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LETHAL AND SUBLETHAL EFFECTS OF SELECTED PPCPs ON THE FRESHWATER ROTIFER, *PLATONUS PATULUS*

Diana A. Martinez Gomez[†], Sarah Baca[†], and Elizabeth J. Walsh^{*,†,‡}

[†]Environmental Science Program, University of Texas at El Paso, El Paso, Texas, USA

[‡]Department of Biological Sciences, University of Texas at El Paso, El Paso, Texas, USA

Abstract

Pharmaceuticals and personal care products (PPCPs) have been reported in surface waters around the world. The continuous input of these pollutants into freshwaters and their potential effects on aquatic life are of increasing concern. The rotifer *Platonus patulus*, a basal member of riverine food webs, was used to test acute and chronic toxicity of 4 PPCPs (acetamidophenol, caffeine, fluoxetine, triclosan). A population from a remote site in Mexico (reference population) and one from an urbanized stretch of the Rio Grande were exposed. Acute toxicity tests show that both populations were more sensitive to fluoxetine. Chronic exposure to acetamidophenol (10mg/L, 15mg/L, and 20 mg/L) inhibited reference population growth, whereas Rio Grande population growth was inhibited only at 15 mg/L and 20mg/L. Population growth was inhibited at 200 mg/L and 300mg/L of caffeine for both populations. Chronic exposure to fluoxetine (0.020mg/L) significantly inhibited population growth for the Rio Grande population only. Triclosan (0.05 mg/L, 0.075mg/L, 0.10 mg/L) had the most deleterious effects, significantly reducing both populations' growth rates. Sublethal effects of chronic exposure to PPCPs included decreased egg production and increased egg detachment. A mixed exposure (6 PPCPs, environmentally relevant concentrations) did not affect population growth in either population. However, the continuous introduction of a broad suite of PPCPs to aquatic ecosystems still may present a risk to aquatic communities.

Keywords

Emerging pollutants; Acute toxicity; Chronic toxicity; Aquatic invertebrates; Rotifer

INTRODUCTION

Surface waters are commonly the most susceptible water bodies to pollution, because both natural (precipitation, 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,

*Address correspondence to ewalsh@utep.edu.

Data Availability—Data, associated metadata, and calculation tools for the present study are publicly available on request from the authors (ewalsh@utep.edu).

All Supplemental Data may be found in the online version of this article.

and nutrients that can degrade aquatic ecosystem function and impair their usage for drinking and recreational purposes [1–5]. The Rio Grande, which serves as an international boundary between the United States and Mexico and spans the cities of El Paso (Texas, USA) to Ciudad Juárez (Chihuahua, Mexico) to the Gulf of Mexico in the Brownsville/Matamoros area [6], 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), Ciudad Juárez (primary treatment), and irrigation return flows [7,8].

The presence of “emerging” pollutants in surface waters that receive effluent from wastewater treatment plants is of recent concern. One group of these compounds, known as pharmaceuticals and personal care products (PPCPs), is broadly used in our daily activities and are applied internally or externally to humans or to domestic animals [9]. The PPCP toxicants include a variety of chemicals that are grouped based on their common use, such as analgesics, anticonvulsants, antimicrobials, lipid regulators, antibiotics, nonsteroidal anti-inflammatory drugs, and synthetic hormones, among others [9,10]. The PPCP contaminants are mainly derived from human or livestock excretion of these compounds or their metabolites, improper disposal of outdated medication, and untreated hospital and veterinary wastes entering domestic sewage systems through overflow or leakage from storage structures or land application [4,11–13]. Attention of the scientific community to these pollutants arose in the late 1990s with extensive reviews of PPCPs in the environment [9,10,14,15]. 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 nanogram per liter to microgram per liter range nearly worldwide [11,16]. Although these unregulated chemicals have been present in the environment for decades, until recently they were not recognized as potentially significant water contaminants [12,17]. The PPCPs are not completely removed from sewage after treatment, allowing these compounds to enter and persist in surface and groundwaters [11,18,19]. The continuous introduction of PPCPs into aquatic environments may produce negative effects to these ecosystems [12], especially to nontarget organisms [18]. Some of the detrimental effects that have been observed include changes in sex ratios, changes in biochemical cycles, and anatomical malformations in a wide range of organisms at higher trophic levels [18].

Ecotoxicological studies have been conducted on a variety of aquatic organisms, including algae, invertebrates such as *Daphnia magna* and *Ceriodaphnia dubia*, and some species of fishes and amphibians, to determine the potential risks of PPCPs released into aquatic systems [20]. Performing studies in other aquatic groups such as rotifers is equally important because of their prevalence and importance within aquatic communities [21]. In a recent review, Dahms et al. [21] summarized the advantages of using monogonont rotifers as model organisms in toxicological research, including their high population growth rates, which contributes to their role in energy transfer and nutrient cycling in these systems [22], and their short life cycles, which allow for the study of multigenerational effects in short periods as compared with other test species such as *Daphnia* [23]. Rotifers are also considered sensitive bioindicators because they have been shown to be susceptible to an extensive range of pollutants [24] and are highly sensitive to changes in water quality [21]. In addition, their primarily parthenogenetic reproduction allows for testing genetically identical individuals [21].

Because of these attributes and its common occurrence in the Rio Grande, the freshwater monogonont rotifer *Platyonus patulus* was selected to assess the toxicity and sublethal effects of 4 selected PPCPs (acetamidophenol, caffeine, fluoxetine, and triclosan). In addition, this species has been recognized as a test standard species by the American Public Health Association [25] and has been used as a model for other toxicological studies [25–27]. 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 [11] and the solubility of the chemical in culture medium.

For acute toxicity tests (48 h), the median lethal concentration (LC50), the maximum concentration of the test chemical that produces no statistical harmful effects compared with control (NOEC), and the lowest concentration of the test compound that has a statistically significant detrimental effect compared with control (LOEC) were selected as toxicological endpoints. Chronic toxicity of target compounds was assessed by conducting population growth studies, with the intrinsic rate of population increase (r) as the endpoint. These studies will aid in better understanding how selected PPCPs may impact aquatic communities, particularly rotifer populations.

MATERIALS AND METHODS

Test lineages

Two geographically distinct *P. patulus* populations were tested. The first population, referred to in the present study as the reference population, was collected from a tinaja located in a remote area of Mexico, south of Big Bend National Park on 31 July, 2008 (29.75111° N; –102.58305° W; 505m). This population was used as the reference population because the site is assumed to be free of PPCP pollutants because it is primarily filled by rainfall and access is difficult. The second population, referred as the Rio Grande population, was collected from the Rio Grande near the Fabens Port of Entry, El Paso County, Texas (31.430277°N, –106.142220° W; elevation 1096 m). 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/d [28]. This population was collected on 12 August, 2010. The *P. patulus* were collected using a 64- μ m mesh plankton net. Both populations were cultured under standard laboratory conditions for more than 20 generations before toxicity testing. Neither population was intentionally started from a single individual, and sexual reproduction, although not common, did occur under laboratory conditions sporadically.

The *P. patulus* populations were cultured in synthetic, moderately hard water (US Environmental Protection Agency [USEPA] media [96 mg NaHCO₃, 60 mg CaSO₄–2 H₂O, 122 mg SO₄–7 H₂O, 4mg KCl in 1 L Milli-Q water], [29]) 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 The Culture Collection of Algae at the University of Texas at Austin, strain 30]). Algae were cultured in modified Marine Biological Laboratory (MEL) medium [30] and incubated at $25 \pm 1^\circ\text{C}$ in 24-h light.

Chemicals

Rotifers from each population were exposed to 7 selected PPCP compounds (acetamidophenol, caffeine, ciprofloxacin, erythromycin, fluoxetine, sulfamethazine, triclosan). Brands and CAS registry numbers are shown in Supplemental Data, Table S1. Stock solutions for tested toxicants were prepared in USEPA medium. 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.

Acute toxicity tests

Range finder tests were conducted for each compound (see Table 1) by exposing both populations to a broad range of concentrations determined from values in the literature for similar species. Concentrations for LC50 tests, as described later, were based on these results.

Ovigerous amictic females were isolated for 4 h to 6 h in USEPA medium containing excess algal food (2.5×10^5 cells/mL) and were incubated at $25 \pm 1^\circ\text{C}$ with 24 h light. Cultures were checked after 4 h to 5 h; neonates were separated into USEPA medium with food and allowed to feed for approximately 1 h before the acute exposure assay. At least 6 concentrations of the test solution were prepared by making dilutions from a stock solution (Table 1).

American Society for Testing and Materials (ASTM) International acute toxicity methods for *Brachionus* [31] were used, with the following modifications. All tests were conducted in disposable, sterile 24-well tissue culture plates. The setup consisted of 1 control solution (USEPA medium) and diluted test concentrations with 6 (2mL) replicates per treatment. After eggs hatched, 1 to 5 neonates (2+ h old; initial number was dependent on total number of individuals hatching within 6 h) were placed into each well. The number of deaths and other effects (e.g., qualitative decrease in swimming speed, immobilization, egg detachment) were recorded after 24 h and 48 h.

Chronic toxicity tests

Six-day population growth studies were carried out to assess the chronic toxicity of the 4 selected PPCP pollutants on the intrinsic rate of population increase (r). Both populations were tested at the same concentrations (Table 2), with 3 individuals per replicate and 5 replicates per treatment. For these experiments, a protocol modified from Snell and Moffat [23] was followed.

One day before testing, individuals were transferred into fresh USEPA medium with excess algal food. On the test day, 4 h to 6 h before initiation of the experiment, ovigerous females were isolated and placed in an incubator at $25 \pm 1^\circ\text{C}$ in 24 h light. *Chlamydomonas reinhardtii* was concentrated by centrifuging it at $15\,344\,g$ for 10 min and resuspended in USEPA medium. Algal density was estimated by using a Neubauer hemocytometer. The algal suspension was diluted to 2.5×10^5 cells/mL. Under these conditions, generation time is approximately 4 d to 6 d. The test pollutant was then diluted in this solution into 3 test concentrations from a stock solution (Table 2). Concentrations for each compound were

based on the LC50 value (lowest LC50 from either *P. patulus* population) and were no higher than 70% of the LC50 value.

The design for the present study consisted of 3 test concentrations and 1 control, 5 replicates each. Control replicates consisted of the algal suspension in USEPA medium and rotifers. Test tubes were filled with 12mL of each test concentration, and 3 neonates (<2 h old) were transferred into each tube. The test tubes were placed on a rotator at 8 rpm to 10 rpm to ensure that algae, rotifers, and compounds remained in suspension during the duration of the experiment. Cultures were incubated at $25 \pm 1^\circ\text{C}$ in the dark for 6 d. Media with food and compound was replaced daily. The number of individuals, their reproductive status (asexual vs sexual; fertilized vs unfertilized), deformities, and any changes in behavior were recorded daily. For acetamidophenol and triclosan, a second set of concentrations (1 mg/L, 5 mg/L, and 10 mg/L) was tested, because these concentrations are more likely to be environmentally relevant [11,32].

Mixture exposure.—An additional 6-d population growth study was conducted to evaluate the chronic toxicity of a mixture of 6 PPCP pollutants (acetamidophenol, caffeine, ciprofloxacin, erythromycin, fluoxetine, and sulfamethazine) at the ng/L range. Tested concentrations (ng/L) were: acetamidophenol, 1.05; caffeine, 57.92; ciprofloxacin, 40.98; erythromycin, 8.03; fluoxetine, 6.65; and sulfamethazine, 1.49. The selection of test concentrations was based on the occurrence of these compounds in surface water samples collected from the Rio Grande during a complementary study in which the presence and occurrence of 9 selected PPCPs was surveyed.

For the present study, the same chronic toxicity protocol was followed. The design consisted of 1 test concentration (mixture) and 1 control, with 5 replicates each. Every replicate was initiated with 3 individuals. For the mixed treatment, a stock solution was prepared for each compound. From each stock solution, a series of dilutions were made to obtain the desired concentration.

Data analysis

The LC50 value for each of the 4 selected PPCP pollutants was determined by Probit analysis in the statistical program PASW (Ver 18.0). The LC50 values for the reference and Rio Grande populations were compared to determine significant differences in the tolerance level to tested toxicants by running nonlinear mixed model analyses in the statistical program SAS (Ver 9.2). 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 versus concentration in PASW (Ver 18.0).

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 live 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 (3), and T is the time in days (1–6 d). The average of the intrinsic rate of population increase (r) per day per concentration was obtained as well as the standard error based on 5 replicates for most of the compounds. Intrinsic rates of population increase (r) for each compound were analyzed by General Linear Mixed Model in SAS (Ver 9.2) to determine whether a significant relationship existed between endpoint and toxicant concentration.

RESULTS

Acute toxicity studies

Acetamidophenol 48-h exposures.—In acute toxicity tests for acetamidophenol, the Rio Grande population was less tolerant than the reference population according to the LC50 values (Figure 1A, D). The 48-h LC50 for the reference population was 319 mg/L ($z = -3.98$; $p < 0.001$), whereas for the Rio Grande population the LC50 was 121 mg/L ($z = -7.138$; $p < 0.0001$), almost 3 times lower than that of the reference population (Table 3). After a Dunnett's post hoc test, the estimated NOEC for the reference population was 200 mg/L, with an observed mortality of 11%, and the LOEC was 300 mg/L, with a mortality of 39% ($F = 11.71$; $p < 0.001$). The Rio Grande population had a NOEC of 75 mg/L with an observed mortality of 12% and a LOEC of 100 mg/L with a mortality of 33% ($F = 64.4$; $p < 0.001$).

Caffeine 48-h exposures.—Among the 4 tested PPCP compounds, caffeine was the least toxic chemical (Figure 1B, E; Supplemental Data, Table S2). The reference population LC50 was 580 mg/L ($z = -2.42$; $p = 0.01$) versus 419 mg/L ($z = -4.44$; $p < 0.0001$) for the Rio Grande population. The estimated NOEC for the reference population was 450 mg/L with 25% mortality, and the LOEC was 525 mg/L with a 50% mortality ($F = 4.43$; $p < 0.05$). The NOEC for the Rio Grande population was 300 mg/L with 22% of mortality when the LOEC was 350 mg/L ($F = 9.13$; $p < 0.001$) with 34% mortality. A qualitative increase in mobility was observed after 24 h of exposure to caffeine at 400 mg/L and 450 mg/L as compared with the test control in the Rio Grande population. However, a decrease in mobility was observed for this population at 250 mg/L and 300 mg/L after 48 h of exposure.

Fluoxetine 48-h exposures.—Fluoxetine was the most toxic chemical for both populations (Table 3), with an estimated LC50 of 0.06 mg/L ($z = -4.17$; $p < 0.001$) for the reference population and 0.19 mg/L ($z = -7.12$; $p < 0.001$) for the Rio Grande population (Figure 1C, F). The NOEC for the reference population was 0.025 mg/L with an observed mortality of 0% and LOEC of 0.050 mg/L with an observed mortality of 67% ($F = 20.90$; $p < 0.001$). The Rio Grande population had a NOEC of 0.075 mg/L, with an observed mortality of 4%, and a LOEC of 0.10 mg/L with an observed mortality of 21% ($F = 48.17$; $p < 0.001$) according to a Dunnett's post hoc test. Fluoxetine caused a decrease in mobility in *P. patulus* at 40 mg/L after 24 h of exposure, whereas mortality was recorded after 48 h.

Triclosan 48-h exposures.—A series of acute exposures for the reference population were conducted, but results obtained were not statistically significant. The estimated 48-h

LC50 for the Rio Grande population was 0.3 mg/L (Figure 1G). The NOEC was 0.25 mg/L with no observed mortality and an LOEC of 0.30 mg/L with an observed mortality of 37% ($z = -3.38$; $p = 0.001$). Decreased mobility was observed in the Rio Grande population after 48 h of exposure at 0.20 mg/L, 0.25 mg/L, and 0.30 mg/L.

Table 3 shows the 48-h LC50, NOEC, and LOEC for each *P. patulus* population when exposed to all compounds. These tests showed that fluoxetine was the most toxic compound for the reference population with an LC50 of 0.06 mg/L ($z = -4.17$; $p < 0.001$) and for the Rio Grande population with an LC50 of 0.19 mg/L ($z = -7.12$; $p < 0.001$). Conversely, the least toxic compound for both *P. patulus* populations was caffeine, with an estimated LC50 of 580 mg/L ($z = -2.42$; $p = 0.011$) and 419 mg/L ($z = -4.44$; $p < 0.001$) for the reference and Rio Grande populations, respectively.

Chronic toxicity studies

Reproduction of both populations was generally inhibited, causing a decrease in population growth with increasing concentrations of acetamidophenol, caffeine, triclosan, and fluoxetine. Of the 4 toxicants, tested concentrations of triclosan were shown to have more deleterious effects on both *P. patulus* populations. Some of the observed sublethal effects for acetamidophenol, 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 with the control treatment, except for 0.020 mg/L, which inhibited population growth as compared with the control treatment. Intrinsic rates of population increase (r) for each tested compound for both populations are shown in Figure 2.

Acetamidophenol 6-d exposures.—Six-day exposure studies to this compound for the reference population showed significant differences in population growth among increasing concentrations of acetamidophenol over time ($F = 20.96$; $p < 0.0001$). Significant differences in population growth as compared with the control treatment occurred at all tested concentrations. For instance, at 10 mg/L, no growth was observed at day 3 of exposure ($t = 3.32$; $p = 0.0014$), whereas 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 with 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 with control treatment over time of exposure (Figure 2A).

Significant differences were observed in population growth among concentrations of acetamidophenol over time ($F = 43.04$; $p < 0.0001$) 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 with the control treatment (Figure 2E). On days 3 and 4, a significant difference was seen between the control treatment and 15 mg/L, where no growth was observed ($t = 8.11$; $p < 0.0001$), whereas 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 ($t = 14.43$; $p < 0.0001$).

Sublethal effects to both *P. patulus* populations produced by the 6-d exposure to acetamidophenol are listed in Supplementary Data (Table S2). These effects were observed at 10 mg/L, 15 mg/L, and 20 mg/L acetamidophenol concentrations but not at the 2 lowest concentrations (1 mg/L and 5 mg/L). Reproduction of both populations was negatively affected by exposure to 15 mg/L and 20 mg/L treatments of acetamidophenol, decreasing egg production as compared with 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 that resulted in the production of unviable embryos in most cases. This effect started on day 4 for both populations.

Caffeine 6-d exposures.—Intrinsic rates of increase (r) over days of exposure to caffeine for both populations are shown in Figure 2. Chronic exposure to caffeine for the reference population resulted in significant differences in the population growth among increasing concentrations over time as compared with 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 with the control treatment and with a negative rate of increase on day 6 ($t = 3.35$; $p = 0.0017$; Figure 2B). 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 6 d of caffeine exposure, the Rio Grande population showed significant differences in population growth among increasing concentrations over days of exposure ($F = 11.42$; $p < 0.0001$). Significant differences were observed on days 3, 4, 5, and 6 for 200 mg/L and 300 mg/L as compared with the control treatment. At 200 mg/L, significant differences were observed on day 3, where population growth was inhibited 86% ($t = 5.26$; $p < 0.0001$) as compared with the control. At this concentration, population growth was also inhibited on days 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 with the control, respectively. Significant differences were also obtained at 300 mg/L on days 3, 4, and 6, where negative growth was observed whereas on day 5 population growth was inhibited 99% ($t = 8.36$; $p < 0.0001$) as compared with population growth of the control treatment (Figure 2F).

Decreased egg production was observed as a result of chronic caffeine exposure. Egg production was slowed at 200 mg/L for both populations starting on day 3, whereas egg production was inhibited at 300 mg/L for both populations starting also on day 3. Similar to acetamidophenol, chronic exposure to caffeine caused egg detachment from females, leading to unviable embryos in both populations (Supplemental Data, Table S2). 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.

Fluoxetine 6-d 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% on day 3 ($t=7.25$; $p<0.0001$), 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 with the control treatment. No effects were seen in the reference population. Rates of intrinsic population increase for this treatment are shown in Figure 2C, 2G. Besides reduced population growth, no other sublethal effects were observed for either population as a result of the chronic exposure to fluoxetine.

Triclosan 6-d exposures.—Significant differences in the population growth of the reference population were determined among increasing concentrations of triclosan over time as compared with the control treatment ($F=10.06$; $p<0.0001$). Effects of this toxicant to rates of population increase (r) were observed at all tested concentrations. At 0.05 mg/L, population growth was inhibited 72% ($t=3.50$; $p=0.0008$) on day 4 of exposure, whereas on day 5 it was inhibited 94% ($t=5.86$; $p<0.0001$) as compared with control treatment. On day 6, a negative rate of population growth was determined ($t=6.43$; $p<0.0001$). At 0.075 mg/L, negative rates of growth 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 ($t=6.71$; $p<0.0001$) and 5 ($t=9.23$; $p<0.0001$), and day 6 ($t=9.25$; $p<0.0001$; Figure 2G).

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 with 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 with the control. For the second tested concentration, 0.075 mg/L, no growth was observed as compared with the control treatment through days of exposure. For the highest tested concentration of the first experiment, 0.10 mg/L, no growth was observed on day 3 ($t=6.59$; $p<0.0001$) or day 4 ($t=9.70$; $p<0.0001$), whereas on days 5 and 6, negative population growth was determined (Figure 2H).

Sublethal effects to *P. patulus* from both populations caused by 6-d exposure to triclosan are shown in Supplemental Data, Table S2. Effects were observed at 0.05 mg/L, 0.075 mg/L, and 0.10 mg/L of triclosan-tested concentrations. Reproduction of both *P. patulus* populations was affected at 0.05 mg/L, 0.075 mg/L, and 0.10 mg/L as a result of chronic exposure to triclosan by inhibiting or slowing egg production as compared with 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. In addition, triclosan had a negative effect on mobility of both *P. patulus* populations. Mobility was generally slowed at 0.05 mg/L, 0.075 mg/L, and 0.10 mg/L as compared with control treatments.

Mixed exposure.—Chronic exposure to a mixture of PPCP pollutants did not produce a significant effect on the intrinsic rate of increase (r) for the reference population ($F = 0.81$; $p = 0.5547$) or for the Rio Grande population ($F = 0.81$; $p = 0.5471$) at the ng/L range. Intrinsic rates of increase (r) over time for the present study are shown in Figure 3A and 3B for both populations. No sublethal effects were observed as a consequence of the 6-d mixture exposure to either population.

DISCUSSION

The LC50s for acetamidophenol, caffeine, fluoxetine, and triclosan do not show a consistent pattern according to the source population of *P. patulus*. For instance, the reference population was more tolerant of the analgesic acetamidophenol and the stimulant caffeine. For both populations, fluoxetine (an antidepressant) was the most toxic compound, with the Rio Grande and reference population having LC50s of 0.19 mg/L and 0.06 mg/L, respectively. These observations are contrary to our predictions, because the reference population was expected to be more sensitive than the Rio Grande population to the acute exposure of these toxicants.

The LC50 values for both populations show a higher level of tolerance to acetamidophenol (reference population LC50 = 319 mg/L; Rio Grande population LC50 = 121 mg/L) and caffeine (reference population LC50 = 580 mg/L; Rio Grande population LC50 = 419 mg/L) as compared with the values reported in the literature for the cladoceran *D. magna*, with an LC50 of 6 mg/L for acetamidophenol and 182 mg/L for caffeine [11,16]. However, tolerance to caffeine appears to be lower in *P. patulus* than in *Brachionus calyciflorus* (LC50 1018 mg/L [24 h]; 104 mg/mL [48 h]) [33]. The LC50 values reported for *D. magna* for fluoxetine and triclosan toxicity [20,34] indicate that this organism is more tolerant to these toxicants than *P. patulus* as shown in Supplemental Data, Table S3.

Although no obvious trends were obtained in the present study regarding the levels of tolerance to the exposure of these toxicants between *P. patulus* and *D. magna*, rotifers are good model organisms in acute studies because of their easy cultivation in the laboratory, 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 with the tolerance of *D. magna*. Isidori et al. [35] determined the acute toxicity of 6 antibiotics, including erythromycin and sulfamethoxazole to the rotifer *B. calyciflorus*, *D. magna*, and other aquatic invertebrates; they found that *B. calyciflorus* was more tolerant than the cladoceran to 5 of these toxicants, with the exception of ofloxacin, to which the LC50 obtained for the rotifer was 29.88 mg/L versus 31.75 mg/L for *D. magna*.

Results from chronic exposure to acetamidophenol, 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 with control treatments.

Intrinsic rates of increase (r) obtained from test controls from chronic exposures for all 4 tested toxicants for both *P. patulus* populations showed that in general the Rio Grande population had higher population growth over time as compared to population growth of reference population. Mean intrinsic rates of population increase per day for the Rio Grande population control treatments were generally 1 to 4 times higher than those obtained for the reference population (Figure 2). 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, because it was collected in a highly urban and industrial stretch of the Rio Grande. This difference may also indicate a genetic variation between the 2 populations and may influence the response that this population had to each exposed toxicant as compared with the reference population.

Results obtained from chronic exposure to acetamidophenol showed a significant inhibition in population growth at 10 mg/L for the reference population, whereas 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, whereas 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 with 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 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 with the control treatment whereas 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 with 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 acetamidophenol, caffeine, and triclosan for both *P. patulus* populations were decreased population growth, 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. [36], in which the toxicity of 3 lipid regulators (bezafibrate, fenofibrate, and gemfibrozil) were evaluated on *B. calyciflorus* population growth over 48 h of exposure; they found that reproduction was inhibited by all tested compounds. In a similar study, the chronic toxicity of ranitidine, a histamine H₂-receptor antagonist that inhibits stomach acid production, was assessed on population growth of *B. calyciflorus* over 48-h exposure; they found that this toxicant also inhibited population growth with an NOEC of 0.31 mg/L and an

LOEC of 0.63 mg/L [37]. Ferrari et al. [38] tested chronic toxicity of carbamazepine, clofibric acid, and diclofenac to *B. calyciflorus* (48-h 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.

As previously discussed, environmental concentrations of PPCPs in surface waters worldwide have been reported to occur in the nanograms per liter to micrograms per liter range [11,32,38]. Although LC50 values obtained in the present study for acetamidophenol, caffeine, fluoxetine, and triclosan are much higher than these values, PPCP toxicants do not occur as single chemicals but as complex mixtures in the environment [34]. The occurrence of mixtures within aquatic ecosystems may lead to different effects than those obtained by single toxicants. As shown in an acute study, the cladoceran *D. magna* was exposed to a mixture of 36 µg/L fluoxetine and 100 µg/L clofibric acid, which caused significant mortality and malformation, whereas no observed effects were determined by the exposure of single toxicants at the same concentrations [34]. This observation may indicate that mixtures of PPCP toxicants display additive effects resulting in a greater toxicity than that obtained for single toxicants [34]. In addition, chronic exposure to these toxicants represents a risk because of their near constant introduction to aquatic ecosystems. Chronic exposure to lower concentrations of PPCP toxicants may lead to detrimental effects such as reproduction inhibition and decrease in mobility as determined in the present study during the 6-d population growth studies of *P. patulus*.

Acetamidophenol 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 [34]. Acute toxicity tests have been conducted in algae, water fleas, fish embryos, luminescent bacteria, and ciliates. *Daphnia magna* was determined to be the most sensitive species, with an EC50 value of 50 mg/L [34]. *Platyonus patulus* was less sensitive than the cladoceran to the acute exposure of this toxicant under our exposure conditions in the present study.

In raw sewage, concentrations of caffeine have been reported to range from 20 µg/L to 300 µg/L and from 0.1 µg/L to 20 µg/L in treated wastewater effluents [39]. In surface waters, this toxicant has been reported to range from 3 ng/L to 1500 ng/L [39] and up to 9700 ng/L in the Somes River [40]. In the present 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, whereas it has been reported to occur in sewage treatment plant influents from 0.4 ng/L to 18.7 ng/L and in sewage treatment plant effluent from 0.12 ng/L to 8.4 ng/L [34]. Sublethal effects observed in invertebrates include stimulation of reproduction as observed in the crustaceans *C. dubia* as exposed to 56 µg/L and increase in total number of offspring produced after 30 d exposure to 36 µg/L in *D. 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 [34]. In the present 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, whereas sewage treatment plant effluent ranges from 0.1 µg/L to 2.7 µg/L [16]. Although chronic exposure to triclosan in *D. magna* resulted in an LOEC value of 200 µg/L after 21 d of exposure, the algae *Pseudokirchneriella subcapitata* appears to be the most sensitive trophic group in which growth was affected at concentrations less than 1 µg/L [20]. In the present study, the lowest tested concentration of triclosan (0.5 µg/L) did not inhibit population growth or produce sublethal effects in *P. patulus*.

The PPCPs were found to be toxic to this rotifer species only at concentrations higher than those typically occurring in freshwater environments. In addition, these toxicants occur as complex mixtures and not singly in nature. Thus, additional studies of mixtures of PPCPs at environmental concentrations are needed to better understand additive and synergistic effects of these pollutants.

In our experiments, responses to acute and chronic exposures of acetamidophenol, caffeine, fluoxetine, and triclosan elicit a variety of responses even between populations of the same species, *P. patulus*. Responses also differed from those reported for other test species such as *D. 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 6-d population growth studies allowed for the assessment of chronic toxicity of 4 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, conducting chronic studies along with acute studies to determine the possible impacts and effects that these pollutants may have on the aquatic system is important.

Major challenges for future researchers include the assessment of mixtures of these chemicals among different trophic levels of aquatic environments as well as better understanding of how these toxicants bind to sediments, suspended matter, and sludge and how they bioaccumulate or biomagnify 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.

CONCLUSIONS

Chronic exposure of PPCPs may reduce rotifer population growth and therefore negatively influence energy transfer to higher trophic levels by decreasing productivity of the aquatic system. The presence of these chemicals may present a risk for the zooplankton community over the long term because of the continuous introduction to aquatic ecosystems via treated wastewater effluent.

A major challenge for future researchers is not only the assessment of mixtures of these chemicals among different trophic levels of aquatic systems but also the development of

techniques for the removal of PPCPs from effluent along with the control of emission from direct and indirect sources of these chemicals.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Percentage of survivorship for the reference and Rio Grande populations of the rotifer *Plationus patulus* as a function of increasing concentrations of selected pharmaceuticals and personal care products. (A, D) Acetamidophenol; (B, E) caffeine; (C, F) fluoxetine; (G) triclosan.

Figure 2.

Rates of population increase (r) over time for the reference and the Rio Grande populations exposed to different concentrations of 4 pharmaceuticals and personal care products. Mean $\pm$ SE are based on 5 replicates. (A, E) Acetamidophenol; (B, F) caffeine; (C, G) fluoxetine; (D, H) triclosan.

Figure 3.
Rates of population increase (r) over time for the reference and Rio Grande populations exposed to mixed concentrations of 6 pharmaceuticals and personal care products. Mean $\pm$ SE are based on 5 replicates.

Concentrations tested for acute toxicity of selected pharmaceutical and personal care products for the rotifer *Platyonus patulus*

Table 1.

Compound	48-h exposure ^a	
	Reference population concentration (mg/L)	Rio Grande population concentration (mg/L)
Acetamidophenol	0, 100, 200, 300, 400, 500	0, 75, 100, 150, 175, 200, 250
Caffeine	0, 450, 525, 600, 675, 750	0, 250, 300, 350, 400, 450
Fluoxetine	0, 0.025, 0.05, 0.075, 0.10, 0.20	0, 0.05, 0.075, 0.10, 0.20, 0.40
Triclosan	ND	0, 0.15, 0.20, 0.25, 0.30, 0.35, 0.40

^a 6 replicates per treatment, 2–5 individuals per replicate.

ND = not determined.

Table 2.

Pharmaceutical and personal care product (PPCP) concentrations tested for chronic toxicity studies on *Platyonus patulus*^a

6-d exposure	
Compound	PPCP concentration (mg/L)
Acetamidophenol	10, 15, 20
Caffeine	100, 200, 300
Fluoxetine	0.005, 0.010, 0.020
Triclosan	0.05, 0.075, 0.10

^a5 replicates with 3 individuals per replicate.

Table 3.

Median lethal concentration (LC50) values (48 h) for *Platyonus patulus* as determined by Probit analysis^a

Compound	Reference population			Rio Grande population		
	LC50	NOEC	LOEC	LC50	NOEC	LOEC
Acetamidophenol	319 mg/L	200 mg/L	300 mg/L	121 mg/L	75 mg/L	100 mg/L
Caffeine	580 mg/L	450 mg/L	525 mg/L	419 mg/L	300 mg/L	350 mg/L
Fluoxetine	0.06 mg/L	0.025 mg/L	0.05 mg/L	0.19 mg/L	0.075 mg/L	0.10 mg/L
Triclosan	ND	ND	ND	0.32 mg/L	0.25 mg/L	0.30 mg/L

^aThe no observed effect concentration (NOEC) and lowest observed effect concentration (LOEC) values were determined by Dunnett's post hoc tests. ND = not determined.