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Contamination profiles and mass loadings of macrolide antibiotics and illicit drugs from a small urban wastewater treatment plant

Bommanna Loganathan [a], Malia Phillips [a], Holly Mowery [a],
Tammy L Jones-Lepp[b*]

[a] Department of Chemistry and Center for Reservoir Research Murray State University,
Murray, KY 42071-3346, USA
[b] USEPA/ORD/NERL-ESD, P.O. Box 93478, Las Vegas, NV 89193-3478, USA
email: jones-lepp.tammy@epa.gov; fax 702-798-2142

*Author to whom correspondence should be addressed.

ABSTRACT

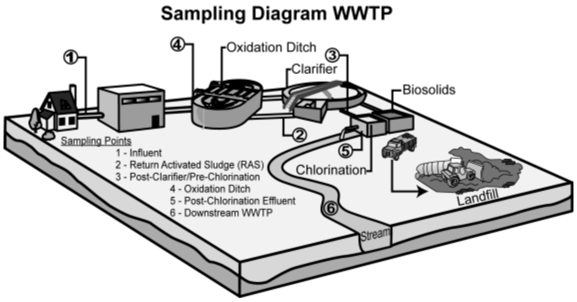
Information is limited regarding sources, distribution, environmental behavior, and fate of prescribed and illicit drugs. Wastewater treatment plant (WWTP) effluents can be one of the sources of pharmaceutical and personal care products (PPCP) into streams, rivers and lakes. The objective of this study was to determine the contamination profiles and mass loadings of urobilin (a chemical marker of human waste), macrolide antibiotics (azithromycin, clarithromycin, roxithromycin), and two drugs of abuse (methamphetamine and ecstasy), from a small (< 19 mega liters day^{-1}, equivalent to < 5 million gallons per day) wastewater treatment plant in southwestern Kentucky. The concentrations of azithromycin, clarithromycin, methamphetamine and ecstasy in wastewater samples varied widely, ranging from non-detects to 300 ng L^{-1}. Among the macrolide antibiotics analyzed, azithromycin was consistently detected in influent and effluent samples. In general, influent samples contained relatively higher concentrations of the analytes than the effluents. Based on the daily flow rates and an average concentration of 17.5 ng L^{-1} in the effluent, the estimated discharge of azithromycin was 200 mg day^{-1} (range 63 to 400 mg day^{-1}). Removal efficiency of the detected analytes from this WWTP were in the following order: urobilin > methamphetamine > azithromycin with percentages of removal of 99.9%, 54.5% and 47% respectively, indicating that the azithromycin and methamphetamine are relatively more recalcitrant than others and have potential for entering receiving waters.

Keywords: PPCPs, macrolide antibiotics, methamphetamine, ecstasy, wastewater, Kentucky

1. Introduction

The occurrence of pharmaceutical and personal care product (PPCP) residues in the environment has received considerable attention in the recent years as these compounds have been implicated for negative effect on biota and the ecosystem. PPCPs are considered emerging environmental pollutants, and have been detected in groundwater, surface water and municipal wastewater, fish and biosolids (Snyder et al., 2001; Kolpin et al., 2002; Mottaleb et al., 2004; Osemwengie and Gerstenberger, 2004; Petrovic et al., 2006; Pedrouzo et al., 2007; Cuderman and Heath, 2007; Kinney et al. 2006; Jones-Lepp and Stevens 2007). Earlier studies have implicated the presence of PPCPs in the development of antibiotic resistant bacteria, feminization of male fish, and acute toxicity and genotoxicity in aquatic organisms (Jobling et al., 1998; Daughton and Ternes, 1999; Daughton, 2001; Schwartz et al., 2003; Nash et al., 2004; Isidori et al., 2005; Jobling et al., 2006; Schwartz et al., 2006; Horii et al., 2007; Kostich and Lazorchak, 2008). Many pharmaceuticals are designed to be persistent and lipophilic so that they can retain their chemical structure in the organism (usually human or domesticated animals) long enough to do their therapeutic work. Consequently, after they are excreted such chemicals can persist in the environment and enter the food chain through bioaccumulation and biomagnification (Daughton and Ternes, 1999). For example, macrolide antibiotics, drugs that are used for therapeutic treatment of infectious disease in humans, have been reported in wastewaters, surface waters, sediments, biosolids, and in aquatic organisms (Hirsch et al. 1999; McArdel et al., 2003; Jones-Lepp et al., 2004; Kim and Carlson, 2007; Jones-Lepp and Stevens, 2007; Ramirez et al., 2007). While the ecotoxicological significance of drugs in environmental matrices, particularly water, has not been closely examined, it can only be surmised that these substances have the potential to adversely affect biota (e.g., bacteria, fish, amphibians, etc.) that are continuously exposed, even at very low levels. Further, the occurrence of antibiotic-resistant bacteria in waters receiving wastewater effluents is of great concern (Miyabara et al., 1995; Schwartz et al., 2003; Schwartz et al., 2006).

Very few studies have examined the contamination profiles and mass loadings of wastewater treatment plants (WWTPs) for prescribed and illicit drugs. For human-use antibiotics, WWTPs are considered the major source of release into the environment due to the partial removal efficiency in the treatment process (Kim and Carlson, 2007). The objectives of this research were to measure the contamination profiles and environmental loadings of three antibiotics and two illicit drugs from a small urban wastewater treatment plant in Kentucky. The target compounds were the macrolide antibiotics azithromycin, clarithromycin, and roxithromycin, and two illicit drugs methamphetamine and ecstasy (3,4-methylene dioxymethamphetamine, MDMA). We also measured urobilin as a chemical marker of human waste. Wastewater samples were collected during different times of the year to determine if seasonal differences occur in concentrations of the target analytes, and from different points within the WWTP to examine the removal efficiency of target compounds during the treatment process, and to determine the final amount of these compounds entering into receiving waters. The samples collected for this study were comprised of influent, effluent, return activated sludge (RAS), the oxidation ditch, and before and after chlorination, see figure 1. <Insert Figure 1. Simplified diagram WWTP>



The WWTP used in this study has a capacity to process 20 megaliters day^{-1} (MLd) of wastewater per day. Primarily, the combined sources of wastewater into this treatment plant are from homes, a hospital, university dormitories, and a small fraction of commercial and industrial sewage. This town has a local population of approximately 15,099 people (2002 U.S. Census), throughout the year (MCC Community Profile, 2007), a considerable portion of which are retirees, and then an influx of university students (10,275 students, academic year 2006-2007) during the academic year (mid-August to mid-May). Significant population fluctuations (about 40%) occur during a calendar year, especially during winter and summer breaks, during which the student population is at its minimum. Considering the nature of population, including elderly (retirement community) and university students, the use of antibiotics and illicit drugs are highly possible. According to recent Murray State statistics, twenty two possessions of marijuana, twenty possession of drug paraphernalia, one drug trafficking within 1000 yards of the university were reported, and drug-related violations are showing an increasing trend since 2005 (second only to theft) (Phelps, 2008a; Phelps 2008b).

The six compounds studied, fig 2, were chosen for their amenability to the methodologies used, and for socially-related reasons. Azithromycin is the most widely prescribed antibiotic (of any kind) in the United States (U.S.), and has been in the top 10 for the last six years (2001-2007) (http://drugtopics.modernmedicine.com/drugtopics/data/articlestandard//drugtopics/072008/491181/article.pdf and www.rxlist.com, top 200 prescribed drugs); clarithromycin is in the top 200 prescribed drugs (www.rxlist.com, top 200 prescribed drugs); roxithromycin, while not prescribed in the US, is widely used in Latin America and Europe, thereby lending itself as a marker of the importation of drugs by other than traditional means. The two illicit drugs (methamphetamine and MDMA) were chosen because of their reported use and limited environmental occurrence data (Zuccato et al., 2005; Jones-Lepp et al., 2004) and verifiable usage in the United States, especially MDMA amongst young adults (last accessed 31 July 2007, http://www.usdoj.gov/dea/pubs/ states/kentucky.html). Urobilin, previously studied as a chemical marker of human waste, was measured throughout the study for correlation to the extraction efficiencies, and was helpful in understanding removal efficiency of the WWTP (Jones-Lepp and Stevens, 2007).

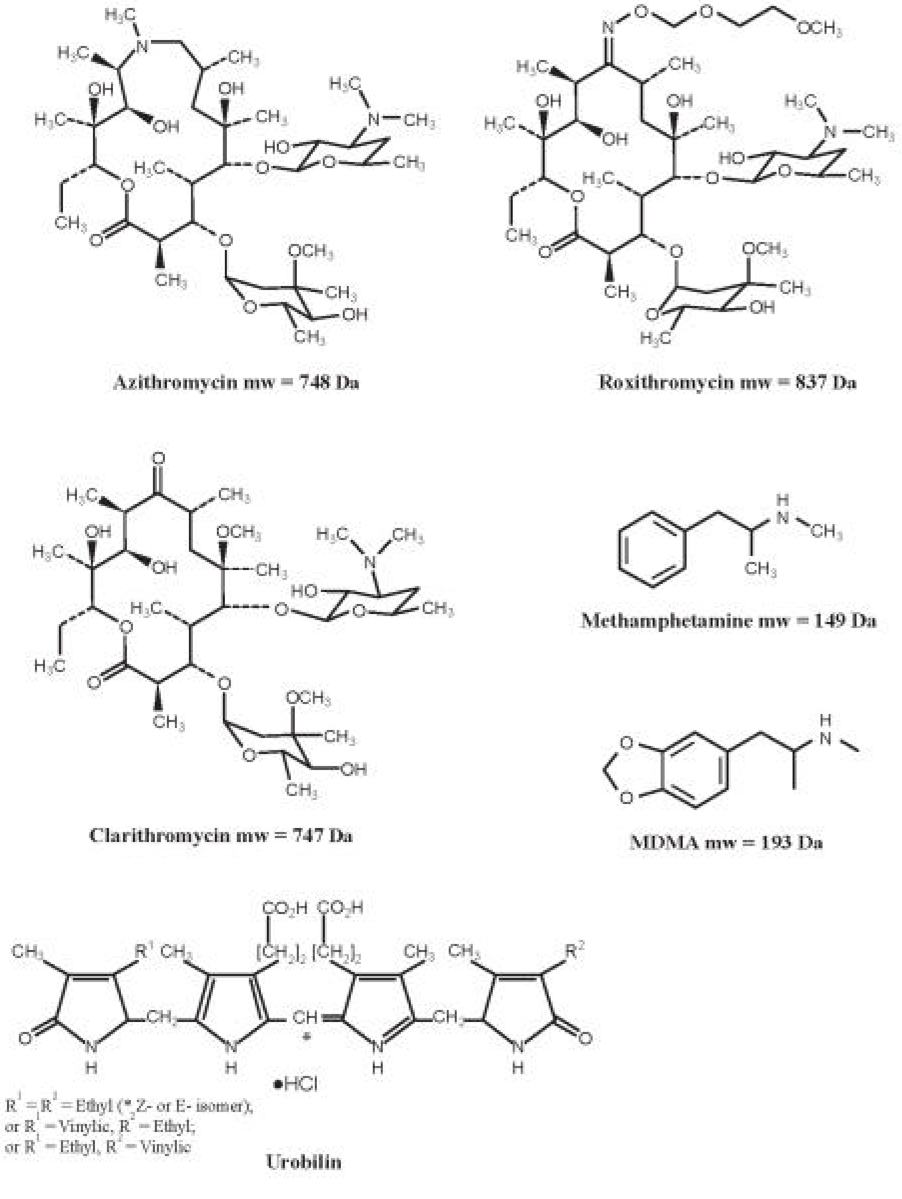
2.0 Materials and Methods

2.1 *Materials*

2.1.1 Drug standards.

Azithromycin [(2_R_-(2_R_*,3_S_*,4_R_*,5_R_*,8_R_*,10_R_*,11_R_*,12_S_*,13_S_*,14_R_*)]-13-[(2,6-dideoxy-3-C-methyl-3-O-methyl-a-L-*ribo*-hexopyranosyl)oxy]-2-ethyl-3,4,10-trihydroxy-3,5,6,8,10,12,14-heptamethyl-11-[[3,4,6-trideoxy-3-(dimethylamino)-ß-D-*xylo*-hexopyranosyl]oxy]-1-oxa-6-azacyclopentadecan-15-one; CASRN 83905-01-5], roxithromycin [3R, 4S, 5S, 6R, 7R, 9R, 11S, 12R, 13R,14R)-6-[(2S,3R,4S,6R)-4-dimethylamino-3-hydroxy-6-methyl-oxan-2-yl]oxy-14-ethyl-7,12,13-trihydroxy-4-[(2S,4R,5S,6S)-5-hydroxy-4-methoxy-4,6-dimethyl-oxan-2-yl]oxy-10-(2-methoxyethoxymethoxyimino)-3,5,7,9,11,13-hexamethyl-1-oxacyclotetradecan-2-one; CASRN 80214-83-1], and clarithromycin [6-(4-dimethylamino-3-hydroxy-6-methyl-tetrahydropyran-2-yl)oxy-14-ethyl-12,13-dihydroxy-4-(5-hydroxy-4-methoxy-4,6-dimethyl-tetrahydropyran-2-yl)oxy-7-methoxy-3,5,7,9,11,13-hexamethyl-1-oxacyclotetradecane-2,10-dione; CASRN 81103-11-9] were obtained from U.S. Pharmacopeia (Rockville, MD, USA) and Sigma-Aldrich (St. Louis, MO, USA). Methamphetamine [(a_S_)-_N_,a-Dimethylbenzeneethanamine; CASRN 537-46-2], and MDMA [_N_,a-Dimethyl-1,3-benzodioxole-5-ethanamine (also known as 3,4-methylene dioxymethamphetamine; CASRN 42542-10-9] were obtained from Cerilliant Corporation (formerly Radian Corp., Round Rock, TX).

d-Urobilin IX hydrochloride [(21H-biline-8,12-dipropanoic acid, 3,18-diethyl-1,4,5,15,16,19,22,24-octahydro-2,7,13,17-tetramethyl-1,19-dioxo-monohydrochloride, (4R, 16R)- (9Cl); CASRN 28925-89-5] was obtained from Frontier Scientific (Logan, UT, USA).



Azithromycin mw = 748 Da

Roxithromycin mw = 837 Da

Clarithromycin mw = 747 Da

Methamphetamine mw = 149 Da

MDMA mw = 193 Da

$R^1 = R^2$ = Ethyl (*, Z- or E- isomer);
or R^1 = Vinylic, R^2 = Ethyl;
or R^1 = Ethyl, R^2 = Vinylic

Urobilin

Fig 2. Chemical structures of target analytes

Stock standard solutions were individually prepared in HPLC-grade methanol (Burdick & Jackson, Muskegon, MI, USA, or equivalent) and stored in darkness at 4°C. A high-level standard mix containing the macrolide antibiotics, urobilin, and the illicit drugs (at 10 or 20 ng μL^{-1}), in methanol, was prepared quarterly, and a calibration standard mix was prepared weekly at environmentally relevant concentrations (0.5 to 1 ng μL^{-1}) in 99% methanol:1% acetic acid.

2.2 *Sampling sites and collection*

The WWTP chosen for this study is relatively small and has a capacity to process about 19 mega liters (5 million gallons) of wastewater per day. Wastewater influent includes domestic sewage combined with a small fraction of commercial and industrial sewage. Aqueous grab samples were collected from various stages of the WWTP treatment process: influent, effluent, return activated sludge (RAS), oxidation ditch, pre- and post-chlorination. Figure 1 shows a generalized schematic of the wastewater treatment processes and sample collection points. Descriptively, influent is sewage collected after entering the WWTP, but after the large "grit" has been removed, but before processing; effluent is sewage that has gone through the full treatment process; RAS is wastewater that has entered the aeration chamber, and it is mixed and oxygen is provided to the microorganisms, the mixed "liquor" then flows into a clarifier or settling chamber where most microorganisms settle to the bottom of the clarifier and a portion are pumped back to the incoming wastewater at the beginning of the plant, this returned semi-liquid material is the RAS; an oxidation ditch is a modified activated sludge biological treatment process that utilizes long solids retention times (SRTs) to remove biodegradable organics (the

RAS returns into the oxidation ditch), this is also a semi-liquid material. The hydraulic retention time for this particular WWTP is 25 to 27 hours.

Aqueous grab samples were also collected from upstream (200 m) and downstream (20 m) of Bee Creek from the WWTP discharge point (Bee Creek discharges into the Clarks River). The Bee Creek is a small creek that primarily carries the effluents from the WWTP to the Clarks River, which ultimately joins the Ohio River. Upstream of Bee Creek is primarily storm runoff from an undeveloped forested area and soccer field. The sampling details including dates collected, average WWTP flows, and weather for the dates collected, are listed in Table 1.

Sampling occurred during the summer and fall of 2006, and the winter and spring of 2007. Approximately 250 mLs of water were collected using high-density polyethylene (HDPE) bottles. The samples were placed in a cooler, transported to the laboratory, and stored at < -20º C until extraction.

2.3. *Chemical Analysis*

2.3.1 Sample Extraction

All samples (aqueous and semi-liquid) were filtered using 10-µm glass fiber filters, and extracted using a solid-phase extraction method, as outlined by Jones-Lepp (2006). Briefly, OASIS HLB cartridges, 6-mL capacity, 0.2 g, 30 µm, (Waters Corporation, Milford, MA), were preconditioned with 5 mL methanol, followed by 2 x 5 mL rinses with DI water. The cartridges were loaded with 250 mL (sometimes less, dependent upon solids present) of wastewater, that was pH adjusted to <3 using 12N HCl. A constant flow of 3 to 4 mL per minute was maintained throughout loading. The analytes were eluted using 4 x 5 mL methanol/1% acetic acid. Using a gentle stream of nitrogen gas, the eluant volume was reduced to 1 mL, then further microconcentrated to 0.5 mL using a TurboVap [Caliper Life Sciences (formerly Zymark

Corporation) Hopkinton, MA USA]. The water bath was set at 25 ºC and the nitrogen flow at 4 psi. The walls of the extraction tubes were rinsed 2 to 3 times using 99% methanol and 1% acetic acid during the volume reduction procedure.

2.3.1.1 Quality Control

Using the above methodology (based on method published in Jones-Lepp 2006), extraction recoveries of azithromycin, roxithromycin, and clarithromycin, spiked into a representative wastewater, averaged (n=3) 6%, 1%, and 13 %, respectively. In comparison to wastewater the extraction recoveries of azithromycin, roxithromycin, and clarithromycin, from spiked de-ionized water, averaged (n=4) 49%, 42%, and 36 %, respectively. Extraction recovery experiments on RAS and influent samples were not performed.

One de-ionized water blank was extracted along with the samples. Also, an upstream water sample from Clark's river was considered a background sample. Two samples, one influent and one effluent, were collected as duplicates, QA data is reported in table 3.

2.3.2. HPLC-ESI-ITMS analysis

2.3.2.1. Liquid chromatography

The separations were performed using a Varian Pursuit XRs 3µm C_{18} 100 x 2 mm (Varian Inc., Lake Forest, CA), with a Varian guard column (MetaGuard 2.0mm Pursuit XRs 3µm C_{18}, Varian Inc., Lake Forest, CA) on the front end. The gradient elution conditions were: 90:10% (Mobile phase A:Mobile phase B) to 10:90% (A:B) over a 15-min gradient, hold for 2-min, then back to 90:10% in 3-min, with a 15 min equilibrium between runs. Mobile phase A: de-ionized water/0.5% formic acid; mobile phase B: 82% methanol/18% acetonitrile/0.5% formic acid.

2.3.2.2. Electrospray-ion trap mass spectrometry

The extracts were analyzed with a Varian 500MS (Walnut Creek, CA USA), µ-liquid chromatography-electrospray-ion trap mass spectrometry (µ-HPLC-ESI-ITMS), configured with a liquid chromatograph and an electrospray ion source. The 500MS uses an ion trap mass spectrometer (ITMS) detector that performs real-time mass analyses of LC eluents over a mass-to-charge ratio range of 50 to 2000. The 500MS was operated in the collision-induced dissociation (CID) positive ionization mode, the voltage applied to the ES needle was 5.0 kV, the heated capillary was set at 200°C, and the sheath gas was set dependent upon the optimized response of the ions of interest. CID was used for both confirming and quantifying the analytes.

2.3.2.3 MS/MS - Collision induced dissociation (CID)

The 500MS can be used to perform CID experiments (referred to as MS/MS) in the ion trap, such that a “fingerprint” spectrum can be made and aid in identification of analytes in complex wastewater matrices. The precursor ion(s) of interest is isolated in the ion trap, where these trapped ions constantly collide with each other and helium in the trap. As the translational energy to the trapped precursor ions is increased this induces more energetic collisions and subsequently product ions are produced. For each analyte of interest capillary voltages (volts) and RF loadings (%) were experimentally determined so as to give the most fragmentation information without loss of sensitivity, other values (e.g., excitation storage and excitation amplitude) were set by the instrument's optimization software. See Table 2 for a listing of the ions for the analytes of interest, detected both in full-scan and MS/MS mode.

2.3.2.4. Calibration, blanks, and HPLC-ES-ITMS quantitation

For each set of HPLC-ES-ITMS analyses, a calibration curve consisting of duplicate or triplicate standard solutions was produced. Two standards were analyzed at the beginning of each day of operation; then a series of solvent blanks [until no carryover was detected, or the signal was well below the limits-of-detection (LOD)], then samples (field blanks and samples) and a final standard were analyzed in that order. An external standard calibration procedure was used, this procedure is outlined in EPA's Solid Waste-846 manual, 8000B, section 7.4.2.1 [available at http://www.epa.gov/epaoswer/hazwaste/test/pdfs/8000b.pdf]. A calibration standard mix was prepared weekly at an environmentally relevant concentration (0.5 and 1 ng μL^{-1}, analyte dependent concentration). This calibration standard was checked periodically for linearity and consistency against a 3-, or 4-point calibration curve. An individual ion using the product ion from CID was used for quantitation purposes. The areas under the most abundant product ion in the ion chromatogram peaks are quantified using a manual algorithm provided by the Varian software.

2.3.2.5. Limits-of-detection

LOD is defined as the lowest concentration of an analyte that an analytical process can detect and is located at 3s (s = standard deviation) above the signal measured at the lowest concentration measured. We defined the limit-of-quantitation (LOQ) as 10 x the LOD. Four different concentrations (ranging from 0.25 to 5.0 ng uL^{-1}) were analyzed in triplicate. The ESI-ITMS LODs and LOQs were determined for the analytes of interest using linear regression to determine the slope from the four concentration levels, see Table 2. (MacDougall, et al., 1980)

3. Results and discussion

3.1 Concentrations of selected macrolide antibiotics, illicit drugs and urobilin in wastewater

WWTPs use a variety of treatment processes (Loganathan et al., 2007), and dependent upon the processes employed the concentrations and fate of prescribed and illicit drugs in WWTPs may vary. In addition, specific sampling methods (grab versus 24-hr composite) and sampling times (e.g. before or after rainfall) can influence the concentration of analytes detected from the WWTP processes. Other influences upon analyte concentration variation can also be from the lag time between sewage entering and exiting the WWTP, realizing that the flow time through any particular WWTP can be up to several hours, depending upon plant flow conditions and processes employed. The results presented here are for grab samples collected at a single time point for each season of the year. In this study influent and effluent grab samples were collected on the same day, at approximately the same time (within an hour or so of each other).

Among the macrolide antibiotics analyzed, azithromycin was detected in all WWTP samples analyzed, with concentrations ranging from 4 ng L^{-1} to 300 ng L^{-1}. In general, influent samples contained relatively higher concentrations of azithromycin than the effluent samples, however, the return activated sludge (RAS) contained the highest concentrations (300 ng L^{-1}) of azithromycin. Clarithromycin was detected in one influent sample (110 ng L^{-1}), in the pre- and post-chlorination samples (65 ng L^{-1} and 35 ng L^{-1} respectively), but not in the final effluent, collected during February 2007. Roxithromycin was not detected in any of the samples analyzed. Methamphetamine was detected in all influents and some effluent samples. MDMA was barely detected in four influent and one effluent sample, but not detected in other samples. Urobilin, a chemical marker of human waste, was found in all influent samples at several orders of magnitude higher than the macrolides and illicit drugs, but barely detectable or non-detected in most effluent samples. Twenty meters downstream from the WWTP, in Bee Creek, we did not detect any of the analytes, except azithromycin. Nothing was detected upstream of the WWTP in Bee Creek.

3.2. Seasonal variation in concentrations of macrolides and illicit drugs

The samples were collected over an eight month period, which during this time the population fluctuated between 15,000 to 25,000 people. The influx of 10,000 students occurs in mid-August, with a decrease around the winter break (mid-December to mid-January), and then increasing from mid-January until the academic year ends in early June. During the summer months the student population is < 2000. Table 3 shows the concentrations (ng L^{-1}) of macrolide antibiotics (azithromycin, roxithromycin, clarithromycin), illicit drugs (methamphetamine, MDMA) and urobilin in wastewater samples collected over 8 months, spanning the four seasons during the years 2006 and 2007.

Considering the population fluctuation, as well as factors such as variations in drug use (due to difference in number of prescriptions due to seasonal illnesses) during different months, and precipitation fluctuation, all of these factors can contribute to seasonal variations in the concentrations of the analytes. Azithromycin was consistently detected in both influent and effluent in all seasons analyzed. In general, the concentration of azithromycin was comparatively higher in the winter months (December and February) than summer, fall, and spring. Azithromycin’s concentration was slightly lower in the influent and twice the amount in the effluent during the September sampling event. This variation could be due to statistical variations occurring during the extraction process, or another possibility was that the raw sewage was overflowing into the outfall, as on that sampling date (9/22/06) 4.5" of rain fell. Clarithromycin was detected in one influent sample and the pre- and post-chlorination samples, but not in the effluent sample, during the February sampling (2/09/07). It is interesting to note that clarithromycin was only seen in the late-winter sampling cycle, possibly linked to an increase in viral/bacterial infections during this time frame (late winter) and; therefore, an increase in prescriptions for different antibiotics, especially for upper respiratory infections.

The amount of methamphetamine detected ranged from not detected (nd) to 35 ng L^{-1} (December sample). MDMA was barely detectable or not detected in most of the samples analyzed. No seasonal variation is discernible for the analytes with low or uncertain concentrations. Overall, in reviewing the data, there were slight seasonal variations in antibiotics and illicit drug use.

3.3 Removal efficiencies of macrolides and illicit drugs

Table 4 shows influent and effluent data in milligram amounts (concentrations in Table 3 were multiplied with total volume processed and converted to mg).

Azithromycin is not efficiently removed during the WWTP treatment processes. To support this observation, the samples taken from the return activated sludge (RAS) line and oxidation ditch (OD), showed elevated levels of azithromycin, compared to the pre-chlorination (pre-Cl), post-chlorination (post-Cl), and influent and effluent samples. In explanation, an oxidation ditch is a modified activated sludge biological treatment process that utilizes long solids retention times (SRTs) to remove biodegradable organics. Preliminary treatment, such as bar screens and grit removal, normally precedes the oxidation ditch, flow to the oxidation ditch is aerated and mixed with return sludge from a secondary clarifier via the RAS (Fig. 2). There are large amounts of suspended solid material in both the RAS and oxidation ditch, which would help explain the greater concentration of azithromycin detected in both areas. The data (Table 3) indicate that the oxidation ditch and RAS treatment processes elevate the azithromycin levels back into the waste stream, allowing some of the azithromycin to entrain onto the sludge. If there was no entrainment into the sludge then the levels at the effluent would be very elevated compared to the influent, but instead they are slightly decreased. Jones-Lepp and Stevens (2007) has shown that both azithromycin and clarithromycin can be found in biosolids, at 51 and 18 ng g^{-1} dry weight, respectively, showing that partitioning of these antibiotics to biosolids can occur.

Clarithromycin was detected in only one influent sample, and in the pre- and post-chlorination samples, but not in the final effluent during the February sampling event (2/09/07). This may be due to the efficient removal of clarithromycin during the treatment process, or the hydraulic retention time between influent and effluent. Since MDMA was not detected in significant concentrations, removal efficiency can not be determined. Urobilin and methamphetamine data reveal that they are efficiently removed during the wastewater treatment process. Urobilin and methamphetamine were detected in the influent, less often in the effluent. indicating a more efficient removal process for these small molecules (compared to the macrolide azithromycin). (Table 3, Figure 3)

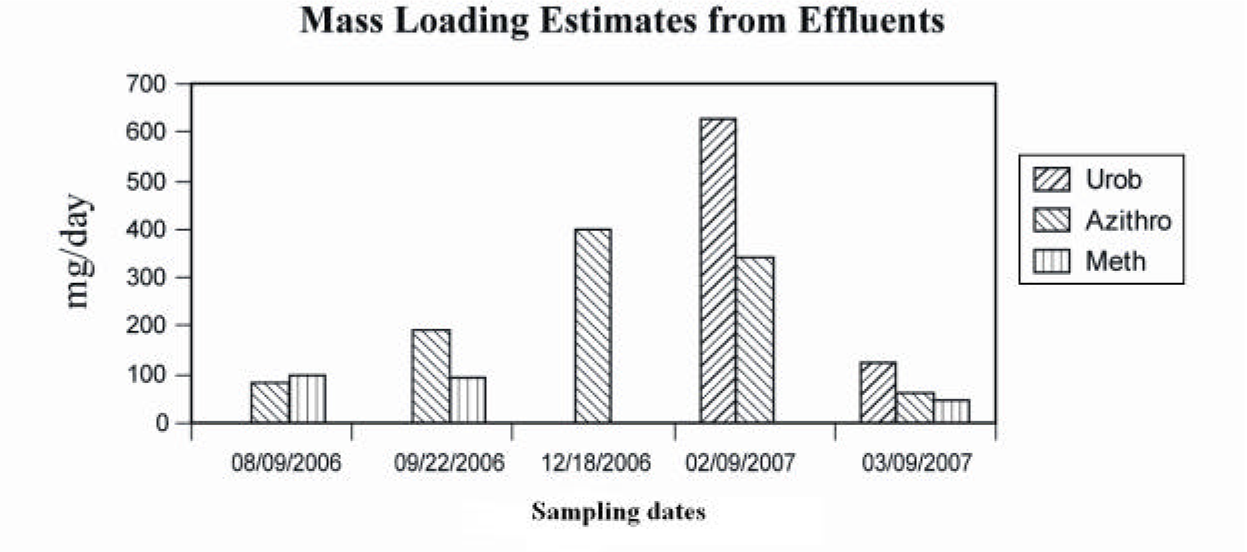


Fig 3. Mass loading estimates of urobilin, azithromycin, and methamphetamine from effluents.

3.4 Loading estimates from this WWTP

Concentrations of macrolide antibiotics, methamphetamine and ecstasy in effluent samples were multiplied with total volume of effluent to obtain the amount of these compounds entering the receiving waters. In figure 3, we compare the mass loadings in the effluent of the study analytes; azithromycin was consistently present, followed by methamphetamine and urobilin. We know of only two studies that have quantitated these specific compounds in US WWTP effluents. In comparing the data between studies, concentrations of azithromycin and methamphetamine (from this study, 17.5 ng µL^{-1} and 5 ng µL^{-1}, respectively) are consistent with the effluent concentrations (allowing for variation in WWTP flow rates and population input from those two studies) that were collected from a variety of WWTPs in the Southwest and Northeastern US (avg. 27 ng µL^{-1} azithromycin, 5 ng µL^{-1} methamphetamine) (Jones-Lepp et al., 2004, Jones-Lepp 2006).

From this one small WWTP facility, using an average value of 17.5 ng L^{-1} (an average of the effluent concentrations) and an average of 4 mgd (approx 15 million liters per day, Table 1) wastewater flow rate, we calculate an annual environmental loading of 0.10 kg yr^{-1} of azithromycin. According to the U.S. EPA's 2008 Clean Water Report to Congress, there are 2,771 WWTPs in the output range of 1 to 10 mgd (USEPA 2008). Using the following formula, we can estimate an annual U.S. loading of azithromycin: using the value of 0.10 kg yr^{-1} x 2,771WWTPs equals approximately 277 kg yr^{-1}; add in the number of plants from 10 to 100+ mgd, 544 WWTPs and estimate that their average azithromycin output at 0.5 kg yr^{-1} (conservatively), we calculate a value of 272 kg yr^{-1}. Adding the two values together gives us approximately 550 kg of azithromycin is being released annually into U.S. streams. As a way of groundtruthing these values, we can calculate a predicted environmental occurrence (PEC) for azithromycin, using the formula's provided by Kostich (Kostich and Lazorchak, 2008) Using the following facts: azithromycin is prescribed in 500 mg doses for approximately 5 days (Rxlist.com), and the annual sales in 2007 were $1,302,635,000 (equated to 45,279,000 prescriptions, see http://drugtopics.modernmedicine.com/drugtopics/data/articlestandard//drugtopics/102008/500218/article.pdf), we can calculate the amount of active pharmaceutical ingredient (API), which for azithromycin equates to 219,430 kg yr^{-1}. This is only a rough estimate, and needs to be used with caution, since the samples are from one small WWTP, with one time sampling events (grab samples) and treatment process vary with different WWTPs, as well as the chemical characteristics of the prescribed and illicit drugs. The PEC is equal to the API activity introduced annually divided by the annual wastewater volume [6.8 x 10^{13} L $year^{-1}$ (USEPA 2008a,b)], we reach a value of 3000 ng L^{-1}. Now if we examine the mass loading value we calculated earlier, using what we found in the environment, and correcting for recovery, we have approximately 200 ng L^{-1}, a 10x fold difference between what we predicted and what we observed in the samples, obviously, there must be other environmental sinks for this antibiotic. In partial explanation for this discrepancy between predicted and observed, the recovery of azithromycin from wastewater is low (approximately 6%, using the extraction methodology as outlined in the experimental section), and it is known that azithromycin partially partitions into biosolids/sludges (Jones-Lepp and Stevens, 2007), as well as sediments and wetland plants (Jones-Lepp ACS Regional meeting, Tucson, Arizona, 2006). We also need to consider that many prescriptions go unused or partially used and the rest may get disposed of into the landfill (Ruhoy and Daughton, 2008), another source of error.

4. Conclusions

Although this study was conducted using grab sampling method, which has its own inherent limitations (Loganathan et al., 2007; Horii et al., 2007; Heidler and Halden, 2008), the data obtained provide evidence that detectable concentrations of macrolide antibiotics and illicit drugs can be found in wastewater treatment plant influent and effluent samples, and that the wastewater treatment processes can remove certain compounds more efficiently than others. Removal efficiency of the detected analytes from this WWTP were in the following order: urobilin > methamphetamine > azithromycin with percentages of removal of 99.9%, 54.5% and 47% respectively. Both azithromycin and methamphetamine are relatively more recalcitrant, as well as pseudopersistant (constantly present due to consistent human use), and can enter into aquatic ecosystems that receive the effluent from wastewater treatment plants. From this one small WWTP facility, using an average value of 17.5 ng L^{-1} (an average of the effluent concentrations) for azithromycin we calculated an annual environmental loading of 0.10 kg yr^{-1}.

Among the three-macrolide antibiotics analyzed in the WWTP samples, azithromycin was detected consistently during the summer, fall, winter, and spring seasons, with a slight elevation in the winter months (December and February). The levels of both illicit drugs remained fairly consistent in the influent throughout the 8 month sampling period. Further study, with more samples collected, including composite sampling method will be needed in order to statistically delineate population fluctuation effects, temporal trends and for more statistically sound environmental loading estimates.

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Figure Captions

1. Simplified diagram of Murray WWTP and sampling points
2. Chemical structures of target analytes
3. Mass loading estimates of urobilin, azithromycin and methamphetamine from effluents

Tables

1. Table 1. Details of wastewater treatment plant (WWTP) samples and Bee Creek samples collected and analyzed for selected macrolide antibiotics and illicit drugs.

2. Table 2. List of precursor and product ions for selected macrolide antibiotics and illicit drugs detected in full-scan and MS-MS mode.

3. Table 3. Concentrations (ng L^{-1}) of selected macrolide antibiotics and illicit drugs in wastewater treatment plant samples and Bee Creek.

4. Table 4. Comparison of total mass (mg day^{-1}) of macrolide antibiotics and illicit drugs in wastewater treatment plant influent and effluent samples

Table 1. Details of wastewater treatment plant (WWTP) samples and Bee Creek samples collected and analyzed for selected macrolide antibiotics and illicit drugs.

Date of Sampling	Precipitation	Volume Processed MLd (mgd†)
8/9/2006	0.2”	14.2 (3.74)
9/22/2006	4.5”	15.8 (4.16)
12/18/2006	Trace	17.2 (4.55)
2/9/2007	None	17.0 (4.48)
3/9/2007	None	15.7 (4.15)
3/9/2007	None	15.7 (4.15)

† Average monthly flow at outfall of WWTP; data from EPA’s Environfacts warehouse: http://www.epa.gov/enviro/html/pcs/pcs_query_java.html

Table 2. List of precursor and product ions for selected macrolide antibiotics and illicit drugs detected in full-scan and MS-MS mode.

Analyte CAS #	Molecular weight (daltons)	Precursor ions	Product ions	LOD[†] ng, on-column
Azithromycin (83905-01-5)	748.5	749.5 $(M+H)^+$	591.4 $(M+H-C_8H_{16}O_2N)^+$	0.5
Roxithromycin (80214-83-1)	837.1	859.4 $(M+Na-H)^+$	755.4 $(M+Na-C_4H_9O_3)^+$	1
Clarithromycin (81103-11-9)	747.3	748.4 $(M+H)^+$	590.1 $(M+H-C_8H_{16}O_2N)^+$	1
Methamphetamine (537-46-2)	149.3	150 $(M+H)^+$	119 $(M+H-CH_3NH_2)^+$	1.5
MDMA (69610-10-2)	193	194 $(M+H)^+$	163.0 $(M-CH_3NH_2+H)^+$	1
Urobilin (28925-89-5)	626	591 $(M + H - HCl)^+$	343 $(M - 2(C_7H_{10}NO) + H - HCl)^+$ 466 $(M - C_7H_{10}NO - HCl)^+$	0.4

[†] See LOD section, 2.3.2.5, for further explanation. Ten microliters is a typical injection volume on-column.

Table 3. Concentrations (ng $\mathrm{L^{-1}}$) of select macrolide antibiotics and illicit drugs in wastewater treatment plant samples and Bee Creek.

Date of Sampling	Sample Type	Concentration (ng $\mathrm{L^{-1}}$)					
		Azit.	Roxi.	Clar.	Meth.	MDMA	Urob.
8/9/2006	Influent	17	ND	ND	20	<1[a]	6630
	Effluent	6	ND	ND	(7) <10[b]	ND	ND
	RAS	300	ND	ND	ND	ND	26
	Post-Chlorination	28	ND	ND	< 1[a]	ND	ND
9/22/2006	Influent	6	ND	ND	(6) <10[b]	<1[a]	3610
	Effluent	12	ND	ND	(6) <10[b]	ND	ND
	RAS	120	ND	ND	ND	ND	96
	Post-Chlorination	9	ND	ND	10	ND	ND
12/18/2006	Influent	37	ND	ND	34	<10[b]	6980
	Influent dup	35	ND	ND	35	<10[b]	312
	Effluent	23	ND	ND	ND	ND	ND
	RAS	200	ND	ND	ND	ND	45
	Oxidation Ditch	75	ND	ND	<10[b]	ND	61
	Pre-Chlorination	11	ND	ND	ND	ND	ND
	Post-Chlorination	13	ND	ND	ND	ND	4
2/9/2007	Influent	53	ND	112	22	<1[a]	39600
	Effluent	20	ND	ND	ND	ND	37
	Effluent duplicate	40	ND	ND	ND	ND	ND
	RAS	140	ND	ND	ND	ND	107
	Oxidation Ditch	82	ND	ND	ND	ND	161
	Pre-Chlorination	38	ND	65	ND	ND	69
	Post-Chlorination	47	ND	35	ND	ND	55
3/9/2007	Influent	(4.5) <5[a]	ND	ND	(8) <10[b]	ND	11300
	Effluent	(4) <5[a]	ND	ND	(3) <10[b]	ND	8
3/9/2007	Upstream Bee Creek	ND	ND	ND	ND	ND	ND
	Downstream Bee Creek	6	ND	ND	ND	ND	ND
	De-ionized water blank	(2) <5[a]	ND	ND	ND	ND	ND

Azit.= Azithromycin; Roxi.= Roxithromycin; Clar.= Clarithromycin; Meth= methamphetamine; MDMA (3,4-methylene dioxymethamphetamine, ecstasy); Urob.= Urobilin (a biomarker of human waste); ND=not detected, [a]Spectrally present, but below LOD: 1 ng $\mathrm{L^{-1}}$; [b]Spectrally present, but below LOQ: 10 ng $\mathrm{L^{-1}}$, values in parentheses are estimated amounts.

Table 4. Comparison of total mass (mg day^{-1}) of macrolide antibiotics and illicit drugs in the wastewater treatment plant influent and effluent samples

Sample collection date	Sample Type	Urobilin (mg)	Azithromycin (mg)	Clarithromycin (mg)	Methamphetamine (mg)
08/09/06	Influent	94300	240	0	280
08/09/06	Effluent	0	85	0	100
09/22/06	Influent	57100	95	0	95
09/22/06	Effluent	0	190	0	95
12/18/06	Influent	121000	640	0	590
12/18/06	Effluent	0	400	0	0
02/09/07	Influent	674000	900	1900	375
02/09/07	Effluent	629	340	0	0
03/09/07	Influent	177000	63	0	130
03/09/07	Effluent	126	63	0	47