BACKGROUND

Contaminants of emerging concern (CECs) are non-point source aquatic contaminants that include pharmaceuticals and personal care products (PPCPs). Due to the exponential increase in pharmaceutical consumption in recent years, pharmaceutical compounds have been detected at elevated concentrations globally in surface water, finding their way into waterways primarily through sewage discharges containing un-metabolized drugs and improper drug disposal. However, little research has been done on the toxicological implications of PPCPs—especially in complex mixtures—on non-target organisms. Moreover, molecular mechanisms of toxicity are poorly understood. Largely as a result, the EPA lacks any cohesive regulation that deals with CECs. Therefore, the overarching objective of this research was to elucidate the toxicological implications of pharmaceutical-derived aquatic contaminants on both a morphological as well as a molecular level, which has never been examined before.

Triamterene and gemfibrozil were the two experimental compounds used for this study. Both are cardiac-specific medications that have been detected at low concentrations in waterways not only in the Puget Sound but globally—however, research regarding the aquatic toxicity of triamterene is nonexistent and very limited for gemfibrozil. Triamterene is a potassium-sparing diuretic, used to treat high blood pressure by inhibiting epithelial sodium channels in the liver. Gemfibrozil, on the other hand, is a fibrate use to treat patients with high lipid levels that increases the activity and synthesis of an enzyme known as lipase, which catabolizes lipids in the blood and thereby lowers lipids levels in the patient. These drugs' distinct mechanisms of action as well as their under-researched nature makes them ideal experimental compounds for simulating environmental conditions.

METHODOLOGY

In order to better understand PPCP toxicity, zebrafish embryos (<48 hpf) were obtained through breeding and exposed to single-chemical trials of gemfibrozil and triamterene as well as binary mixture trials with the drugs combined. Effects on morphology were observed by imaging the embryos at 48 hours as well as taking video feeds of their cardiac functioning. ImageJ software was used to extract morphometric measurements from embryo images for various toxicological endpoints (eye area, yolk sac area, whole-body length, cardiac structure) and video feeds were analyzed for a host of cardiac defects/structural morphology malformations. From these measurements, dose-response curves were generated for single-chemical triamterene and gemfibrozil exposures. Next, based on these single-chemical trials, the response addition (RA) model was used to predict what the additive mixture toxicity should be. These predictions were then compared to the actual mixture toxicity to assess whether mixture toxicity was additive, synergistic, or antagonistic. The following RA equation was used for calculating predicted additive mixture response:

$mixture\ response = (probability\ A + probability\ B) - (probability\ A * probability\ B)$, where A and B are endpoints from single-chemical exposures.

In order to determine potential toxic effects on a molecular level, lipid content was examined in the embryos. Currently, HPLC is the standard for detecting lipids in fish; however, the method is impractical for zebrafish embryos due to their low tissue mass as it would require over a 1000 embryos. Thus, a novel methodology coupling thin layer chromatography with flame ionization detection (TLC-FID) was developed to sensitively quantify not only total lipid content but also triglyceride/cholesterol levels within exposed zebrafish embryos, utilizing only 300 embryos, which is a fraction of HPLC.

RESULTS

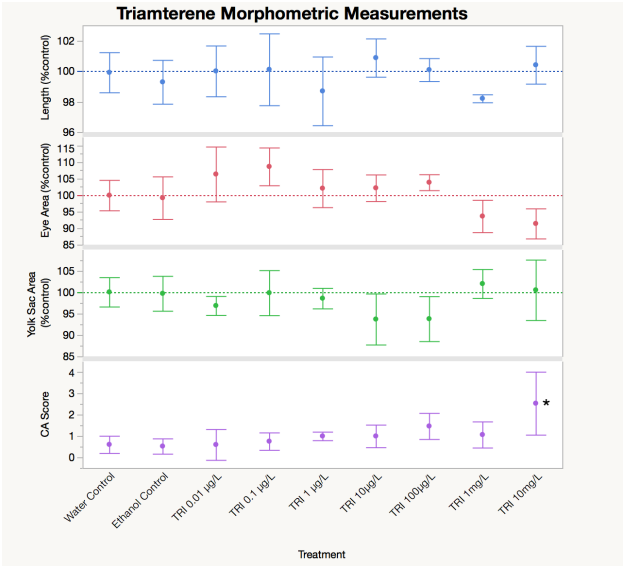
Triamterene did not induce any statistically significant change in length, eye area, or yolk sac area. However, increased cardiac abnormalities were observed in a dose-dependent manner. The terminal injury phenotype showed marked cardiac defects, with adverse changes in heart morphology and functioning observed, such as pericardial blood pooling, cardiac failure (cardiostasis), and edema.

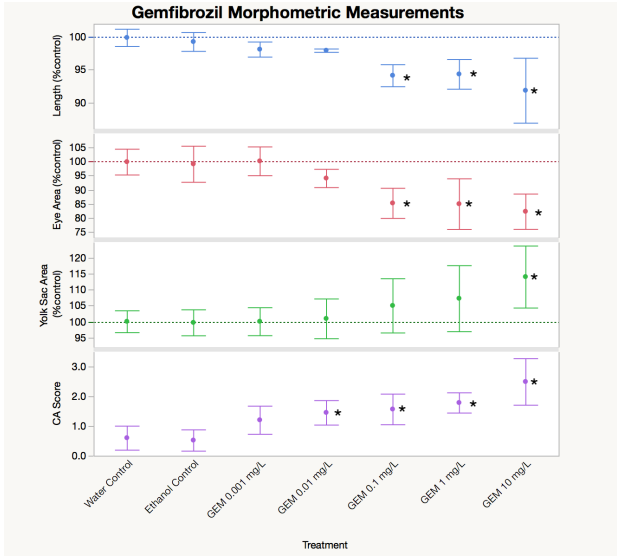
Gemfibrozil induced a similar dose-dependent increase in observed cardiac abnormalities. Additionally, significant effects were seen in extracardial toxicological endpoints, with gemfibrozil inducing a dose-dependent decrease in whole-body length and eye area as well as a converse dose-dependent increase in yolk sac area. Pericardial blood pooling as well as severe blood regurgitation were sensitive measures of exposure. A small eye phenotype (microphthalmia) was prominent across all concentrations.

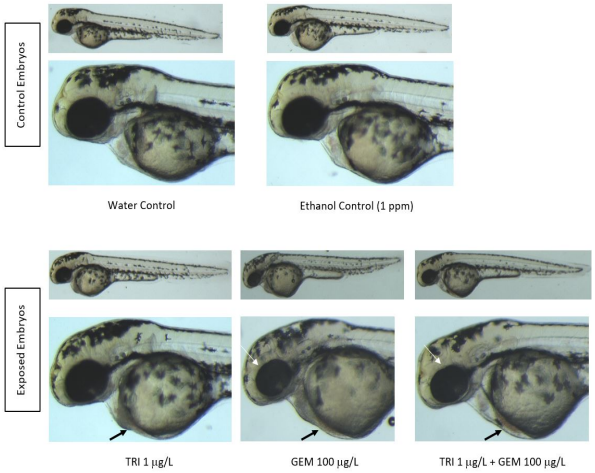
The predicted additive toxic response generated by the RA model was largely consistent with observed mixture toxicity across the eye area, whole-body length, and cardiac abnormalities (CA) score endpoints, indicating additive toxicity in those endpoints. However, with yolk sac area, observed toxicity was consistently above predicted values for all mixture concentrations, suggesting that the drugs might be metabolically interact to produce synergistic toxicity in terms of yolk sac.

On a molecular level, TLC-FID results indicated that gemfibrozil-exposed embryos had significantly higher total lipid content as compared to water control values. Embryos exposed to triamterene and a mixture of gemfibrozil and triamterene did not exhibit any significant change in lipid content as compared to control values.

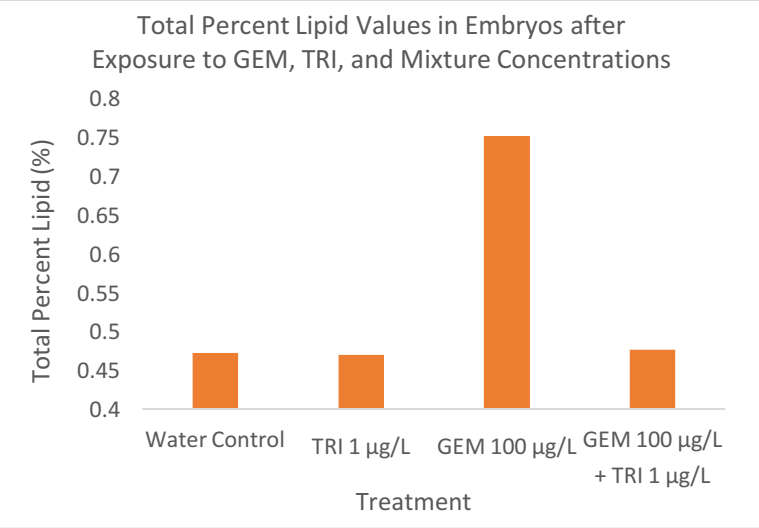
Lipid subclass breakdown analysis revealed that gemfibrozil-exposed embryos had higher cholesterol and triglyceride levels than water control embryos. Triamterene-exposed embryos did not have significantly different cholesterol or triglyceride content compared to water control values. Mixture-exposed embryos showed lower triglyceride levels and higher cholesterol levels than control embryos.

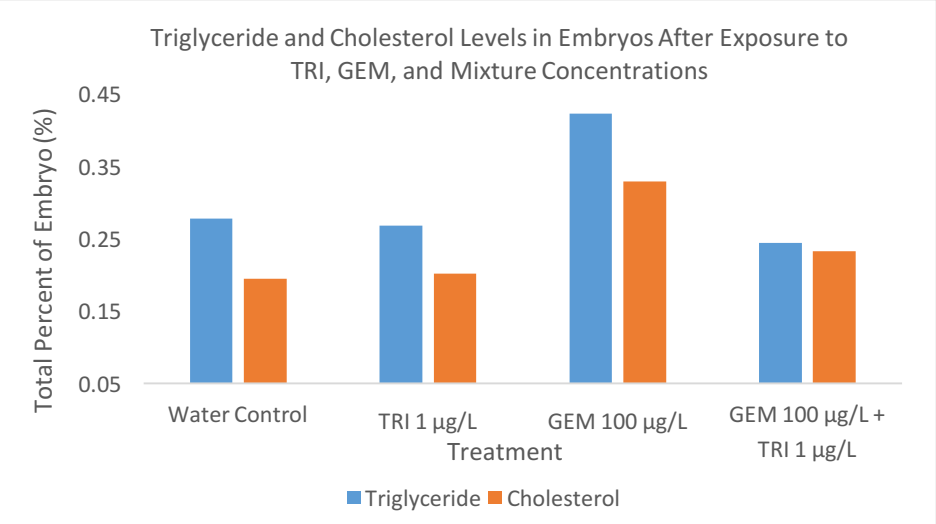






Results indicate that, taken singly, triamterene and gemfibrozil induce sub-lethal toxic effects in early life stage fish. Increased observed heart physiological abnormalities suggest that triamterene is toxic to the developing fish heart. Gemfibrozil induced a similar dose-dependent increase in cardiac defects as well





as extracardial abnormalities, suggesting that gemfibrozil is both cardiotoxic and also induces a developmental delay during zebrafish embryogenesis.

TLC-FID method allowed for novel examination of metabolic pathway disruption in exposed embryos. Results suggest that gemfibrozil blocks lipid metabolism in exposed embryos, as total percent lipid content in gemfibrozil-exposed embryos was significantly higher compared to control values. In humans, gemfibrozil increases lipase activity and therefore increases the catabolism of lipids, lowering lipid levels; however, a converse effect was observed in fish. Results therefore suggest that gemfibrozil may be acting through the same metabolic pathway as it does in humans but, instead of increasing lipase activity, it may be inhibiting lipase function, thereby leading to a buildup of lipids within the fish. As the yolk sac is the developing fish's only source of food at this life stage, the inability to metabolize lipids in the yolk sac has serious implications for the growth and development of the embryo.

Total lipid percent values for mixture-exposed embryos were not significantly different from control values. However, while total lipid content was the same, triglyceride and cholesterol levels differed from control values, indicating that lipid composition in mixture-exposed embryos was distinct from both control embryos as well as gemfibrozil-exposed embryos. This suggests that, when taken together, triamterene and gemfibrozil may be synergistically interacting to induce a distinct, alternative metabolic pathway compared to the lipase-mediated pathway induced by single chemical exposure.

Overall, this is one of the first studies to examine the toxicological implications of PPCPs, especially in mixtures. Results provide some of the first evidence that PPCPs pose as environmental stressors for non-target organisms and can potentially induce sub-lethal toxicity which cannot be detected by crude mortality assays. Morphometric analysis, however, allowed for novel examination of this sub-lethal toxicity. Moreover, mixture results using RA model indicate that PPCPs in waterways could induce additive toxic effects and, for certain endpoints, could metabolically interact to produce synergistic toxicity. This study also highlights the need for toxicity assays to take into account the effect of complex PPCP mixtures in order to more accurately predict environmental effects. TLC-FID lipid analysis provides novel insight into the molecular mechanisms of PPCP toxicity and confirms for the first time that PPCPs could be disrupting metabolic pathways in non-target organisms. This study also lays the foundation for increased evidence-based policy regulating the disposal of pharmaceutical waste.