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A Survey Of Selected Pharmaceuticals And Personal Care Products In A Binational River And Their Effects On A Member Of Its Zooplankton Community, Plationus patulus (Rotifera)

Diana Angelica Martinez Gomez
University of Texas at El Paso, prunusserrulata@hotmail.com

A SURVEY OF SELECTED PHARMACEUTICALS AND PERSONAL CARE PRODUCTS IN A BINATIONAL RIVER AND THEIR EFFECTS ON A MEMBER OF ITS ZOOPLANKTON COMMUNITY, *Plationus patulus* (ROTIFERA)

DIANA ANGÉLICA MARTÍNEZ GÓMEZ

Environmental Science Program

APPROVED:

_____
Elizabeth J. Walsh, Ph.D., Chair

_____
Vanessa Lougheed, Ph.D.

_____
Wen-Yee Lee, Ph.D.

_____
Benjamin C. Flores, Ph.D.
Interim Dean of the Graduate School

DEDICATION

To my supportive and beloved husband to whom I really thank.

To my parents who have always been there for me, giving me their love, support, and advice through the course of my life.

To my beloved sister and brother.

A SURVEY OF SELCTED PHARAMCEUTICALS AND PERSONAL CARE PRODUCTS IN A BINATIONAL RIVER AND THEIR EFFECTS ON A MEMBER OF ITS ZOOPLANKTON COMMUNITY, *Plationus patulus* (ROTIFERA)

By

Diana Angélica Martínez Gómez, B. Sc.

THESIS

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CHAPTER 1: INTRODUCTION TO WATER QUALITY ASSESSMENT AND AQUATIC TOXICOLOGY

Surface waters are commonly the most susceptible water bodies to pollution since both natural (precipitation inputs, erosion) and anthropogenic (urban, industrial, and agricultural activities) factors affect their quality. Surface waters such as rivers typically receive wastewater inputs, which can potentially contain pathogens, trace organics, heavy metals and nutrients that can degrade aquatic ecosystem function and impair their usage for drinking and recreational purposes (Simeonov *et at*., 2003; Singh *et al*., 2004; Ouyang *et al*., 2006; Watkinson *et al*., 2007; Oelsner *et al*., 2007). The Rio Grande, which serves as an international boundary between the United States and Mexico from the cities of El Paso (TX, USA) - Ciudad Juárez (Chihuahua, MX) to the Gulf of Mexico in the Brownsville/Matamoros area (IBWC, 2010), is no exception. Flow of the Rio Grande in the El Paso/Ciudad Juárez metroplex consists mainly of treated municipal wastewater from El Paso (tertiary and secondary treatment), and Ciudad Juárez (primary treatment) or irrigation return flows (IBWC, 1998; Rodriguez and Lougheed, 2010). Consistent water quality monitoring by the United States and Mexican sections of the International Boundary and Water Commission (IBWC) in this stretch of the river has been carried out since 1992 when both agencies agreed to screen water in the river and its tributaries in order to characterize the occurrence and effects of toxic substances (IBWC, 1998). This agreement consisted of a three phase study plan in which 46 sections of the river from the El Paso/Ciudad Juárez area through the Brownsville/Matamoros region were monitored. In the first phase of the study, a total of 48 toxic chemicals were detected in the El Paso/Ciudad Juárez area of which 30 exceeded screening (used to evaluate concerns for pollutants without specific numerical criterion) levels at some sites. During the second phase a total of 38 toxic substances

were detected in water, sediment and fish tissue of which 28 exceeded criteria (defined concentrations for the protection of aquatic life and human health) levels (IBWC, 1998). Toxic substance exceedances for the second phase within this stretch included arsenic, copper, nickel, chloride and ammonia (IBWC, 1998) while during the third phase arsenic and ammonia were found above screening levels (IBWC, 2004). The presence of heavy metals (Cr, Cu, Ni, Pb, Zn) and As in the El Paso/Ciudad Juárez stretch was detected in the water column in a study conducted by Ríos-Arana *et al*. (2003). In addition, IBWC reports that state levels for chloride, bacterial pollution, sulfate and total dissolved solids were exceeded in 2010 (IBWC, 2010). In 2011, bacterial pollution was again elevated above recommended levels while parameters of concern included chlorophyll-a, nitrate, total phosphorus, and ammonia (IBWC, 2011).

In arid and semi-arid areas like the El Paso/Ciudad Juárez region, the reuse of water is a common practice since water supplies are limited and demands for urban and agricultural activities must be met (Oelsner *et al.*, 2007). This is where the importance in determining and reporting the water quality of any region lies. Of recent concern is the presence of "emerging" pollutants in surface waters that receive effluent from wastewater treatment plants. Regulatory requirements for the majority of these toxicants have not been established and because of this, periodic monitoring by water treatment facilities is not conducted (Teijon *et al*., 2010). One group of these compounds, known as Pharmaceuticals and Personal Care Products (PPCPs), is broadly used in our daily activities and is applied internally or externally to humans and/or to domestic animals (Murray *et al.*, 2010). PPCP toxicants include a variety of chemicals that are grouped based on their common use such as analgesics, anti-convulsants, anti-epileptic drugs, anti-microbials, lipid regulators, polycyclic musks, non-steroidal anti-inflammatory drugs, and synthetic hormones among others (Jjemba, 2008; Murray *et al.*, 2010). PPCP contaminants are

mainly derived from human or livestock excretion of these compounds and/or their metabolites into sewage, improper disposal of outdated medication in sewage systems, and untreated hospital and veterinary wastes entering domestic sewage systems through overflow or leakage from storage structures or land application (Kolpin *et al.*, 2002; Ellis, 2006; Watkinson *et al*., 2007; Tong *et al*., 2011). Attention of the scientific community to these pollutants arose in the late 1990s with extensive reviews of PPCPs in the environment (Daughton and Ternes, 1999; Williams, 2005; Jjemba, 2008; Miège *et al*., 2009; Murray *et al*., 2010). Since then, the environmental occurrence of these toxicants in surface waters and influent and effluent from wastewater treatment plants has been documented to occur in the ng/L to µg/L range nearly worldwide (Waiser *et al*., 2010; Kolpin *et al*., 2002). One of the most notable studies was conducted by Kolpin *et al*. (2002) in 1999 – 2000 in which the occurrence of 95 organic wastewater contaminants including PPCPs was surveyed in 139 streams in the United States; they found that one or more organic wastewater compounds were present in 80% of the surveyed streams in the µg/L range. In a more regional study, the occurrence of 10 antibiotics was surveyed in three sites within the upper stretch of the Rio Grande which receives treated wastewater effluent from Albuquerque (NM) and surrounding communities. Only one of the ten antibiotics, sulfamethoxazole, was found at one site at a concentration of 300 ng/L (Brown *et al.*, 2006). Although these unregulated chemicals have been present in the environment for decades, until recently they were not recognized as potentially significant water contaminants (Ellis, 2006). According to Kolpin *et al.* (2002), Jjemba (2006), and Cooper *et al.* (2008). PPCPs are not completely removed from sewage after treatment, allowing these compounds to enter and persist in surface and ground waters. The continuous introduction of PPCPs into aquatic environments may produce negative effects to these ecosystems (Ellis, 2006), especially to non-

target organisms (Jjemba, 2006). Some of the detrimental effects that have been observed include changes in sex ratios and changes in biochemical cycles as well as anatomical malformations in a wide range of organisms at higher trophic levels (Jjemba, 2006).

Ecotoxicological studies have been conducted on a variety of aquatic organisms including algae, invertebrates such as *Daphnia magna* and *Ceriodaphnia dubia*, as well as some species of fishes and amphibians, in order to determine the potential risks of PPCPs released into aquatic systems (Brausch and Rand, 2011). It is equally important to perform studies in other aquatic groups such as rotifers due to their prevalence within aquatic communities (Dahms *et al.* 2011). Rotifers are considered to be among the smallest animals, ranging from <20 – 3500 µm (Wallace and Snell, 2010). According to Segers (2007), the phylum Rotifera includes about 2030 known species that are classified into three main groups: the marine Seisonida (3 species), the Monogononta (1570 species) and the Bdelloidea (461 species). In a recent review, Dahms *et al.* (2011) summarized the advantages of using monogonont rotifers as model organisms in toxicological research. Monogononts are quite common in aquatic ecosystems and have high population growth rates, which contributes to their role in energy transfer and nutrient cycling in these systems. Their rapid reproduction facilitates their cultivation in laboratories (Wallace and Snell, 2010; Dahms *et al.*, 2011). In addition, their short life cycles allow the study of multigenerational effects in short periods of time as compared to other test species such as *Daphnia* (Snell and Moffat, 1992). Rotifers are also considered sensitive bioindicators since they have shown to be susceptible to an extensive range of pollutants (Snell and Joaquim-Justo, 2007) and are highly sensitive to changes in water quality (Dahms *et al.* 2011). Additionally, their primarily parthenogenetic reproduction allows for testing genetically identical individuals (Dahms *et al.* 2011). In ecotoxicological studies, impacts and effects on rotifers are

quantitatively measured by mortality and reproduction rates and results are reported as the LC_{50} (concentration of the test chemical that kills 50% of the population), EC_{50} (concentration of the test chemical that produces a response in 50% of the population), NOEC (maximum concentration of the test chemical that produces no statistical harmful effect compared to control) and LOEC (lowest concentration of the test compound that has a statistically significant detrimental effect compared to control) values for reproductive and/or behavioral endpoints (Dahms *et al.* 2011). Two of the most common ecotoxicological assessments performed using rotifers are acute toxicity studies that are usually conducted over 48 hours and chronic studies or life cycle tests which range from 2 – 7 days of exposure (Dahms *et al.* 2011). Ferrari *et al.* (2003) tested the chronic toxicity of three pharmaceuticals (carbamazepine, clofibric acid, and diclofenac) to the rotifer *Brachionus calyciflorus* and they found it to be more tolerant to these compounds than the cladoceran *Ceriodaphnia dubia*.

Because of concerns of PPCPs in US waterways and the lack of knowledge of their presence in the middle Rio Grande, this study provides a first survey of the occurrence and concentrations of these chemicals along the El Paso, TX/Ciudad Juárez stretch along with information on basic water quality parameters. In addition, effects to a basal member of the riverine system, the monogonont rotifer *Plationus patulus*, were determined by conducting acute and chronic toxicity studies. These studies will aid in better understanding how selected PPCPs may impact aquatic communities, particularly, rotifer populations. This study will also contribute to understanding and potentially controlling Rio Grande pollution by providing useful information for resource managers for decision making regarding wastewater management. It is also of great importance to provide results obtained in this study to the El Paso/Ciudad Juárez

communities to show them the risks that the improper disposal of these kinds of pollutants can have on our water resources.

1.1 Research Objectives

This study comprises the following key objectives: (1) To determine the occurrence and baseline concentrations of 9 selected PPCPs at four sites along the middle Rio Grande using high performance liquid chromatography combined with tandem mass spectrometry (HPLC MS/MS). This information is complemented with other water quality indicators such as pH, dissolved oxygen, conductivity, nutrients, turbidity, hardness, and chlorophyll-a. (2) To evaluate the toxicity and possible effects that four PPCPs pollutants (caffeine, acetaminophen, fluoxetine, and triclosan) have on the rotifer *Plationus patulus* (a basal member of aquatic food webs) from the Rio Grande. For this purpose, an additional *P. patulus* population was used as a reference in order to compare the level of tolerance to these compounds between the two populations. Effects were determined by conducting 48 hr exposure studies in which LC_{50} is obtained as well as the NOEC and LOEC values. Chronic toxicity of each compound was also determined by conducting six-day population growth studies. (3) Develop a species list and photographic guide to rotifers species occurring in the Rio Grande derived from this study and past collections made by our laboratory.

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CHAPTER 2: ASSESSMENT OF PHARMACEUTICALS AND PERSONAL CARE PRODUCTS IN THE MIDDLE RIO GRANDE BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY COUPLED TO TANDEM MASS SPECTROMETRY

2.1 Abstract

The presence of 9 selected PPCPs (acetaminophen, caffeine, cotinine, codeine, fluoxetine, erythromycin, ciprofloxacin, sulfamethazine, and trimethoprim) was surveyed in water samples collected at four sites along the middle Rio Grande during two flow regimes (irrigation and non-irrigation). Water collection for PPCPs analyses were conducted from August 2010 through September 2011. Three of the sites were located upstream, in, and downstream of the El Paso, TX/Ciudad Juárez, MX metroplex. Site selection was based on their proximity to the metroplex and due to the fact that the Rio Grande in this region is an effluent receiver water system (IBWC, 2011). The fourth site was selected as a reference site since it is located approximately 160 km upstream of El Paso, TX and receives water from the Elephant Butte and Caballo reservoirs for irrigation purposes. EPA Method 1694 was used as a guide for analysis of PPCPs by HPLC MS/MS. Selected PPCPs were detected at least once at each sampling site although for some dates they were detected below limits of quantification. Concentrations of these PPCPs were found in the ng/L range with concentrations from 0.004 ng/L to 42.8 ng/L. Ciprofloxacin was found at the highest concentration in the Rio Grande at the American Dam sampling site during the 2011 irrigation season. Cotinine and codeine were consistently found in all four sampled sites during both flow regimes. Results from this study demonstrate the ubiquitous occurrence of some PPCPs and provides information to water resource managers to better protect our aquatic systems.

2.2 Introduction

As discussed in Chapter 1, PPCPs are unregulated chemicals that are grouped based on their common use including human and veterinary antibiotics, analgesics and anti-inflammatory drugs, fragrances, antiseptics, and others (Ellis, 2006). Within the last twenty years, the need for analytical measures of these emerging pollutants has been of increasing importance in order to determine and understand their fate and degradability in the environment (Ferrer *et al*., 2010; Jjemba 2006). For these reasons, several methods have been developed in order to analyze their occurrence and concentrations in water, wastewater, soil, and sludge matrices. Most of these methods are based on the principles of chromatography, either gas chromatography (GC) in which analytes of interest are vaporized and eluted in a stream of gas (mobile phase) through a column or liquid chromatography (LC) in which the mobile phase is a liquid (Jjemba, 2008). However, high-performance liquid chromatography (HPLC) offers better separation of analytes through smaller size columns and higher mobile phase pressures as compared to LC (Buchberger, 2011; Jjemba, 2008). Analysis and detection of these toxicants have been largely improved by combining chromatography principles with tandem mass spectrometry (MS/MS) techniques (Ferrer *et al*., 2010) in order to minimize background noise such as that produced from organic matter typically present in environmental samples (Jjemba, 2008).

In this study, the Middle Rio Grande was surveyed for PPCP pollutants using EPA Method 1694: Pharmaceuticals and Personal Care Products in Water, Soil, Sediment, and Biosolids by High Performance Liquid Chromatography Combined with Tandem Mass Spectrometry (HPLC MS/MS) as a guide. This method was followed for river water samples and field blank preparation and analysis. EPA Method 1694 recommends analysis of target analytes using the HPLC Waters 2690 or equivalent coupled to a Waters Quattro Ultima triple quadrupole

MS or equivalent (EPA, 2007) but in this study, the Eksigent NanoLC-1D Plus™ system coupled to the Thermo LTQ-ETD/XL was used. Instruments used in this study allowed for reduction of solvent usage and sample injection volume as compared to EPA Method guidelines. Analyte extraction from water samples was conducted through solid-phase extraction (SPE) followed by analysis of extracted analytes by high performance liquid chromatography combined with tandem mass spectrometry. Target compounds for this study were selected based on their frequency of detection in U.S. streams (Kolpin *et al*., 2002) and the commercial availability of native and labeled compound needed for standardization of results.

2.3 Objectives

(1) To determine the occurrence of 9 selected PPCPs (Table 2) at four sites along the middle Rio Grande under two flow regimes (irrigation and non-irrigation) and,

(2) To establish baseline concentrations of interest compounds.

2.4 Hypothesis

The occurrence and concentrations of 9 selected PPCPs are expected to be higher:

a) during the non-irrigation flow regime since previous studies have shown that occurrence and concentrations of these toxicants are related to dilution effects which increase with higher flows, and,

b) at sampling sites 2 – 4 located within the El Paso/Ciudad Juárez area, an urban stretch with substantial wastewater input.

2.5 Materials and Methods

2.5.1 Study area

The Rio Grande, one of the 23 river and coastal basins of Texas, has been and is of great importance to the United States and Mexico (IBWC, 1998). Its headwaters are in the San Juan Mountains of southern Colorado, it flows southward through New Mexico and reaches Texas about 32 km northwest of El Paso/Ciudad Juárez area where it forms the international boundary between these two countries (IBWC-EPA 1998). The Rio Grande near the El Paso, TX/Ciudad Juárez, MX metroplex provides water for agriculture, industry and municipal needs such as drinking water (IBWC, 2011; Rodriguez and Lougheed, 2010). Downstream of this area, water in the river mainly consists of agricultural return flows, wastewater effluent, and raw or partially treated sewage (IBWC, 2011). Water flow in the river is generally low during the non-irrigation season (fall and winter) due to alluvial seepage at delayed water returns from irrigation while during the irrigation season flows are higher (spring and summer) when water is released from Elephant Butte and Caballo reservoirs for agricultural irrigation purposes (Brinegar, 2009; Bilbe, 2006). Crops in this basin include potatoes, alfalfa, chiles, and pecans among others (Brinegar, 2009; Bilbe, 2006). In this study water samples were collected at four sites along the middle Rio Grande (Figure 1) in order to evaluate water quality in terms of PPCP pollutants complemented with basic water chemistry parameters (summarized in Appendix A). Three sites are located in the highly impacted urban stretch and one site is upstream of urbanization and intensive dairy operations.

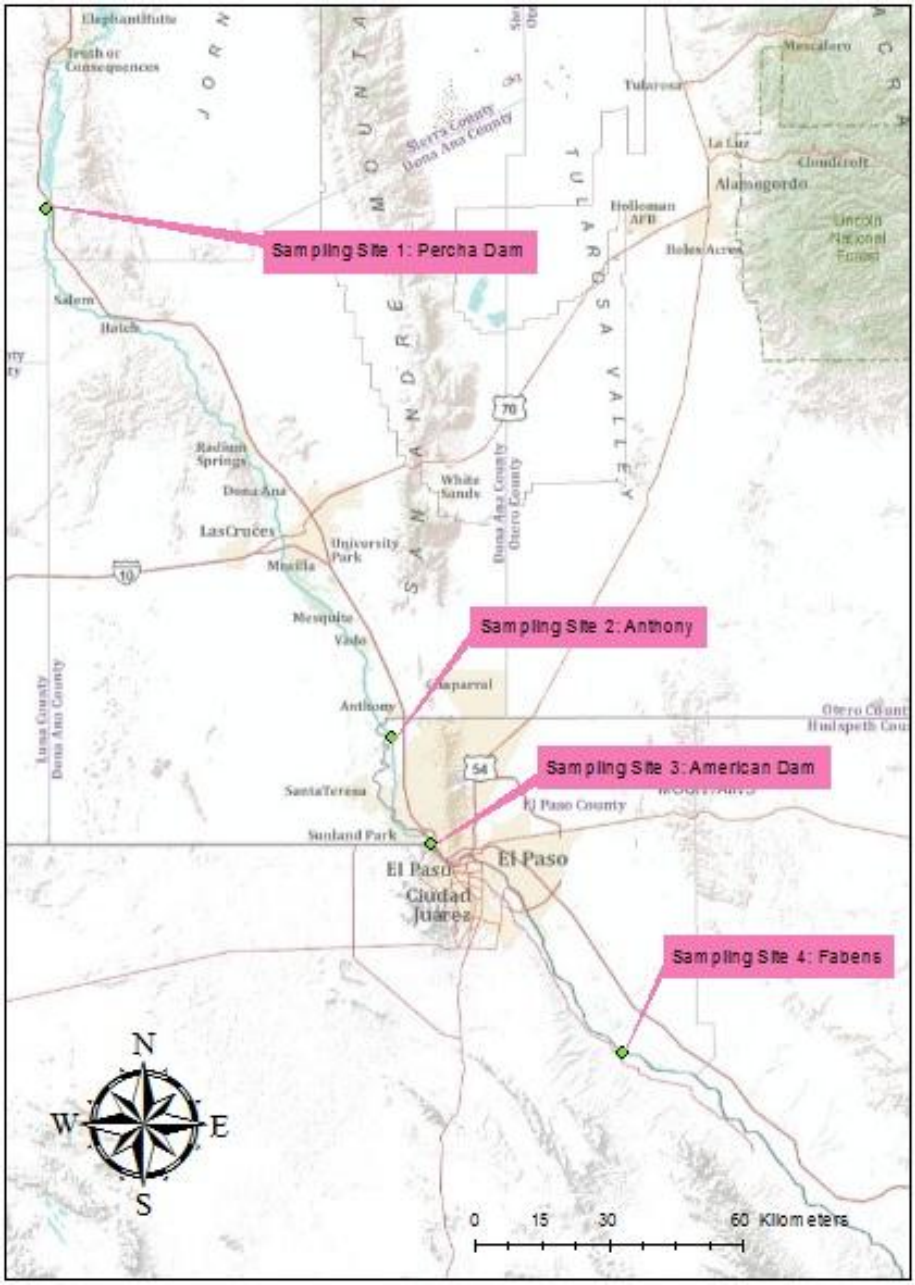


Figure 1. Middle Rio Grande sampling sites surveyed in this study from August 2010 through September 2011 (UTEP – Regional Geospatial Service Center).

The first site was located in Percha Dam State Park, Sierra Co., NM (32° 52.109' N; 107° 18.262' W; elevation: 1256 m; (Figure 1)). Water collection was conducted just below Percha Dam, a diversion structure located on the Rio Grande which receives water from the Elephant

Butte and Caballo reservoirs and redirects part of it through the Rincon Valley Main Canal for irrigation purposes (National Park Service – U.S. Department of the Interior, 2012). Vegetation at this site mainly consists of salt cedar (*Tamarix* spp.), cottonwood (*Populus wislizeni*), and Russian olive (*Elaeagnus angustifolia*) trees while recreational activities primarily comprise fishing and bird watching (New Mexico State Parks Division, 2012). This site is located about 14 km downstream from Caballo Reservoir, Sierra Co., NM and is referred to as Percha Dam in this study. Figure 2 shows typical flow in this site during non-irrigation and irrigation seasons.

Figure 2. Sampling site 1 at Percha Dam State Park, NM showing typical flows during (A) non-irrigation and (B) irrigation seasons.

The second site was located in Anthony, El Paso Co., TX (31° 58.098' N, 106° 36.421' W; elevation: 1145 m; (Figure 1)). Water collection at this site was conducted downstream of the boundary of New Mexico and Texas. Vegetation at this site is predominately salt cedar (*Tamarix* spp.) and grasses. This site is a water quality station in which quarterly routine monitoring is conducted by the International Boundary Water Commission (IBWC). Typical flows for non-irrigation and irrigation seasons are shown in Figure 3.

Figure 3. Anthony sampling site showing typical flows during (A) non-irrigation and (B) irrigation seasons.

The third sampling site is situated upstream of American Dam, (31° 47.308' N, 106° 31.611' W; elevation: 1145m; (Figure 1)), a diversion dam located in El Paso Co., TX. The Rio Grande at this site receives treated effluent from the Northwest Wastewater Treatment Plant (Northwest WWTP) which treats approximately 17.5 million gallons per day of wastewater from residential and industrial sources (El Paso Water Utilities, 2012). For this sampling site, water collection was conducted in the zone where water from the river and treated wastewater from the canal that conveys treated effluent to the Rio Grande mix. Additional samples were collected in and downstream of this effluent canal for three of the sampling events during the 2011 irrigation season. This site will be referred to as the American Dam site. Figure 4 shows representative flows during both flow regimes in this site.

Figure 4. American Dam sampling site showing typical flows during (A) non-irrigation and (B) irrigation seasons.

The furthest downstream location is near Fabens Port of Entry, El Paso Co., TX (31.430277° N, -106.142220° W: elevation 1096 m; Figure 1). Upstream of this point, the Rio Grande receives treated effluent from the Roberto R. Bustamante Wastewater Treatment Plant which has a treatment capacity of approximately 39 million gallons per day (El Paso Water Utilities, 2012). Vegetation at this site mainly includes salt cedar (*Tamarix* spp.), common cattail (*Typha latifolia*), and grasses. All sampling events at this site were coordinated with the IBWC as a safety precaution. This site is also a water quality station in which quarterly routine monitoring is conducted by the IBWC. Figure 5 shows typical flow at the Fabens sampling station during non-irrigation and irrigation seasons.

Figure 5. Sampling site near Fabens Port of Entry showing typical flows during (A) non-irrigation and (B) irrigation seasons.

2.5.2 Water collection

Each location was sampled seven or eight times total, distributed during both the irrigation and non-irrigation season. The irrigation season typically extends from April to September and the non-irrigation season runs from October through March. Mean daily flow in

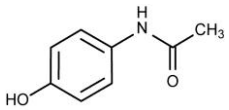
the Rio Grande in the El Paso, TX area during the irrigation season was recorded at 29.70 m^3/s on 08/12/2010 and during the non-irrigation season at 0.69 m^3/s on 11/29/10 and 12/16/10 sampling events (IBWC, 2012). Sampling was initiated in August 2010 and finalized in September 2011 (Table 1).

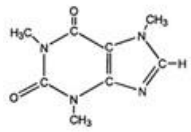
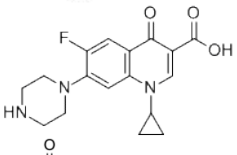
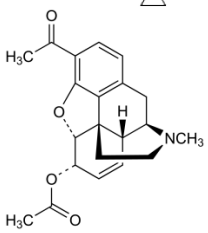
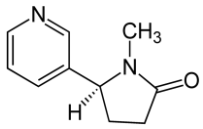
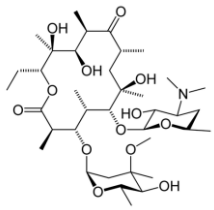
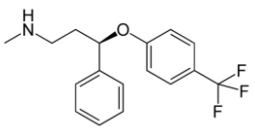
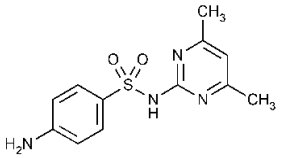
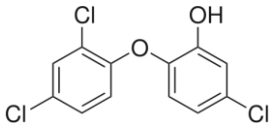
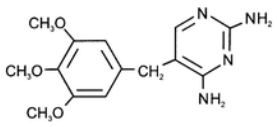
Table 1. Sampling events conducted in the middle stretch of the Rio Grande for this study.

Sampling site	2010					2011							
	Irrigation season		Non-irrigation season					Irrigation season				Non-irrigation season	
	Aug	Sept	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	Jun	Jul	Sept
Percha Dam			10/27	11/29		01/23		03/27	04/25		06/27	07/28	
Anthony	08/12			11/29	12/16	01/23	02/20	03/27		05/30	06/27		
American Dam			10/27		12/16		02/20	03/27	04/25	05/20, 05/30	06/27		
Fabens	08/12	09/29			12/20	01/24		03/28			06/28		09/27

At each sampling event, a grab water sample was collected in pre-cleaned 1 L amber bottles (2 L total) in order to determine the occurrence and concentration of the 9 target compounds (Table 2). One field blank (1 L total in pre-cleaned amber bottle) was included at every sampling event in order to detect any possible contamination during the collection and transport of the samples.

Table 2. Target PPCPs surveyed in this study. Modified from Murray *et al*., 2010; Jjemba, 2006; Kolpin *et al*., 2002. *Acute and chronic toxicity assessed on the freshwater rotifer *Plationus patulus* (Chapter 3). Environmental occurrence not surveyed (a).

Compound	Structure	Use/origin
Acetaminophen*		Analgesic/Anti-pyretic/Pain reliever

Caffeine*		Stimulant
Ciprofloxacin		Antibiotic/Anti-microbial
Codeine		Analgesic/Anti-tussive/Anti-diarrheal
Cotinine		Metabolite of nicotine
Erythromycin		Antibiotic/Anti-microbial
Fluoxetine*		Anti-depressant
Sulfamethazine		Antibiotic/Anti-microbial
Triclosan*[a]		Anti-bacterial/Anti-fungal
Trimethoprim		Antibiotic/Anti-microbial

2.5.3 Water Sample Preparation

Water samples were processed according to Environmental Protection Agency Method 1694 (EPA 2007) as follows: water samples collected for PPCPs analysis were kept in the dark at 4 °C immediately after sampling in order to prevent degradation of analytes. Within 48 hr of collection, each sample was filtered three times. For the first and second filtration, glass microfiber filters (GF/F 70 mm) were used in order to remove larger particles whereas for the third filtration Millipore® nitrocellulose membrane filters (0.22 µm) were used to remove fine particles. After each sample was filtered, the pH was adjusted to 2 ± 0.5 with hydrochloric acid (6N HCl). 500 mg of EDTA, a chelating agent, was added to 1 L of sample one or two hours before running it through Solid-phase Extraction (SPE; Oasis HLB 20 cc/1g extraction cartridges) in order to release analytes from interferences present in the sample. A shaking water bath was used during this waiting time since occasional agitation is recommended following the addition of EDTA. Samples were spiked with standard isotopes of known concentrations (Table 3) and then ran through SPE in order to extract the selected analytes from the water samples followed by elution with 12 mL of methanol.

Table 3. Labeled compounds used as internal controls for HPLC MS/MS analyses.

Compound	Concentration	Volume Spiked
Fluoxetine-d_5	1 µg/mL	50 µL
$^{13}C_3$ Caffeine	3 µg/mL	10 µL
Cotinine-d_3	2 µg/mL	15 µL

The eluent was then collected and later solvents were evaporated and analytes were concentrated by putting the sample under nitrogen flow in a water bath at 50 ± 5 °C. The extract was then transferred to an amber glass micro-vial with 95 % LC-MS grade water and 5% grade Acetonitrile for a total volume of 100 µl for HPLC MS/MS analysis (EPA 2007).

2.5.4 Analytes Analysis

Standards, all processed samples and field blanks were run through HPLC/MS/MS. The Eksigent NanoLC-1D Plus™ system coupled to a linear trap quadrupole (LTQ XL) mass spectrometer was used for qualitative and quantitative analysis of the target analytes.

One µL of each extract was injected by the Eksigent NanoLC-1D syringe pump into a resine-C18 analytical column. Within this column, the sample was divided into its different elements and then passed to the LTQ XL mass spectrometer which consists of an atmospheric pressure ionization (API; where ionization of the sample takes place) source, ion optics, mass analyzer, and ion detection system (Thermo Electron Corporation, 2006). The LTQ XL MS instrument was set up in positive electrospray ionization mode (ESI+). Transmitted mass-to-charged ratios produced within the ionization phase were then measured by the LTQ XL system (Thermo Electron Corporation, 2006).

All the samples and standards were run with a flow rate of 300 nL/min. The mobile phases consisted of 0.3% formic acid, 0.1% Ammonium Formate solvent for mobile phase A, and 100% LC-MS grade Acetonitrile was used for mobile phase B.

Standards were prepared by making dilutions from a stock solution containing a mix of the 9 target compounds. Dilutions were made to final concentrations of 1 fmol/µL, 10 fmol/µL, 100 fmol/µL, 200 fmol/µL, 400 fmol/µL, 600 fmol/µL, and 800 fmol/µL.

After initial conditions were obtained by running standards through HPLC, calibration curves were constructed for each target compound. Determination of the analytes in surface water samples was based on the obtained retention times (RT) for each compound and also by considering the mass-to-charged ratios (m/z) of the fragmented ions from parent ions of the corresponding standards.

Peak area selection for each native and labeled compound in water samples and field blanks was also based on the signal to noise ratio produced by each peak. In order to determine the presence of target analytes in an environmental sample, the signal to noise ratio needed to be greater than 3 for each peak and greater than 10 for quantification as recommended in EPA Method 1694 (2007). Determination of peak areas for each native and labeled compound contained in river samples, field blanks and standards was estimated using Xcalibur 2.0.7 Software (Thermo Electron Corporation, 2006).

Calculations of final concentrations of target analytes in water samples were determined by using slope intercept equations obtained from standard calibration curves. For river samples, concentrations determined in field blanks were subtracted from the corresponding water sample values.

2.5.5 Data Analysis

Concentrations for each compound at each site were analyzed using a General Linear Mixed Model in the statistical program SAS® (version 9.2) to determine significance effects of flow regimes type (irrigation and non-irrigation) and of site type on concentrations of each target PPCP analyte. These analyses were conducted by Dr. Julia Bader (BBRC Statistical Consulting Laboratory, UTEP).

2.6 Results

2.6.1 Detection Limits and Standard Calibration Curves

The detection limits of methods employed in this study were set to encompass values ranging from 0.001 pmol/µL to 0.800 pmol/µL. Calibration curves for standards were created for each native and labeled compound by plotting peak areas (Figure 6 is representative for cotinine) obtained from chromatograms as determined with Xcalibur 2.0.7 S software (Thermo Electron Corporation, 2006). Remaining calibration curves are shown in Appendix B.

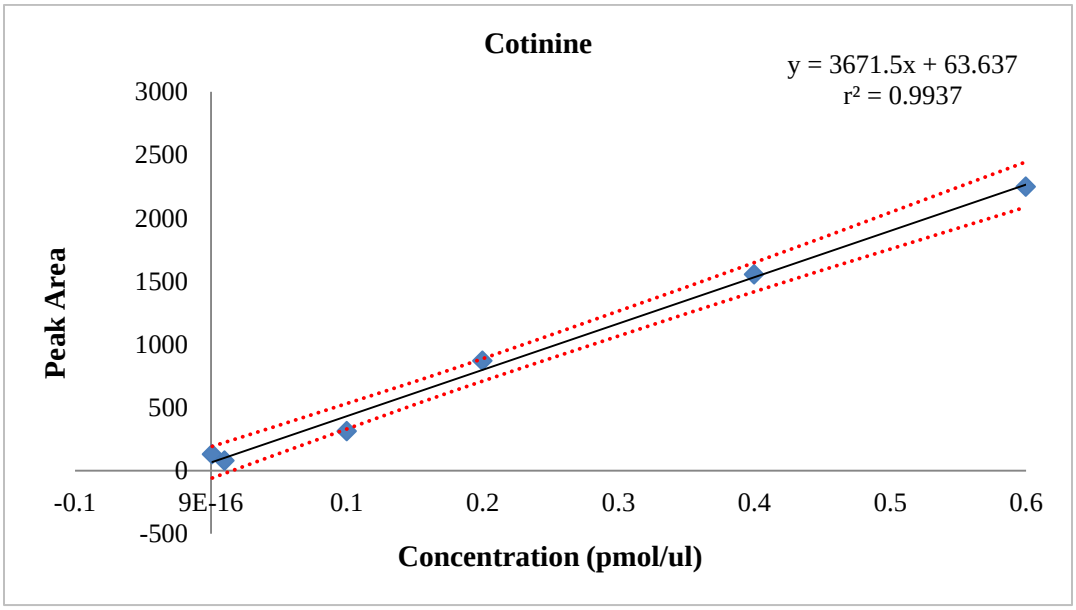


Figure 6. Calibration curve for cotinine showing peak areas over a range of concentrations. Dotted lines represent the confidence interval of the calibration curve.

For all standard calibration curves the slope intercept equation was obtained with a coefficient of determination (r^2) value greater than 0.92 (Table 4). For caffeine, two peaks were obtained. The first peak was detected at 16.09 ± 0.49 min. while the second peak was observed at 28.41 ± 0.32 min.

Table 4. Coefficient of determination values (r^2) obtained from slope intercept equations for each calibration curve used in calculations of final concentrations of PPCPs in river samples.

Compound	Coefficient of Determination Value (r^2)
Acetaminophen	0.9412
Cotinine	0.9937
Caffeine (16 min.)	0.9645
Caffeine (28 min.)	0.9885
Averaged caffeine	0.9891
Sulfamethazine	0.9878
Trimethoprim	0.9247
Codeine	0.9445
Fluoxetine	0.9606
Ciprofloxacin	0.9266
Erythromycin	0.9229

Identification of target analytes in chromatograms from river samples and field blanks was based on retention times and mass-to-charge ratios of fragmented ions obtained in standards. Retention times and mass-to-charge ratios of fragmented ions from parent ions used for selection of peak areas are shown in Table 5.

Table 5. Retention times and mass-to-charge ratios obtained from standards used for identification and selection of peak areas of PPCP target analytes in river samples. Retention time in min. (RT); mass-to-charge ratio (*m/z*).

Compound	Concentration (fmol/µL)							
		1	**10**	**100**	**200**	**400**	**600**	**800**
Acetaminophen	**RT**	15.90	15.25	16.06	15.74	16.06	16.06	15.58
	m/z	110.73	110.67	110.81	110.70	110.92	110.27	110.15
Cotinine	**RT**	15.24	15.89	16.05	16.21	16.21	15.73	15.89
	m/z	98.98	-	98.91	98.19	98.14	98.20	98.23
Caffeine	**RT**	15.60	15.60	15.76	15.76	16.09	16.09	16.09
	m/z	138.96	138.23	138.18	138.22	138.21	138.17	138.16
	RT	28.57	28.57	28.73	28.57	28.41	28.57	28.41
	m/z	138.26	138.19	138.34	138.24	138.24	138.22	138.14
Sulfamethazine	**RT**	29.73	29.57	29.89	29.89	29.71	29.57	29.73
	m/z	156.15	156.19	156.19	156.20	156.21	156.21	156.21
Trimethoprim	**RT**	29.55	29.39	29.55	29.55	29.54	29.55	29.55
	m/z	230.18	230.18	230.17	230.19	230.18	230.17	230.17
Codeine	**RT**	30.18	29.20	28.56	28.72	29.20	28.56	28.72
	m/z	-	152.91	152.92	152.99	152.95	152.96	159.92
Fluoxetine	**RT**	35.57	35.40	35.25	35.24	35.21	35.24	35.24
	m/z	148.19	148.24	148.21	148.21	148.23	148.22	148.21
Ciprofloxacin	**RT**	29.74	30.06	30.55	29.74	30.20	30.06	30.07
	m/z	314.73	314.44	315.01	314.38	314.40	314.37	314.46
Erythromycin	**RT**	ND	ND	32.83	32.67	32.64	32.67	33.00
	m/z	-	-	158.52	158.33	158.22	158.19	158.27

2.6.2 Occurrence and concentrations of target PPCPs in river samples

Sampling was conducted from August 2010 to September 2011 during two flow regimes, irrigation and non-irrigation seasons. The occurrence of target analytes for each flow regime per sampled site is shown in Figure 7. In determining the occurrence of analytes, compounds

detected below limits of quantification were included. The presence of a target analytes was detected at least one time during the irrigation or non-irrigation seasons.

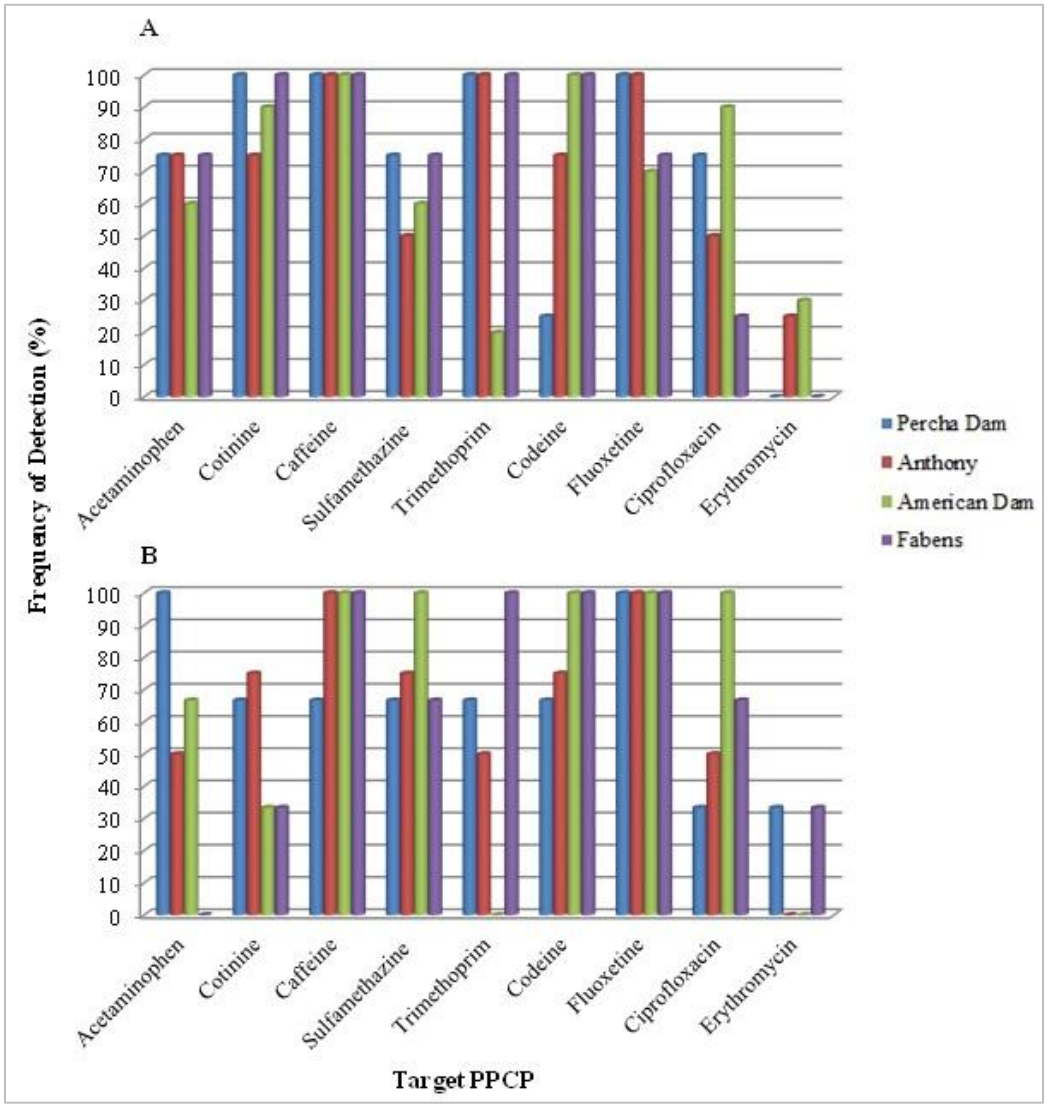


Figure 7. Frequency of detection (%) of target PPCP analytes for all sampled sites in the Rio Grande during (A) irrigation and (B) non-irrigation flow regimes.

The occurrence of acetaminophen, cotinine, caffeine, and trimethoprim at all sites was higher (17%, 39%, 8%, and 26%, respectively) during the irrigation as compared to the non-irrigation season while the occurrence of sulfamethazine, codeine, fluoxetine, ciprofloxacin, and

erythromycin was higher (12%, 10%, 14%, 2%, and 3%, respectively) during the non-irrigation season as compared to the irrigation season.

The presence of all target analytes except erythromycin was detected in all sampled sites for the irrigation season. Erythromycin was only detected at Anthony and American Dam for this flow regime.

For the non-irrigation season, cotinine, caffeine, sulfamethazine, codeine, fluoxetine, and ciprofloxacin were detected to occur in all sampled sites while the presence of acetaminophen and trimethoprim was not detected at the Fabens or American Dam site. On the other hand, erythromycin was detected at Percha Dam and Fabens only.

Caffeine was detected below limits of quantification for most sampling events during the irrigation season while fluoxetine was also mostly detected below limits of quantification during the non-irrigation season.

According to the results obtained from the General linear mixed model, no significant effects of flow regime on analyte concentrations were found. No significant effects of site type were found either on analyte concentrations with the exception of fluoxetine, which concentrations were found to be significantly higher in the Rio Grande at Percha Dam sampling station ($F = 5.62$; 0.0040) than those obtained from the other three downstream sites.

2.6.2.1 Percha Dam, Sierra Co., NM

Four sampling events were conducted in Percha Dam during the 2011 irrigation season while three sampling events were conducted for the 2010 non-irrigation season. Detected concentrations for target analytes are shown in Table 6.

Table 6. Concentrations of PPCPs found in the Rio Grande at Percha Dam (Sierra Co., NM) reported in ng/L. Below limit of quantification (BLQ). Not detected (ND).

		Compound									
		Date of collection	Acetaminophen (ng/L)	Cotinine (ng/L)	Caffeine (ng/L)	Sulfamethazine (ng/L)	Trimethoprim (ng/L)	Codeine (ng/L)	Fluoxetine (ng/L)	Ciprofloxacin (ng/L)	Erythromycin (ng/L)
Season	Irrigation	03-27-11	BLQ	**2.7**	**BLQ**	**0.1**	BLQ	BLQ	**3.1**	**5.2**	ND
		04-25-11	ND	BLQ	**7.6**	ND	BLQ	ND	BLQ	ND	ND
		06-27-11	BLQ	**1.7**	BLQ	**0.1**	BLQ	ND	**3.5**	BLQ	ND
		07-28-11	BLQ	**2.4**	BLQ	BLQ	BLQ	ND	**3.2**	BLQ	ND
	Non-irrigation	10-27-10	**1.0**	**1.9**	BLQ	ND	ND	ND	**6.6**	**1.8**	**8.0**
		11-29-10	BLQ	ND	ND	**0.8**	BLQ	**1.3**	BLQ	ND	ND
		01-23-11	BLQ	BLQ	**2.1**	BLQ	BLQ	**2.6**	BLQ	ND	ND

Acetaminophen, trimethoprim, and erythromycin were detected at this site but they were below limits of quantification for most of the sampling events during the irrigation season while during the non-irrigation season acetaminophen and erythromycin were found at 1.0 ng/L and 8.0 ng/L respectively.

Cotinine and fluoxetine were frequently detected during the irrigation season while during the non-irrigation season they were detected in one sampling event (Figure 8).

Caffeine was only detected in one sampling event for the irrigation and non-irrigation seasons at 7.6 ng/L and 2.1 ng/L respectively.

Sulfamethazine was found at 0.1 ng/L during two sampling events while it was found at 0.8 ng/L during the non-irrigation season.

Codeine was below limits of quantification most of sampling events during the irrigation season while it highest concentration in this site was 2.6 ng/L during the non-irrigation season.

Ciprofloxacin was found in one sampling event during the irrigation season and in one sampling event during the non-irrigation season at 5.2 ng/L and 1.8 ng/L respectively.

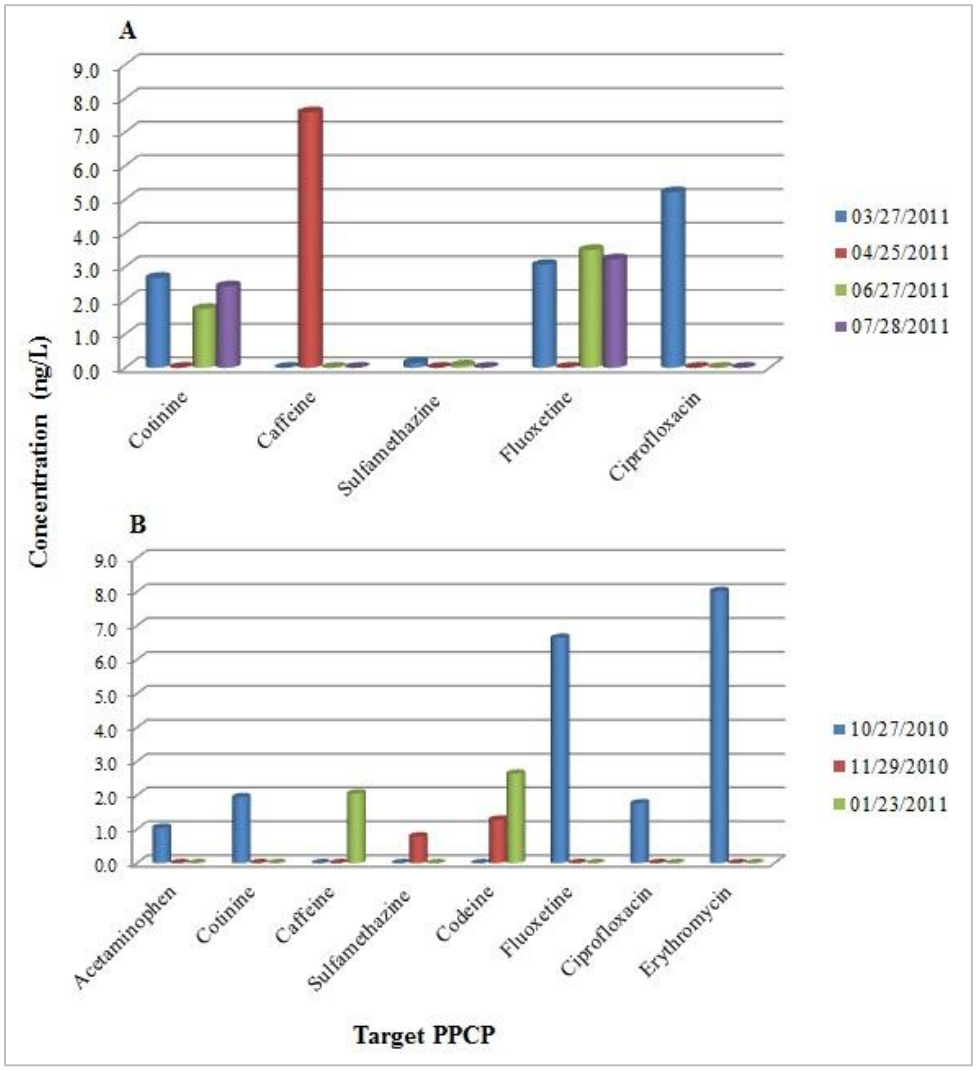


Figure 8. Target PPCP concentrations detected in the Rio Grande at Percha Dam, Sierra Co., NM for (1A) irrigation and (1B) non-irrigation seasons.

2.6.2.2 Anthony, El Paso, Co., TX

For the Anthony sampling site, one sampling event was conducted during the 2010 irrigation season while three sampling events were conducted during the 2011 irrigation season. For the 2010 non-irrigation season, four sampling events were conducted. Table 7 shows concentrations for target PPCPs.

Table 7. Concentrations of PPCPs found in the Rio Grande at Anthony, El Paso Co., TX, reported in ng/L. Below limit of quantification (BLQ). Not detected (ND).

		Compound									
		Date of collection	Acetaminophen (ng/L)	Cotinine (ng/L)	Caffeine (ng/L)	Sulfamethazine (ng/L)	Trimethoprim (ng/L)	Codeine (ng/L)	Fluoxetine (ng/L)	Ciprofloxacin (ng/L)	Erythromycin (ng/L)
Season	Irrigation	08-12-10	ND	ND	**6.5**	ND	BLQ	**5.0**	BLQ	ND	BLQ
		03-27-11	BLQ	**1.1**	**24.3**	**1.5**	BLQ	**1.1**	BLQ	**22.6**	ND
		05-30-11	BLQ	**2.3**	BLQ	**0.2**	BLQ	**1.9**	**1.6**	BLQ	BLQ
		06-27-11	BLQ	**0.4**	**2.0**	ND	BLQ	ND	BLQ	ND	ND
	Non-irrigation	11-29-10	BLQ	**2.0**	BLQ	BLQ	ND	ND	**2.0**	ND	ND
		12-16-10	BLQ	**0.5**	BLQ	ND	BLQ	**3.2**	BLQ	ND	ND
		01-23-11	ND	ND	BLQ	BLQ	**0.1**	**18.1**	BLQ	**3.1**	ND
		02-20-11	ND	**0.2**	BLQ	**0.1**	ND	**0.1**	BLQ	**1.1**	ND

Acetaminophen and erythromycin were mostly below limits of quantification or not detected at this site during both flow regimes.

Caffeine and codeine were found at 6.5 ng/L and 5.0 ng/L for the 2010 irrigation season while the rest of compounds were either below limits of quantification or below limits of detection.

For the 2011 irrigation season, cotinine ranged from 0.4 ng/L to 2.3 ng/L while codeine was found at 1.1 ng/L and 1.9 ng/L. During the non-irrigation season, cotinine ranged from 0.2 ng/L to 2.0 ng/L codeine was found at 0.1 ng/L to 18.1 ng/L (Figure 9).

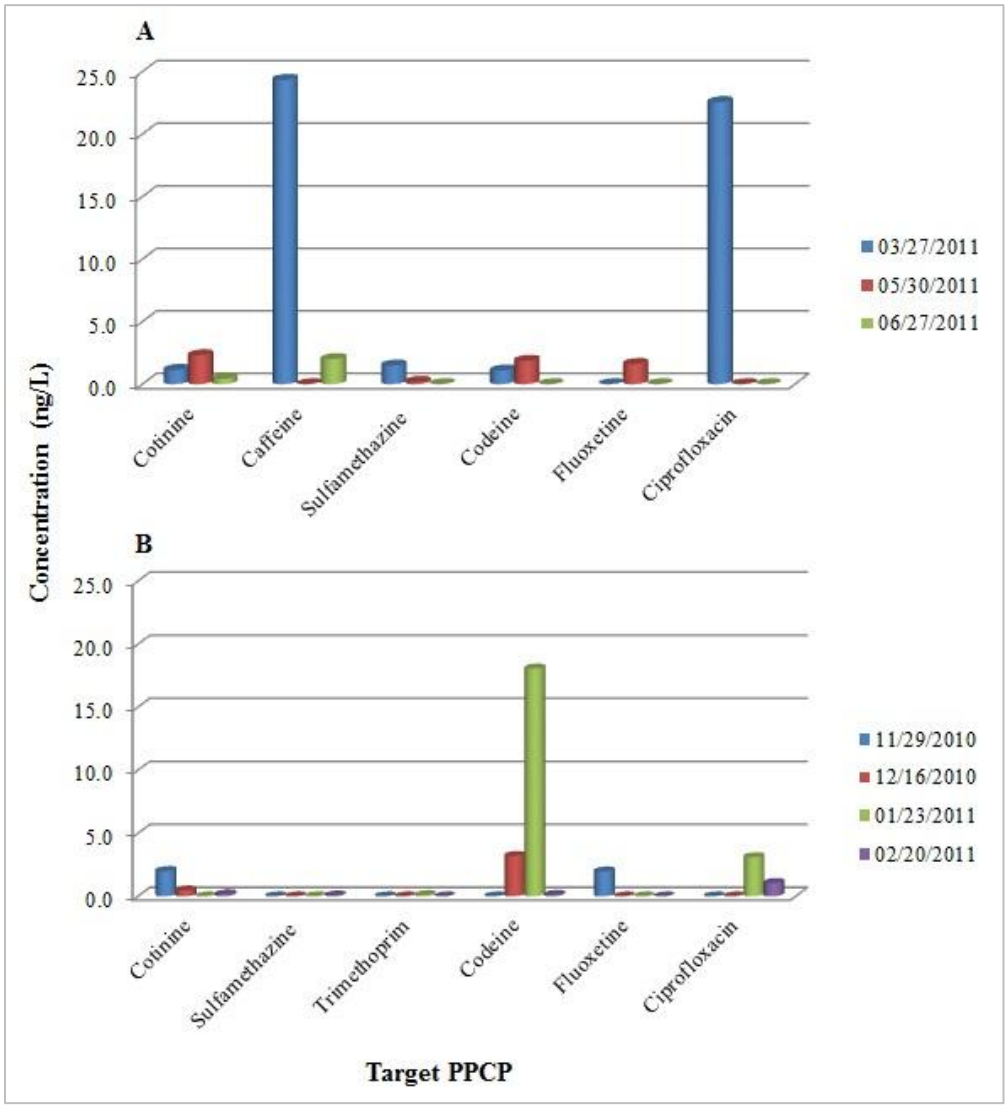


Figure 9. Target PPCP concentrations detected in the Rio Grande at Anthony, El Paso Co., TX during (1A) irrigation and (1B) non-irrigation seasons.

Caffeine was the compound occurring at higher concentrations in this site with 24.3 ng/L followed by ciprofloxacin with 22.6 ng/L for the irrigation season while during the non-irrigation season codeine was found at the highest concentration (18.1 ng/L).

2.5.2.3 American Dam, El Paso, Co., TX

For this sampling site, five sampling events were conducted during the 2011 irrigation season while three water collection events were conducted for the 2010 non-irrigation season. Concentrations for this site are shown in Table 8.

Generally, acetaminophen, fluoxetine, and trimethoprim were below limits of quantification or below limits of detection during both flow regimes.

During the irrigation season, cotinine ranged from 0.004 ng/L to 2.4 ng/L while during the non-irrigation season it was detected at 0.4 ng/L (Figure 10).

Caffeine concentrations ranged from 0.9 ng/L to 9.8 ng/L during the irrigation season while for the non-irrigation season it was below limits of quantification (Figure 10).

Sulfamethazine was detected as high as 0.3 ng/L during the irrigation season while the lower detected concentration was 0.04 ng/L for the non-irrigation season.

Codeine concentrations during the irrigation season ranged from 1.0 ng/L to 10.3 ng/L while for the non-irrigation season the highest concentration obtained for this toxicant was 5.8 ng/L.

Table 8. Concentrations of PPCPs found in the Rio Grande at American Dam, El Paso, TX, reported in ng/L. Below limit of quantification (BLQ). Not detected (ND).

		Compound									
		Date of collection	Acetaminophen (ng/L)	Cotinine (ng/L)	Caffeine (ng/L)	Sulfamethazine (ng/L)	Trimethoprim (ng/L)	Codeine (ng/L)	Fluoxetine (ng/L)	Ciprofloxacin (ng/L)	Erythromycin (ng/L)
Season	Irrigation	03-27-11	BLQ	**2.4**	BLQ	ND	ND	**4.2**	BLQ	**9.9**	ND
		04-25-11	BLQ	**1.6**	**0.9**	**0.3**	BLQ	**2.6**	BLQ	**13.1**	**4.1**
		05-20-11	ND	BLQ	BLQ	**0.2**	ND	**3.9**	BLQ	ND	ND
		05-20-11 *Downstream	BLQ	**0.1**	**6.3**	BLQ	ND	**5.5**	BLQ	**41**	**0.3**
		05-20-11 *Canal	BLQ	**0.1**	**9.8**	ND	ND	**10.3**	ND	**25.8**	ND
		05-30-11	ND	ND	BLQ	ND	ND	**7.3**	BLQ	**42.8**	ND
		05-30-11 *Downstream	BLQ	**0.2**	**1.1**	**0.1**	ND	BLQ	ND	**21.7**	ND
		06-27-11	ND	BLQ	BLQ	BLQ	ND	BLQ	ND	BLQ	ND
		06-27-11 *Downstream	ND	**0.004**	BLQ	ND	ND	**4.8**	BLQ	BLQ	ND
		06-27-11 *Canal	BLQ	**1.4**	BLQ	**0.1**	BLQ	**1.0**	BLQ	BLQ	**5.1**
	Non-irrigation	10-27-10	BLQ	**0.4**	BLQ	**0.04**	ND	**5.8**	BLQ	BLQ	ND
		12-16-10	BLQ	ND	BLQ	BLQ	BLQ	**3.0**	BLQ	**26.6**	ND
		02-20-11	ND	ND	BLQ	**0.07**	BLQ	**2.7**	BLQ	**11.5**	ND

Indicates that sample was collected downstream of the effluent canal (*Downstream); Indicates that sample was collected in the effluent canal (*Canal)

The highest concentrations of ciprofloxacin were observed at American Dam. Concentrations of this toxicant ranged from 9.9 ng/L to 42.8 ng/L during the irrigation season while the highest concentration detected for the non-irrigation season was 26.6 ng/L. During the

irrigation season, the highest concentration for this chemical (42.08 ng/L) was detected upstream of effluent canal while the next highest concentration (41 ng/L) was detected downstream of the effluent canal.

During the irrigation season, erythromycin was detected on three water samples collected upstream of the wastewater effluent canal (4.1 ng/L), downstream of the canal (0.3 ng/L) and from the canal (5.1 ng/L). This compound was not detected during the non-irrigation season. Cotinine and codeine concentrations were slightly higher in the effluent canal than concentrations detected upstream and downstream of the canal (Figure 10).

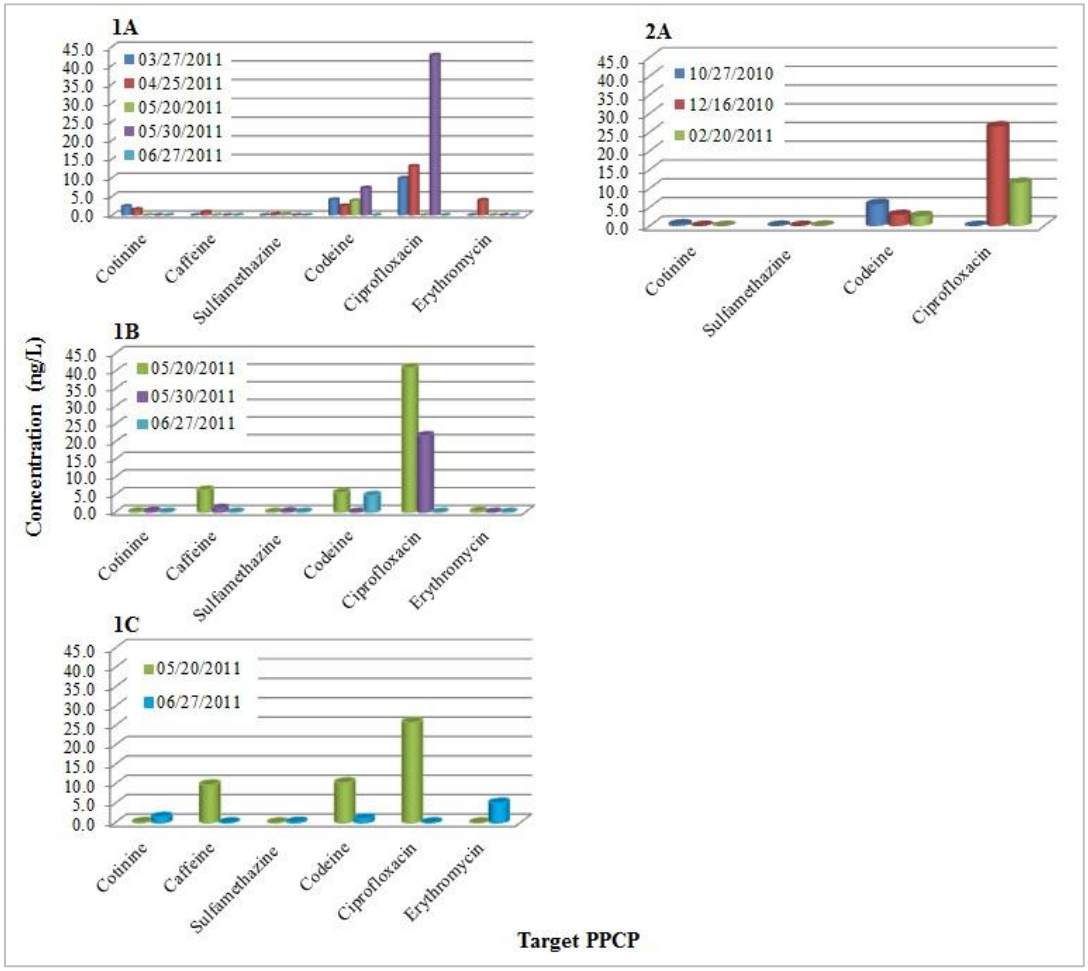


Figure 10. Target PPCP concentrations detected in the Rio Grande at American Dam, El Paso Co., TX during irrigation and (2A) non-irrigation seasons. For irrigation season (1A) presents concentrations at upstream of effluent canal, (1B) downstream of effluent canal, and (1C) effluent canal.

Overall, ciprofloxacin was the compound with the highest detected concentrations for this site for the irrigation and non- irrigation season.

2.6.2.4 Fabens, El Paso Co., TX

For the Fabens site, two sampling events were conducted during the 2010 irrigation season as well as for the 2011 irrigation season while for the 2010 non-irrigation season two sampling events were conducted and one more for the 2011 non-irrigation season. Concentrations for this site are shown in Table 9.

Table 9. Concentrations of PPCPs found in the Rio Grande at Fabens, TX, reported in ng/L. Below limit of quantification (BLQ). Not detected (ND).

		Compound									
		Date of collection	Acetaminophen (ng/L)	Cotinine (ng/L)	Caffeine (ng/L)	Sulfamethazine (ng/L)	Trimethoprim (ng/L)	Codeine (ng/L)	Fluoxetine (ng/L)	Ciprofloxacin (ng/L)	Erythromycin (ng/L)
Season	Irrigation	08-12-10	BLQ	BLQ	**10.0**	BLQ	BLQ	**3.6**	ND	ND	ND
		09-29-10	ND	BLQ	**1.1**	BLQ	BLQ	**9.2**	ND	ND	ND
		03-28-11	BLQ	**0.1**	**3.9**	ND	BLQ	**6.3**	BLQ	ND	ND
		06-28-11	BLQ	BLQ	BLQ	**0.6**	BLQ	**6.8**	BLQ	BLQ	ND
	Non-irrigation	12-20-10	ND	ND	BLQ	BLQ	BLQ	**2.6**	BLQ	BLQ	ND
		01-24-11	ND	ND	BLQ	**0.7**	BLQ	**11**	BLQ	ND	BLQ
		09-27-11	ND	BLQ	BLQ	ND	BLQ	**3.0**	BLQ	**13.1**	ND

Cotinine was detected in this site during the 2011 irrigation season at 0.1 ng/L as well as caffeine at 3.9 ng/L. Caffeine was also detected during the 2010 irrigation season at 1.1 ng/L and 10.0 ng/L. Sulfamethazine was detected only during the 2011 irrigation season 0.6 ng/L and during the 2010 non-irrigation season at 0.7 ng/L while ciprofloxacin was found at 13.1 ng/L during the 2011 non-irrigation season. Codeine concentrations ranged from 3.6 ng/L to 9.2 ng/L for both 2010 and 2011 irrigation seasons while it ranged from 2.6 ng/L to 11.0 ng/L during both 2010 and 2011 non-irrigation seasons. Acetaminophen, trimethoprim, fluoxetine, and erythromycin in this site were either below limits of quantification or below limits of detection in both seasons. Ciprofloxacin was found as high as 13.1 ng/L during the 2011 non-irrigation season followed by codeine (11.0 ng/L) and caffeine (10.0 ng/L) during the 2010 non-irrigation season and 2010 irrigation season respectively.

2.6.2.5 Irrigation and Non-irrigation seasons

According to results obtained from the general linear mixed model, there were no significant differences on PPCP target analytes concentrations between the irrigation and non-irrigation seasons. Figure 11 shows mean concentrations of target analytes for 2011 irrigation season and 2010 non-irrigation season.

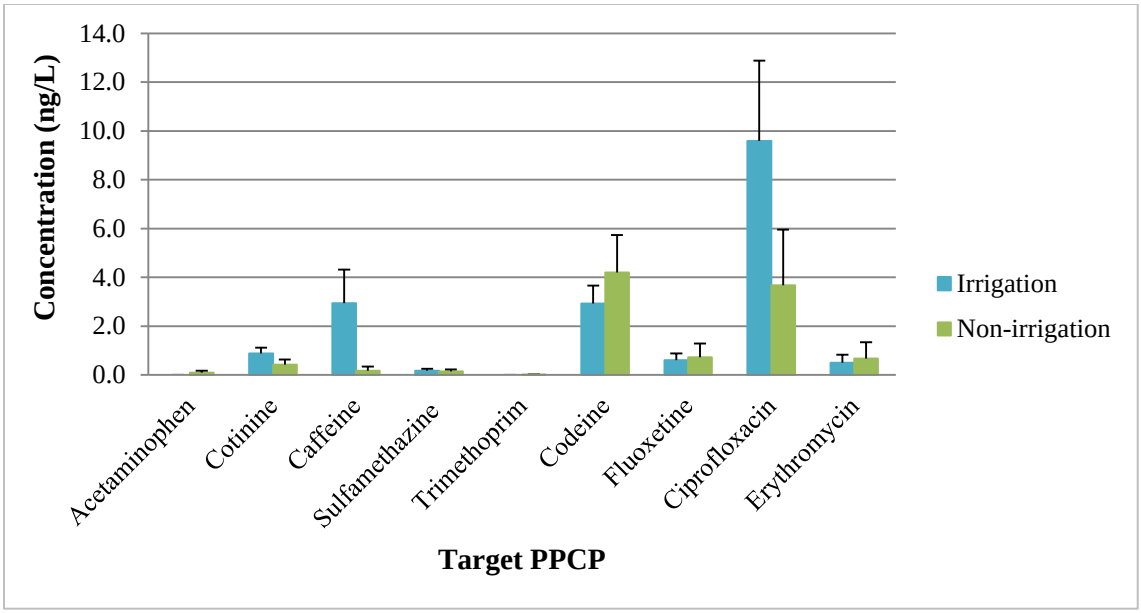


Figure 11. Mean concentrations of target PPCP analytes in the Middle Rio Grande during irrigation and non-irrigation seasons. Standard error bars are indicated.

2.7 Discussion

The range of concentrations of target analytes found in water samples from the middle Rio Grande in this study (ng/L) agree with similar findings reported in literature from studies conducted elsewhere in the U.S., Canada, and Europe (Waiser *et al*., 2011; Ferrer *et al*., 2010; Ellis, 2006; Moldovan, 2006; Boyd *et al*., 2003). Concentrations of target analytes in this study ranged from 0.004 ng/L to 42.8 ng/L. Generally, concentrations of acetaminophen, caffeine, codeine, cotinine, fluoxetine, sulfamethazine, trimethoprim and erythromycin were found below 10 ng/L while ciprofloxacin was found as high as 42.8 ng/L. These results agrees with those of Ellis (2006) who reported that according to the European Union 5th Poseidon Project, PPCPs and their metabolites are frequently found below 10 ng/L levels and rarely above 100 ng/L in surface waters.

Even though the occurrence of acetaminophen (an analgesic), cotinine (metabolite of nicotine), caffeine (stimulant) and trimethoprim (antibiotic) was higher during the irrigation season, the presence of acetaminophen and trimethoprim was always found below limits of quantification. This finding might be due to high rates of degradation by hydrolysis that increase with increasing temperatures in the case of cotinine while for caffeine, acetaminophen and trimethoprim degradation may be occurring by photo or bio-degradation (Waiser *et al*., 2011; Ziylan and Ince, 2011). On the other hand, the occurrence of sulfamethazine (antibiotic), codeine (analgesic), fluoxetine (anti-depressant), ciprofloxacin (antibiotic) and erythromycin (antibiotic) was higher during the non-irrigation season. Non-irrigation water samples collection was conducted from October 2010 to February 2011, a period in which water temperatures are colder and significantly lower from the irrigation season (Table 20, Appendix A), which decreases hydrolysis of these chemicals (Waiser *et al*., 2011).

Although the occurrence and concentrations of nine selected PPCP toxicants was expected to be higher during the non-irrigation season, results obtained from the general linear mixed model indicated that there was not a significant difference in concentrations of target analytes between the irrigation and non-irrigation seasons. Mean concentrations for both flow regimes are shown in Figure 11. This observation disagrees with findings reported by Kolpin *et al*. (2004) in which PPCPs occurrence was higher in water bodies with low flow conditions in which urban contributions of PPCPs and other organic compounds decrease as flow conditions increase likely due to dilution effects.

General linear mixed model results also indicated that there were no significant differences in concentration of target analytes by site with the exception of fluoxetine, which concentrations were found to be significantly higher in the Rio Grande at Percha Dam, ($F = 5.62$;

p = 0.0040) as those obtained from the other three downstream sites. This observation also disagrees with our hypothesis that lower concentrations would be found in the Rio Grande at the Percha Dam sampling station due to its relative remoteness from urbanization.

Target analytes were generally higher in concentrations at American Dam, El Paso Co., TX. This finding agrees with our hypothesis of higher occurrence and concentrations of target analytes in sampling sites located within the El Paso/Ciudad Juárez metroplex. Additionally, this may be explained by the fact that the Rio Grande at this point is continuously receiving treated wastewater from the Northwest WWTP. The highest concentrations detected in this study were found at this site. For instance, concentrations of ciprofloxacin were as high as 42.8 ng/L upstream of the effluent canal while the second highest concentration (41.0 ng/L) was found in a water sample collected downstream of the effluent canal. These concentrations were found during the irrigation season and were collected on May 2011. Along with this study, a study in which the occurrence and concentrations of the same PPCP target analytes in wastewater influent and effluent from the Northwest WWTP located in El Paso, Co., TX was conducted. As previously discussed, the Northwest WWTP discharges treated wastewater effluent in the Rio Grande at the American Dam sampling station. Mean concentrations of target analytes in effluent samples from the Northwest WWTP ranged from 0.34 ± 0.37 ng/L to 9.73 ± 5.42 ng/L. Mean concentrations from the Northwest WWTP treated effluent were found to be higher as compared to those collected at the American Dam sampling station with the exception of ciprofloxacin that was higher in samples collected from the river [Northwest WWTP = 1.39 ± 2.58 ng/L; American Dam sampling station = 12.99 ± 15.08 ng/L (Guerrero, 2011]). Additional studies conducted in the Rio Grande include that conducted by Brown *et al*. (2006) in the Albuquerque, NM area in which the occurrence of 10 antibiotics (trimethoprim and ciprofloxacin among others) was

surveyed in three sites of the river. One study site was located upstream of a WWTP while the other two were located 3.2 and 6.4 km downstream of the WWTP. Only one antibiotic, sulfamethoxazole, was found and it was detected (300 ng/L) in both sites downstream of the WWTP while ciprofloxacin and trimethoprim were not detected.

Comparing by analyte, cotinine and codeine were consistently found in all four surveyed sites during both irrigation and non-irrigation seasons. Cotinine levels ranged from 0.004 ng/L to 2.7 ng/L while codeine levels ranged from 0.1 ng/L to 18.1 ng/L. The constant found of cotinine may be explained by urban activities conducted in the El Paso/Ciudad Juárez area, specifically by the consumption of tobacco in which 70 – 80% of absorbed nicotine is metabolized into cotinine and then excreted (Martínez Bueno *et al*., 2011). Codeine is an analgesic frequently detected in surface waters (Moldovan, 2006) as reported in a study conducted at four sites in the Somes River in Romania where this chemical was found at 27 - 54 ng/L at three of the sites and was undetected at a fourth site (Moldovan, 2006).

From this study, methods for future analysis of PPCPs in surface waters can be refined. The collection of additional samples is recommended since collected samples in this study may have not integrated concentration over time or flow due to the limited duration and frequency of sample collection as well as variation in flow and other environmental conditions. In addition, in order to obtain a more representative sample, the use of composite samplers and passive samplers is recommended. Passive samplers for PPCPs in water matrices provide additional information over longer periods of time (days to weeks) as compared to the results obtained by collecting grab samples and will reflect concentrations of target analytes over a longer sampling interval (Buchberger, 2011). The collection of sediment samples is also suggested since it has been shown that lipophilic PPCP chemicals tend to accumulate in sediment or suspended matter

(Santos *et al*., 2010) which may influence concentrations detected in water matrices. The survey of other PPCP toxicants such as ibuprofen, an anti-inflammatory, is recommended due to its highly detection in surface waters and in treated wastewater effluent nearly worldwide (Santos *et al*., 2010; Waiser *et al*., 2011). The survey of this toxicant is also recommended due to its chronic toxicity at environmental relevant concentrations as observed in the freshwater amphipod *Gammarux pulex* which locomotion and feeding success was inhibited at concentrations ranging from 10 ng/L to 100 ng/L (Santos *et al*., 2010; Waiser *et al*., 2011). Also, the survey of metabolites is highly suggested since, as previously discussed in Chapter 1, part of these toxicants is metabolized before excretion.

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CHAPTER 3: EFFECTS OF SELECTED PHARMACEUTICALS AND PERSONAL CARE PRODUCTS (PPCPS) ON THE FRESHWATER ROTIFER *Plationus patulus*

3.1 Abstract

The occurrence of PPCPs has been reported to occur in surface waters around the world, thus, aquatic systems are of great concern due to the continuous input of these pollutants. The freshwater rotifer *Plationus patulus,* a basal member of riverine food webs, was used to test acute and chronic toxicity of 4 selected PPCPs (acetaminophen, caffeine, fluoxetine, and triclosan). Two geographically distinct *P. patulus* populations were tested. The first population was collected in a remote site of Mexico, south of Big Bend National Park, TX, and was used as a reference population since it was assumed that this site is free of PPCPs toxicants. The second population was collected in a highly urban reach of the Rio Grande within the El Paso, TX/Ciudad Juárez metroplex. Results from acute toxicity tests show that the *P. patulus* reference population is more sensitive to triclosan (LC_{50} = 0.13 mg/L) while the Rio Grande population was more sensitive to acetaminophen (LC_{50} = 121 mg/L). Both populations showed a similar LC_{50} for caffeine (Reference population LC_{50} = 423 mg/L; Rio Grande population LC_{50} = 419 mg/L). LC_{50} values for both populations indicate that *P. patulus* is less sensitive to acute exposure to acetaminophen and caffeine as compared to *Daphnia magna* while this rotifer species was more sensitive to fluoxetine and triclosan. In chronic exposures, there was generally a decrease in population growth for all four tested PPCPs in both *P. patulus* populations. For instance, chronic exposure to acetaminophen (10 mg/L, 15 mg/L, and 20 mg/L) in the reference population inhibited population growth at all tested concentrations starting on day 3 through day

6 of exposure while for the Rio Grande population growth was inhibited only at 15 mg/L and 20 mg/L starting on day 3 and continuing through day 6 of exposure. A second set of lower acetaminophen concentrations were tested in both populations (1 mg/L, 5 mg/L, and 10 mg/L) for which no significant population growth inhibition was determined for either population with the exception of the 10 mg/L treatment which inhibited population growth in the reference population as previously shown. For tested concentrations of caffeine (100 mg/L, 200 mg/L, and 300 mg/L), population growth was inhibited in 200 mg/L and 300 mg/L treatments for both populations. For the reference population, growth was inhibited on days 5 and 6 at 200 mg/L while at 300 mg/L negative rates of population increase were seen on days 4 through 6. Population growth of the Rio Grande population was inhibited at 200 mg/L on days 3 (86%), 4 (45%), 5 (44%), and 6 (52%) as compared to control treatment. Further at 300 mg/L, negative rates of population increase were seen on days 3, 4, and 6 while on day 5 population growth was inhibited 99% as compared to the control treatment. Chronic exposure to fluoxetine (0.005 mg/L, 0.010 mg/L, and 0.020 mg/L) was only tested in the Rio Grande population. This toxicant caused significant population growth inhibition at 0.020 mg/L on days 3 (26%), 4 (15%, 5 (15%), and 6 (16%) as compared to control treatment. Tested concentrations of triclosan (0.05 mg/L, 0.075 mg/L, and 0.10 mg/L) had the most deleterious effects on both populations. In the reference population, population growth was affected at all tested concentrations with growth inhibition at 0.05 mg/L over days 4 and 5 and with negative growth rates at 0.075 mg/L and 0.10 mg/L over days 4 – 6 of exposure. For the Rio Grande population, negative growth rates or no growth was observed at all tested concentrations. Sub-lethal effects observed for chronic exposure to acetaminophen, caffeine, and triclosan included decreases in egg production and increased incidence of egg detachment from ovigerous females. Overall, the reference population was

more sensitive to the chronic exposure of selected PPCPs as compared to control treatments than the Rio Grande population as compared to control treatments. Although tested concentrations of selected PPCPs are higher than those occurring in the environment, the continuous introduction of these toxicants to aquatic ecosystems may still present a risk. In addition, additive effects of mixtures of these toxicants have shown greater toxicities as those determined for single chemicals. Thus the impacts of these toxicants need further investigation.

3.2 Introduction

The quantity and composition of pharmaceuticals and personal care products (PPCPs) released into the environment by anthropogenic means is of increasing concern. Of all the environments, aquatic systems are of the greatest concern since they receive most of these pollutants (Jjemba 2008, Kolpin *et al*. 2002). Ecotoxicology studies allow for the understanding of how toxicants impact individuals, populations, communities, and ecosystems by testing chemicals at lethal and sublethal concentrations (Jjemba, 2008). The assessment of their effects can be evaluated by performing field and laboratory studies (Jjemba, 2008). One of the advantages of conducting laboratory studies is that they allow for the assessment of single toxicants using standardized methods in which a wide variety of organisms can be used for the evaluation of acute and chronic toxicity of pollutants (Jjemba, 2008).

As previously discussed (Chapter 1), rotifers have been used extensively in toxicity testing due to their susceptibility to a diverse range of pollutants (Snell and Joaquim-Justo, 2007) and due to their sensitivity to water quality changes (Dahms *et al.*, 2011). In addition, their prevalence in aquatic communities allows for a better understanding of the effects that these

pollutants may have on aquatic ecosystems (Dahms *et al.*, 2011). Due to these attributes and its common occurrence in the Rio Grande, *Plationus patulus* (Figure 12), a freshwater rotifer, was selected in order to assess the toxicity and sub-lethal effects of four selected PPCPs. This species has been recognized along with *Brachionus calyciflorus* as a test standard species by the American Public Health Association (Sarma *et al.*, 2008).

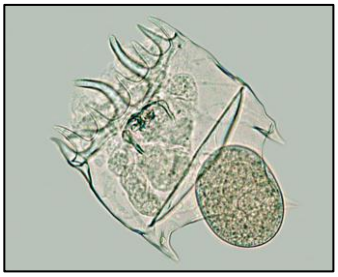

Figure 12. The freshwater rotifer *Plationus patulus*.
Photo by E.J. Walsh.

For acute toxicity tests (48 hr), the median lethal concentration (LC_{50}), the maximum concentration of the test chemical that produces no statistical harmful compared to control (NOEC), and the lowest concentration of the test compound that has a statistically significant detrimental effect compared to control (LOEC) were selected as toxicological endpoints. Chronic toxicity of target compounds was assessed by performing population growth studies, with the intrinsic rate of population increase (r) as the endpoint. In population growth studies, the evaluation of adaptation to specific toxicants is possible since individuals from all ages are being tested at the same time within the same created environment (Sarma *et al.*, 2008; Sarma *et al.*, 2006).

3.3 Objectives

To evaluate the toxicity and possible effects of four selected PPCPs pollutants (acetaminophen, caffeine, fluoxetine, and triclosan) on the freshwater rotifer *Plationus patulus*, from the Rio Grande. An additional *P. patulus* population collected from a remote location in Mexico was used as a reference for comparison of tolerance levels.

The criteria for selection of test compounds included their frequency of detection in surface waters (Kolpin *et al*., 2002) and the solubility of the chemical in culture medium.

3.4 Hypothesis

The reference population is expected to be less tolerant than the Rio Grande population to the acute and chronic exposure of selected PPCP toxicants.

3.5 Materials and Methods

3.5.1 Test Lineages

Two geographically distinct *P. patulus* populations were tested. The first population, referred in this study as the reference population, was collected from a tinaja located in a remote area of Mexico, south of Big Bend National Park on 07/31/2008. This population was used as the reference population because it is assumed that the site is free of PPCP pollutants since this tinaja is primarily rainfall filled and human access to this water body is difficult. The second population, referred as the Rio Grande population, was collected from the Rio Grande at

sampling site four, located near the Fabens Port of Entry described in Chapter 2. This population was collected on 08/12/2010. *P. patulus* were collected using a 64 µm mesh plankton net. Both populations were cultured under standard laboratory conditions. Toxicity studies were conducted after more than 20 generations of laboratory culture for each population.

P. patulus populations were cultured in EPA medium (EPA, 1993), a synthetic moderately hardwater with pH adjusted to 7.5 ± 0.02, and were fed with a mixture of algae (*Chlamydomonas reinhardtii* [UTEX strain 90] and *Chlorella vulgaris* [UTEX strain 30]). Cultures were incubated at 25 ± 1°C in 24 hr light.

Rotifers from each population were exposed to four selected PPCP compounds (acetaminophen, caffeine, fluoxetine, and triclosan; Table 2, Chapter 2) in order to evaluate the toxicity and determine the chronic effects of these pollutants.

The *P. patulus* reference population was maintained in optimum conditions by Sarah Baca, an undergraduate student in the Environmental Science Program at the University of Texas at El Paso. Exposure studies for this population were also carried out by her.

Compounds were obtained from a licensed distributor in powdered form. Brands and CAS registry numbers are shown in Table 10.

Table 10. Chemical Abstracts Service Registry Numbers and brands of tested toxicants.

Compound	Powdered form	CAS RN	Laboratory
Acetaminophen	4-Acetamidophenol,98%	103-90-2	Acros Organics
Caffeine	Caffeine, Anhydrous	58-08-2	MP Biomedicals, LLC
Fluoxetine	Fluoxetine hydrochloride	56296-78-7	Sigma-Aldrich
Triclosan	2,4,4'-Trichloro-2' hydroxydiphenyl ether	3380-34-5	TCI America

Effects were determined by conducting 48 hr exposure studies in which the LC_{50} was obtained as well as the NOEC and LOEC values for each of the four PPCPs compounds. Chronic toxicity of each compound was also determined by conducting 6-day population growth studies.

Stock solutions for tested toxicants were prepared in EPA medium with a pH of 7.5 ± 0.02 for acute and chronic toxicity studies. For each toxicant, the solubility in water was considered before determining the final concentration of the solution. For chronic exposures stock solutions were prepared daily.

3.5.2 Acute Toxicity Studies

In order to find the LC_{50}, range finder studies were conducted for each of the four compounds by exposing each *P. patulus* population to a broad range of concentrations of each chemical. These ranges were determined from values in the literature for similar species (e.g., other rotifer species, *Daphnia*).

Before the start of LC_{50} tests for each pollutant, ovigerous females were isolated for 4 – 6 hr in EPA medium containing excess algal food and were incubated at 25 ± 1 °C with full light. Cultures were checked after 4 – 5 hrs; neonates were transferred into a separate Petri dish containing EPA medium with food and were left there for about one hour before the exposure study started. Concentrations of the test solution were prepared by making dilutions from a stock solution. Up to 6 test concentrations (Table 11) were diluted to the appropriate concentration.

Table 11. Concentrations tested for acute toxicity of selected PPCPs.

48 hr exposure			
***Plationus patulus* population**		**Reference population**	**Rio Grande population**
Concentration (mg/L)	Acetaminophen	0, 100, 200, 300, 400, & 500	0, 75, 100, 150, 175, 200, & 250
	Caffeine	0, 250, 300, 350, 400, & 450	0, 250, 300, 350, 400, & 450
	Fluoxetine	Not tested	0, 0.05, 0.075, 0.10, 0.20, & 0.40
	Triclosan	0, 0.08, 0.11, 0.14, 0.17, & 0.20	0, 0.15, 0.20, 0.25, 0.30, 0.35, & 0.40
Replicates/compound		6	6
Number of individuals/replicate		1 - 3	3 - 5

All tests were conducted in disposable, sterile 24-well tissue culture plates. The set up consisted of one control solution (EPA medium) and diluted test concentrations with 6 replicates per treatment. For each test concentration, 2 ml were pipetted in each well. After the eggs hatched, 1 - 5 neonates (+ 2 hr old) were placed onto each well (Figure 13). The number of deaths and other effects (e.g. slow swimming speed, immobilization) were recorded after 24 and 48 hours.

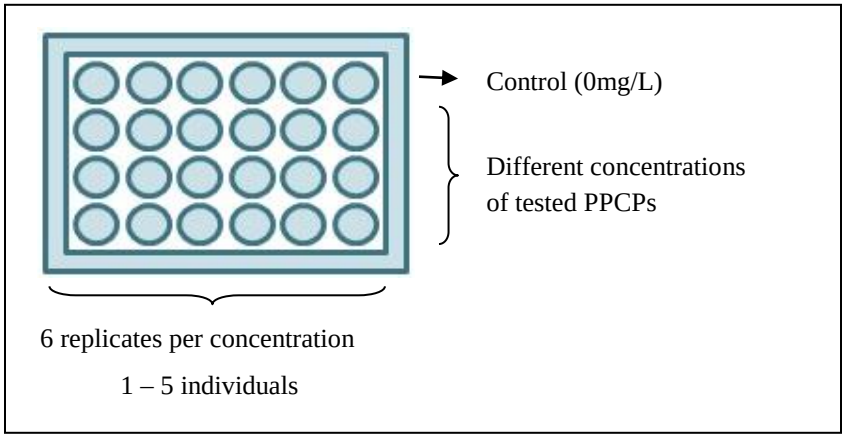

Figure 13. Set up of 24-well tissue culture plates used for acute exposures of the rotifer *Plationus patulus* to selected PPCPs.

3.5.3 Chronic Toxicity Tests

Six-day population growth studies were carried out in order to assess the chronic toxicity by determining the effects that each of the four selected PPCPs pollutants has on the intrinsic rate of population increase (r) of each *P. patulus* population. Both populations were tested at the same concentrations. For these studies, a modified protocol from Snell and Moffat (1992) was followed.

One day before each study was performed, all the individuals were transferred into fresh EPA medium with excess algal food. On the test day, 4 - 6 hrs before initiation of the experiment, ovigerous females were isolated and placed in an incubator at 25 ± 1 ºC in full light. *Chlamydomonas reinhardtii* was concentrated by centrifuging it at 10,000 rpm for 10 min. After this, pellets were collected and re-suspended in EPA medium. Algal density was estimated by using a Neubauer hematocytometer. The algal suspension was diluted to 2.5 X 10^5 cells/ml. The

test pollutant was then diluted in this solution into 3 test concentrations from a stock solution (Table 12).

Table 12. PPCPs concentrations tested for chronic toxicity studies on *Plationus patulus*. Replicates (Rep.); Individuals (ind.). Not conducted on reference population (*).

Six-day exposure						
PPCP	**Acetaminophen**		**Caffeine**	**Fluoxetine***	**Triclosan**	
	1st study	2nd study			1st study	2nd study*
Concentration (mg/L)	10, 15, & 20	1, 5, & 10	100, 200, & 300	0.005, 0.010, & 0.020	0.05, 0.075, & 0.10	0.0005, 0.005, & 0.05
Rep./concentration	5	5	5	5	5	5
Initial number of ind./replicate	3	3	3	3	3	3

Selection of tested concentrations for each compound was based on the LC_{50} value (lowest LC_{50} from either *P. patulus* population). Concentrations for each compound were no higher than 70% of the LC_{50} value.

The design for this study consisted of 3 test concentrations and one control, 5 replicates each. Control replicates consisted of EPA medium with the algal suspension and rotifers. Test tubes were filled with 12 ml of each test concentration and 3 neonates (+ 2 hr old) were transferred on each tube. The test tubes were placed on a rotator at 8 to 10 rpm and were incubated at 25 ± 1 ºC in dark for 6 days. Media with food and compound was replaced daily. The number of individuals and their reproductive status (asexual versus sexual; fertilized versus

unfertilized) were recorded daily. Deformities and any changes in behavior were also recorded daily.

At the end of the experiment, the number of *P. patulus* individuals was counted as well as the number of unviable eggs.

For acetaminophen and triclosan, a second set of concentrations (1 mg/L, 5 mg/L and 10 mg/L) was tested. These concentrations are more likely to be environmental relevant concentrations since PPCPs have been generally reported to occur in the ng/L to µg/L range in surface waters (Kolpin *et al*., 2002; Flaherty and Dodson, 2005).

3.5.4 Data Analysis

The LC_{50} value for each of the four selected PPCPs pollutants was determined by Probit Analysis in the statistical program IBM SPSS (version 17.0). LC_{50} values for reference and Rio Grande populations were compared in order to determine significant differences in the tolerance level to tested toxicants by running a Non-Linear Mixed Model Analyses in the statistical program SAS® (version 9.2). In order to determine the NOEC and LOEC values, an analysis of variance (ANOVA) followed by a Dunnett´s Post Hoc test was run to compare the survival rate response vs. concentration in the statistical program IBM SPSS (version 17.0).

For population growth studies, the intrinsic rate of population increase (r) for each concentration at a given day was calculated according to the following formula:

$$r = (\ln N_t - \ln N_0)/T$$

where $\ln N_t$ is the natural log of number of alive rotifers in the test tube at each sampled day, $\ln N_0$ is the natural log of initial number of rotifers in the test tube (three), and T is the time in days (one through six). The average of the intrinsic rate of population increase (r) per day per

concentration was obtained as well as the standard error based on five replicates for most of the compounds.

Intrinsic rates of population increase (r) for each compound were analyzed by General Linear Mixed Model (GLMM) in the statistical program SAS® (version 9.2) in order to determine if there was a significant relationship of endpoint and toxicant concentration. GLMM analyses were conducted by Dr. Julia Bader from the BBRC Statistical Consulting Laboratory (UTEP).

3.6 Results

3.6.1 Acute toxicity studies

Acute toxicity tests showed that triclosan was the most toxic compound for the reference *P. patulus* population with an obtained LC_{50} of 0.13 mg/L ($z = -3.327$; $p = 0.001$). However, for the Rio Grande population the most toxic compound was fluoxetine with an LC_{50} of 0.19 mg/L ($z = -7.119$; $p < 0.001$). On the other hand, the least toxic compound for both *P. patulus* populations was caffeine with an estimated LC_{50} of 423 mg/L ($z = -3.449$; $p = 0.001$) for the reference population and an LC_{50} of 419 mg/L ($z = -4.437$; $p < 0.001$) for the Rio Grande population.

3.6.1.1 Acetaminophen 48 hr exposures

In acute toxicity tests of acetaminophen, the Rio Grande population was less tolerant than the reference population according to the LC_{50} values (Figures 14 and 15).

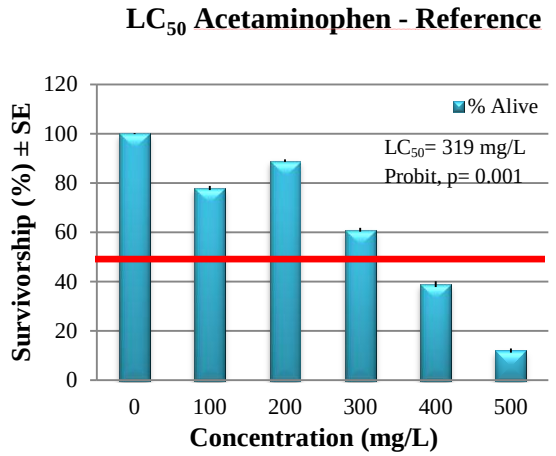

Figure 14. Percent survivorship of a reference population of the rotifer *Plationus patulus* as a function of acetaminophen increasing concentrations. Red line shows survivorship of 50% of the population over increasing concentrations of acetaminophen.

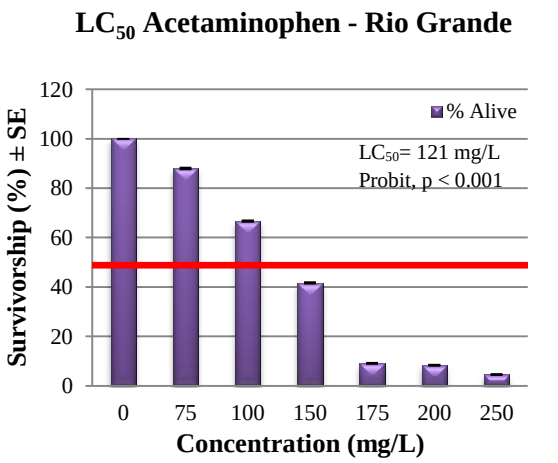

Figure 15. Percent survivorship of the Rio Grande population of the rotifer *Plationus patulus* as a function of acetaminophen increasing concentrations. Red line shows survivorship of 50% of the population over increasing concentrations of acetaminophen.

For acetaminophen, the 48 hr LC_{50} for the *P. patulus* reference population according to Probit analysis was 319 mg/L ($z = -3.984$, $p < 0.001$) while for the Rio Grande population the LC_{50} was 121 mg/L ($z = -7.138$; $p < 0.0001$), almost three times lower than that of the reference population (Table 22; Appendix C). The estimated no-observed effect concentration and the lowest-observed effect concentration for the Rio Grande population after a Dunnett´s Post Hoc test was 75 mg/L ($F = 64.354$; $p < 0.001$) with an observed mortality of 12% and 100 mg/L ($F = 64.354$; $p = < 0.001$) with a mortality of 33%.

3.6.1.2 Caffeine 48 hr exposures

The caffeine LC_{50} values for both *P. patulus* populations were similar as shown in Figures 16 and 17. Among the four tested PPCPs compounds, caffeine was the least toxic chemical (Table 22; Appendix C). For the reference population the LC_{50} was 423 mg/L ($z = -3.449$, $p = 0.001$) versus 419 mg/L ($z = -4.437$; $p < 0.0001$) for the Rio Grande population. The NOEC for the Rio Grande population was 300 mg/L ($F = 9.129$; $p < 0.001$) with 22% of mortality while the LOEC was 350 mg/L ($F = 9.129$; $p < 0.001$) with 34% mortality.

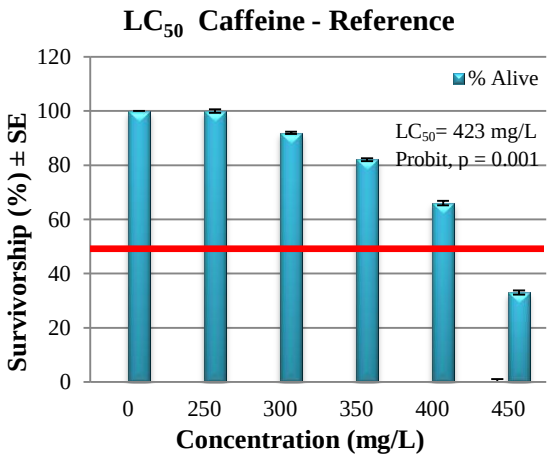

Figure 16. Percent survivorship of a reference population of the rotifer *Plationus patulus* as a function of increasing caffeine concentration. Red line shows survivorship of 50% of the population over increasing concentrations of caffeine.

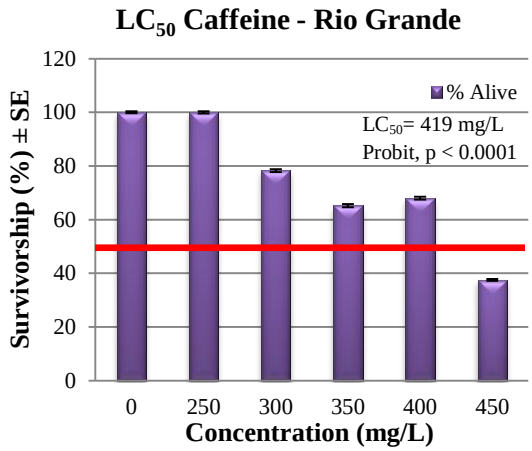

Figure 17. Percent survivorship of the Rio Grande population of the rotifer *Plationus patulus* as a function of increasing caffeine concentration. Red line shows survivorship of 50% of the population over increasing concentrations of caffeine.

For the Rio Grande population, an increase in mobility was observed after 24 hr of exposure to caffeine at 400 mg/L and 450 mg/L as compared to the test control. However, a decrease in mobility was observed also for this population at 250 mg/L and 300 mg/L after 48 hr of exposure.

3.6.1.3 Fluoxetine 48 hr exposures

Fluoxetine was tested only in the Rio Grande population and it was the most toxic chemical for this population (Table 22; Appendix C) with an estimated 48 hr LC_{50} of 0.19 mg/L ($z = -7.119$; $p < 0.001$). The NOEC was 0.075 mg/L and the LOEC was 0.10 mg/L according to Dunnett´s Post Hoc test ($F = 48.172$; $p < 0.001$). Fluoxetine caused a decrease in mobility in *P. patulus* at 40 mg/L after 24 hr of exposure while mortality was recorded after 48 hr. A mortality of 4% as compared to control treatment was recorded at 0.075 mg/L, of 21% at 0.10 mg/L, of 52% at 0.20 mg/L and of 86% at 0.40 mg/L treatments (Figure 18).

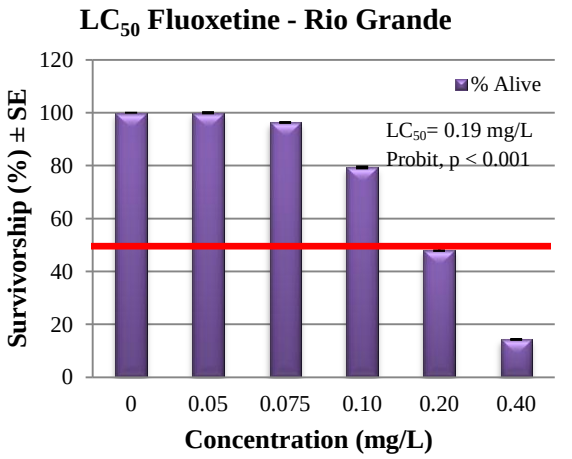

Figure 18. Percent survivorship of the Rio Grande population of the rotifer *Plationus patulus* as a function of fluoxetine exposure. Red line shows survivorship of 50% of the population over increasing concentrations of fluoxetine.

3.6.1.4 Triclosan 48 hr exposures

According to the LC_{50}, triclosan is the most toxic compound for the reference *P. patulus* population (Table 22; Appendix C) with an estimated 48 hr LC_{50} of 0.13 mg/L (z = -3.327; p = 0.001) while for the Rio Grande population the 48 hr LC_{50} was 0.32 mg/L (z = -3.382; p = 0.001) as shown in Figures 19 and 20.

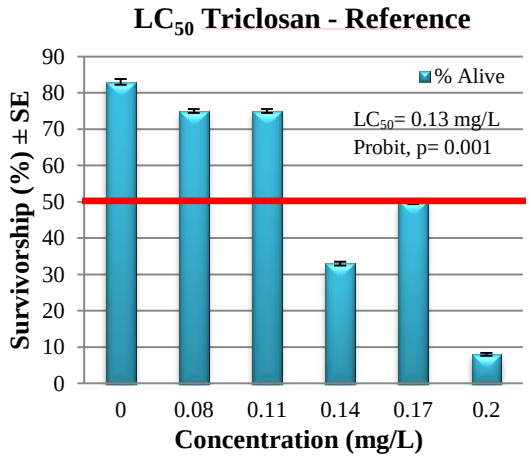

Figure 19. Percent survivorship of a reference population of the rotifer *Plationus patulus* as a function of triclosan exposure. Red line shows survivorship of 50% of the population over increasing concentrations of triclosan.

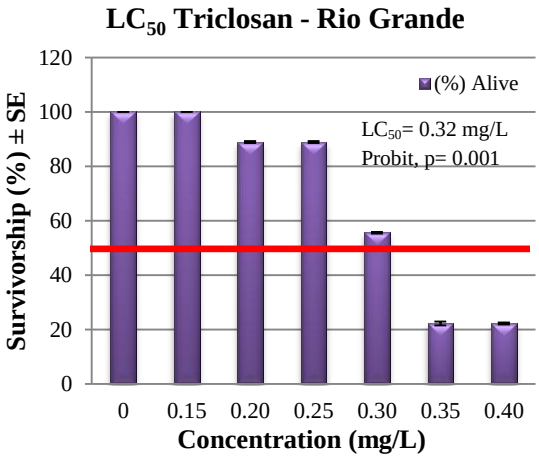

Figure 20. Percent survivorship of the Rio Grande population of the rotifer *Plationus patulus* as a function of triclosan exposure. Red line shows survivorship of 50% of the population over increasing concentrations of triclosan.

Decrease in mobility was observed for triclosan after 48 hr of exposure at 0.20 mg/L, 0.25 mg/L, and 0.30 mg/L in the Rio Grande population.

Table 13 shows the 48 hr LC_{50}, NOEC, and LOEC for each compound for the reference and Rio Grande *P. patulus* populations.

Table 13. 48 hr LC_{50} values for *P. patulus* as determined by Probit analysis. The no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) values were determined by Dunnett´s Post Hoc tests. Not determined (ND).

	Reference population			**Rio Grande population**		
Compound	**LC_{50} (mg/L)**	**NOEC (mg/L)**	**LOEC (mg/L)**	**LC_{50} (mg/L)**	**NOEC (mg/L)**	**LOEC (mg/L)**
Acetaminophen	319	200	300	121	75	100
Caffeine	423	ND	ND	419	300	350
Fluoxetine	ND	ND	ND	0.19	0.075	0.10
Triclosan	0.13	ND	ND	0.32	0.25	0.30

The reference population was more tolerant than the Rio Grande population to acetaminophen ($t = 7.49$; $p < 0.0001$) as shown in Table 13, which resulted as well in higher NOEC and LOEC values. Similar LC_{50} values were determined for both populations as exposed to increasing concentrations of caffeine which resulted to be no significantly different ($t = 0.41$; $p = 0.6881$). NOEC and LOEC values were not determined for the reference population. Acute toxicity of fluoxetine was only tested on the Rio Grande population due to time constraints. LC_{50} values obtained from acute exposure to triclosan resulted to be significantly different between the two populations ($t = -10.06$; $p < 0.0001$) showing that the Rio Grande population is more tolerant to this toxicant than the reference population.

3.6.2 Chronic Toxicity Studies

Reproduction of both *P. patulus* populations was generally inhibited causing a decrease in population growth with increasing concentrations of acetaminophen, caffeine, and triclosan. Out of the four assessed toxicants, tested concentrations of triclosan showed to have more deleterious effects on both *P. patulus* populations. Some of the observed sublethal effects for acetaminophen, caffeine, and triclosan were decreased egg production and egg detachment from females leading to unviable embryos in most cases. The tested concentrations for fluoxetine in the Rio Grande population showed a similar intrinsic rate of increase as compared to the control treatment except for the 0.020 mg/L treatment which showed to inhibit population growth as compared to control treatment.

Intrinsic rates of population increase (r) for each tested compound for both populations are shown below.

3.6.2.1 Acetaminophen 6-day exposures

Six-day exposure studies to acetaminophen for the reference population showed significant differences in population growth among increasing concentrations of acetaminophen over time ($F = 20.96$; $p < 0.0001$). Significant differences in population growth as compared to the control treatment were determined to occur at all tested concentrations. For instance, at 10 mg/L, no growth was observed at day 3 of exposure ($t = 3.32$, $p = 0.0014$) while for days 4, 5, and 6, population growth was inhibited 83% ($t = 5.77$, $p < 0.0001$), 92% ($t = 11.46$, $p < 0.0001$), and 89% ($t = 11.05$, $p < 0.0001$) respectively as compared to the control treatment. At 15 mg/L,

no population growth was observed at day 3 ($t = 3.32$, $p = 0.0014$) whereas for day 4 a negative rate of population increase was observed ($t = 8.11$, $p < 0.0001$) and population growth was inhibited 98% at days 5 ($t = 12.16$, $p < 0.0001$) and 6 ($t = 12.18$, $p < 0.0001$). Population growth was not observed at 20 mg/L as compared to control treatment over time of exposure (Inset of Figure 21).

Significant differences were observed in population growth among concentrations of acetaminophen over time (F value = 43.04; $p = < 0.0001$) for the Rio Grande population. Significant differences were observed specifically on days 3, 4, 5, and 6 for 15 mg/L and 20 mg/L as compared to the control treatment (Figure 21). On days 3 and 4, there was a significant difference between the control treatment and 15 mg/L where no growth was observed ($p = < 0.0001$) while for this same treatment, population growth was inhibited 70% ($t = 9.79$, $p = < 0.0001$) and 67% ($t = 9.51$, $p = 0.0001$) on days 5 and 6 respectively. Significant differences were also observed between the control treatment and the 20 mg/L treatment where a negative intrinsic rate of increase was obtained through days 3 ($t = 9.37$, $p < 0.0001$), 4 ($t = 13.81$, $p < 0.0001$), 5 ($t = 14.29$, $p < 0.0001$), and 6 ($t = 14.43$, $p < 0.0001$).

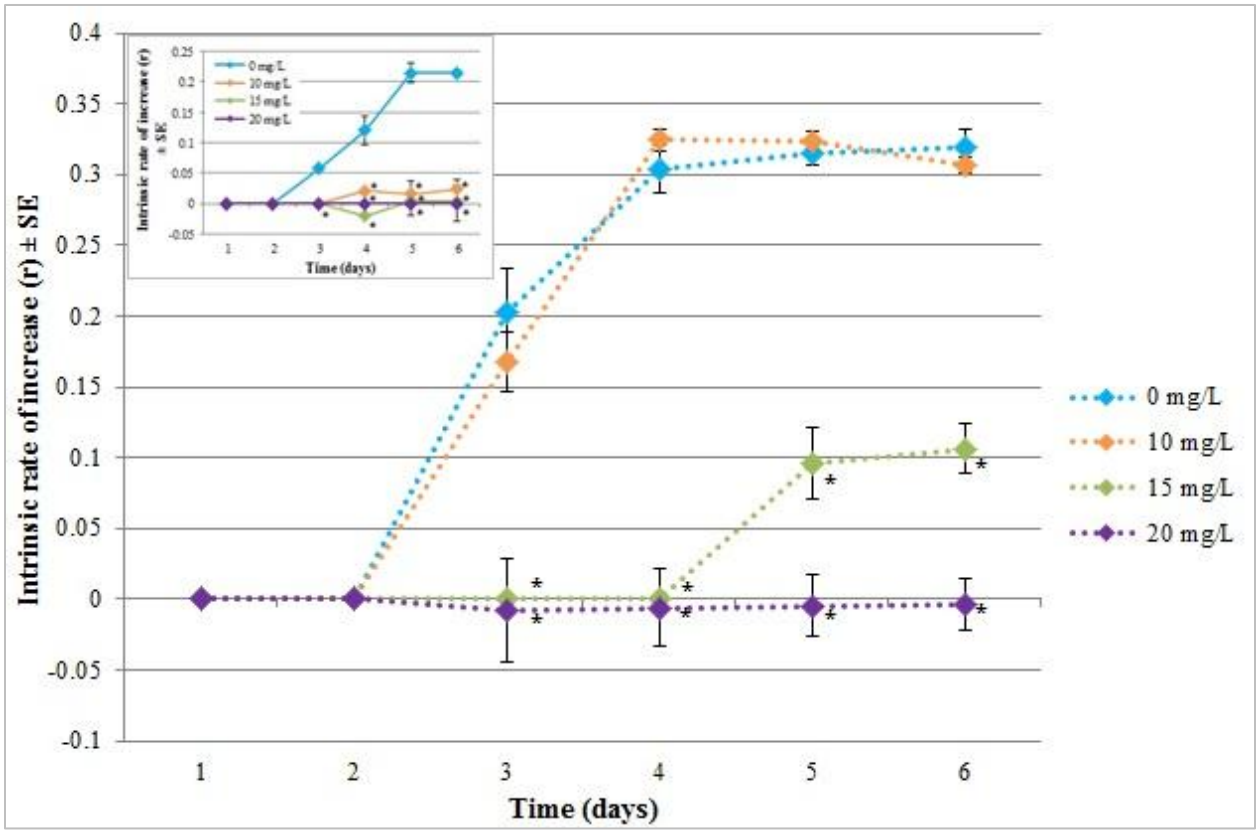

Figure 21. Rates of population increase (r) over time for the reference population (inset) and the Rio Grande population as exposed to different concentrations of acetaminophen. Mean ± standard error are based on five replicates. * Indicates significant effects on population growth.

According to the results obtained from the GLMM analysis, there was not a significant effect on the reference population´s intrinsic rate of increase (r) for the second set of tested concentrations ($F = 0.64$; $p = 0.8303$) nor for the Rio Grande population ($F = 0.51$; $p = 0.9266$). Intrinsic rates of increase (r) over time for this study are shown in Figure 27 in Appendix D for both populations.

Sublethal effects to both *P. patulus* populations produced by the six-day exposure to acetaminophen are listed in Table 14. These effects were observed at 10 mg/L, 15 mg/L, and 20 mg/L acetaminophen concentrations but not at the two lowest concentrations (1 mg/L and 5 mg/L).

Table 14. Sublethal effects observed on *P. patulus* as a response to acetaminophen exposure.

Six-day acetaminophen exposure		
Concentration (mg/L)	Observed effect	
	Egg production slowed/inhibited	Egg detachment
1	-	-
5	-	-
10	✓ (For reference population only)	✓
15	✓	✓
20	✓	✓

Reproduction of both populations was negatively affected by exposure to 15 mg/L and 20 mg/L treatments of acetaminophen, decreasing egg production as compared to the control treatment of each population. This effect was also observed at 10 mg/L for the reference population. Egg production was affected starting on day 3 of exposure for both populations. Egg detachment from females was another observed effect which results in the production of unviable embryos in most cases. This effect started on day 4 for both populations.

3.6.2.2 Caffeine 6-day exposures

Chronic exposure to caffeine on the reference population resulted in significant differences in the population growth among increasing concentrations over time as compared to the control treatment ($F = 6.63$; $p < 0.0001$). Significant differences from exposure to caffeine were observed at 200 mg/L on day 5 where population growth was inhibited 79% ($t = 2.26$, $p = 0.0293$) as compared to the control treatment and with a negative rate of increase on day 6 ($t = 3.35$, $p = 0017$). For the 300 mg/L treatment, a negative rate of population increase was obtained for days 4 ($t = 3.08$, $p = 0.0036$), 5 ($t = 3.89$, $p = 0.0003$), and 6 ($t = 7.60$, $p < 0.0001$).

In response to six-days of caffeine exposure for the Rio Grande population, significant differences were obtained in the population growth among increasing concentrations over days ($F = 11.42$; $p = < 0.0001$). Significant differences were observed on days 3, 4, 5, and 6 for 200 and 300 mg/L as compared to the control treatment. For the 200 mg/L treatment, significant differences were observed on day 3 where population growth was inhibited 86% ($t = 5.26$, $p = < 0.0001$) as compared to population growth in the control. At this concentration, population growth was also inhibited on day 4, 5, and 6 with 45% ($t = 4.42$, $p = < 0.0001$), 44% ($t = 4.66$, $p = < 0.0001$), and 52% ($t = 4.18$, $p = 0.0002$) of growth as compared to the control respectively. Significant differences were also obtained at 300 mg/L on day 3, 4, and 6 where negative growth was observed while on day 5 population growth was inhibited 99% ($t = 8.36$, $p = < 0.0001$) as compared to population growth of control treatment.

Intrinsic rates of increase (r) over days of exposure to caffeine for both populations are shown in Figure 22.

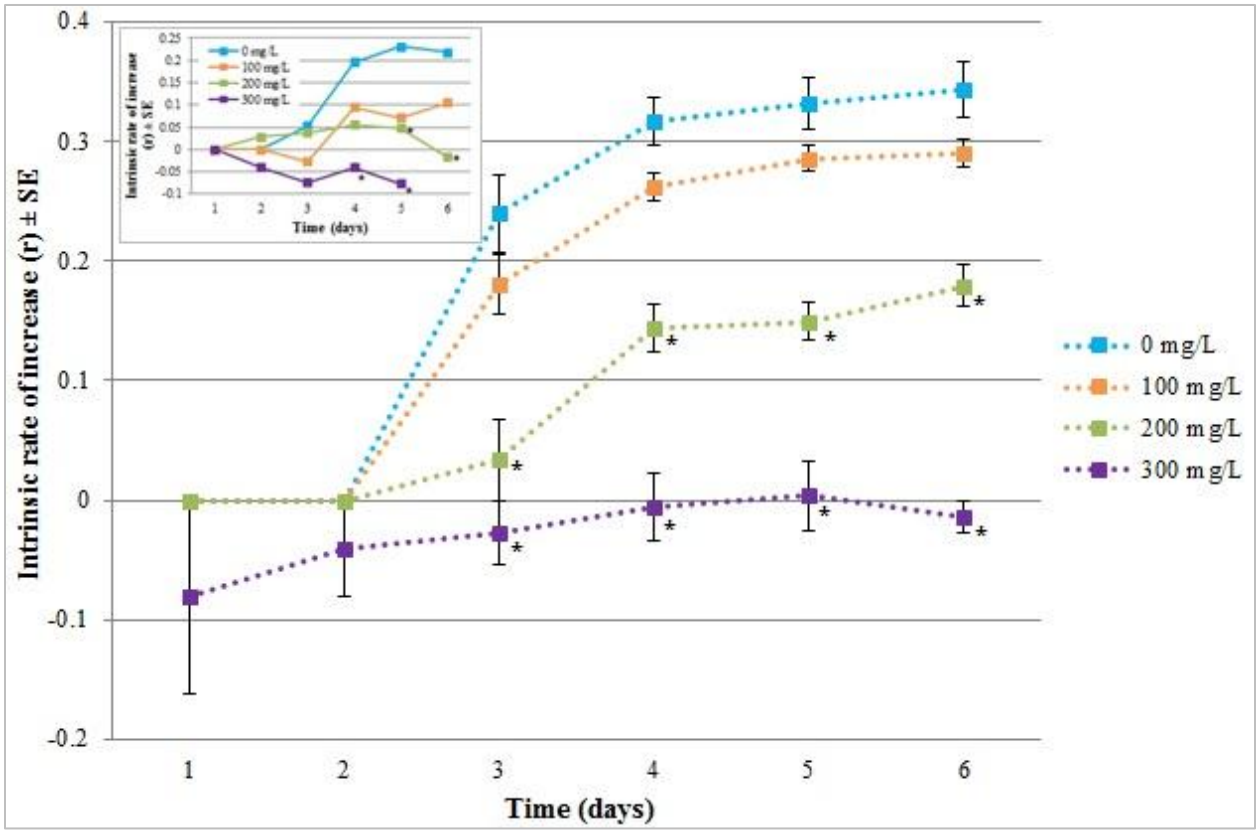

Figure 22. Rates of population increase (r) over time for the reference population (inset) and the Rio Grande population as exposed to different concentrations of caffeine. Mean ± standard error are based on five replicates. * Indicates significant effects on population growth.

Table 15 shows the predominant sublethal effects observed in both *P. patulus* populations as a result of a six-day exposure to caffeine.

Table 15. Sublethal effects observed in the rotifer *P. patulus* in response to caffeine exposure.

Six-day caffeine exposure		
	Observed effect	
Concentration (mg/L)	Egg production slowed/inhibited	Egg detachment (Start date)
100	-	-
200	✓	✓ (day 4)
300	✓	✓ (day 3)

Decreased egg production was also observed as a result of chronic caffeine exposure. Egg production was slowed at 200 mg/L for both populations starting on day 3 while egg production was inhibited at 300 mg/L for both populations starting also on day 3. Similar to acetaminophen, chronic exposure to caffeine caused egg detachment from females leading to unviable embryos in both populations. Egg detachment started on day 4 at 200 mg/L and on day 3 for 300 mg/L. No detached eggs were observed in the control treatment over the course of the experiment.

3.6.2.3 Fluoxetine 6-day exposures

Chronic exposure to fluoxetine for the Rio Grande population resulted in significant differences in the population growth between the control treatment and the 0.020 mg/L concentration ($F = 6.77$; $p < 0.0001$). Population growth was inhibited 26% ($t = 7.25$, $p < 0.0001$) on day 3, 15% on days 4 ($t = 4.60$, $p < 0.0001$) and 5 ($t = 4.44$, $p < 0.0001$), and 16% on

day 6 ($t = 5.04$, $p < 0.0001$) as compared to the control treatment. Rates of intrinsic population increase for this population as exposed to this toxicant are shown in Figure 23.

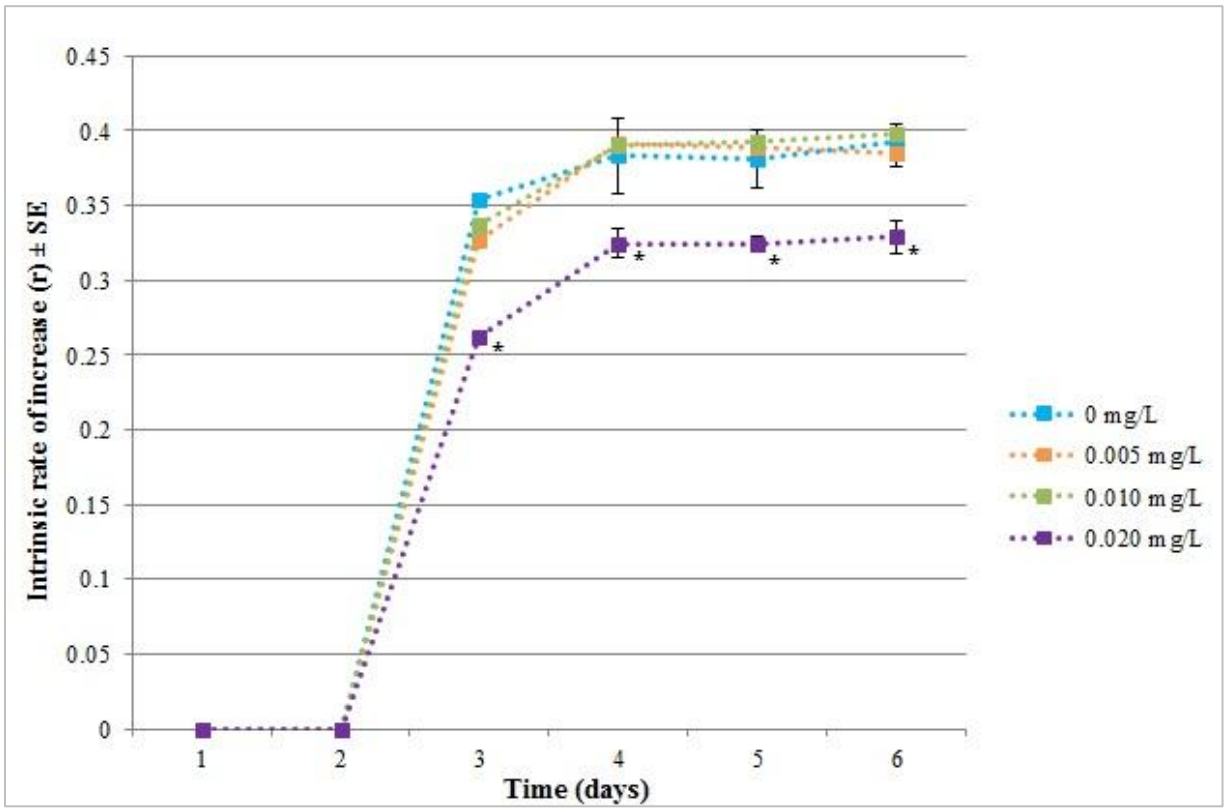

Figure 23. Rate of population increase (r) of Rio Grande population as exposed to different concentrations of fluoxetine. Mean ± standard error are based on three replicates for test control and first treatment, four for second treatment and five for third treatment. * Indicates significant effects on population growth.

No sublethal effects were observed for the Rio Grande population as a result of the chronic exposure to fluoxetine.

3.6.2.4 Triclosan 6-day exposures

Significant differences in the population growth of the reference population were determined among increasing concentrations of triclosan over time as compared to the control treatment ($F = 10.06$; $p < 0.0001$). Effects of this toxicant to rates of population increase were observed at all tested concentrations. At the 0.05 mg/L treatment, population growth was inhibited 72% ($t = 3.50$, $p = 0.0008$) on day 4 of exposure while on day 5 it was inhibited 94% ($t = 5.86$, $p<0.0001$) as compared to control treatment. On day 6, a negative rate of population increase was determined ($t = 6.43$, $p < 0.0001$). At the 0.075 mg/L treatment, negative rates of population increase were determined for this population on days 4 ($t = 5.55$, $p < 0.0001$), 5 ($t = 6.76$, $p < 0.0001$), and 6 ($t = 6.74$, $p < 0.0001$). In the highest tested concentration, 0.10 mg/L, negative rates of population increase were also observed on days 4 ($t = 6.71$, $p < 0.0001$), day 5 ($t = 9.23$, $p < 0.0001$), and day 6 ($t = 9.25$, $p < 0.0001$).

Chronic exposure to triclosan for the Rio Grande population also showed significant differences in the population growth among increasing concentrations over time ($F = 29.39$; $p = <0.0001$) as compared to the control treatment. For the lowest tested concentration, 0.05 mg/L, a negative intrinsic rate of population increase (r) was obtained on day 1 ($t = 2.58$, $p = 0.0141$), day 3 ($t = 6.84$, $p < 0.0001$), day 4 ($t = 9.89$, $p < 0.0001$), day 5 ($t = 10.91$, $p < 0.0001$), and on day 6 ($t = 10.99$, $p < 0.0001$) as compared to the control. For the second tested concentration, 0.075 mg/L, no growth was observed as compared to the control treatment through days of exposure. For the highest tested concentration of the first study, 0.10 mg/L, no growth was observed on day 3 ($t = 6.59$, $p = < 0.0001$) or day 4 ($t = 9.70$, $p < 0.0001$) while on days 5 and 6, negative population growth was determined. Figure 24 shows intrinsic rates of increase (r) over time for the first triclosan exposure study for both populations.

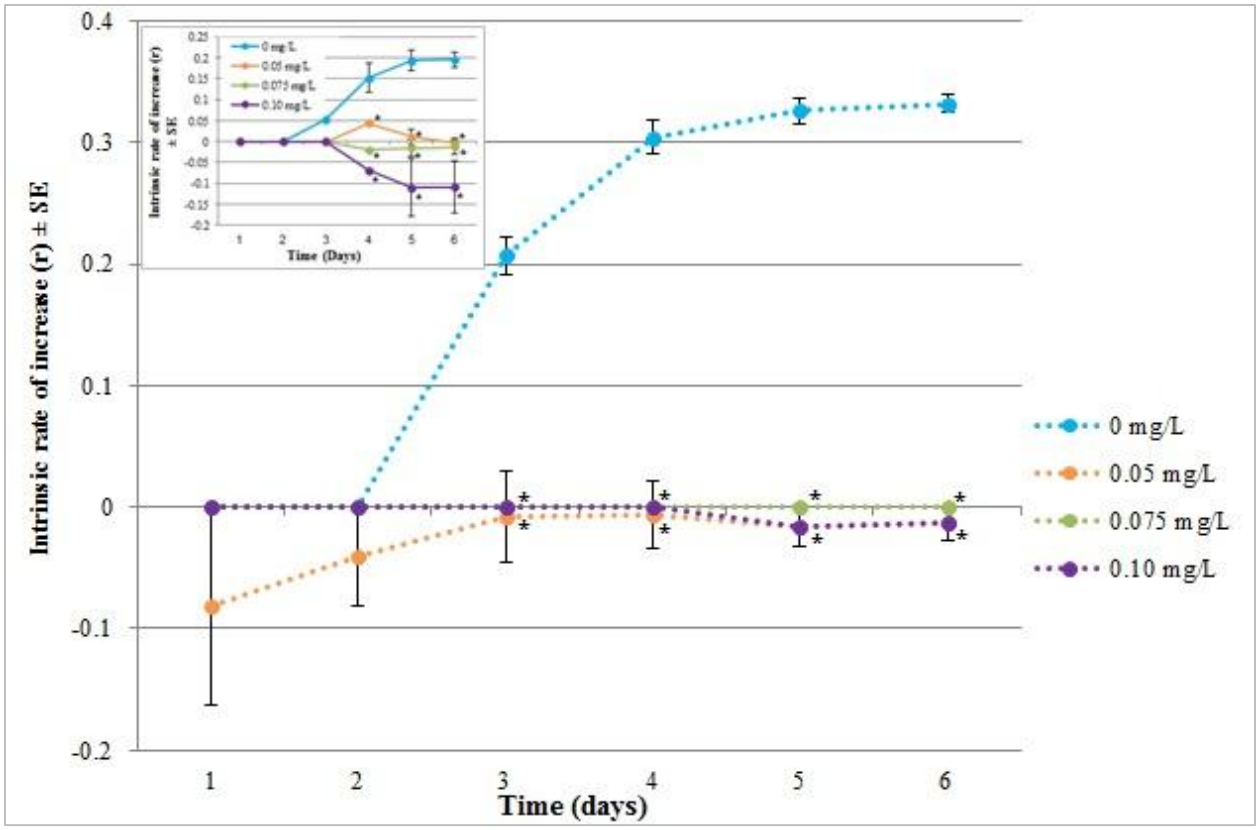

Figure 24. Rates of population increase (r) over time for the reference population (inset) and for the Rio Grande population exposed to three concentrations of triclosan. Mean ± standard error are based on five replicates. * Indicates significant effects on population growth.

Results obtained from the second study in which lower concentrations of triclosan were tested to the Rio Grande population resulted in significant differences in population growth among increasing concentrations over time ($F = 100.64$; $p < 0.0001$) as compared to the control treatment. Significant differences were observed specifically on days 3, 4, 5, and 6 for 0.005 and 0.05 mg/L treatments as compared to the control. At the 0.005 mg/L treatment, population growth was inhibited 14% on day 3 ($t = 2.69$, $p = 0.0089$) as compared to growth obtained in the control treatment. On the other hand, significant differences as compared to the control were observed for 0.05 mg/L treatment on days 3 were no growth was obtained ($t = 19.91$, $p <$

0.0001) while on days 4, 5, and 6 a negative r was determined. Intrinsic rates of increase over time obtained from this study are presented in Figure 25.

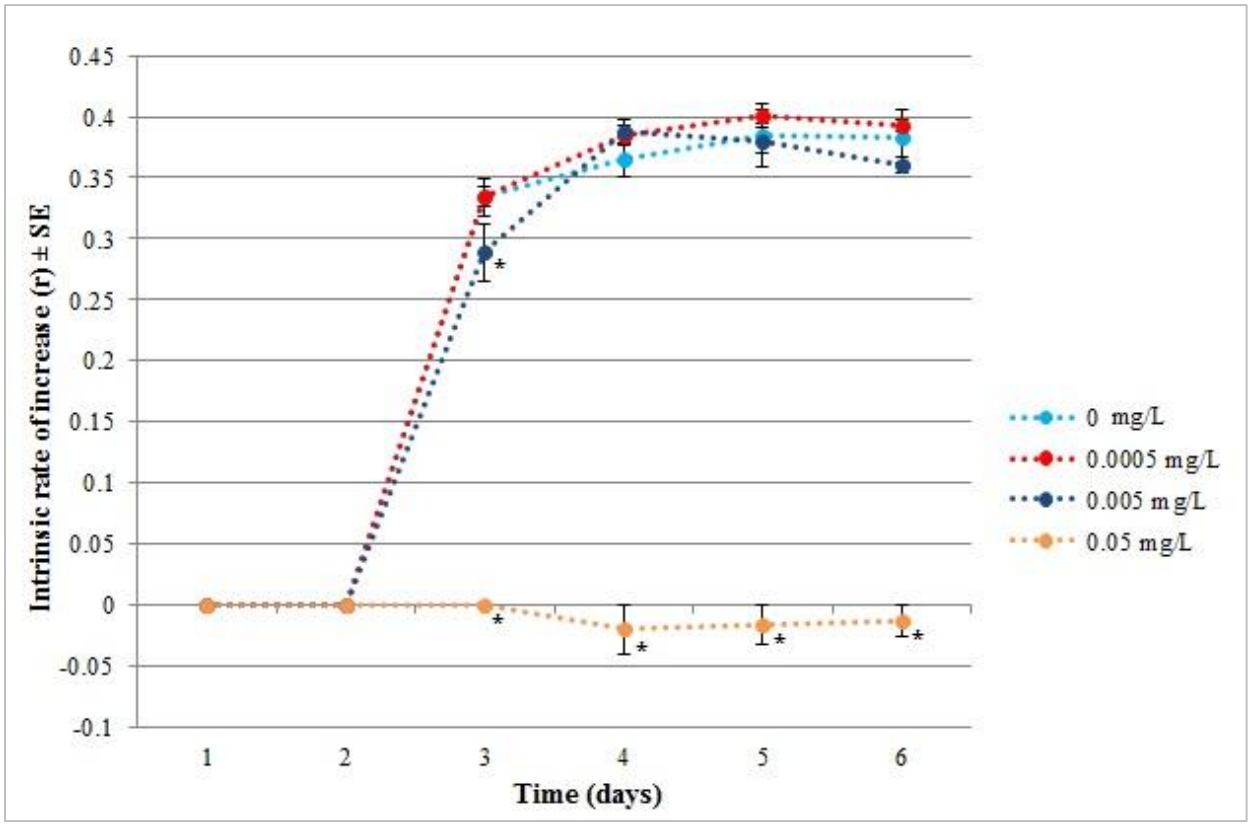

Figure 25. Rate of population increase (r) over time for the Rio Grande population exposed to concentrations of triclosan. The mean ± standard error are based on five replicates. * Indicates significant effects on population growth.

Sublethal effects to *P. patulus* from both populations caused by six-day exposure to triclosan are listed in Table 16. Effects were mainly observed at 0.05, 0.075, and 0.10 mg/L triclosan tested concentrations.

Table 16. Sublethal effects observed on *P. patulus* as a response to triclosan exposure

Six-day triclosan exposure			
	Observed effect		
Concentration (mg/L)	Egg production slowed/inhibited	Egg detachment (Start date)	Mobility slowed
0.0005	-	-	-
0.005	-	-	-
0.05	✓	✓ (day 3)	✓
0.075	✓	✓ (day 3)	✓
0.10	✓	✓ (day 3)	✓

Reproduction of both *P. patulus* populations was affected at 0.05, 0.075, and 0.10 mg/L as a result of chronic exposure to triclosan by inhibiting or slowing egg production as compared to controls. This sublethal effect was observed in both populations along with egg detachment. Effects on reproduction and egg detachment started on day 3 for both populations. Additionally, triclosan had a negative effect on mobility of both *P. patulus* populations. Mobility was generally slowed at 0.05, 0.075, and 0.10 mg/L as compared to control treatments.

3.7 Discussion

3.7.1 Acute toxicity studies

Median lethal concentrations (LC_{50}) obtained in acute studies for acetaminophen, caffeine, fluoxetine, and triclosan do not show a pattern regarding levels of tolerance between *P. patulus* reference population and *P. patulus* Rio Grande population. For instance, the reference population showed to be more tolerant to acetaminophen, an analgesic used for prevention or reduction of fever while for caffeine, a stimulant, a similar 48 hr LC_{50} was obtained for both populations. Results obtained for triclosan, an antimicrobial present in personal care products including shampoo, toothpaste and hand soap (Waiser *et al*., 2011), showed that the reference population was less tolerant to this toxicant than the Rio Grande population with a 48 hr LC_{50} of 0.13 mg/L. For the Rio Grande population, fluoxetine (an antidepressant) was the most toxic compound with a LC_{50} of 0.19 mg/L. These observations disagree with our hypothesis since the reference population was expected to be more sensitive than the Rio Grande population to the acute exposure of these toxicants.

LC_{50} values obtained for both *P. patulus* populations show a higher level of tolerance to acetaminophen (Reference population LC_{50} = 319 mg/L; Rio Grande population LC_{50} = 121 mg/L) and caffeine (Reference population LC_{50} = 423 mg/L; Rio Grande population LC_{50} = 419 mg/L) as compared to the values reported in literature for *Daphnia magna* with an LC_{50} of 6 mg/L for acetaminophen and 182 mg/L for caffeine (Waiser *et* al., 2011; Kolpin *et al*., 2002). However, LC_{50} values reported for *Daphnia magna* regarding fluoxetine and triclosan toxicity indicate that this organism is more tolerant to these toxicants (Brausch and Rand, 2011; Santos *et al*., 2010) as shown in Table 17.

Table 17. Median lethal concentration (LC_{50}) for tested PPCPs. Not determined (ND).

Compound	This study		Literature	
	P. patulus **Reference population**	***P. patulus*** **Rio Grande population**	***Daphnia magna***	
	48 hr LC_{50} (mg/L)			**Reference**
Acetaminophen	319	121	6	Kolpin *et al*., 2002
Caffeine	423	419	182	Waiser *et al*., 2011
Fluoxetine	ND	0.19	0.82	Santos *et al*., 2010
Triclosan	0.13	0.32	0.39	Brausch & Rand, 2011

Although no obvious trends were obtained in this study regarding the levels of tolerance to the exposure of these toxicants between *P. patulus* and *Daphnia magna*, rotifers are good model organisms in acute studies due to their easy cultivation in the lab and easy handling and their increased sensitivity to certain toxicants.

Acute toxicity values obtained in previous studies with rotifers exposed to antibiotics also showed a higher tolerance to these toxicants as compared to the tolerance of the crustacean *Daphnia magna*. Isidori *et al.* (2005) determined the acute toxicity of six antibiotics including erythromycin and sulfamethoxazole to the rotifer *Brachionus calyciflorus*, *Daphnia magna* and other aquatic invertebrates; they found that *B. calyciflorus* was more tolerant than the cladoceran to five of these toxicants with the exception of ofloxacin to which the LC_{50} obtained for the rotifer was 29.88 mg/L versus 31.75 mg/L for *D. magna*.

3.7.2 Chronic toxicity studies

Results from chronic exposure to acetaminophen (1st set of tested concentrations), caffeine, and triclosan generally showed an inhibition in the reproduction of both *P. patulus* populations causing a decrease in population growth. Population growth inhibition ranged from 79% to 98% in the reference population and from 44% to 99% in the Rio Grande population as compared to control treatments.

Intrinsic rates of increase (r) obtained for tests controls from chronic exposures for all four tested toxicants for both *P. patulus* populations showed that in general the Rio Grande population had higher population growth over days as compared to population growth of the reference population. Mean intrinsic rates of population increase per day for the Rio Grande population´s control treatments were generally one to four times higher as those obtained for the reference population (Figures 21, 22, 24, and 27). The difference observed among intrinsic rates of increase of control treatments for the Rio Grande population may indicate that this population is subjected to environmental toxic stress since it was collected in a highly urban and industrial stretch of the Rio Grande. This difference may also indicate that there is a genetic variation between the two populations and may influence the response that this population had to each exposed toxicant as compared to the reference *P. patulus* population.

Results obtained from chronic exposure to acetaminophen showed a significant inhibition in population growth at 10 mg/L for the reference population while for the Rio Grande population there was no significant population growth inhibition at this concentration. Another difference in the tolerance under chronic exposure to this compound was observed at 15 mg/L on days 5 and 6 in which the Rio Grande population growth was inhibited 70% and 67% respectively while for the reference population growth was inhibited 98% on both days.

For caffeine, significant responses from both populations were observed at 200 mg/L on days 5 and 6 where the reference population growth was inhibited 79% on day 5 and negative rates of population increase were observed on day 6. Population growth of the Rio Grande population was inhibited 56% on day 5 and 48% on day 6 as compared to control treatment. Negative intrinsic rates of population increase were obtained for both populations at 300 mg/L for days 4, 5, and 6 for the reference population and for days 4 and 6 for the Rio Grande population.

Tested concentrations of triclosan for the first study resulted in negative rates of population increase at 0.05 mg/L for the Rio Grande population on days 3, 4, 5, and 6 as compared to the control treatment while for the reference population a negative rate of population increase was obtained only at day 6. No population growth was observed for either population as compared to control treatments at 0.075 mg/L and 0.10 mg/L during days 3, 4, 5, and 6 of exposure.

The sublethal effects observed as a result of chronic exposure to acetaminophen, caffeine, and triclosan for both *P. patulus* populations were decreased egg production and increased egg detachment from ovigerous females.

Additional studies with rotifers in which the chronic toxicity of PPCPs was assessed include that by Isidori *et al.* (2007) in which the toxicity of three lipid regulators (benzafibrate, fenofibrate, and gemfibrozil) was evaluated on *Brachionus calyciflorus* population growth over 48 hr of exposure; they found that reproduction was inhibited by all tested compounds. In a similar study, the chronic toxicity of ranitidine, a histamine H_2-receptor antagonist that inhibits stomach acid production, was assessed on population growth of *B. calyciflorus* over 48 hr exposure; they found that this toxicant also inhibited population growth with a NOEC of 0.31

mg/L and a LOEC of 0.63 mg/L (Isidori *et al*., 2009). Ferrari *et al*. (2003) tested chronic toxicity of carbamazepine, clofibric acid, and diclofenac to *B. calyciflorus* (48 hr exposure). They found inhibition in growth population with LOEC values of 754 µg/L for carbamazepine, 740 µg/L for clofibric acid, and 25,000 µg/L for diclofenac.

3.7.3 Environmental occurrence and concentrations for tested PPCP toxicants

As previously discussed, environmental concentrations of PPCPs in surface waters worldwide have been reported to occur in the ng/L to µg/L range (Flaherty and Dodson, 2005; Ferrari *et al*., 2003; Kolpin *et al*., 2002). Although LC_{50} values obtained in this study for acetaminophen, caffeine, fluoxetine, and triclosan are much higher than these values, it is well documented that PPCP toxicants do not occur as single chemicals but they occur as complex mixtures in the environment (Santos *et al*., 2010). The occurrence of mixtures within aquatic ecosystems may lead to different effects as those obtained by single toxicants as shown in an acute study where the cladoceran *Daphnia magna* was exposed to a mixture of 36 µg/L of fluoxetine and 100 µg/L of clofibric acid which caused a significant mortality and malformation while no observed effects were determined by the exposure of single toxicants at the same concentrations (Santos *et al*., 2010). This observation may indicate that mixtures of PPCP toxicants display additive effects resulting in a greater toxicity than the one obtained for single toxicants (Santos *et al*., 2010). In addition, chronic exposure to these toxicants represents a risk due to their near constant introduction to aquatic ecosystems. Chronic exposure to lower concentrations of PPCPs toxicants may lead to detrimental effects such as reproduction inhibition and decrease in mobility as determined here during the six-day population growth studies of *Plationus patulus*.

Acetaminophen is reported in surface waters up to 78.17 µg/L (Danube River in Serbia) and up to 4.3 µg/L in sewage treatment plants (STP) (Santos *et al*., 2010). Acute toxicity tests have been conducted in algae, water fleas, fish embryos, luminescent bacteria and ciliates. *Daphnia magna* showed to be the most sensitive species with an EC_{50} value of 50 mg/L (Santos *et al*., 2010). *Plationus patulus* showed to be less sensitive than the cladoceran to the acute exposure of this toxicant under our exposure conditions.

In raw sewage, concentrations of caffeine have been reported to range from 20 to 300 µg/L and from 0.1 to 20 µg/L in treated wastewater effluents (Sauvé *et al*., 2012). In surface waters this toxicant has been reported to range from 3 to 1500 ng/L (Sauvé *et al*., 2012) and up to 9700 ng/L in the Somes River (Moldovan, 2006). In this study, tested concentrations of caffeine (100 mg/L, 200 mg/L and 300 mg/L) inhibited population growth by causing a decrease in egg production and an increase in egg detachment.

The environmental occurrence of fluoxetine has been reported at 12 ng/L in surface water of the United States while it has been reported to occur in STP influents from 0.4 to 18.7 ng/L and in STP effluent from 0.12 – 8.4 ng/L (Santos *et al*., 2010). Sub-lethal effects observed in invertebrates include stimulation of reproduction as observed in the crustaceans *Ceriodaphnia dubia* as exposed to 56 µg/L and increase in total number of offspring produced after 30 days of exposure to 36 µg/L in *Daphnia magna*. Sublethal effects have also been reported in the development of *D. magna* embryos (neonate length) when exposed to 31 µg/L of this toxicant (Santos *et al*., 2010). In this study, tested concentrations of fluoxetine (5 µg/L, 10 µg/L and 20 µg/L) to the rotifer *P. patulus* did inhibit population growth at 20 µg/L but no sublethal effects were observed.

Triclosan has been reported to occur in surface waters as high as 2.3 µg/L in North America and Europe while STP effluent ranges from 0.1 to 2.7 µg/L (Waiser *et al*., 2011). Although chronic exposure to triclosan in *D. magna* resulted in a LOEC value of 200 µg/L after 21 days of exposure, the algae *Pseudokirch-neriella subcapitata* appears to be the most sensitive trophic group in which growth was affected at concentrations less than 1 µg/L (Brausch and Rand, 2011). In this study, the lowest tested concentration of triclosan (0.5 µg/L) to *P. patulus* did not inhibit population growth or produced sub-lethal effects but the second lowest concentration (5 µg/L) did inhibit population growth (13% on day 3 of exposure) as compared to control treatment in the Rio Grande population.

Acute and chronic exposure of the rotifer *Plationus patulus* to mixtures of acetaminophen, caffeine, fluoxetine, and triclosan is highly recommended as the next step to better understand their additive and synergistic effects at more likely environmental concentrations. In addition, the exposure of *P. patulus* to ciprofloxacin, codeine, cotinine, and sulfamethazine is suggested since these PPCP toxicants were consistently found in this study in samples collected within the middle Rio Grande as discussed in Chapter 2. Also, for future studies, the toxicity assessment of extracted PPCP analytes obtained from river water samples preparation (Chapter 2) is recommended since extracts contain a mixture of PPCPs at concentrations found in the Rio Grande which may provide a closer insight of sublethal effects occurring in the environment.

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CHAPTER 4: ROTIFERS FROM THE RIO GRANDE

4.1 Abstract

Rotifers are mainly microscopic aquatic invertebrates recognized for being important members of freshwater zooplankton communities. In this chapter, the species richness of four sites along the middle Rio Grande is characterized. Zooplankton samples were collected from August 2010 through September 2011. Zooplankton samples were sorted within 24 hr after collection under Leica and Zeiss dissecting scopes. A total of 24 species of monogonont rotifers were found during the study, representing 12 families. *Philodina megalotrocha* and other bdelloids were also found. *Euchlanis dilatata* and *Lecane luna* were found in all four sampled sites. This study provides a list of rotifer species found along with a photographic guide that will be useful for future researchers in the middle Rio Grande aquatic systems.

4.2 Introduction

Rotifers are among the most common aquatic invertebrates. They are mainly microscopic, with sizes ranging from <20-3500 µm and classified in the Phylum Rotifera (Dahms *et al*., 2011; Wallace and Snell, 2010). This group includes three major clades: Seisonidea, Bdelloidea, and Monogononta, which differ morphologically, genetically and in their mode of reproduction and life-cycles (Dahms *et al*., 2011; Wallace and Snell, 2010). The marine Seisonidea comprises only three species and are characterized by possessing gamogenetic reproduction (Ricci *et al*., 1993). Bdelloidea comprises about 400 species and have only a parthenogenetic reproductive phase while the third clade, Monogononta, consists of approximately 2000 species (Segers, 2007) and show a complex life cycle with a parthenogenetic, amictic phase and a mictic phase (Dahms *et al*., 2011). The mictic phase in monogonont rotifers include sexual reproduction and the presence of small, haploid males (Wallace and Snell, 2010).

Rotifers are common members of freshwater zooplankton communities (Wallace and Snell, 2010), are easy to collect due to their high natural densities and can be collected from their natural habitats using plankton nets and other routine water sampling devices (Dahms *et al*., 2011).

The importance of rotifers in arid zone rivers is due to their role within the food web since they are the primarily food source of invertebrate predators, planktivorous fish and some water birds (Walsh, 1995, Walsh *et al*., 2006, Shiel *et al*., 2006). Rotifers, along with other microfauna are also important phytoplankton grazers in rivers of arid regions (Shiel *et al*., 2006).

In this study, freshwater rotifers occurring in the middle Rio Grande were collected for identification during sampling events for PPCPs analyses on dates shown in Table 1 (Chapter 2).

Bdelloid rotifers were not identified to species level due to difficulties discerning taxonomic features.

4.3 Objectives

The main objectives were to develop a species list and a photographic guide to rotifers species occurring in the middle Rio Grande.

4.4 Materials and Methods

In the field, two zooplankton samples were collected at each of the four sampled sites (Figure 1) using a 64 µm mesh plankton net. Zooplankton were concentrated by throwing the plankton net ten times per sample through the water column. Basic water chemistry parameters were taken at the time of collection (Table 20, Appendix A).

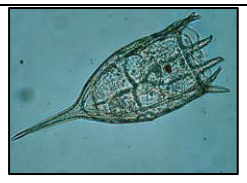
In the lab, zooplankton samples were examined within 24 hr after collection. Rotifers were sorted under Leica and Zeiss dissecting scopes and identified to species using a Zeiss Axioskop. Digital images were taken with a SPOT INSIGHT camera (Diagnostic Instruments, Inc.) mounted on the Axioskop and using the SPOT image analysis software, version 4.6 (Diagnostic Instruments, Inc.). Species identification was based on the following keys: Koste (1978), Nogrady *et al*. (1993), Segers (1995a, b), Nogrady *et al*. (1995), De Smet (1996), De Smet & Pourriot (1997), and Nogrady and Segers (2002).

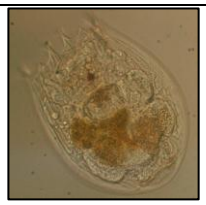
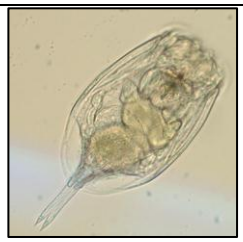
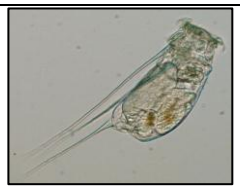
Twenty-nine sampling events were conducted from August 2010 through September 2011 (Table 1, Chapter 2).

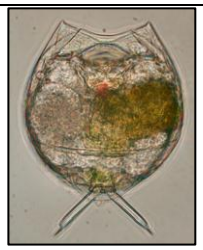
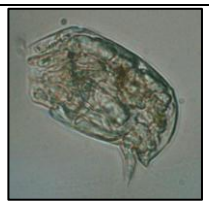
4.5 Results

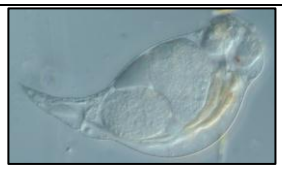
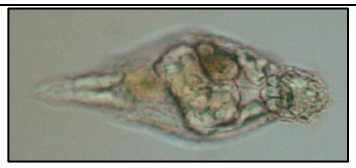
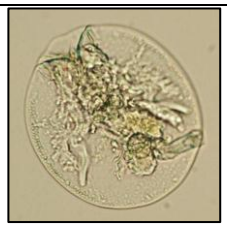
Rotifers occurring in the middle Rio Grande are listed below (Table 18). Total monogonont species richness was 24, representing 12 families and 17 genera. *Philodina megalotrocha* and several other bdelloids were found.

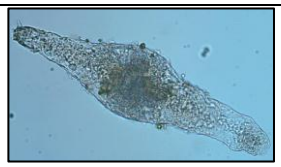
Table 18. Rotifers from the Middle Rio Grande collected from 4 Sites (August 2010 - September 2011).

Family	Species	Illustration
Brachionidae	*Brachionus bidentata*	
	Brachionus calyciflorus	
	Brachionus quadridentatus	
	Keratella cochlearis	

	Notholca acuminata	
	Notholca squamula	
	Plationus patulus	
	Platyias quadricornis	
Euchlanidae	*Euchlanis dilatata*	
Filiniidae	*Filinia terminalis*	
Lecanidae	*Lecane bulla*	

	Lecane luna	
	Lecane quadridentata	
Lepadellidae	*Lepadella patella*	
Mytilinidae	*Mytilina mucronata*	
Notommatidae	*Cephalodella catelina*	
	Cephalodella forficula	
	Cephalodella gibba	

	Cephalodella sterea	
	Notommata allantois	
Philodinidae	*Philodina megalotrocha*	
Proalidae	*Proales similis*	
	Proales daphnicola	
Synchaetidae	*Polyarthra dolichoptera*	
Testudinellidae	*Testudinella patina*	

Trichotriidae	*Trichotria tetractis*	
	Bdelloids (unidentified)	

Occurrence of rotifers at the four sampled sites (Percha Dam, Anthony, American Dam, and Fabens) during two flow regimes is shown in Table 19.

Table 19. Occurrence of rotifers at 4 stations in the Middle Rio Grande from August 2010 through September 2011. Irrigation (A) and non-irrigation (B) flow regimes.

	Study site							
	Percha Dam		**Anthony**		**American Dam**		**Fabens**	
Species	**A**	**B**	**A**	**B**	**A**	**B**	**A**	**B**
Brachionus bidentata				✓				
Brachionus calyciflorus				✓				
Brachionus quadridentatus							✓	
Keratella cochlearis	✓			✓	✓			
Notholca acuminata				✓				
Notholca squamula		✓						
Plationus patulus					✓		✓	
Platyias quadricornis							✓	
Euchlanis dilatata		✓	✓	✓	✓		✓	✓
Filinia terminalis				✓				

Lecane bulla							✓	
Lecane luna	✓		✓	✓	✓		✓	✓
Lecane quadridentata							✓	
Lepadella patella							✓	
Mytilina mucronata							✓	
Cephalodella catelina				✓				
Cephalodella forficula					✓		✓	
Cephalodella gibba	✓					✓		
Cephalodella sterea							✓	
Notommata allantois							✓	
Philodina megalotrocha			✓				✓	
Proales similis				✓		✓		
Proales daphnicola				✓				
Polyarthra dolichoptera		✓						
Testudinella patina							✓	
Trichotria tetractis		✓		✓				
Bdelloids (unidentified)			✓		✓		✓	

Euchlanis dilatata and *Lecane luna* occurred in the middle Rio Grande at all four sampling sites in this study. The presence of *E. dilatata* was observed during the irrigation and non-irrigation seasons at the Anthony and Fabens sites while at the Percha Dam site it was observed during the non-irrigation season and during the irrigation season at the American Dam site. *L. luna* was also observed during the irrigation and non-irrigation seasons at the Anthony and Fabens sampling stations and during the irrigation season at the Percha Dam and American Dam sites.

The highest species richness of rotifers in the middle Rio Grande was observed at the Fabens site (16 out of 24 recorded) followed by the Anthony sampling station (12 species) as shown in Figure 26.

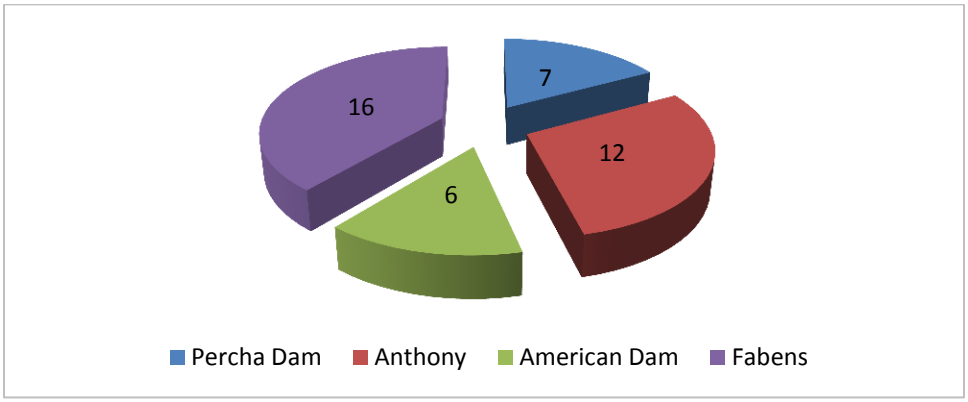


Figure 26. Rotifer species richness observed at each sampling site during this study.

Rotifer species occurring in the Rio Grande found in this study and in other conducted sampling events by our lab at other locations in the vicinity of El Paso, Caballo Reservoir, Elephant Butte Reservoir, Bosque del Apache, near Williamsburg, NM and several locations in Big Bend National Park, Brewster Co., TX are shown in Table 23 (Appendix E).

4.6 Discussion

The species richness of rotifers observed during this study is much higher than previously reported in the Rio Grande at the El Paso area (Williams, 1962). In this study, a total of 24 monogonont species, *Philodina megalotrocha* and other bdelloids were found while Williams

(1962) only reported the presence of the *Brachionus*, *Keratella*, and *Trichocerca* genera. Further he stated that this area exhibited low rotifer diversity as compared with other sites in his survey of the Rio Grande. Although the species richness was higher in this study, the presence of *Trichocerca* was not observed from August 2010 through September 2011.

The presence of the brachionid *Plationus patulus* was previously reported in the Rio Grande at the El Paso area by Rios-Arana *et al.* (2005) in a study in which this rotifer was cultured and exposed to concentrations of arsenic and heavy metals. Its occurrence was observed in this study at the American Dam and Fabens sampling stations.

Euchlanis dilatata and *Lecane luna* are common species (Sarma and Nandini, 2009) occurring in water bodies throughout the world (Sarma and Nandini, 2009; Walsh *et* al., 2008; García-Morales and Elías-Gutiérrez, 2007; Wallace *et al*., 2005; Lair, 2005) which may explain their presence within all four sampled sites of the middle Rio Grande.

The highest species richness of rotifers in the middle Rio Grande during this study was observed at the Fabens site during the irrigation season (warmer water temperatures recorded; See Table 20 in Appendix A) which agrees with Williams (1962) who stated that rotifers populations fluctuate seasonally, sometimes being absent during winter (16 out of 24 recorded) and with Lair (2005) who stated that during high water temperatures rotifers reach their highest numbers.

Other studies in which other areas of the Rio Grande were surveyed for rotifers occurrence include that by Wallace *et al.* (2005). In this study, rotifers from the Chihuahuan Desert, specifically from springs, streams, ponds, tanks, and huecos and tinajas from Big Bend National Park (TX) were surveyed. Wallace *et al*. (2005) reported the presence of *Brachionus* spp. in the Rio Grande within the Big Bend National Park area.

Surveys of rotifer species richness within major rivers of the world include that by García-Morales and Elías-Gutiérrez (2007) in which rivers from Guatemala (5 rivers) and Belize (2 rivers) were surveyed for rotifer occurrence. They found the presence of 37 species in Guatemala´s rivers and 23 species in Belize´s rivers including *Euchlanis dilatata* and *Lecane luna*.

In other study conducted by Lair (2005) in the Middle Loire River in France in which rotifers composition was surveyed during low flow conditions the presence of 13 rotifer families was reported including 6 families found in this study (Brachionidae, Euchlanidae, Lecanidae, Notommatidae, Synchaetidae, and Testudinellidae). Within the 13 rotifer families, 25 genera and 61 species were identified.

Overall the rotifer species richness occurring in the middle Rio Grande during this study shows that warm water temperatures influence the presence of this group of microinvertebrates within riverine systems.

Although the development of this rotifer species list along with the photographic guide will aid future researchers when conducting studies in this river, further sampling needs to be conducted in order to obtain a complete species list for the Rio Grande.

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CHAPTER 5: CONCLUSIONS

The occurrence of target PPCP toxicants in the middle Rio Grande at the El Paso,TX/Ciudad Juárez, MX metroplex during two flow regimes was detected in the ng/L range as in many other water bodies receiving wastewater treated effluent around the world. The presence of antibiotics such as ciprofloxacin and sulfamethazine, a nicotine metabolite (cotinine), and the stimulant caffeine was consistently found during this study probably as a consequence of urban and agricultural activities as well as intensive dairy operations that are located within this area. This is the first study of these compounds in this large, urbanized stretch of the Rio Grande and confirms the presence of these emerging compounds at detectable levels. However, no significant differences in concentrations of target analytes between the irrigation and non-irrigation seasons or among sites were found, contrary to expectations. This may be a consequence of the water collection techniques used (grab sampling of limited duration and frequency). It may be more appropriate to use passive, composite samplers for the survey of these toxicants. In addition, it has been recently shown that lipophilic PPCPs tend to bind to sediments and other suspended matter which may influence the distribution of these toxicants in water matrices. Therefore, the use of complementing sampling devices is recommended for PPCPs analysis in future studies along with collection of sediment samples which may provide additional information about the fate of these toxicants in the environment.

As observed here, acute and chronic studies allowed to determine the toxicity of four PPCP chemicals to the rotifer *Plationus patulus* from two different populations. PPCPs were found to be toxic to this rotifer species only at concentrations higher than those typically occurring in freshwater environments. Additionally, these toxicants occur as complex mixtures and not singly in nature. Thus additional studies in which mixtures of PPCPs at environmental

concentrations be tested are needed to better understand additive and synergistic effects of these pollutants.

In our studies, responses to acute and chronic exposures of acetaminophen, caffeine, fluoxetine, and triclosan showed a variety of responses to these chemicals even between populations of the same species, *Plationus patulus*. Responses also differed from those reported for other test species such as *Daphnia magna* demonstrating the importance of using a variety of aquatic model organisms even within the same trophic level in toxicology research. In addition, the use of six-day population growth studies allowed for the assessment of chronic toxicity of four PPCPs over multiple generations. The chronic exposure of these toxicants to rotifers affected their reproductive potential causing inhibition in population growth by decreasing egg production and increasing egg detachment from egg-carrying females. Thus reducing energy transfer to higher trophic levels, and having an overall impact in productivity of the aquatic system. Therefore, it is of great importance conducting chronic studies along with acute studies in order to determine the possible impacts and effects that these pollutants may have on the aquatic system.

Major challenges for future researchers include the assessment of mixtures of these chemicals among different trophic levels of aquatic environments as well as to better understand how these toxicants bind to sediments, suspended matter and sludge and how they bioaccumulate and/or bioconcentrate in organisms at different trophic levels. Also, the development of techniques for the removal of these pollutants from treated effluent along with the control of emission from direct and indirect sources of these chemicals remains a crucial task.

APPENDIX A: Water Quality Parameters in the Middle Rio Grande during this study

Along with water collection for PPCPs analyses, basic water chemistry parameters at each sampled location were recorded. Parameters included: Water temperature (˚C), pH, Conductivity (µS/cm), Total Dissolved Solids (TDS), Dissolved Oxygen (DO % and mg/L), the Oxidation-Reduction Potential (ORP), and Salinity. These water chemistry parameters were measured by using a handheld YSI 556 multiprobe system. The YSI multiprobe was calibrated prior to each sampling event for pH, ORP, conductivity and DO in the lab. Chlorophyll-a (µg/L) and phycocyanin (RU) concentrations were determined by using a handheld Turner Instruments Fluorometer. Readings for these pigments were taken in triplicate.

At each sampling site, two samples (500 ml) for water chemistry analysis were collected in order to determine the concentration of dissolved nutrients (nitrite, nitrate, phosphate, sulfate, ammonia, silica, and chloride), alkalinity, hardness, turbidity and color. Water chemistry was determined using Palintest® water chemistry kits according to manufacturer's instruction for each parameter. Prior to testing, all samples were filtered by using a Nalgene® filter and Whatman™ glass microfiber filters (GF/C 47 mm). Concentrations for these parameters were determined using an YSI 9000 field photometer.

Water quality parameters obtained for all four sampled sites during both flow regimes are shown in Table 20.

Table 20. Water chemistry parameters (mean ± SE) for all sampling dates during this study. Not determined (ND).

Season	Variable	Percha Dam	Anthony	American Dam	Fabens
Irrigation	Temperature (° C)	20.6 ± 2.9	25.0 ± 2.7	23.8 ± 2.2	23.3 ± 1.5
	pH	8.1 ± 0.1	8.2 ± 0.2	8.1 ± 0.2	8.2 ± 0.2
	Specific conductance (µS/cm)	743.5 ± 28.7	784.5 ± 52.3	1066 ± 142.4	1417.3 ± 93
	O_2 (mg/L)	8.1 ± 0.3	7.7 ± 0.1	7.6 ± 0.4	8.2 ± 0.5
	Total Dissolved Solids (g/L)	0.483 ± 0.019	0.537 ± 0.026	0.682 ± 0.095	ND
	Nitrite as NO_2^- (mg/L)	0.002 ± 0.001	0.005 ± 0.002	0.08 ± 0.05	0.05 ± 0.02
	Alkalinity as $CaCO_3$ (mg/L)	161.7 ± 8.3	156.7 ± 4.3	153.5 ± 10.1	212.7 ± 17.4
	Hardness as $CaCO_3$ (mg/L)	231.2 ± 24.1	263.7 ± 19.3	258.7 ± 46.4	351.2 ± 59.3
	Silica as SiO_2 (mg/L)	16.0 ± 3	15.3 ± 1.3	27.5 ± 5.6	23.1 ± 1.5
	Chloride as Cl^- (mg/L)	54.5 ± 18.2	72 ± 9.6	97.3 ± 38.4	161 ± 16.3
	Sulfate as SO_4 (mg/L)	70 ± 13.8	102 ± 6.2	200.7 ± 51	232.5 ± 29.3

	Phosphate as PO_4 (mg/L)	0.04 ± 0.01	0.04 ± 0.01	0.65 ± 0.49	0.48 ± 0.15
	Color	45 ± 2.9	27.5 ± 4.8	27.5 ± 6.3	32.5 ± 8.5
	Turbidity	27.8 ± 10.1	63.7 ± 31.3	16.2 ± 3.3	128 ± 79.4
	Chlorophyll-a (µg/L)	13 ± 2	25.4 ± 8.1	19.4 ± 4.6	23 ± 8
	Phycocyanin (RU)	0.307 ± 0.059	0.553 ± 0.148	0.389 ± 0.107	0.469 ± 0.199
Non-irrigation	Temperature (° C)	13.9 ± 4.4	10.6 ± 2.4	18.5 ± 1.8	12.2 ± 3.5
	pH	7.1 ± 0.6	7.8 ± 0.6	8.1 ± 0.2	7.8 ± 0.3
	Specific conductance (µS/cm)	1083.3 ± 13.1	2165.5 ± 413.1	3799.3 ± 1359.6	2015 ± 235.8
	O_2 (mg/L)	9.9 ± 1	12.8 ± 1.3	10.9 ± 1.5	9.9 ± 0.9
	Total Dissolved Solids (g/L)	0.704 ± 0.009	1.408 ± 0.269	2.463 ± 0.886	ND
	Nitrite as NO_2^- (mg/L)	0.04 ± 0.002	0.2 ± 0.1	0.2 ± 0.1	0.2 ± 0.1
	Alkalinity as $CaCO_3$ (mg/L)	290 ± 21.8	239 ± 5.1	274.3 ± 43.8	235 ± 12.6
	Hardness as $CaCO_3$ (mg/L)	875 ± 539.9	621.2 ± 185.3	1145 ± 615.4	356.7 ± 18.8
	Silica as SiO_2	23.2 ± 2.2	19.1 ± 3.6	35.2 ± 1.7	30.9 ± 2.7

	(mg/L)				
	Chloride as Cl^- (mg/L)	87.3 ± 5.5	177.5 ± 8.7	264.7 ± 36.4	214.3 ± 24.18
	Sulfate as SO_4 (mg/L)	143.3 ± 18.6	295 ± 8.7	456.7 ± 92.1	280 ± 17.3
	Phosphate as PO_4 (mg/L)	0.02 ± 0.004	0.22 ± 0.05	0.27 ± 0.1	0.87 ± 0.43
	Color	16.7 ± 3.3	32.5 ± 8.5	36.7 ± 8.8	50 ± 5.8
	Turbidity	1.7 ± 1.7	11.7 ± 1.9	16 ± 3.6	65 ± 30.8
	Chlorophyll-a (µg/L)	6.8 ± 0.78	27 ± 5.5	11.3 ± 2.3	12.4 ± 2.7
	Phycocyanin (RU)	0.163 ± 0.026	0.5 ± 0.050	0.252 ± 0.046	0.347 ± 0.087

Statistically significant effects of season (irrigation and non-irrigation) on physicochemical chemical parameters were determined by GLMM (SAS® version 9.2) and are presented in Table 21.

Mean temperature was significantly higher during the irrigation season (23.2 ± 1.1 °C; $F = 7.51$; $p = 0.0145$) as compared to the non-irrigation season (13.6 ± 1.6 °C). This was expected since water collection during the irrigation season was conducted during the spring and summer months of March through July 2011 while the non-irrigation season occurs in the winter months. Significant differences in specific conductance ($F = 29.75$; $p < 0.0001$) and total dissolved solids

(F = 12.41; p = 0.0031) were also found. Mean values for specific conductance and total dissolved solids were higher during the non-irrigation season (2258.1 ± 399.9 µS/cm and 1.513 ± 0.296 g/L, respectively) as compared to the irrigation season (1002.8 ± 80.4 µS/cm and 0.570 ± 0.035 g/L, respectively). These differences in specific conductance and total dissolved solids are likely associated to water flow in the river, with higher values found during low flows.

Dissolved oxygen levels (mg/L) were observed to be significant different (F = 30.26; p = 0.0002) between the irrigation and non-irrigation seasons. Mean concentrations of O_2 were higher during the non-irrigation season (11 ± 0.6 mg/L) as compared to the irrigation season (7.9 ± 0.2) which may due to lower turbidity during the non-irrigation season which would support higher levels of photosynthesis.

Significant differences were also determined for dissolved nutrients ((nitrite (F = 9.44; p = 0.0128), silica (F = 16.26; p = 0.0026), chloride (F = 31.26; p < 0.0001), and sulfate (F = 30.34; p < 0.0001)) between the irrigation and non-irrigation seasons. Mean concentrations of dissolved nutrients were higher during the non-irrigation season ((nitrite (0.16 ± 0.04 mg/L), silica (26.5 ± 2.2 mg/L), chloride (185.3 ± 20.1 mg/L), and sulfate (293.8 ± 36.1 mg/L)) as compared to the irrigation season ((nitrite (0.03 ± 0.01 mg/L), silica (20.5 ± 1.9 mg/L), chloride (96.2 ± 14.7 mg/L), and sulfate (151.3 ± 22 mg/L)). Differences in nutrient concentrations are likely due to releases of water from Elephant Butte during the irrigation season while during the non-irrigation season water in the river mainly consists of agricultural return flows and wastewater effluent which have been found to be important source of nutrient loadings in the river (IBWC, 2011; Rodriguez and Lougheed, 2010).

Table 21. General linear mixed model results for physicochemical parameters by season (irrigation and non-irrigation) in the middle stretch of the Rio Grande (August 2010 – September 2011).

Model Class	Variable	*DF*	*F value*	*Pr>F*
Season	Temperature (° C)	16	7.51	0.0145
	Specific conductance (µS/cm)	16	29.75	< 0.0001
	O_2 (mg/L)	11	30.26	0.0002
	Total Dissolved Solids (g/L)	15	12.41	0.0031
	Nitrite (mg/L)	9	9.44	0.0128
	Alkalinity as $CaCO_3$ (mg/L)	21	54.25	< 0.0001
	Hardness as $CaCO_3$ (mg/L)	21	12.28	0.0021
	Silica as SiO_2 (mg/L)	10	16.26	0.0026
	Chloride as Cl^- (mg/L)	21	31.26	< 0.0001
	Sulfate as SO_4 (mg/L)	21	30.34	< 0.0001

Overall, the Rio Grande at Percha Dam, Anthony, and Fabens sampling stations met Texas Surface Water Quality Standards (IBWC, 2011; Reference in Chapter 1). The Rio Grande at American Dam presented high concentrations of chloride (264.7 ± 36.4 mg/L (State criterion: 250 mg/L)) and sulfate (456.7 ± 92.1 mg/L (State criterion: 450 mg/L)) as well as a high amount of total dissolved solids (2,463 ± 886 mg/L (State criterion: 1,400 mg/L)) as compared to those set by the State during sampling events in this study.

APPENDIX B: Calibration Curves (Peak areas over Concentration) of target analytes

This appendix contains multipoint calibration curves obtained from standards used for final determination of PPCP analyte concentrations. Peak areas were determined by using Xcalibur 2.0.7 Software (Thermo Electron Corporation, 2006).

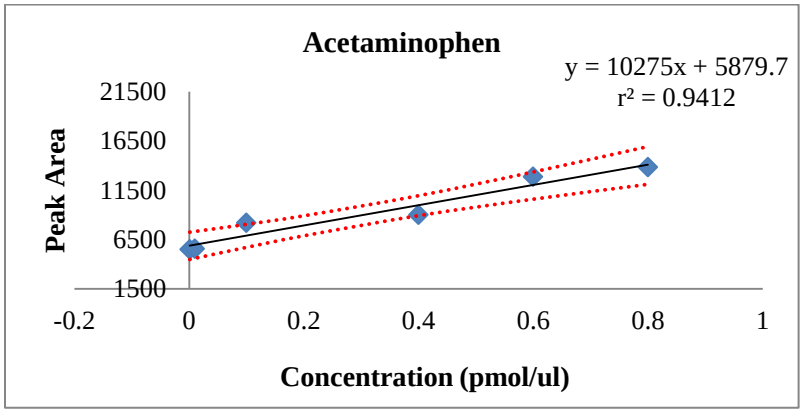

Figure 28. Calibration curve for acetaminophen showing peak areas over a range of concentrations. Dotted lines represent the confidence interval of the calibration curve.

For caffeine, two peaks were obtained, the first showed at a retention time of 16 min. and the second one at 28 min. Thus Figure 29 shows the averaged calibration curve for caffeine.

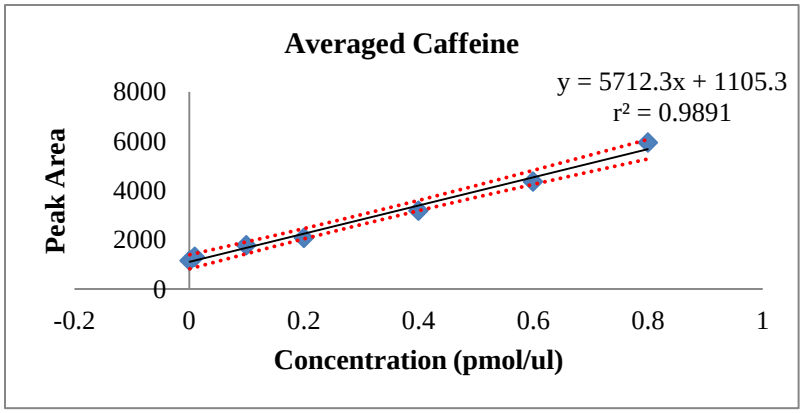

Figure 29. Calibration curve for averaged caffeine showing peak areas over a range of concentrations. Dotted lines represent the confidence interval of the calibration curve.

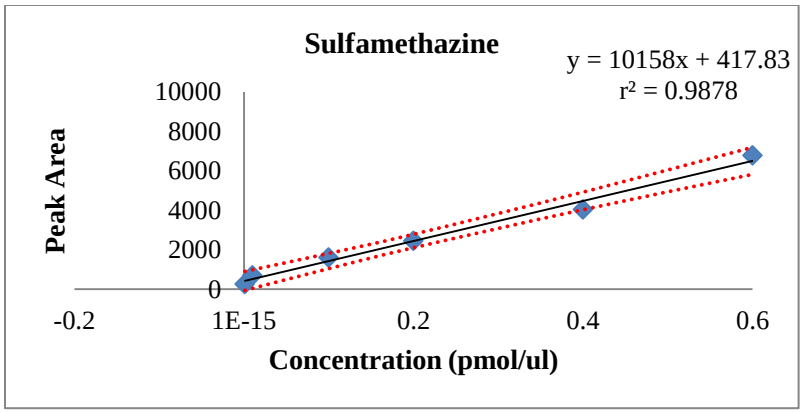

Figure 30. Calibration curve for sulfamethazine showing peak areas over a range of concentrations. Dotted lines represent the confidence interval of the calibration curve.

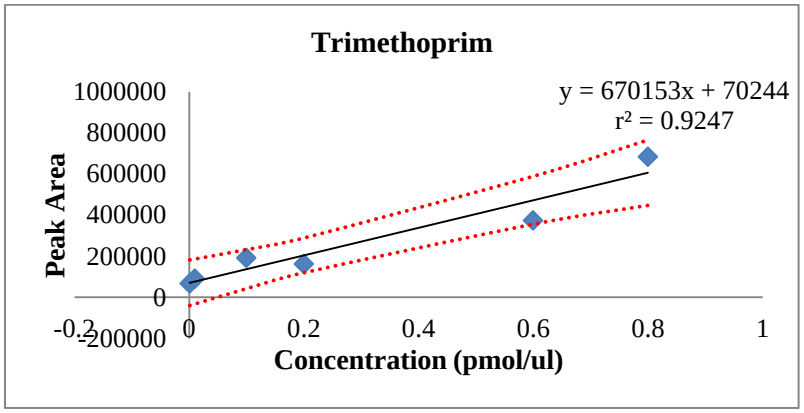

Figure 31. Calibration curve for trimethoprim showing peak areas over a range of concentrations. Dotted lines represent the confidence interval of the calibration curve.

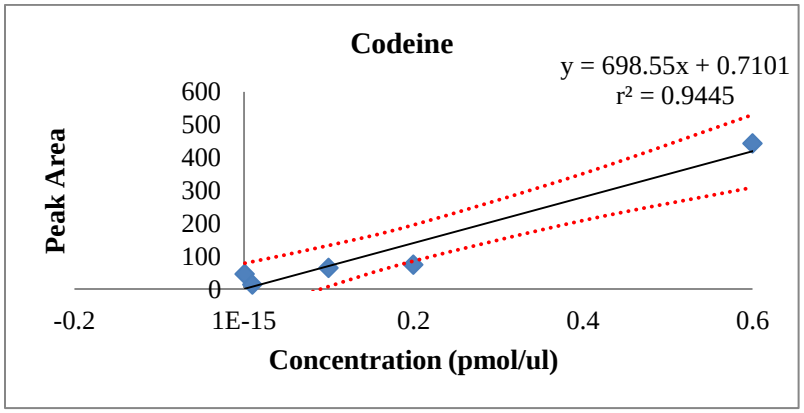

Figure 32. Calibration curve for codeine showing peak areas over a range of concentrations. Dotted lines represent the confidence interval of the calibration curve.

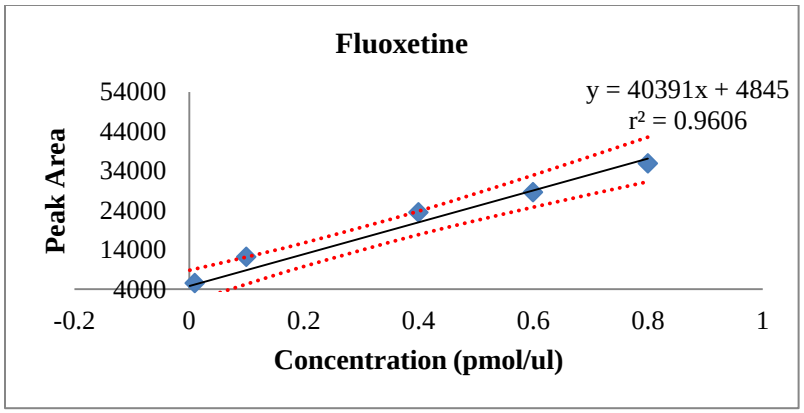

Figure 33. Calibration curve for fluoxetine showing peak areas over a range of concentrations. Dotted lines represent the confidence interval of the calibration curve.

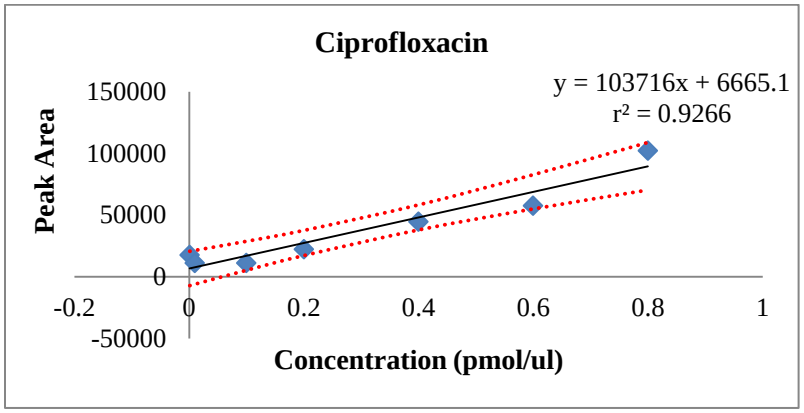

Figure 34. Calibration curve for ciprofloxacin showing peak areas over a range of concentrations. Dotted lines represent the confidence interval of the calibration curve.

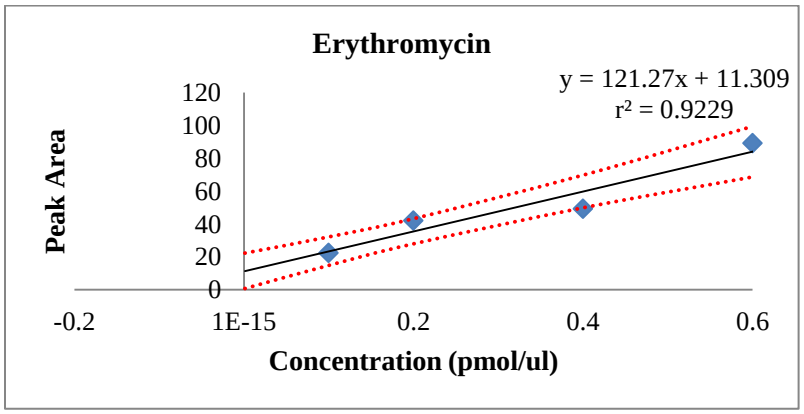

Figure 35. Calibration curve for erythromycin showing peak areas over a range of concentrations. Dotted lines represent the confidence interval of the calibration curve.

APPENDIX C: Median Lethal Concentrations (LC_{50}) for *Platonus patulus* (Reference and Rio Grande populations)

Table 22 shows 48 hr LC_{50} values for both *P. patulus* populations determined by Probit analysis. The no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) values were determined by Dunnett's Post Hoc tests. LC_{50} values, NOEC and LOECs are presented in pmol/L for comparison purposes.

Table 22. 48 hr LC_{50} values in pmol/L of PPCPs for *P. patulus* as determined by Probit analysis. Not determined (ND).

Compound	Reference population			Rio Grande population		
	LC_{50} (pmol/L)	**NOEC (pmol/L)**	**LOEC (pmol/L)**	**LC_{50} (pmol/L)**	**NOEC (pmol/L)**	**LOEC (pmol/L)**
Acetaminophen	2.11×10^{9}	1.44×10^{6}	1.98×10^{9}	8.00×10^{8}	4.96×10^{5}	6.61×10^{8}
Caffeine	2.14×10^{9}	ND	ND	2.12×10^{9}	1.52×10^{9}	1.77×10^{9}
Fluoxetine	ND	ND	ND	6.14×10^{5}	2.42×10^{5}	3.23×10^{5}
Triclosan	4.48×10^{5}	ND	ND	1.10×10^{6}	8.63×10^{5}	1.03×10^{6}

APPENDIX D: Chronic exposure of *P. patulus* to lower concentrations of acetaminophen

As discussed in Chapter 3, a second set of concentrations was tested for acetaminophen. Intrinsic rates of population increase (r) over time for both populations are shown in Figure 27.

For the reference population no significant differences in population growth over time were observed among lower concentrations of acetaminophen (1 mg/L and 5 mg/L) as compared to the control treatment ($F = 0.64$; $p = 0.8303$). Significant interactions for the 10 mg/L treatment over days were previously discussed (Chapter 3). Similarly, for the Rio Grande population, no significant differences in population growth rate over time occurred among lower concentrations ($F = 0.51$; $p = 0.9266$).

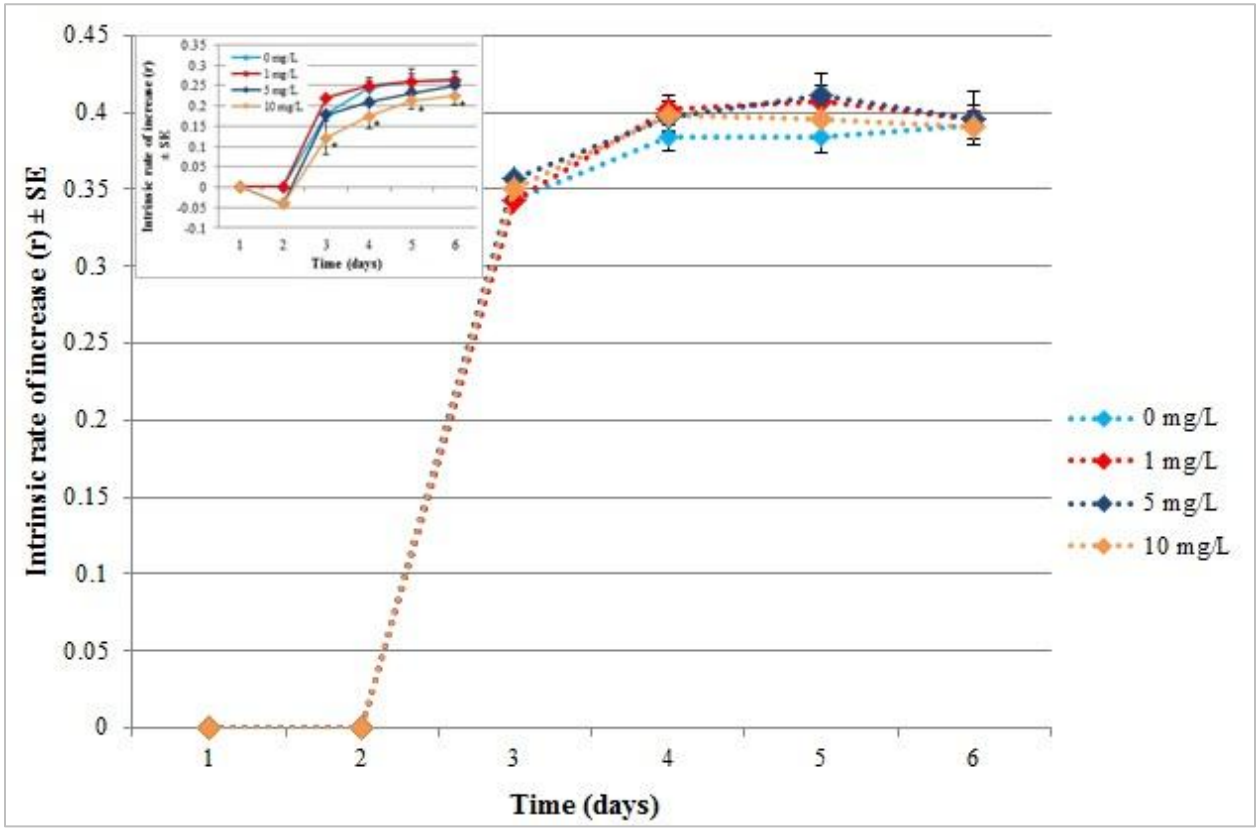

Figure 27. Rates of population increase (r) over time for the reference population (inset) and Rio Grande population exposed to low concentrations of acetaminophen. Mean ± standard error are based on five replicates. * Indicates significant effects on population growth.

APPENDIX E: List of species found in the Rio Grande during this study and during additional sampling events conducted by our lab

Table 23 shows the rotifer species that have been found in the Rio Grande. These species were found at four sites in this study (Figure 1) from August 2010 through September 2011 as well as at other locations surveyed by our lab including sites in the vicinity of El Paso, Caballo Reservoir, Elephant Butte Reservoir, Bosque del Apache, near Williamsburg, NM and several locations in Big Bend National Park, Brewster Co., TX, and the Wild Scenic stretch of the Rio Grande. These locations have been sporadically sampled since 1996 using similar collection methods.

Table 23. Preliminary species list of Rotifera in the Rio Grande.

Family	**Number**	**Species**
Brachionidae	1	*Brachionus angularis*
	2	*Brachionus bidentata*
	3	*Brachionus calyciflorus*
	4	*Brachionus plicatilis*
	5	*Brachionus quadridentatus*
	6	*Brachionus urceolaris*
	7	*Brachionus variabilis*
	8	*Keratella americana*
	9	*Keratella cochlearis*
	10	*Notholca acuminata*

	11	*Plationus patulus*
	12	*Platyias quadricornis*
Asplanchnidae	13	*Asplanchna priodonta*
Dicranophoridae	14	*Encentrum putorius*
Epiphanidae	15	*Epiphanes chihuahuaensis*
Euchlanidae	16	*Euchlanis calypida*
	17	*Euchlanis dilatata*
	18	*Euchlanis triquetra*
Filiniidae	19	*Filinia longiseta*
	20	*Filinia terminalis*
Flosculariidae	21	*Sinantherina socialis*
Lecanidae	22	*Lecane bulla*
	23	*Lecane luna*
	24	*Lecane quadridentata*
Lepadellidae	25	*Lepadella patella/ovalis*
	26	*Colurella uncinata*
Mytilinidae	27	*Mytilina mucronata*
Notommatidae	28	*Cephalodella catellina*
	29	*Cephalodella forficula*
	30	*Cephalodella gibba*
	31	*Cephalodella cf. graciosa*
	32	*Cephalodella megalocephala*
	33	*Cephalodella cf. misgurnus/pachyodon*

	34	*Cephalodella sterea*
	35	*Eosphora najas*
	36	*Notommata allantois*
Philodinidae	37	*Philodina megalotrocha*
Proalidae	38	*Proales daphnicola*
	39	*Proales similis*
Synchaetidae	40	*Synchaeta cylindrica/tavina*
	41	*Polyarthra dolichoptera*
Testudinellidae	42	*Testudinella patina*
Trichotriidae	43	*Trichotria tetractis*
	44	Bdelloids (unidentified)

CURRICULUM VITAE

Diana Angélica Martínez Gómez was born in Ciudad Juárez, Chihuahua, México. In 2001, she entered the Biology Program at Universidad Autónoma de Ciudad Juárez (UACJ). As an undergraduate, she conducted herpetology and field biology research at La Escondida Mountain Range, Nuevo Casas Grandes, Chihuahua, Mexico (2004 – 2006). She graduated in June 2006 with a Bachelor´s of Science degree in Biology receiving the Top Ten Class of the State of Chihuahua award. After she graduated, she taught Biology and Chemistry to high school students. In 2007, she obtained a position as a Microbiology Technician in the medical device industry where she conducted environmental monitoring in clean rooms. In Fall 2009, she entered to the Environmental Science Masters Program at the University of Texas at El Paso where she obtained a teaching assistant position. By the end of Summer 2010, she join the lab of Dr. Elizabeth Walsh in the Department of Biological Sciences where she conducted research focused on a water quality assessment of the middle Rio Grande specifically looking at the occurrence of Pharmaceuticals and Personal Care Products (PPCPs) and evaluating the toxicity of these toxicants to the freshwater rotifer *Plationus patulus*. In April 2011, she was awarded the Cotton Memorial Scholarship (UTEP). She presented preliminary results at the Sustainability on the Border Conference held at UTEP (May 2011), the Ecological Society of America annual meeting held in Austin, TX (August 2011), the SETAC Mexico Focused Topic Meeting held in Merida, Mexico (August 2011), and the SETAC North America 31st annual meeting held in Boston, MA (November 2011).

Contact information:

damartinezgomez@miners.utep.edu or diana180@prodigy.net.mx