Pharmaceuticals and personal care products alter the holobiome and development of a medically important mosquito

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ABSTRACT

The increasing demand for fresh water has forced many countries to use reclaimed wastewater for agricultural purposes. This water contains pharmaceuticals and personal care products (PPCPs) that remain biologically active following passage through wastewater treatment plants. Run-off from farms and contaminated water from treatment facilities exposes aquatic ecosystems to PPCPs. This study examined the effects of PPCPs on a lower trophic organism. Culex quinquefasciatus larvae were reared in water contaminated with environmentally relevant concentrations of common PPCPs. Acetaminophen alone and a mixture of contaminants were found to increase developmental time of larvae. Susceptibility to Bti increased in larvae exposed to antibiotics, acetaminophen, or a mixture of PPCPs. Antibiotics, hormones, and the mixture altered the mosquito bacterial microbiome. Overall, the results indicate that at environmentally relevant concentrations, PPCPs in reclaimed water can have biologically important effects on an ecologically and medically important lower trophic level insect.

1. Introduction

Use of pharmaceuticals and personal-care products (PPCPs) has been increasing over the past 30 years, doubling or tripling in the past 12 years (National Center for Health Statistics, 2014; Schumock et al., 2014). Common PPCPs include, but are not limited to acetaminophen, mental stimulants, and treatments for heartburn, allergies, and bacterial infections. Since these products are excreted from the human body with little or no changes to the chemical structure (Wright et al., 2012), they frequently show up in wastewater treatment plants.

Common wastewater treatment facilities are not equipped to remove all PPCPs and therefore discharge a wide range of concentrations into surface waters (Carballa et al., 2004; Joss et al., 2006, 2005). Untreated water from treatment plants can be released into the environment during powerful storms, when the overflow drains directly into nearby aquatic systems (Phillips et al., 2012). A study by Kolpin et al. (2002) found over 90 PPCPs in U.S.

watersheds. In other countries, the concentrations of PPCPs in wastewater treatment plant effluents are even higher (Liu and Wong, 2013; Mutiyar and Mittal, 2014). In response to an increasing demand for fresh water, it has been proposed to use reclaimed wastewater for agricultural purposes (Wu et al., 2012). Unfortunately, PPCPs tend to be relatively long-lived and mobile in both the soil and water (Alvarez et al., 2013; Gan et al., 2012; Kinney et al., 2006). Therefore, runoff from agricultural fields irrigated with reclaimed water, or fertilized with reclaimed biosolids, is likely to contain increased concentrations of PPCPs.

There is minimal information available regarding the effects of PPCPs on insects in aquatic environments. However, some research has described PPCP effects on bacterial communities in aquatic environments and on aquatic plants. Kołodziejska et al. (2013) demonstrated that antibiotic contaminates commonly released from fisheries reduced growth of green algae (Scenedesmus vacuolatus) and duckweed (Lemna minor), as well as viability of the crustacean Daphnia magna. Rosi-Marshall et al. (2013) showed the stimulant, caffeine, significantly reduced respiration rates of stream biofilms, but had no effect on their bacterial composition. They also showed that an antihistamine, diphenhydramine, caused both a reduced respiration rate and a significant change in the bacterial community of the biofilms. These compounds were not designed or

intended to affect bacterial communities. Thus, these variable impacts make predicting how chemicals will affect unintended target organisms difficult.

PPCPs usually cause toxicity because they have similar chemical structures to an organism's natural signaling compounds, resulting in expression or blockage of the organism's receptors. For example, mammalian hormones, commonly used in birth control and hormone therapy, have been linked to endocrine disruption in reptiles, birds, and some arthropods (Gonzalez et al., 2001; Hoffmann and Kloas, 2012; Jindra et al., 2013; Tompsett et al., 2013, 2012; Watts et al., 2003, 2001). They also have been shown to alter primary and secondary sexual characteristics such as gonads and mating and courtships behaviors (Gonzalez et al., 2001; Hoffmann and Kloas, 2012; Tompsett et al., 2012).

Arthropods rely on various hormones for much of their coloration, production of eggs, mating behaviors, and immature development (Jindra et al., 2013; Knowles and Carlisle, 1956; Martín et al., 2001). While many insect hormones do not specifically match mammalian hormones, the structure of mammalian sex hormones and ecdysone (the insect molting hormone) are similar. In crustaceans, mammalian sex hormones have been shown to cause infertility, increase molting events, and inhibit chitobiase. Chitobiase plays a role in digestion of the cuticle during a molt (Rodríguez et al., 2007; Yang et al., 2008; Zou and Fingerman, 1997). Ecdysone is an insect hormone important for initiating the molting process when larvae are transitioning between instars, and is structurally similar to estrone. Bisphenol A (BPA), a xenoestrogen, has the ability to bind and express not only the estrogen-binding proteins in mammals, but also to the ecdysone-binding protein in *Chironomus riparius* (Planelló et al., 2008). Exposure of the non-biting midge, *C. riparius*, to estrogenic compounds has been shown to cause mouth deformities, decreased fecundity, and increased developmental time if administered over multiple generations (Jindra et al., 2013; Watts et al., 2003, 2001).

Culex quinquefasciatus (southern house mosquitoes) are aquatic arthropods in their larval instars, and terrestrial as adults. Eggs are laid in rafts on the water's surface and the larvae hatch directly into the water. From their first to fourth instar, most mosquito larvae feed on detritus and thereby recycle nutrients back into the environment (Walker et al., 1991). Once they reach the fourth instar, larvae cease feeding and prepare to molt into a non-feeding pupal stage. Mosquitoes, like many insects, rely on endosymbionts (microorganisms which provide nutrients or other beneficial functions) to grow and develop (Chouaia et al., 2012; Clark et al., 2010; Kafil et al., 2013; Kuechler et al., 2013; Moran et al., 2005b). For example, bacteria in the genus *Buchnera* are commonly endosymbionts of aphids and provide the aphid with essential amino acids (Douglas, 1998). Similarly, bacterial symbionts in the genus *Asaia* have been shown to be crucial in the development of the mosquito, *Anopheles stephensi* (Chouaia et al., 2012). Not surprisingly, mosquitoes treated with antibiotics to eliminate bacteria took significantly longer to develop than untreated control larvae. However mosquitoes "rescued" by a subsequent introduction of the bacteria following antibiotic exposure showed no difference in development (Chouaia et al., 2012).

Currently, there is little information available on the effects of PPCPs at environmentally relevant concentrations on aquatic invertebrates, on the effects of PPCPs on the holobiome of insects, or on the effects of PPCPs on efficacy of *Bacillus thuringiensis* subsp. *israelensis* (Bti), a bacterial insecticide commonly used to control larvae of mosquitoes. Joint exposure to a pollutant and a microbial larvicide can be used to detect sublethal physiological stress (Mogren et al., 2013). Further, since 1997, 1000s of hectares of floodwater mosquito breeding sites have been treated with Bti (Becker et al., 2003). Thus Bti is likely to coexist with PPCPs in aqueous environments. Therefore, we used a series of bioassays to evaluate the effects of PPCPs on development and mortality of the mosquito, *C. quinquefasciatus*, which is a lower trophic level arthropod found worldwide and native to the Southern U.S. (Barr, 1952). This species is a vector of encephalitides including West Nile Virus and the nematode, *Wuchereria bancrofti,* which causes filariasis in the tropics and subtropics (Agrawal and Sashindran, 2006). Thus, any potential effects would have interesting implications from both ecological and medical perspectives.

2. Materials and methods

2.1. Chemicals

To determine the effect of PPCPs on *C. quinquefasciatus* a series of representative compounds were chosen based on Kolpin et al. (2002) and Mutiyar and Mittal (2014); acetaminophen, caffeine, three antibiotics, and four estrogenic steroidal hormones. Acetaminophen (10 μg/L), caffeine (6 μg/L), estrone (0.112 μg/L), 19-norethindrone (0.872 μg/L), 17β-estradiol (0.2 μg/L), 17α-ethynylestradiol (0.831 μg/L), lincomycin (0.73 μg/L), and oxytetracycline (72.9 μg/L) concentrations were chosen based on the maximum concentrations measured by Kolpin et al. (2002). Ciprofloxacin (31,000 μg/L) concentration was chosen from the maximum effluent discharge reported by Mutiyar and Mittal (2014). Six treatments were examined: acetaminophen, caffeine, an antibiotic mixture (lincomycin, oxytetracycline, and ciprofloxacin), hormones (estrone, 19-norethindrone, 17β-estradiol, and 17α-ethynylestradiol), a mixture of all chemicals, and a control, consisting of only Crystal Geyser® Natural Alpine Spring Water (CGSW) (C G Roxane, Olancha, CA).

The chemicals used were purchased as follows: acetaminophen with a purity of ≥90%; (MP Biomedicals, LLC, Santa Ana, CA); caffeine at laboratory grade purity (Fisher Scientific, Hanover Park, IL); lincomycin, oxytetracycline, and ciprofloxacin with purities of ≥98% (Alfa Aesar, Ward Hill, MA); estrone, 19-norethindrone, 17β-estradiol, and 17α-ethynylestradiol at ≥98% purity (Sigma–Aldrich, St. Louis, MO); and *B. thuringiensis* subsp. *israelensis* with 37.4% active ingredients of fermentation solids and solubles (Gnatrol® WDG Biological Larvicide, Valent® Professional Products, Walnut Creek, CA). Hydrochloric acid was obtained from Fisher Scientific at 12.1 M concentrate. Sodium hydroxide was acquired from Sigma–Aldrich (St. Louis, MO) as anhydrous pellets. Stock solutions were prepared by adding powdered chemicals to deionized water. Approximately 5 mL 80% ethanol was added to 250 mL steroidal hormone solutions to facilitate dissolution. Hydrochloric acid (1 M) was added to antibiotic chemical solutions to facilitate dissolution and pH was adjusted using NaOH (1 M) to a pH of 4.00. In all experiments, preparations and concentrations were identical.

2.2. Insect rearing

C. quinquefasciatus mosquito egg rafts were obtained from a parental colony maintained at the University of California, Riverside using the procedures described by Wirth et al. (2004). Rafts were maintained in shallow porcelain pans (30 × 20 × 5 cm) containing 3 L water or water and one of the PPCP treatments. Water was maintained at pH 7.00 ± 0.50 for all experimental procedures. Following emergence, larvae were kept in an incubator (model 818: Precision Scientific Inc., Buffalo, NY) at 28 °C, approximately 70% RH, and a light: dark cycle of 16:8.

Second instars were transferred individually by disposable pipette to a bioassay container consisting of a 29.57 mL plastic cup with clear plastic lid containing 15 mL of CGSW. Each mosquito larva was given 67.0 μL of diet on day 1 and 33.5 μL of diet every

other day thereafter. Diet was prepared as in Sorensen et al. (2006); briefly a 3:1 (wt/wt) mixture of ground mouse chow: brewers yeast was rehydrated by 50 mL of CGSW for 4 g of dry mixture. Bioassay containers were treated with stock solutions to ensure environmentally relevant concentrations of PPCPs and/or the correct concentration of Bti before larvae were transferred. Volume was checked periodically throughout the experiments with no noticeable difference. This methodology was used for all experiments.

2.3. Determination of a chronic LC_{50}

To determine the chronic LC_{50} (dose required to prevent 50% of the population from reaching the adult stage) of Bti for *C. quinquefasciatus*, mosquito larvae were treated with one of seven concentrations (3.075, 6.15 12.3, 24.6, 50, 100, or 200 ng Bti/mL) and an untreated control. Initially, doses of Bti were chosen by dividing a 24-h acute LC_{50} (123.0 ng/mL), determined by Mogren et al. (2013), by ten. Based on the resulting mortalities, an eight-dose concentration range was developed to determine the chronic LC_{50} covering the time span from second instar through adult eclosion. Second instars were transferred from pans to bioassay cups and given an 8-h acclimation period. If a larva died before the end of the acclimation period, it was replaced. Sixty individuals were used for each replicate, with three replicates per treatment. This replication was used throughout all experiments. After treatment, larvae were maintained in incubators as previously described. Larvae were monitored daily until all larvae died or eclosed. The resulting chronic LC_{50} (10.20 ng/mL) of Bti on *C. quinquefasciatus* was then used as a standard Bti concentration in all subsequent treatments with Bti and/or PPCPs and Bti.

2.4. Growth and development

In order to determine the effect of PPCPs on growth and development of *C. quinquefasciatus,* larvae were reared in CGSW treated with each of the five PPCP treatments, or an untreated control. Mosquito larvae were reared and transferred to their respective bioassay containers as stated previously. Larvae were monitored daily for growth, developmental stage, mortality, and number of days to pupation.

2.5. Susceptibility assay

To discern the effects PPCPs on susceptibility to Bti, larvae were treated with the PPCPs as before and an additional trial was conducted with both PPCPs and chronic LC_{50} of Bti (10.20 ng/mL). Individuals were monitored daily for growth, development, and mortality.

2.6. Effects of PPCPs on the mosquito as a holobiont

The endosymbiont microbial community of mosquitoes reared under the various PPCP regimes was sequenced and quantified. Mosquitoes were reared in pans as described previously. Three mosquitoes were collected from each treatment when mosquitoes reached the second, third, and fourth instar. Mosquitoes were then twice washed with 95% ethanol to remove any external microorganisms. After washing, larvae were transferred to a sterile 2 mL microcentrifuge tube with 95% ethanol and frozen at -60 ± 3 °C in an ultra cold freezer (Forma Scientific, Inc. Marietta, OH) until DNA extraction. DNA was extracted using a Qiagen DNeasy® Blood and Tissue Kit following the manufacturers protocols with the following amendments. Mosquitoes were crushed by micropestles in a sterile 2 mL microcentrifuge tube and 20 μL of Buffer ATL. After thorough pulverization, 160 μL of the Buffer ATL and the 20 μL of proteinase K

was added. Nucleic acid concentration was quantified using a Nanodrop ND- 2000c Spectrophotometer (Cole-Palmer, Vernon Hills, IL).

A commercial sequencing facility (Molecular Research LP MR DNA, Shallowater, TX) performed Roche 454 bacteria barcoded amplicon pyrosequencing (bTEFAP®). The bTEFAP® procedure used the primer set: 27Fmod (GRGTTTGATCMTGGCTCAG) and 519Rmodbio (GTNTTACNGCGGCKGCTG) in a single-step 30 cycle PCR using HotStarTaq Plus Master Mix (Qiagen, Valencia, CA). PCR was performed using the following conditions: 94 °C for 3 min, followed by 28 cycles of 94 °C for 30 s; 53 °C for 40 s and 72 °C for 1 min; finally an elongation at 72 °C for 5 min. After PCR, all amplicons were mixed in equal concentrations and purified using Agencourt Ampure beads (Agencourt Bioscience Corporation, MA). Samples were sequenced with Roche 454 FLX titanium instruments and reagents following the manufacturer's guidelines.

2.7. Statistical analyses

Statistical analyses were performed in R (the R Foundation for Statistical Computing, version 3.1.1). The chronic LC_{50} was calculated using logistic regression, and a logit link function. Lethal concentrations at 95% confidence intervals were calculated using the dose.p function of the MASS package (Venables and Ripley, 2002). Growth and development were examined using a generalized linear model with a Poisson probability distribution. Individual treatments were examined using linear contrasts with the untreated control. In susceptibility assays, overall significance in mortality was determined using ANOVA; individual significances were determined using a binomial generalized linear model. Bacterial community data from bTEFAP® was examined with respect to instar and treatment using "permutational MANOVA" (PERMANOVA). PERMANOVA is analogous to MANOVA but is robust to nonnormality that is commonly associated with count data (Anderson, 2001; Mcardle and Anderson, 2001). Microbial community data from pyrosequencing was further examined with principal component analysis (PCA) performed in the FactoMineR package (Husson et al., 2010).

3. Results

3.1. Chronic Bti LC_{50}

The percentage of *C. quinquefasciatus* treated with increasing levels of Bti demonstrated a dose dependent response curve and fit a binomial logistic regression line ($y = 1.704\ln(x) - 3.957$; $R^2 = 0.90$). The chronic LC_{50} was determined to be 10.20 ng/mL (95% fiducial limits: 8.81–11.81 ng/mL).

3.2. Growth and development

Both acetaminophen and the mixture of PPCPs had significant effects on the developmental time of *C. quinquefasciatus*. Those individuals treated with acetaminophen alone and the mixture of PPCPs required significantly more time on average to pupate ($X^2 = 19.64$, df = 6, p < 0.05) than the control treatments, but were not significantly different than each other (p > 0.05). The majority of control mosquitoes pupated on days 5 and 6 while the majority of those in the acetaminophen and the PPCP mixture pupated on days 6 and 7 (Fig. 1). When treated with Bti and the PPCP(s), trends were similar. However, compared to the non-Bti-treated mosquitoes, all Bti treatments took on average an extra 1–2 days to pupate ($X^2 = 43.63$, df = 1, p < 0.05) (Fig. 2).
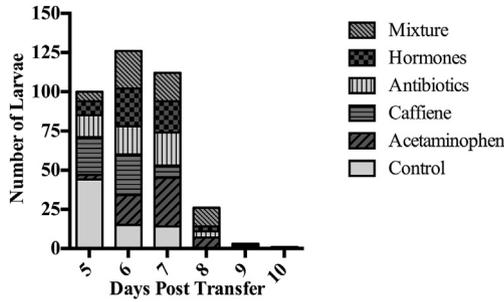
Fig. 1. Number of mosquito larvae that pupated for all PPCP treatments by day.

3.3. Susceptibility to B. thuringiensis subsp. israelensis

Overall mortality varied among treatments ($X^2 = 58.28$; df = 6; $p < 0.01$). Bti-treated mosquitoes exposed to acetaminophen alone, antibiotics alone, and the PPCP mixture were more susceptible to Bti than larvae only exposed to Bti ($p < 0.05$). Mortality of untreated larvae (control) was minimal (3.33%). The mortality of mosquitoes exposed only to Bti was 45%, which was within the 95% confidence limits of the LC_{50} as determined in experiment 1 (Fig. 3).

3.4. Effects of PPCPs on the mosquito as a holobiont

The microbiome of the mosquito was significantly different among the PPCP treatments (PERMANOVA: F = 6.37; df = 5,48; $p < 0.0001$), between instars (PERMANOVA: F = 18.20; df = 1,52; $p < 0.0001$), and there was a significant interaction of PPCP treatment and instar (PERMANOVA: F = 3.19; df = 5,48; $p < 0.0001$). PCA was performed to determine groupings relative to instar. The variable 'instar' loaded onto the first principal component, which explained 28.5% of variation. We tested for differences in the microbiome with respect to instar by examining only the mosquitoes reared in the untreated controls. Overall there was a significant difference among the instars (PERMANOVA: F = 10.33; df = 2,6; $p < 0.001$), with third and fourth instars loading together, separately from second instars. Therefore we tested for a difference in third and fourth instars, which were not significantly different (PERMANOVA: F = 1.91; df = 1,4; $p = 0.22$). Consequently, subsequent analyses were based on combined third and fourth instars.

When third and fourth instar larvae were considered together there was a significant effect of treatment (PERMANOVA: F = 10.80; df = 5,30; $p < 0.0001$). In contrast to findings when controls only were considered, in which third and fourth instars do not differ, there was a significant difference between third and fourth instars
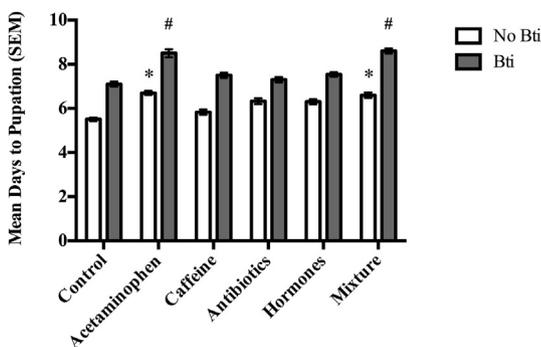

Fig. 2. Average days to pupation from egg hatch of PPCP treated, untreated, and Bti and control mosquitoes. *: Denotes significant difference from the control in the No Bti group. #: Denotes significant difference from the control in the Bti treated group.
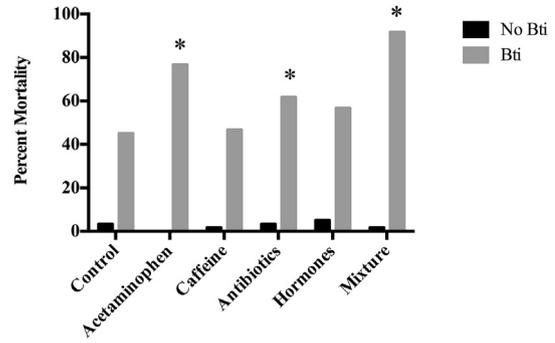

Fig. 3. Percent mortality of untreated larvae and larvae exposed to the chronic LC_{50} (10.20 ng Bti/mL) of Bti and PPCPs. There are no significant differences among No Bti treatment groups ($p > 0.05$). *: Denotes significant difference relative to the control in the treatment groups containing Bti ($p < 0.05$).

when all treatments were included in the analysis (PERMANOVA: F = 16.79; df = 1,34; $p < 0.001$). This was likely due to the interaction between treatments and instar (PERMANOVA: F = 2.54; df = 5,30; $p < 0.001$).

When the microbiome of third and fourth instar larvae was examined with PCA, there were 17 dimensions with an Eigenvalue greater than one. However, the first two dimensions explained 23% of the total variation (Fig. 4). When examined across the first and second principal components (Fig. 4), bacterial communities treated with acetaminophen and caffeine and the controls cluster together, suggesting they are similar. The microbiomes of the mosquitoes in the mixture or antibiotic only treatments are similar to each other. The hormone treated mosquitoes are distinct from all other treatment groups.

There were 30 bacterial families with non-zero contributions to one of the first two principal components. Twenty of these families account for at least 96% of the PPCP-treated mosquitoes' bacterial community and cluster in three distinct groups (Fig. 5). Eight bacterial families each contribute greater than 0.01% to the overall bacterial community in all treatment groups (Table 1). The family
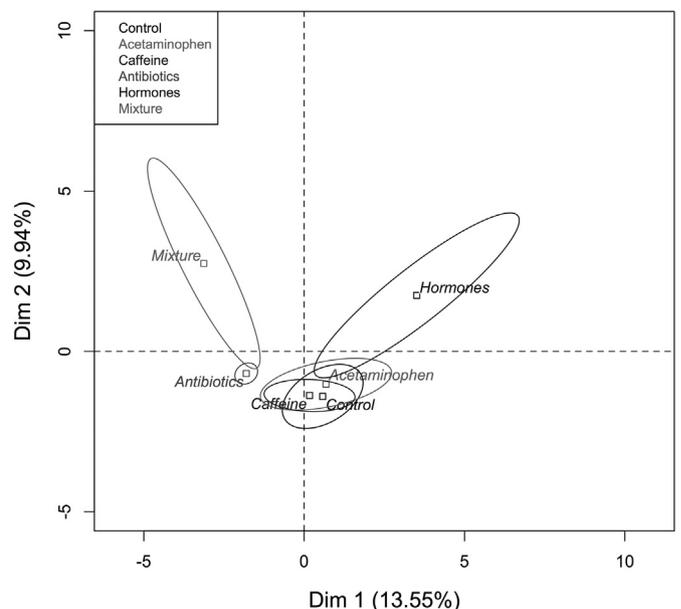

Fig. 4. Ordination of PPCP treatments and control as determined by the first and second principal component dimensions. Squares denote the center of the ellipses.
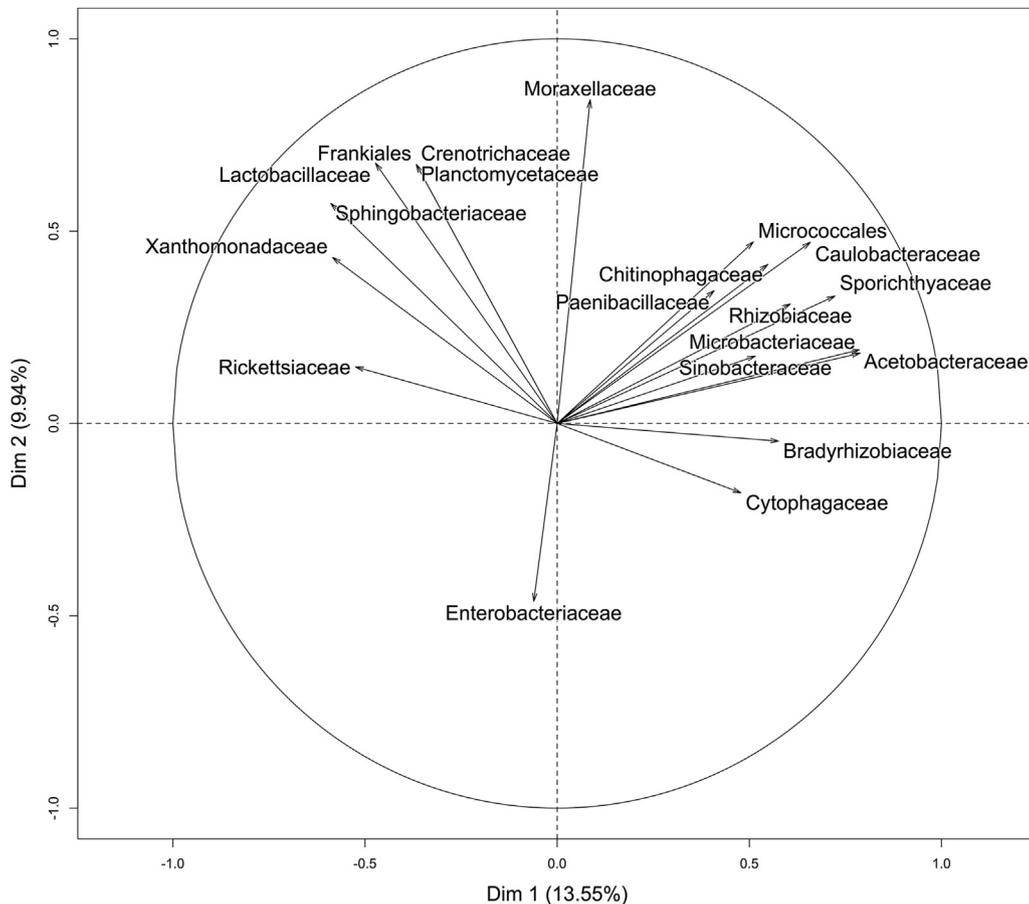
Fig. 5. PCA factor graph displaying the top twenty families with their associated component dimensions.

Enterobacteriaceae is mostly described by the first dimension and is associated with the control, acetaminophen, and caffeine treatments (Figs. 4 and 5; Table 1). *Rickettsiaceae* is the most abundant bacterial family in all of the treatment groups except for the hormone-treated mosquitoes, where it is second most abundant. *Wolbachia pipientis* accounts for >99% of this family (Tables 1 and 2). *Microbacteriaceae* is the most represented bacterial family in the treatment groups (Table 2) and, like *Rickettsiaceae*, has > 9% abundance in all treatment groups (Table 1). However the *Microbacteriaceae* species vary among treatments, but *Rickettsiaceae* bacteria are consistently represented by *W. pipientis* (Table 2).

While the eight families presented in Table 1 account for at least 96% of the bacterial community, the total counts are reduced by 66% in the antibiotic treatment and reduced by 33% the mixture treatments. Thus, while *W. pipientis* has a relatively similar number of counts in all distinct treatments, this species accounts for 86.7% of all bacteria in the antibiotic-treated and 69.1% of bacteria in the mosquitoes exposed to mixtures of PPCPs.

4. Discussion

Previous studies reporting LC_{50} values for *C. quinquefasciatus* exposed to *B. thuringiensis* subsp. *israelensis* (Bti) have only reported acute values (24 h). The dose-dependent toxicity of Bti to *C. quinquefasciatus* documented in our study was used to calculate the first chronic LC_{50} of Bti on *C. quinquefasciatus*. This value of 10.20 ng/mL is much lower than previously reported acute LC_{50}s determined in 24 h tests. For example an acute LC_{50} value was reported at 140.0 ng/mL by Mogren et al. (2013). Thus, although acute

assays are faster to conduct, and can be compared against previously published reports, they overestimate the amounts of Bti needed to kill 50 percent of mosquito populations that are exposed over their entire life spans. Bechmann (1994) noted that in life-table experiments some toxicants, especially pesticides, can drive a population to extinction even at concentrations well below an acute LC_{50}. However, effective mosquito control typically requires suppression of late instar larvae even with only an acute exposure and a relatively high dose of Bti is be needed to achieve that goal.

Mosquitoes treated with PPCPs at environmentally relevant concentrations displayed increased developmental time in the acetaminophen and mixture treatments. As these two treatments were not significantly different, it is possible the effects on mixture treated mosquitoes could be from acetaminophen alone. The majority of these two treatments pupated 1–2 days after the control group. The cause of this delay is difficult to determine and may be due to individual or joint actions of many factors including an effect on the nutrients during rearing, an effect of these specific PPCPs on the mosquito physiology, or some general stress. Stress has been shown to influence insect physiology and behavior (Adamo et al., 2001; Zera and Harshman, 2001). Regardless of the cause, increased developmental time would increase larval exposure to PPCPs and potentially to predation. All mosquitoes treated with Bti exhibited an increase in developmental time of 1–2 days, including those in the acetaminophen and mixture treatments. Since Bti is a larvicide that damages the gut lumen in certain mosquitoes (Bravo et al., 2007), a reduced nutrient absorption could lead to increased developmental time. Longer developmental times caused by PPCPs would increase exposure to contaminants and exposure time to Bti.

Table 1
Mean percentages, location, and purported function of bacterial families that contribute at least 0.01 percent to the overall mosquito holobiont.

Family	Location of bacteria	Purported function	Percentage in control	Percentage in acetaminophen	Percentage in caffeine	Percentage in antibiotics	Percentage in hormones	Percentage in mixture
Acetobacteraceae[a]	Gut, Whole mosquito	Possible plant symbiont, Nitrogen fixators, Secondary symbionts of insects dependent on sugar-based diets	0.5	0.6	0.1	>0.1	1.3	>0.1
Bradyrhizobiaceae[b]	Whole tick	Nitrogen fixation	2.5	5.4	1.4	0	2.5	0.6
Enterobacteriaceae[c]	Whole mosquito, moth midgut, Huhu beetle gut, ant gut, Whole Aphid, Mosquito gut	Insects symbionts both primary and secondary	32.8	27.7	36.8	>0.1	>0.1	0.2
Microbacteriaceae[d]	Whole mosquito, Mosquito gut, Stem-borer gut	Cellulase activity	24.6	34.5	20.3	2.6	58.2	9.9
Rickettsiaceae[e]	Various Tissues and Organs	Reproductive Parasite	33.8	28.9	38.2	86.7	29.5	69.1
Sinobacteraceae[f]	Roots area	Possible xenobiotic metabolic function	2.0	1.3	1.5	0.9	5.5	>0.1
Sphingobacteriaceae[g]	Whole mosquito, Root area	Heparinase activity	>0.1	0.3	>0.1	5.9	>0.1	15.5
Xanthomonadaceae[h]	Gut, whole mosquito	Nitrate/-ite reduction, Chemoorganotrophism, Cellobiose metabolism	0.1	0.1	0.1	3.5	0.1	2.7
		Sum of Percentages	96.4	98.8	98.1	99.6	97.3	98.2
		Total Counts	29140	29937	27234	9829	27142	19579

[a] Reid et al. (2011); Valiente Moro et al. (2013).
[b] Menchaca et al. (2013); Garritty et al. (2005).
[c] Valiente Moro et al. (2013); Xia et al. (2013); Reid et al. (2011); He et al. (2014); Moran et al. (2005a)
[d] Valiente Moro et al. (2013); Coon et al. (2014); Bashir et al. (2013).
[e] Nachappa et al. (2011); Dodson et al. (2014).
[f] Dean et al. (2014); Chen et al. (2014).
[g] Coon et al. (2014); Dean et al. (2014).
[h] Reid et al. (2011); Valiente Moro et al. (2013).

Table 2
Most abundant bacterial species by family in four PPCP treatments as they correlate to PCs.

Family	Control	Mean counts	Percentage of counts
Rickettsiaceae	*Wolbachia pipientis*	4923[a]	33.8
Enterobacteriaceae	*Yersinia mollaretii*	3387[b]	23.3
Microbacteriaceae	*Microbacterium laevaniformans*	3206[c]	22.0
Sphingobacteriaceae	*Pedobacter* spp.	1.2	>0.1
Xanthomonadaceae	*Stenotrophomonas maltophilia*	8.2	0.1
Microbacteriaceae	*Microbacterium testaceum*	0	0
Microbacteriaceae	*Klugiella xanthotipulae*	154.5	1.1
	Antibiotic		
Rickettsiaceae	*Wolbachia pipientis*	4261[a]	86.7
Enterobacteriaceae	*Yersinia mollaretii*	0	0
Microbacteriaceae	*Microbacterium laevaniformans*	0.3	>0.1
Sphingobacteriaceae	*Pedobacter* spp.	291.0[b]	5.9
Xanthomonadaceae	*Stenotrophomonas maltophilia*	174.3[c]	3.5
Microbacteriaceae	*Microbacterium testaceum*	3.7	>0.1
Microbacteriaceae	*Klugiella xanthotipulae*	0	0
	Hormone		
Rickettsiaceae	*Wolbachia pipientis*	4007[b]	29.5
Enterobacteriaceae	*Yersinia mollaretii*	0.3	>0.1
Microbacteriaceae	*Microbacterium laevaniformans*	5288[a]	39.0
Sphingobacteriaceae	*Pedobacter* spp.	1514	>0.1
Xanthomonadaceae	*Stenotrophomonas maltophilia*	7.5	>0.1
Microbacteriaceae	*Microbacterium testaceum*	0	0
Microbacteriaceae	*Klugiella xanthotipulae*	1162[c]	8.6
	Mixture		
Rickettsiaceae	*Wolbachia pipientis*	6764[a]	69.1
Enterobacteriaceae	*Yersinia mollaretii*	17.2	0.2
Microbacteriaceae	*Microbacterium laevaniformans*	250.8	2.6
Sphingobacteriaceae	*Pedobacter* spp.	6764[b]	15.5
Xanthomonadaceae	*Stenotrophomonas maltophilia*	260.7	2.7
Microbacteriaceae	*Microbacterium testaceum*	359.3[c]	3.7
Microbacteriaceae	*Klugiella xanthotipulae*	16.2	0.2

[a] Denotes most abundant.
[b] Denotes second most abundant.
[c] Denotes third most abundant.

Increased mortality would almost certainly occur.

Larvae in the acetaminophen, antibiotic, and mixture treatments were more susceptible to Bti than the larvae exposed to Bti alone. While the increased time to exposure described above may have played a role in the increased mortality, the pyrosequencing results indicated significant changes in the bacterial community of the mosquito. If some of the affected bacterial communities, especially those eliminated, have detoxifying abilities for Bti-toxin this could also play a role. However, more research in the area is warranted. Previous studies show the inability of *Anopheles* mosquitoes to fend off the malaria parasite *Plasmodium falciparum* following treatment with the antibiotic gentamycin (Beier et al., 1994). Contrary to our findings, Broderick et al. (2006) showed the gypsy moth (*Lymantria dispar*) had a reduced susceptibility to *B. thuringiensis* after treatment with antibiotics, which was removed after reintroducing the *Enterobacter* sp. NAB3. As the mosquitoes in our study retained their *Enterobacteria*, the Bti was still activated and the mosquitoes were still susceptible.

In our study, larvae exposed to hormones contained substantially different bacterial communities as compared to controls, suggesting that at least some hormones likely play a role in altering bacterial communities. Overexpression of ecdysone during development could explain why BPA causes mouth deformities and increased pupation time in *C. riparius* (Watts et al., 2003). In mosquitoes (*Aedes aegypti*), the vitellogenin gene is a target for ecdysteroid receptor, which can be modulated by the xenoestrogen BPA (Martín et al., 2001; Rouaulta et al., 2006). This suggests that BPA and other xenoestrogens could have an effect on the production of vitellogenin, the egg yolk protein. This also would lead to altered viability of offspring, which we did not assess.

Acetaminophen and the mixture of PPCPs significantly slowed developmental time of *C. quinquefasciatus*, but antibiotics alone did not. In contrast, Chouaia et al. (2012) described delayed larval development of *A. stephensi* after treatment with the antibiotic rifampicin at 120 μg/mL. The developmental time was rescued by reintroducing bacteria from the genus *Asaia*. In our study, there was relatively little difference in development of immature mosquitoes between the antibiotic treatments and the controls for the *Acetobacteraceae*, to which *Asaia* belongs. It is possible that the antibiotics chosen for this study are not effective in eliminating *Asaia*. Alternatively, the antibiotics in our study were used at doses much lower than the rifampicin tested by Chouaia et al. (2012), and might have achieved similar effects if applied at higher doses.

With a combination of antibiotics, hormones and other constituents that occur in PPCP-contaminated reclaimed water, it is difficult to know exactly which chemical is affecting which bacterial family and which bacteria were responsible for the deleterious effects on developmental time. Notably, the antibiotic treatments had approximately 1/3 of the total number of bacteria relative to the control. Therefore even the approximately 8500 counts of *Rickettsiaceae* (the majority) in the antibiotic treatments are lower in number than the *Enterobacteriaceae* (second highest) found in control treatments. Surprisingly, the substantial loss of bacterial counts (density) in the antibiotic only treatments did not slow development.

Although the antibiotics decreased overall bacterial counts in *C. quinquefasciatus*, *W. pipientis* appeared to be relatively unaffected. *W. pipientis* is susceptible to doxycycline and rifampin (Brenner et al., 2005a). Of the chemicals we tested, oxytetracycline should have had the most impact (based on structural similarity) on *Wolbachia*. However, because the bacterial counts were relatively similar for *W. pipientis* in all treatments, we suspect either the oxytetracycline was too dilute to have an effect, it allowed a non-susceptible strain of *W. pipientis* to dominate, or it is simply ineffective against *W. pipientis*. However the vast majority of the eight bacterial families were greatly reduced in the antibiotic treatments. Of these families, *Enterobacteriaceae* and *Microbacteriaceae* were the most reduced in both the antibiotic and mixture treatments.

The family *Enterobacteriaceae* is highly associated with insect endosymbionts, such as *Buchnera* in pea aphids (Brenner et al., 2005b; Douglas, 1998). *Buchnera* bacteria are known to aid the aphid by supplying essential amino acids lacking from a nutritionally deficient diet. Without these endosymbionts the aphids do not develop properly, but the microorganisms apparently have no other direct biological effect on the aphid (Wilkinson, 1998). The size of the *Enterobacteriaceae* populations (counts) probably had a minimal effect on larval developmental time, as the counts in the acetaminophen treatment are relatively similar to the hormone-treated group. Additionally, the hormone treated group has a substantially reduced count of *Enterobacteriaceae* bacteria compared to the control, but showed no significant changes in developmental time. We suspect, therefore, that the acetaminophen is negatively impacting some other biological system in the larvae, but determination will require additional research.

The use of reclaimed water for crop irrigation and the release of water from waste treatment plants and farm waste ponds into surface waters is occurring and likely to escalate as demand for fresh water increases. While, the research reported here suggests that PPCPs contaminating reclaimed water will have potentially useful effects for mosquito control, if the data can be extrapolated to other insect species, PPCPs will also have unintended negative effects on other aquatic insects. Very little is known regarding how these contaminants might biomagnify or change chemically as they move through the food web. The eventual impact on populations is also unknown. Further, combinations of PPCPs may be more important for some insects than individual components. Additional research is needed not only on aquatic insects living in surface waters, but also on uptake by plants and associated herbivores in terrestrial environments.

Acknowledgments

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