 2). All pharmaceutical treatments, except acetaminophen and carbamazepine, had rhizoid or filamentous margin configurations at 37°C. At 25°C incubation, acetaminophen, caffeine, and cotinine treatments had fungal configurations (hyphae) in addition to carbamazepine treatments under UV-A exposure. All Gram stains prepared were Gram positive across treatments with no Gram negative cells observed. Cellular arrangements ranged from having single coccus and bacillus, diplococcus and diplobacillus, and both streptococcus and streptobacillus. Only carbamazepine and

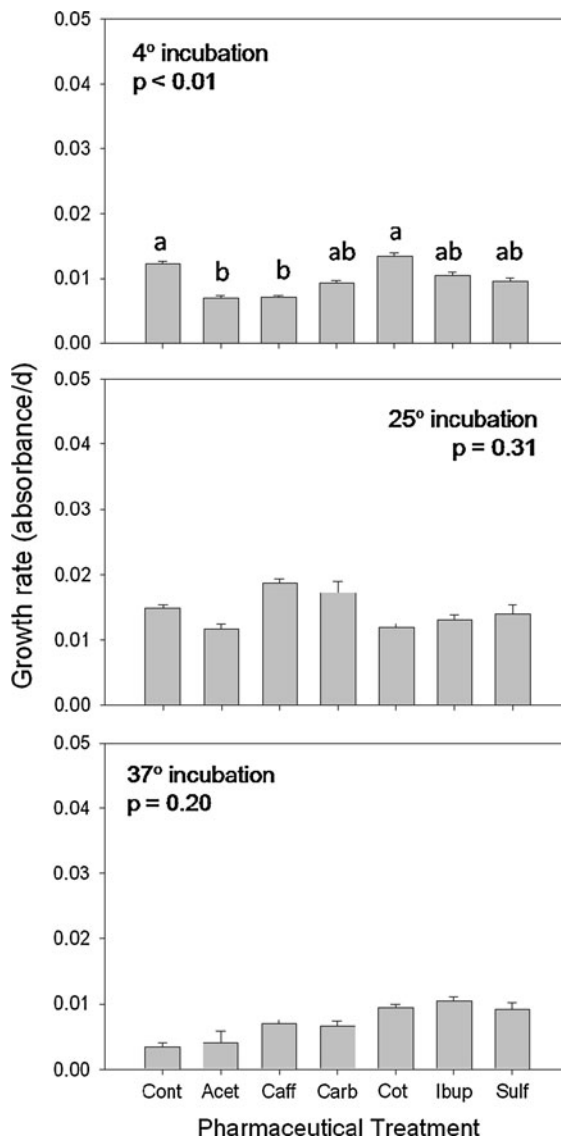


Fig. 2 Differences in microbial growth rates in response to pharmaceutical treatments for temperature incubation treatments of 4, 25, and 37°C. $N = 6 \pm$ S.E. for each bar. Significant differences among pharmaceutical treatments were identified within 4°C incubation treatments ($P < 0.01$)

ibuprofen treatments incubated at 25°C contained spiral-shaped organisms in addition to coccus and bacillus arrangements.

Discussion

Turbidity of a solution measures microbial growth within a medium and can be analyzed via sensitive

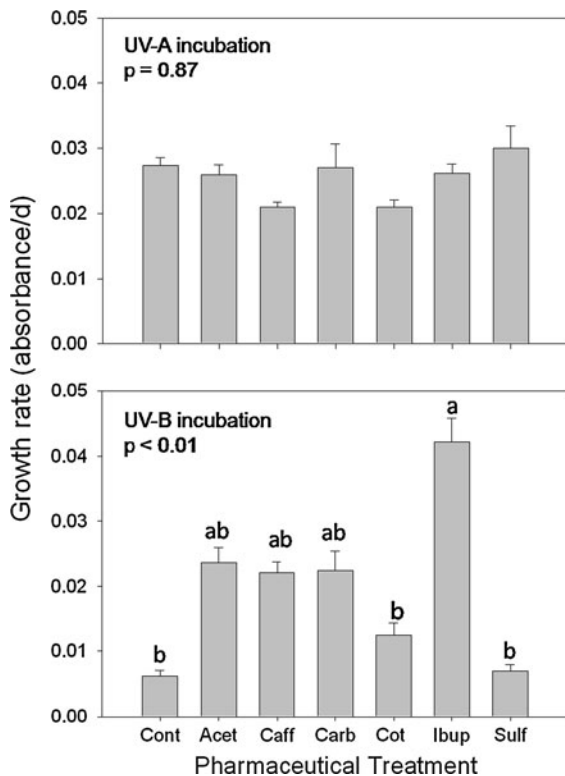


Fig. 3 Differences in microbial growth rates in response to pharmaceutical treatments for UV-A and UV-B incubation treatments. $N = 6 \pm$ S.E. for each bar. Significant differences among pharmaceutical treatments were identified for the UV-B incubation treatment ($P < 0.01$). Significant pairwise comparisons among pharmaceutical treatments denoted by letters

instruments such as spectrophotometers (Talaro 2008). Carbon (e.g., sucrose, glucose) sources were incorporated into basal salt media to promote initial microbial growth. However, amended pharmaceuticals were added in addition to sucrose and glucose to determine microbial growth in the presence of these compounds. Thus, measuring turbidity via absorbance was used as a proxy for analyzing the rate of microbial use of the pharmaceutical compounds.

Changes in microbial growth in response to pharmaceuticals observed in this study, may have been due to toxicity, stimulation, or assimilation of pharmaceutical compounds. Studies investigating toxicological effects of pharmaceuticals on certain microbial organisms (e.g., *Microtox* sp.) have found acute EC_{50} values of over 80 mg/l (Ferrari et al. 2003). However, there is a lack of research in ecotoxicology of pharmaceuticals on microbial communities at environmentally relevant concentrations so low pharmaceutical concentrations

used in this study may potentially be suppressing growth due to toxicity. Conversely, certain pharmaceuticals may not have any ecotoxicological effect and be stimulatory thereby increasing microbial growth. Previous studies have found many microbial species are able to break down pharmaceutical contaminants (Murdoch and Hay 2005; Ogunsetian 1996). For example, *Sphingomonas* sp. can catabolize ibuprofen and use metabolites as a nutritive source (Murdoch and Hay 2005). Similarly, *Pseudomonas putida* isolated from sewage can grow with caffeine as a sole carbon source (Ogunsetian 1996). Multiple microbial species found in freshwater sediment can likely assimilate these novel contaminants due to their ability to quickly adapt, potentially reducing pharmaceutical contamination in freshwater through degradation. The numerous microbial organisms sustained over the incubation period suggest that multiple microbial species in freshwater sediment are not inhibited by pharmaceuticals.

Temperature and UV effects on microbial growth

In agreement with our hypothesis, 25°C incubation treatments had higher microbial growth rates than 37°C incubation treatments suggesting moderate temperatures foster microbial growth more than higher temperatures for these communities. In addition, mean microbial growth rates at 25°C incubation did not differ from growth rates at 4°C incubation. Thus, higher temperatures may reduce microbial growth suggesting that there may be inhibition of growth during summer months. During summer months with higher temperatures, inhibition of microbial activity may foster persistence of pharmaceuticals in aquatic ecosystems. However, other in situ studies have documented higher concentrations of pharmaceuticals in freshwaters during winter relative to other times of the year (Daneshvar et al. 2010; Veach and Bernot 2011). The differences in temperature effects observed in this study relative to previous studies may be due to specific environmental factors not replicated in laboratory experiments. For example, this laboratory experiment selected for a less diverse microbial community through incubation treatments than what would be found in natural environments. Further, stream physiochemical characteristics such as water flow and dissolved oxygen were not maintained in test tube incubations as would be in a stream ecosystem.

Table 2 Colony morphology characteristics of temperature and UV treatments for pharmaceutical amended solid basal salt media

Compound	Treatment	Colony color	Configuration
Acetaminophen	4	White, translucent, yellow	Round, L-form, radiating margin
	25	White, translucent	Round, filamentous, rhizoid
	37	Translucent	Round
	UV-A	White, red	Round
	UV-B	White, translucent	Round, radiating margin, irregular and spreading
Caffeine	4	White, translucent, yellow	Round, L-form, radiating margin
	25	White, translucent	Round, filamentous, rhizoid
	37	Translucent	Round, rhizoid
	UV-A	White, red	Round
	UV-B	White, red	Round, irregular and spreading
Carbamazapine	4	White, translucent	Round, L-form, round with radiating margin
	25	White, translucent	Round
	37	Translucent	Round
	UV-A	White	Round, filamentous
	UV-B	White, red	Round, round with raised margin
Cotinine	4	White, translucent, yellow, red	Round, L-form
	25	White, translucent, red	Round, rhizoid
	37	Translucent	Round, rhizoid
	UV-A	White	Round
	UV-B	White, translucent	Round, raised margin
Ibuprofen	4	White, translucent, yellow	Round, L-form, radiating margin
	25	White, translucent	Round, L-form, radiating margin
	37	Translucent	Round, rhizoid, raised margin
	UV-A	White, red	Round, radiating margin
	UV-B	Yellow, black	Smooth, irregular and spreading, raised margin
Sulfamethoxazole	4	White, translucent, yellow	Round, L-form, radiating margin
	25	White, translucent	Round
	37	White, translucent, black	Round, L-form, rhizoid
	UV-A	White	Round
	UV-B	White, translucent	Round
Control	4	White, translucent, yellow	Round, L-form, radiating margin, raised margin
	25	Translucent	Round
	37	White, translucent	Round
	UV-A	White, red	Round
	UV-B	White, red, yellow	Smooth, raised margin

Table 2 continued

Compound	Treatment	Margin	Cellular arrangement
Acetaminophen	4	Smooth, ciliate	Single, diplo, streptococcus, single diplo, streptobacillus
	25	Smooth, branching, wooly	Single, diplococcus, single, diplococcus, tetrad
	37	Smooth, thread-like	Single, diplo, streptococcus, single diplobacillus
	UV-A	Smooth	Single, diplo, streptococcus, single diplobacillus
	UV-B	Smooth, irregular	Single, diplo, streptococcus, single diplobacillus
Caffeine	4	Smooth, ciliate	Single, coccus, single, diplo, streptobacillus
	25	Smooth, branching, wooly	NA
	37	Smooth, wooly	Single, streptococcus, diplobacillus
	UV-A	Smooth	Single, coccus, diplo, streptobacillus
	UV-B	Smooth	Single, coccus, single, diplococcus
Carbamazapine	4	Smooth, ciliate	Single, diplococcus, single, diplo, streptobacillus
	25	Smooth	Single diplo, streptococcus, single, diplo, streptobacillus, spirochete
	37	Smooth	Single, diplo, streptococcus, single, diplo, streptobacillus
	UV-A	Smooth, wavy, hairlock-like	Single, diplo, streptococcus, single, diplo, streptococcus
	UV-B	Smooth	Single, diplococcus, single, streptobacillus
Cotinine	4	Smooth, wavy	Single, diplo, streptococcus, single, diplobacillus
	25	Smooth, wooly	Single, diplo, streptococcus, single, diplo, streptobacillus
	37	Smooth, wooly, thread-like	Single, diplo, streptococcus, single, diplobacillus, tetrad
	UV-A	Smooth	Single, diplo, streptococcus, single, diplo, streptobacillus
	UV-B	Smooth	Single, diplococcus, single, diplo, streptobacillus
Ibuprofen	4	Smooth, ciliate	Single, diplo, streptococcus, single, diplo, streptobacillus
	25	Smooth	Single, diplo, streptococcus, diplobacillus, spirochete
	37	Smooth, wooly	Single, diplococcus, diplobacillus
	UV-A	Smooth	Single, diplococcus, single, diplo, streptobacillus
	UV-B	Smooth	Single, diplo, streptococcus, single, diplobacillus
Sulfamethoxazole	4	Smooth, ciliate, wavy	Single, diplo, streptococcus, single, streptobacillus
	25	Smooth	Single, diplo, streptococcus, single, diplo, streptobacillus
	37	Smooth, thread-like	Single, diplo, streptococcus, single, diplo, streptobacillus
	UV-A	Smooth	Single, diplo, streptococcus, single, diplobacillus
	UV-B	Smooth	Single, diplococcus, diplobacillus
Control	4	Smooth, ciliate, wavy	Single, diplococcus, single, diplo, streptobacillus
	25	Smooth	Single, diplo, streptococcus, single, diplobacillus
	37	Smooth	Single, diplo, streptococcus, single, diplobacillus
	UV-A	Smooth, wavy	Single, streptococcus, single, diplo, streptobacillus
	UV-B	Smooth	Single, diplo, streptococcus, single, diplo, streptobacillus

UV morphology traits were evaluated at 4 days after plating. Thirty-seven degree celsius morphology traits were evaluated at 5 days after plating. Twenty-five degree celsius morphology traits were evaluated 6 days after plating. Four degree celsius morphology traits were evaluated at 36 days after plating. All pharmaceutical treatments yielded both bacillus and coccus shapes

Across incubation treatments, UV-A exposure stimulated microbial growth more than temperature treatments, though growth under UV-A exposure was comparable to UV-B exposure. Although it has been documented that degradation via photolysis of parent pharmaceutical compounds can yield toxic

metabolites, some parent compounds may degrade into non-toxic, labile carbon compounds (Kummerer 2009). Therefore, parent pharmaceutical compounds in UV light may have been transformed via photo-degradation into labile products potentially allowing for microbes to use transformation products as a

nutritive source. Since the 1970s, large increases in UV radiation of 10–20% per decade (Madronich 1992) have occurred due to stratosphere loss of ozone. This study suggests that an increase in UV radiation due to depletion of ozone over time may foster microbial degradation of pharmaceutical contaminants.

Recalcitrant and labile pharmaceutical compounds

Studies investigating *in vitro* microbial growth of pharmaceutical classes (i.e., non-steroidal anti-inflammatory drugs and stimulants) with concentrations of alternative nutritive sources included in the medium are limited. However, other studies have evaluated growth rates within non sterile environmental water samples (Benotti and Brownawell 2009; Yamamoto et al. 2009). Benotti and Brownawell (2009) found that cotinine, sulfamethoxazole, and carbamazepine are persistent (half-lives >40 d) and resistant to microbial degradation in freshwaters whereas acetaminophen (half-life = 1.2–11 d) and caffeine (half-life = 3.5–13) quickly undergo microbial degradation in freshwater environments. Yamamoto et al. (2009) also found that carbamazepine and ibuprofen were resistant to microbial degradation in river water (half-lives \geq 120 h) but acetaminophen was more labile (half-life <120 h).

In contrast to previous studies, these data show low temperatures result in higher microbial growth rates with exposure to cotinine, relative to both acetaminophen and caffeine treatments (Fig. 2) which have been previously shown to be more labile (Benotti and Brownawell 2009; Winkler et al. 2001). Consistent with this study, Ogunsetian (1996) found that after an incubation period of 2 months at 25°C, caffeine (1 mg/ml) is not degraded when introduced into non-sterile creek water. Although previous studies have documented cotinine as resistant to microbial transformation (Benotti and Brownawell 2009), other studies have found it to be transformed via sediment microbial communities (Bradley et al. 2007). Therefore, under certain conditions, caffeine may be less readily degraded relative to cotinine. However, cotinine exposure yielded lower microbial growth rates when exposed to UV-B; therefore, it is likely that cotinine is easily metabolized only when coupled with photodegradation.

In contrast to previous studies, ibuprofen treatments in this study yielded significantly higher microbial growth under UV-B exposure relative to cotinine and

sulfamethoxazole treatments (Fig. 3). Quintana et al. (2005) found that when ibuprofen was the sole growth substrate, it was not transformed after 28 d. However, when an additional carbon source was added, co-metabolism of ibuprofen was completed at 22 d. Therefore, ibuprofen may be more easily metabolized when other carbon sources are available. Due to the presence of additional carbon sources in the basal salt media, this may explain higher growth rates observed in this study. In addition, transformation products of ibuprofen formed via photolysis may be more labile in comparison to cotinine and sulfamethoxazole potentially facilitating microbial growth.

Microbial acclimation to pharmaceutical input

The presence of pharmaceuticals in the location the sediment inoculum was collected has been previously documented (Veach and Bernot 2011). Pharmaceutical concentrations detected at this location had comparable concentrations (ng/l) to concentrations of pharmaceuticals amended to basal salt media. An acclimation period, defined by the amount of time taken to metabolize a compound after its addition, is a prerequisite before growth resulting from that compound occurs (Wiggins et al. 1987). Thus, microbial communities present in sediment inoculum under UV irradiation may have been acclimated to ibuprofen due to its presence in the aquatic environment. However, caffeine and acetaminophen were also present at the location the sediment inoculum was collected. Consequently, it would be expected for microbial communities to be acclimated to these latter compounds as well. Additionally, carbamazepine was not frequently detected at the sediment inoculum location yet it did not yield lower growth rates indicating that other factors are contributing to the ability of microbial communities to respond to pharmaceuticals.

Conclusions

Microbial growth was significantly influenced by pharmaceutical exposure though incubation treatments confounded effects highlighting the variability of pharmaceutical influence on microbial growth in natural ecosystems. Pharmaceutical concentrations used to amend basal salt media were comparable to field assessments of pharmaceuticals within aquatic

environments; hence, this study shows that low concentrations (ng/l) of pharmaceuticals may in fact alter natural microbial communities. The potential for microbial toxicity, stimulation, degradation, or assimilation of pharmaceuticals in freshwater ecosystems is likely dependent on multiple physiochemical properties of the surrounding environment and more research is needed to identify dominant controls of this important pathway.

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References

- Andreozzi R, Rafaela M, Nicklas P (2003) Pharmaceuticals in STP effluents and their solar photodegradation in aquatic environment. *Chemosphere* 50:1319–1330
- Barnes K, Kolpin D, Furlong E, Zaugg S, Meyer M, Barber L (2008) A national reconnaissance of pharmaceuticals and other organic wastewater contaminants in the United States I) groundwater. *Sci Total Environ* 402:192–200
- Benotti MJ, Brownawell BJ (2009) Microbial degradation of pharmaceuticals in estuarine and coastal seawater. *Environ Pollut* 157:994–1002
- Bradley PM, Barber LB, Kolpin DW, McMahon PB, Chapelle FH (2007) Biotransformation of caffeine, cotinine, and nicotine in stream sediments: implications for use as wastewater indicators. *Environ Toxicol Chem* 26:1116–1121
- Bunch AR, Bernot MJ (2011) Distribution of nonprescription pharmaceuticals in central Indiana streams and effects on sediment microbial activity. *Ecotoxicology* 20:97–109
- Camacho-Muñoz MD, Santos JL, Aparicio I, Alonso E (2010) Presence of pharmaceutically active compounds in DoñanaPark (Spain) main watersheds. *J Hazard Mater* 177:1159–1162
- Daneshvar A, Svanfelt J, Kronberg L, Weyhenmeyer G (2010) Winter accumulation of acidic pharmaceuticals in a Swedish river. *Environ Sci Pollut Res Int* 17:908–916
- Ellis JB (2006) Pharmaceutical and personal care products (PPCPs) in urban receiving waters. *Environ Pollut* 144:184–189
- Ferrari B, Paxeus N, Giudice R, Pollio A, Garric J (2003) Ecotoxicological impact of pharmaceuticals found in treated wastewaters: study of carbamazepine, clofibrac acid, and diclofenac. *Ecotoxicol Environ Safe* 55:359–370
- Focazio M, Kolpin D, Barnes K, Furlong E, Meyer M, Zaugg S, Barber L, Thurman M (2008) A national reconnaissance for pharmaceuticals and other organic wastewater contaminants in the United States II) untreated drinking water sources. *Sci Total Environ* 402:201–216
- Glassmeyer S, Furlong E, Kolpin D, Cahill J, Zaugg S, Werner S, Meyer M, Kryak D (2005) Transport of chemical and microbial compounds from known wastewater discharges: potential for use as indicators of human fecal contamination. *Environ Sci Technol* 39:5157–5169
- Jones OAH, Voulvoulis N, Lester JN (2005) Human pharmaceuticals in wastewater treatment processes. *Crit Rev Environ Sci Technol* 35:401–427
- Jorgensen SE (2000) Drugs in the environment. *Chemosphere* 40:691–699
- Kim I, Tanaka H (2009) Photodegradation characteristics of PPCPs in water with UV treatment. *Environ Int* 35:793–802
- Kolpin DW, Furlong ET, Meyer MT, Thurman EM, Zaugg SD, Barber LB, Buxton HT (2002) Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999–2000: a national reconnaissance. *Environ Sci Technol* 36:1202–1211
- Kolpin DW, Skopec M, Meyer M, Furlong E, Zaugg SD (2004) Urban contribution of pharmaceuticals and other organic wastewater contaminants to streams during differing flow conditions. *Sci Total Environ* 328:119–130
- Kummerer K (2009) The presence of pharmaceuticals in the environment due to human use—present knowledge and future challenges. *J Environ Manage* 90:2354–2366
- Lai H, Hou J (2008) Light and microbial effects on the transformation of four sulfonamides in eel pond water and sediment. *Aquaculture* 283:50–55
- Lawrence J, Swerhone G, Wassenaar L, Neu T (2005) Effects of selected pharmaceuticals on riverine biofilm communities. *Can J Microbiol* 51:655–669
- Madronich S (1992) Implications of recent total atmospheric ozone measurements for biologically active ultraviolet radiation reaching the Earth's surface. *Geophys Res Lett* 19:37–40
- Manzano MA, Perales JA, Sales D, Quiroga JM (1999) The effect of temperature on the biodegradation of a nonyl-phenol polyethoxylate in river water. *Water Res* 33:2593–2600
- Murdoch R, Hay A (2005) Formation of catechols via removal of acid side chains of ibuprofen and related aromatic acids. *Appl Environ Microbiol* 71:6121–6125
- Ogunsetian OA (1996) Removal of caffeine in sewage by *Pseudomonas putida*: implications for water pollution index. *World J Microbiol Biotechnol* 12:251–256
- Quintana JB, Weiss S, Reemtsma T (2005) Pathways and metabolites of microbial degradation of selected acidic pharmaceutical and their occurrence in municipal wastewater treated by a membrane bioreactor. *Water Res* 39:2654–2664
- Sim W, Lee J, Oh J (2010) Occurrence and fate of pharmaceuticals in wastewater treatment plants and rivers in Korea. *Environ Pollut* 158:1938–1947
- Tabak H, Bunch R (1970) Steroid hormones as water pollutants I. Metabolism of natural and synthetic ovulation-inhibiting hormones by microorganisms of activated sludge and primary settler sewage. *Dev Ind Microbiol* 11:367–376
- Talaro KM (2008) Foundations of microbiology. McGraw Hill, New York
- Veach AM, Bernot MJ (2011) Temporal variation of pharmaceuticals in an urban and agriculturally influenced stream. *Sci Total Environ* 409:4553–4563

- Vieno NM, Tuhkanen T, Kronberg L (2005) Season variation in the occurrence of pharmaceuticals in effluents from a sewage treatment plant in the recipient water. *Environ Sci Technol* 39:8220–8226
- Wiggins B, Jones S, Alexander M (1987) Explanations for the acclimation period preceding the mineralization of organic chemicals in the aquatic environment. *Appl Environ Microbiol* 53:791–796
- Winkler M, Lawrence JR, Neu TR (2001) Selective degradation of ibuprofen and clofibric acid in two model river biofilm systems. *Water Res* 35:3197–3205
- Yamamoto H, Nakamura Y, Moriguchi S, Nakamura Y, Honda Y, Tamura I, Hirata Y, Hayashi A, Sekizawa J (2009) Persistence and partitioning of eight selected pharmaceuticals in the aquatic environment: laboratory photolysis, biodegradation, and sorption experiments. *Water Res* 43:351–362
- Zwiener C, Seeger S, Glauner T, Frimmel FH (2002) Metabolites from the bio-degradation of pharmaceutical residues of ibuprofen in biofilm reactors and batch experiments. *Anal Bioanal Chem* 372:569–575