Final Report: Illinois-Indiana Sea Grant Development Project

Title of Project: Estimating the Ecological Impacts of Pharmaceuticals in Lake Michigan

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Problem:

Pharmaceuticals and personal care products (PPCPs) are a new class of emerging contaminants. These compounds include antibiotics, antimicrobials, cholesterol-lowering drugs, anti-inflammatory drugs, anti- epileptic drugs, anti-depressants, hormones, and fragrances. Discharges of sewage wastewater treatment plants (SWWTPs) and runoff from agricultural land-applied with animal wastes are the main sources of these chemicals to the environment. Although each compound is ingested at small concentrations, the population as a whole consumes large quantities. Many of these compounds pass unaltered into feces and urine and end up in the sewage treatment process. In addition many unused pharmaceuticals are disposed of directly through the sewage system. Some PPCPs are also structurally stable and can cross lipid layers, bio-accumulating in fish (Brooks et al. 2005).

Table 1. Maximum concentrations of PPCPs detected from Lake Michigan (Bernot and Lauer 2011).

Chemical	Maximum Concentration in Lake Michigan (µg/L)
Antibiotics	
Trimethoprim	0.01
Tylosin	0.01
Antimicrobials	
Triclosan	0.01
Triclocarban	0.01
Nonsteroidal Anti-inflammatory(NSAID)	
Ibuprofen	0.03
Naproxen	0.03
Analgesics	
Acetaminophen	0.01
Lipid lowering drugs	
Gemfibrozil	0.05
Metabolite of caffeine	
Cotinine	0.006

PPCPs are different from conventional contaminants in many respects. Because they are synthesized to combat specific human diseases after years of pharmaceutical research, they tend to target specific tissues and physiological functions that are well conserved across all vertebrates at very low doses. This, in turn, can translate into high potency. In fact, at environmentally relevant concentrations (i.e., sub-parts-per-billion and parts-per-trillion levels), some can affect growth, reproduction, and behavior of fish and crustaceans (Foran et al. 2004, Han et al. 2010, Waiser et al. 2011). Antimicrobials and antibiotics such as triclosan, triclorban, and tylosin, can affect algae growth at much lower concentrations compared to effects on invertebrates and fish (Brausch and Rand 2011).

As most chemical pollutants, PPCPs occur in complex mixtures. However, only a handful of studies have evaluated the overall health impact of a "typical" pharmaceutical mixture found downstream from a SWWTP. In fathead minnows (*Pimephales promelas*) chronic exposure to environmentally relevant concentrations of seven PPCP most often detected in Canadian SWWTP did not affect survival, growth, or egg production, although it increased deformities in the F_1 generation (Parrott and Bennie 2009). In contrast, toxicity tests with combinations of various PPCPs on aquatic invertebrates and algae revealed stronger effects (on growth and mobility) than expected from the effects measured singly (Cleuvers 2003, 2004). Thus, more studies are needed that evaluate the combined effects of PPCPs on lower trophic aquatic organisms.

Rationale and Specific Aims:

Despite the known presence of PPCPs in Lake Michigan, little to no data is currently available on the ecological impacts of these chemicals in this region. This information is of critical importance to help prioritize monitoring efforts to target those compounds, which may be of greatest threat to both aquatic communities and human health. In addition, limited toxicological data exist for some of the PPCPs recently detected in Lake Michigan like cotinine, and triclocarban, both singly and in mixtures. Cotinine is a metabolic byproduct of nicotine. Triclocarban is an antibacterial agent, commonly found in personal care products and is a suspected endocrine disrupting chemical with the potential to bioaccumulate in a number of organisms (Higgins et al. 2011, Snyder et al. 2011, Prosser et al. 2014). Furthermore, almost no data is available on the toxicity of PPCPs to diatoms. This is of great ecological importance in the Great Lakes, since trophic cascades are dependent on healthy diatom populations. Based on these data gaps, we have chosen to conduct a set of studies that aim to: (1) Test the acute and chronic effects of cotinine, and triclocarban on aquatic organisms including: green algae (*Pseudokirchneriella capricornutum*), water fleas (*Daphnia magna*) and fathead minnows; (2) Test the effects of PPCPs detected in Lake Michigan (all compounds listed in **Table 1**) on diatom (*Cyclotella meneghiniana)* survival and; (3) Conduct a mixture study chronically exposing *D. magna, P.capricornotum, Cyclotella meneghiniana, and P. promelas* to several PPCPs. This mixture will mimic the types and maximum concentrations of PPCPs reported from Lake Michigan by Bernot and Lauer 2011 (**Table 1**).

[Note: Between the time these studies were completed and present time, the Bernot and Lauer 2011 IISG Report was published as a full manuscript with additional data that was not presented in the IISG report (Ferguson et al. 2013) and an additional study by also presented data on PPCPs in Lake Michigan (Blair et al. 2013)].

Methods:

Test chemicals and stock solutions: Trimethoprim (purity > 99.5%), tylosin (purity > 87.9%), triclosan (purity > 99.1%), triclocarban (purity > 99%), ibuprofen (purity > 98%), naproxen (purity > 99.5%), acetaminophen (analytical standard), gemfibrozil (purity > 99.93%), and cotinine (purity > 98%) were obtained from Sigma Aldrich, St. Louis, MO, USA.

Stock solutions for tylosin, ibuprofen, naproxen, acetaminophen, gemfibrozil and cotinine were prepared in ethanol whereas triclosan and trimethoprim were made in methanol, and triclocarban was made in acetone. All test solutions were freshly prepared by diluting the stock solutions using Milli Q water.

Test organisms and culture conditions: Algae were purchased from UTEX, University of Texas at Austin, TX. They were inoculated into Bristol medium (http://web.biosci.utexas.edu/utex/mediaDetail.aspx?mediaID=29) and cultured in an environmental chamber at a constant temperature of 25°C and a light intensity of 86 ± 8.6 μE/m2/s until they reached the logarithmic growth phase before further cultivation. Algae was grown in static cultures and cultures shaken at 100 rpm to prevent algal cells adhering to the culture vessel. Cells were cultured for three generations, and then examined under a microscope

to check for normal morphology. Algae at the logarithmic growth phase were used for all experiments.

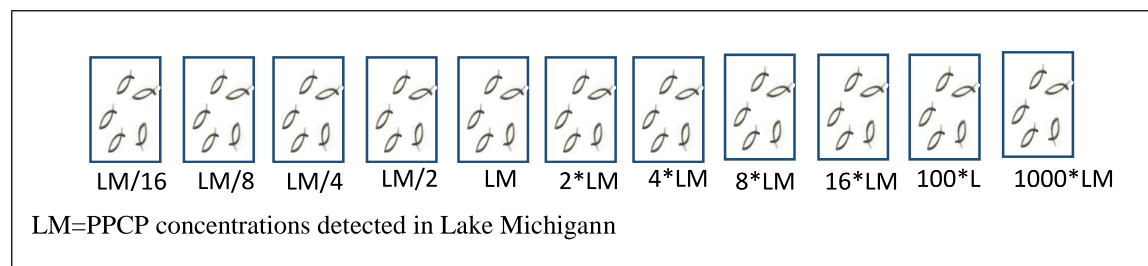
A pure culture of diatoms was purchased from The National Center for Marine Algae and Microbiota and maintained in F/2 medium (Guillard and Ryther 1962, Guillard 1975) at 17°C with continuous illumination at a light intensity of 86 ± 8.6 µE/m2/s. Cells were cultured for a few generations until a healthy population in its growth phase was used for the experiments.

Water fleas were obtained from in-house cultures at Purdue University, West Lafayette, IN and were acclimated in the laboratory for at least 7 days prior to the experiments. Daphnids were cultured in growth medium ($NaCHO_3$ 96 mg/L, $CaSO_4$-$2H_20$ 60 mg/L, $MgSO_4$ 60 mg/L, KCl 4 mg/L) at a constant temperature of 25˚C and a photoperiod of 16 L:8 D. Animals were fed daily with 100 µl of green algae and YCT. Growth medium was renewed once a day.

The strain of fathead minnow used in this study was obtained from the US Environmental Protection Agency (Duluth, MN) in 2009. Breeding pairs (1:1 sex ratio) were maintained in 9.5 L aerated, flow-through tanks (well water) held at 25°C and a photoperiod of 16 L:8 D. Fish were fed frozen brine shrimp (Brine Shrimp Direct, Ogden, UT) daily to satiation. Uneaten food was siphoned from the tanks daily. Each tank was provided with a piece of 3 in. diameter PVC cut in half as breeding substrate which was checked daily for eggs. Temperature and dissolved oxygen (DO) were measured daily using a portable meter (YSI 55, Yellow Springs, OH) prior to water change.

Experimental design: Concentrations of the PPCPs were selected based on the Bernot and Lauer (2011) study, which was the only available data for Lake Michigan at the time these studies were conducted (**Table 1**). For individual exposures, this Lake Michigan concentration was diluted multiple times to achieve the desired target concentrations (**Figure 1**). For the mixture exposures, all the PPCPS were mixed at the level of Lake Michigan concentrations and then diluted.

Figure 1. Test concentrations of pharmaceutical and personal care products (PPCPs) used for the experiments.



PPCP Analysis: An Agilent 1100 series liquid chromatography instrument (Agilent Technologies, Santa Clara, CA) equipped with a thermostatted auto sampler, binary pumping device, and an Agilent diode array detector (DAD) was used for the analysis of PPCPs. Reverse phase liquid chromatography was used to separate the samples. An Agilent Eclipse XDB-C18 with 4.6 x 150 mm, 3.5 µm dimensions was used for the separation. Solvent A consisted of water + 0.1 % formic acid. Solvent B consisted of acetonitrile + 0.1 % formic acid. The flow

rate was 1.0 mL/minute. A sample volume of 10 µL was loaded onto the column. The linear gradient was as follows: time 0 minutes, 5 % B; time 1 minute, 5 % B; time 20 minutes, 95 % B; time 25 minutes, 95 % B; time 26 minutes, 5 % B; time 30 minutes, 5 % B. The analytes were detected by UV absorption (**Table 2**). Samples were evaluated and preprocessed with Agilent ChemStation software.

Toxicity assays: Algae tests were conducted following standardized protocols (USEPA 2002). There were four replicates for each concentration. At test initiation, cell densities were adjusted to 10^4 cells/ml from an algal culture in log phase growth. Test solutions were shaken continuously at 100 cpm on a mechanical shaker to keep the algal cultures in suspension. Because of the continuous illumination of the test flasks, DO (dissolved oxygen) concentration was never a problem during tests and thus no aeration was provided. The acute test was terminated 72 h after initiation and the chronic exposure test was stopped after 7 days. Algal growth was measured using a hemocytometer and counting number of cells manually under a microscope. This method has the advantage of allowing for the direct examination of the condition of the cells.

Diatom tests were conducted following standard protocols (USEPA 2002). Tests were conducted using 50 mL beakers with 4 replicates per treatment group. Cell counts of existing diatom cultures at log max growth were determined with a hemocytometer and diluted to 10,000 cells/mL in sample water. Beakers were covered with parafilm to prevent evaporation and shaken by hand twice daily to suspend the organisms. The acute exposure test was terminated at 72 hours and total cells were counted. For the chronic exposures, the cell count was estimated at the end of 7 days.

Acute toxicity assays with *Daphnia magna* were preformed according to standardized protocols (USEPA 2002). Individual neonates (< 24h old) were placed in 100 mL beakers at 25°C with 10 replicates per concentration under static conditions for 72 hours (acute) or 14 days (chronic). Each beaker was filled with 25 - 50 ml of the test solution and checked daily for mortality. Test solutions were renewed daily. Endpoints for chronic tests were survival and fecundity and for the acute tests, only survival was measured.

For the fathead minnow experiments, newly fertilized (< 24-h old) eggs were collected from each PVC substrate and checked for fertility using a dissecting microscope. Fertilized eggs were then randomly chosen and placed in 50 ml beakers and allowed to acclimate to their new environment for 2–3 h before the start of the dosing experiments. There were 3 replicates of 10 embryos per beaker for each concentration. Exposure solutions were changed daily and any dead embryos or larvae were removed. Survival was recorded at the end of the acute (72 h) as well as chronic (7 days) exposures.

Results:

Detection of PPCPs from the test solutions are shown in **Table 2**.

Table 2. Pharmaceutical and personal care products quantified using high performance liquid chromatography.

Analyte	Stock Concentration	UV Absorbance (nm)
Individual Chemicals		
Cotinine	100 μg/L	256
Triclocarban	100 μg/L	283
PPCP Mixture Chemicals		
Acetaminophen	100 μg/L	283
Gembfibrozil	100 μg/L	270
Ibuprofen	100 μg/L	220
Methy-triclosan	100 μg/L	283
Naproxen	100 μg/L	300
Trimethoprim	100 μg/L	220
Tylosin	100 μg/L	283

Results from the probit analysis of acute and chronic tests are shown in **Tables 3** through **6**.

Table 3. Effective concentration 50% (EC_{50}) and lethal concentration 50% (LC_{50}) (ppm) for pharmaceutical and personal care products obtained with green algae. NOEC = No observable effect concentration; LOEC = Lowest observable effect concentration.

Treatment	Length of Exposure	EC_{50}	95% CI	NOEC	LOEC
Triclocarban	72 h	8.33	7.29-9.37	0.16	1
Triclocarban	7 days	6.83	5.86-7.81	0.16	1
Cotinine	72 h	4.89	4.11 - 5.67	0.48	0.096
Cotinine	7 days	3.90	3.18 - 4.62	0.48	0.096

Table 4. Effective concentration 50% (EC_{50}) and lethal concentration 50% (LC_{50}) (ppm) for pharmaceutical and personal care products obtained with diatoms *Cyclotella meneghiniana*. NOEC = No observable effect concentration; LOEC = Lowest observable effect concentration.

Treatment	Length of Exposure	EC_{50}	95% CI	NOEC	LOEC
Triclocarban	72 h	8.33	7.29-9.37	0.16	1
Triclocarban	7 days	6.83	5.86-7.81	0.16	1
Cotinine	72 h	4.89	4.11 - 5.67	0.48	0.096
Cotinine	7 days	3.90	3.18 - 4.62	0.48	0.096
Triclocarban	72 h	7.02	5.94-8.10	0.16	1
Triclocarban	7 days	7.96	6.95-8.97	0.16	1
Cotinine	72 h	4.92	4.16-5.67	0.048	0.096
Cotinine	7 days	4.13	3.43-4.83	0.96	0.6
Trimethoprim	72 h	2432.82			
Trimethoprim	7 days	3143.65			
Tylosin	72 h	3245.39			
Tylosin	7 days	617.27			
Triclosan	72 h	24.06			
Triclosan	7 days	33.48			
Ibuprofen	72 h	15480			
Ibuprofen	7 days	10110.69			
Naproxen	72 h	25.45			
Naproxen	7 days	27.565			
Acetaminophen	72 h	46199.87			
Acetaminophen	7 days	28635.98			
Gemfibrozil	72 h	8633.205			
Gemfibrozil	7 days	7262.483			

Table 5. Effective concentration 50% (EC_{50}) and lethal concentration 50% (LC_{50}) (ppm) for pharmaceutical and personal care products obtained with *Daphnia magna* and *Pimephales promelas*. NOEC = No observable effect concentration; LOEC = Lowest observable effect concentration.

Treatment	Length of Exposure	Endpoint	EC_{50}	95% CI	LC_{50}	95% CI	NOEC	LOEC
Daphnia magna								
Triclocarban	7 days	Survival			16.33	16.15-16.51	N/A	N/A
Triclocarban	14 days	Survival			4.69	4.59-4.78	0.096	0.6
Triclocarban	14 days	Neonates/adult	4.55	3.54-5.67				
Cotinine	7 days	Survival			15.64	15.49-15.80	N/A	N/A
Cotinine	14 days	Survival			48.52	47.84-49.19	N/A	N/A
Cotinine	14 days	Neonates/adult	34.11	26.7-41.6				
Pimephales promelas								
Triclocarban	72 h	Survival			7.23	7.18-7.28	0.16	1
Triclocarban	7 days	Survival			2.41	2.35-2.46	0.16	1
Cotinine	72 h	Survival			4.01	3.98-4.05	0.006	0.024
Cotinine	7 days	Survival			4.01	3.99-4.04	0.024	0.096

Table 6. Effective concentration 50% (EC_{50}) and lethal concentration 50% (LC_{50}) (ppm) for pharmaceutical and personal care products obtained with algae, diatoms, *Daphnia magna* and *Pimephales promelas* exposed to mixtures. NOEC = No observable effect concentration; LOEC = Lowest observable effect concentration. * LM = times Lake Michigan.

Organism	Length of Exposure	Endpoint	EC_{50} (*LM)	95% CI (*LM)	LC_{50} (*LM)	95% CI (*LM)	NOEC (*LM)	LOEC (*LM)
Algae	7 days	TCC	509.23	379 - 639			2	4
Diatom	7 days	TCC	514.22	380 - 649			0.13	0.25
Daphnia	7 days	Survival			414.63	404-425	16	100
Daphnia	7 days	Neonates/adult	93.08	71 - 257			0.25	1
FHM	7 days	Survival			257.15	252-262	1	10

In green algae the acute exposure, 72-hr EC_{50} (95%CI) values of triclocarban and cotinine were 8.33 (7.29 - 9.37) and 4.89 (4.11 - 5.67) ppm, respectively. The chronic 7-d EC_{50} values for the same compounds were 6.83 (5.86 - 7.81) and 3.90 (3.18 - 4.62) ppm, respectively. In both exposures, a significant decrease in growth rate was observed at ≥ 100 times the concentration found in Lake Michigan (100*LM) (**Figure 2**). When algae were exposed to PPCP mixtures for 7 days, a 25% reduction in the total algal cell count was observed at the Lake Michigan concentration and ~ 70% decrease when exposed to 1000*LM (**Figure 2**).

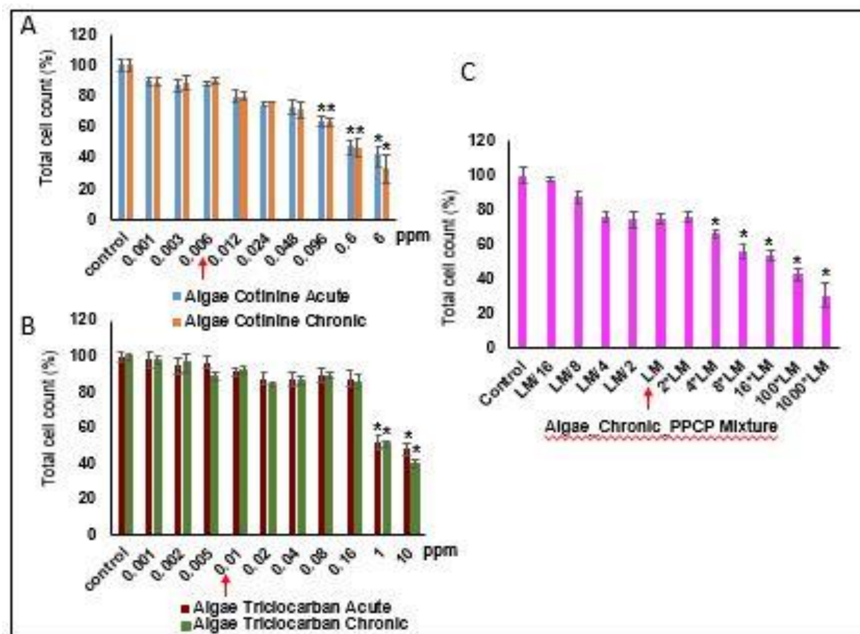


Figure 2. Chronic and acute exposure of S. *capricornutum* to cotinine (A), triclocarban (B) and a pharmaceutical and personal care product mixture (C) (see Table 1 for chemicals used in mixture experiment). Red arrow denotes Lake Michigan concentrations reported by Bernot and Lauer (2011). Asterisk denotes significant difference from controls ($p < 0.05$).

In diatoms, acute, 72-hr EC_{50} values for triclocarban and cotinine were 7.02 (5.94 - 8.10) and 4.92 (4.16-5.67) ppm, respectively. Chronic 7-d EC_{50} values for the same chemicals were 7.96 (6.95 - 8.97) and 4.13 (3.43 - 4.83) ppm, respectively. For the PPCP mixture, there was ~25% drop in total cell numbers at LM concentration and a significant drop (~60 to 70%) was observed at ≥100*LM (**Figure 3**). A similar observation was reported when diatoms were exposed to triclocarban and cotinine separately (**Figure 3**). No significant change in total cell counts were observed when diatoms were exposed to naproxen, trimethoprim, ibuprofen, tylosin, acetaminophen, gemfibrozil and Triclosan (**Table 4**).

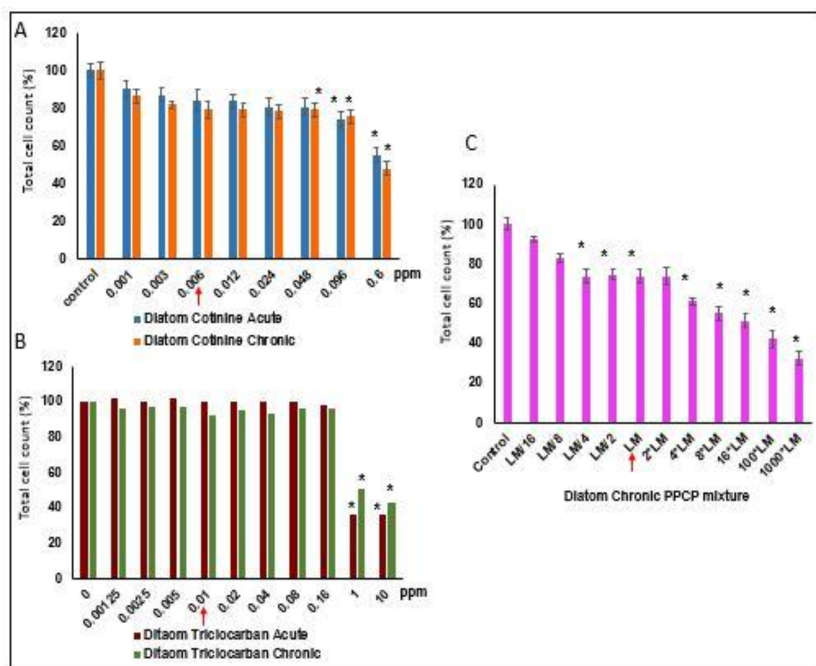


Figure 3. Chronic and acute exposure of *Cyclotella meneghiniana* to cotinine (A), triclocarban (B) and a pharmaceutical and personal care product mixture (C) *(see Table 1 for chemicals used in mixture experiment). Red arrow denotes Lake Michigan concentrations reported by Bernot and Lauer (2011). Asterisk denotes significant difference from controls ($p < 0.05$).

When *D. magna* were exposed to triclocarban for 7 days no significant effect on survival was observed, but none survived for 14 days at the highest concentration tested (10 ppm). In addition, there was a significant decrease (> 90%) in fecundity (neonates produced per adult) in adults exposed to 10 ppm triclocarban. Cotinine had no effect on survival or fecundity of *D. magna* at the highest concentration tested (6 ppm) under both acute and chronic conditions (**Figure 4**). Chronic exposures to a PPCP mixture had no significant effect on survival at the Lake Michigan concentration, but 100% mortality was reported when exposed to 1000*LM. On the contrary, fecundity was reduced by ~ 50% at Lake Michigan concentration, ~99% at 100*LM and no neonates were produced at 1000*LM (**Figure 5**)

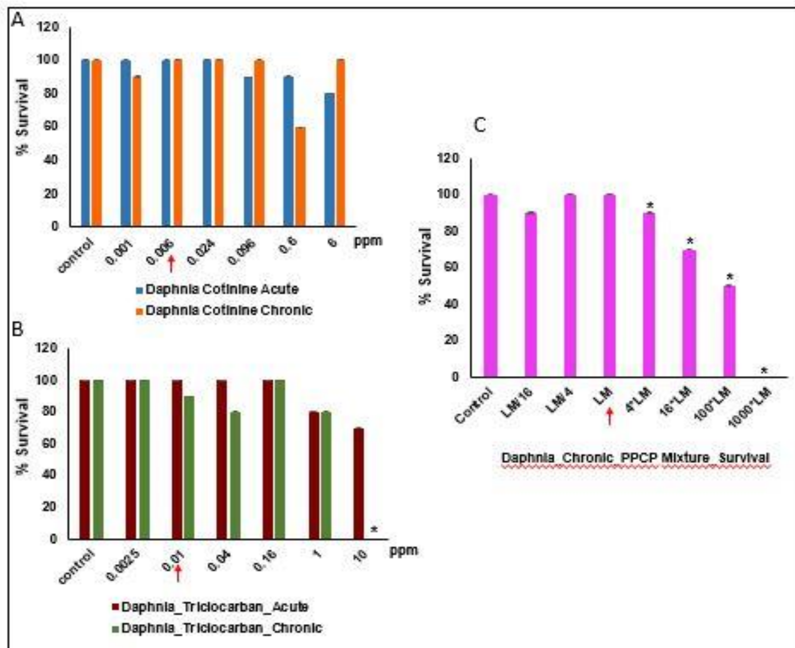


Figure 4. Effects on survival after chronic and acute exposure of *Daphnia magna* to cotinine (A), triclocarban (B) and a pharmaceutical and personal care product mixture (C) *(see Table 1 for chemicals used in mixture experiment). Red arrow denotes Lake Michigan concentrations reported by Bernot and Lauer (2011). Asterisk denotes significant difference from controls ($p < 0.05$).

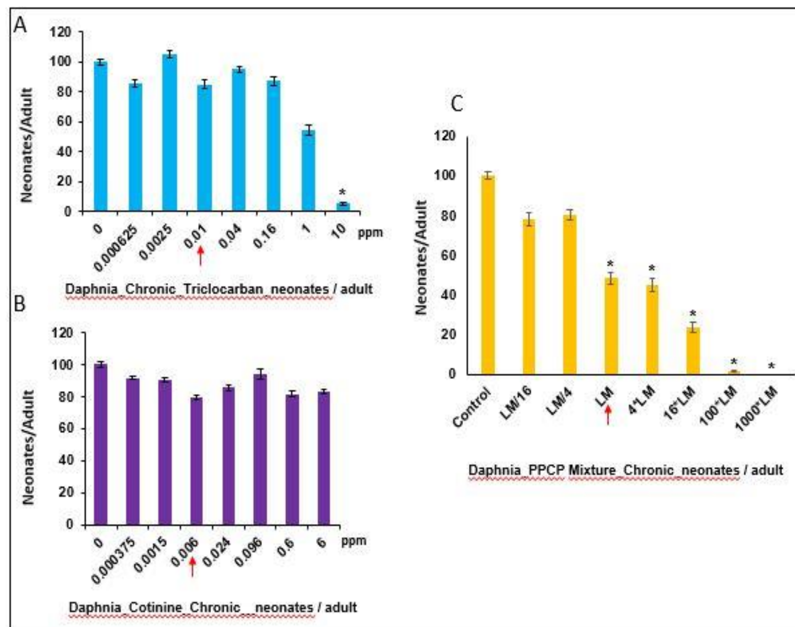


Figure 5. Effects on fecundity after chronic and acute exposure of *Daphnia magna* to cotinine (A), triclocarban (B) and a pharmaceutical and personal care product mixture (C) *(see Table 1 for chemicals used in mixture experiment). Red arrow denotes Lake Michigan concentrations reported by Bernot and Lauer (2011). Asterisk denotes significant difference from controls ($p < 0.05$).

The effect of triclocarban on the survival of fathead minnow embryos decreased with the duration of exposure. Upon chronic exposure to ≥ 1ppm of triclocarban, there was no survival of embryos after 7 days. The survival was reduced to ≥ 80% when exposed to a PPCP mixture at 100*LM and there were no survival at 1000*LM (**Figure 6**).

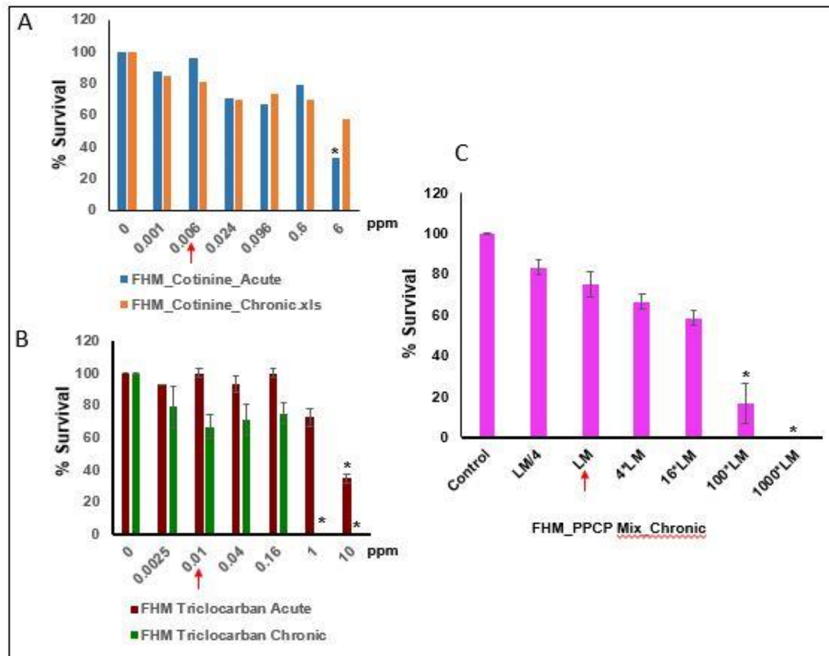


Figure 6. Effects on fecundity after chronic and acute exposure of *Pimephales promelas* to cotinine (A), triclocarban (B) and a pharmaceutical and personal care product mixture (C) *(see Table 1 for chemicals used in mixture experiment). Red arrow denotes Lake Michigan concentrations reported by Bernot and Lauer (2011). Asterisk denotes significant difference from controls ($p < 0.05$).

Conclusions:

In conclusion, PPCP mixtures increased toxicity to diatoms and *D. magna* (fecundity effects) to values below or currently reported from Lake Michigan. These results suggest potential ecological impacts in Lake Michigan due to the presence of these compounds.

The data generated from the proposed studies should help prioritize risks posed by different PPCPs aiding in monitoring efforts.

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