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Morphological and molecular analysis of the toxicity of pharmaceutical-derived aquatic contaminants (PPCPs) in zebrafish

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Morphological and Molecular Analysis of the Toxicity of Pharmaceutical-Derived Aquatic Contaminants (PPCPs)

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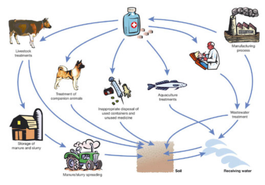
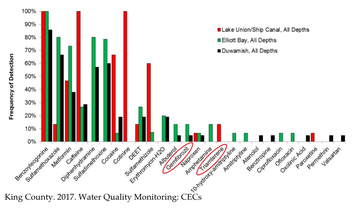
Background

Contaminants of emerging concern (CECs) are ubiquitous 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. As these medications are specifically designed to maximize biological activity at low doses, there is increasing concern that pharmaceutical-derived aquatic contaminants could pose as potential environmental stressors to non-target organisms.



Boxall. Viewpoint, 2005. Environmental effects of medication



King County. 2017. Water Quality Monitoring: CECs

Due to their relative newness, the toxicological implications of pharmaceutical-derived aquatic contaminants, especially in complex mixtures, are poorly understood. Previous research has largely focused on single-chemical exposures—however, field testing data shows that this is not environmentally relevant, as waterways often contain a complex mixture of multiple contaminants. The limited research that has been conducted on the toxicity of PPCP mixtures has focused almost exclusively on drugs with similar mechanisms of action—however, this approach does not account for the inherent diversity in the types of pharmaceutical contaminants found in waterways and thus presents an oversimplified model. Moreover, the molecular mechanisms of potential PPCP toxicity are not well understood, especially for complex PPCP mixtures.

Purpose

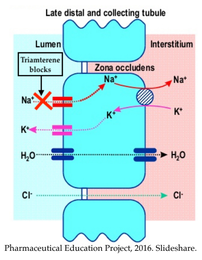
The overarching objective of this study was to elucidate the toxicological implications of pharmaceutical-derived aquatic contaminants. Specifically, the purpose was two-fold: (1) quantitatively characterize toxicological responses to both single-chemical and mixtures of PPCPs in zebrafish embryos through morphometric analysis and (2) conduct molecular analysis of PPCP toxicity in order to better understand potential mechanisms of toxicity.

Thus, the research question was: **What are the toxicological implications of PPCPs both singly and in complex mixtures and can molecular analysis be applied to this specific class of contaminants for enhanced understanding of toxicity?** It was hypothesized that contaminant compounds in a mixture would result in an additive toxic effect which could potentially induce measureable changes on the molecular level.

Experimental Compounds

Triamterene and gemfibrozil are pharmaceutical-derived contaminants that have been detected at elevated concentrations not just in the Puget Sound but globally. However, research regarding the aquatic toxicity of triamterene is nonexistent and very limited for gemfibrozil. The two drugs have distinct mechanisms of action, making them ideal compounds for accurately simulating environmental conditions:

Triamterene is a diuretic primarily used to treat hypertension and edema.

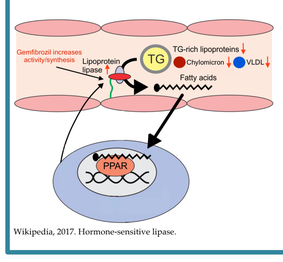


Pharmaceutical Education Project, 2016. Slideshare.

Triamterene Mechanism of Action (MOA)

Inhibition of sodium channels → Increased osmolarity in lumen, decreased osmolarity in interstitium → Decreased osmotic gradient for water reabsorption → Diuresis → Inhibition of potassium excretion

Gemfibrozil is a lipid-regulating fibrate used to treat hypertriglyceridemia.

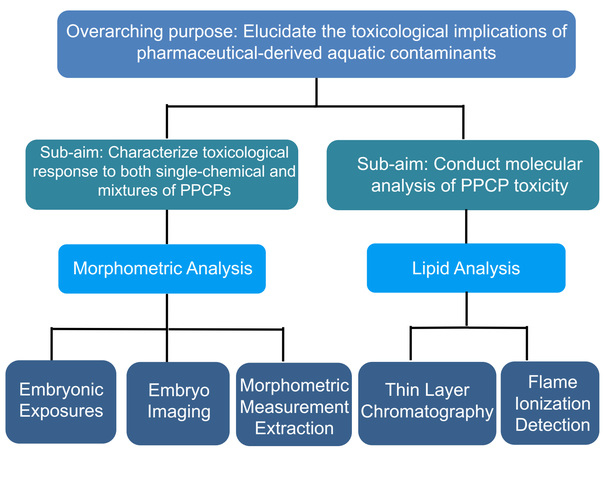


Wikipedia, 2017. Hormone-sensitive lipase.

Gemfibrozil Mechanism of Action (MOA)

PPAR receptor induction (agonist) → Increased lipase activity and synthesis → Increased clearance of triglycerides → Triglyceride levels reduced; cholesterol variably reduced → Overall decrease in lipid levels

Methodology Overview



Morphometric Analysis

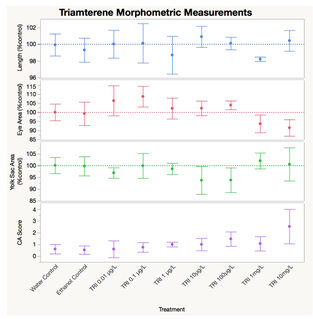
Morphometric measurements were extracted from images of zebrafish embryos (48 hpf) for various toxicological endpoints (eye area, whole-body length, yolk sac size, cardiac abnormalities). Videos were assessed for the following cardiac morphology/functioning endpoints: enlarged atrium, enlarged ventricle, hemorrhaging, blood regurgitation, cardiostasis, tube heart, unlooped heart, pericardial blood pooling, and pericardial/periventral edema. A binary (1, 0) system was used to indicate the presence of a specific condition and the resultant sum was used as a comprehensive indicator of cardiac morphology/functioning output (CA score).

The response addition (RA) model was used to arrive at the predicted mixture response based on single-chemical trials:

$$\boldsymbol{Mixture\ response} = (\boldsymbol{probability\ A} + \boldsymbol{probability\ B}) - (\boldsymbol{probability\ A} * \boldsymbol{probability\ B})$$

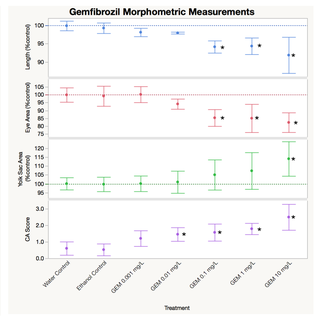
where A and B are endpoints from single-chemical exposures

Single-Chemical Embryonic Exposures



Asterisks denote statistically significant different treatment groups compared to water control values as determined by ANOVA one-way test and Tukey-Kramer post hoc test

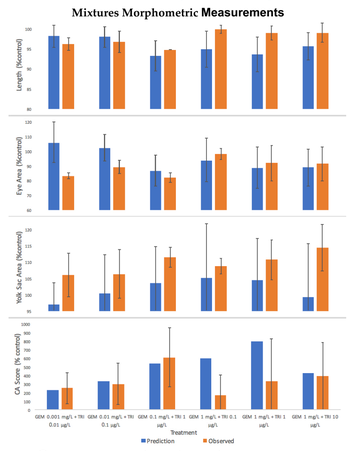
- Triamterene does not induce any statistically significant change in extracardial endpoints: whole-body length, eye area, or yolk sac area.
- However, increased cardiac abnormalities are observed in a dose-dependent manner.



Asterisks denote statistically significant different treatment groups compared to water control values as determined by ANOVA one-way test and Tukey-Kramer post hoc test

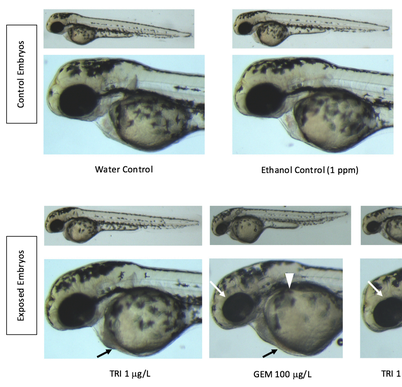
- Gemfibrozil induces a dose-dependent decrease in whole-body length and eye area as well as a converse dose-dependent increase in yolk sac size.
- Moreover, gemfibrozil exposure induces cardiac abnormalities in a dose-dependent manner.

Mixtures Embryonic Exposures



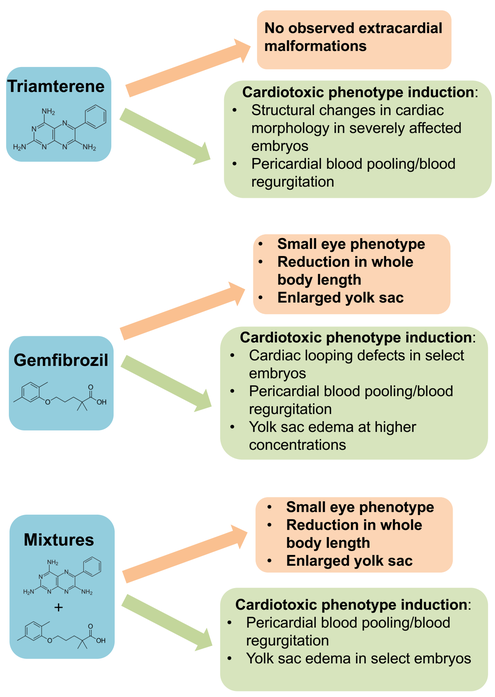
- Observed mixture toxicity is largely consistent with predicted toxicity across all concentrations.
- However, while not significantly different, yolk sac measurements are consistently higher than predicted values.
- Yolk sac area increases dose-dependently when gemfibrozil concentration is kept constant and triamterene concentrations are variable.

Terminal Injury Phenotypes of Exposed Embryos



Black arrows indicate pericardial blood pooling, white arrows indicate small eye phenotype (microphthalmia), and white triangles indicate enlarged yolk sac

- **Triamterene:** Pericardial blood pooling and blood regurgitation as well as adverse changes in heart morphology are observed at certain concentrations. No abnormalities in extracardial endpoints are observed.
- **Gemfibrozil:** Pericardial blood pooling and blood regurgitation, looping defects, and yolk sac edema are observed at some concentrations. In addition to small eye phenotype/reduced length, enlarged yolk sacs are seen, suggesting interference with lipid metabolism.
- **Mixtures:** Severe cardiac abnormalities are seen at certain concentrations, with pericardial blood pooling/blood regurgitation and yolk sac edema being prominent indicators of toxicity. Reduced eye area and whole-body length as well as enlarged yolk sacs are observed across all concentrations.



Lipid Analysis Using Thin Layer Chromatography Coupled with Flame Ionization Detection (TLC-FID)

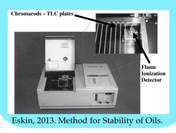
Method Development

Sample preparation
- Extracts concentrated with nitrogen
- Sodium sulfate and magnesium sulfate used to remove water

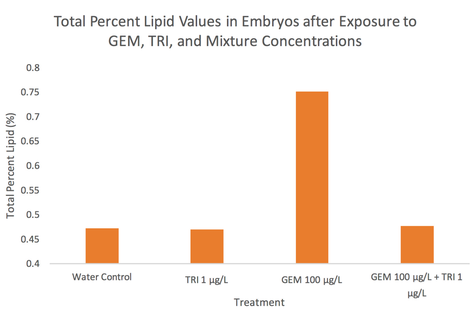
→ **Accelerated Solvent Extractor Cell**
- Embryo composite sample packed into cell
- Lipids extracted using dichloromethane

→ **Thin Layer Chromatography**
- Samples spotted on Silica Chromarods
- Lipid classes separated based on polarity in chromatography tank

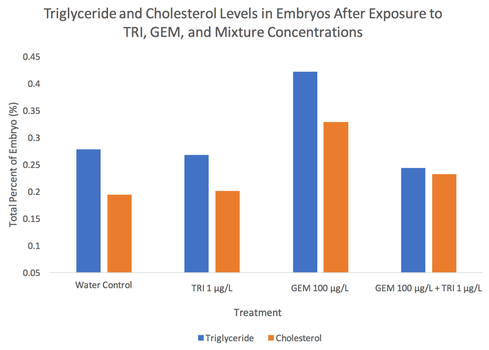
→ **Lipid Subclass Quantification using Flame Ionization Detection (FID)**



Eskin, 2013. Method for Stability of Oils.



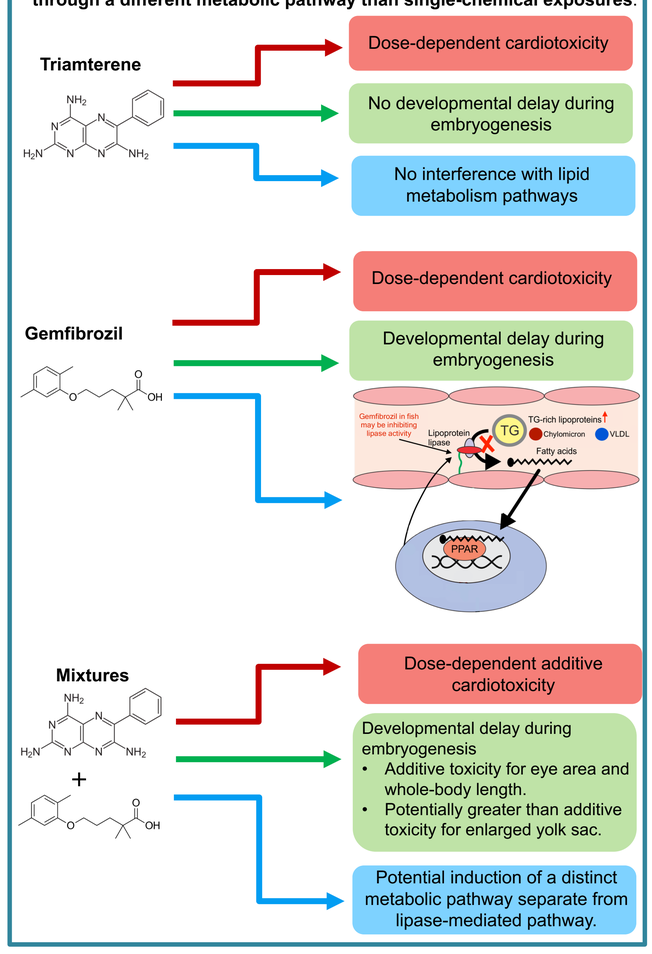
- Lipid levels are significantly elevated following single-chemical gemfibrozil exposure.
- No significant difference is observed between lipid percent values in control embryos and those in triamterene and mixtures treated embryos.



- Cholesterol and triglyceride levels are significantly elevated following gemfibrozil exposure.
- Triamterene-exposed embryos do not have significantly different cholesterol or triglyceride content as compared to water control values.
- Mixture-exposed embryos show lower triglyceride levels and higher cholesterol levels than control embryos.
  - Lipid composition in mixture-exposed embryos is therefore distinct from control embryos.

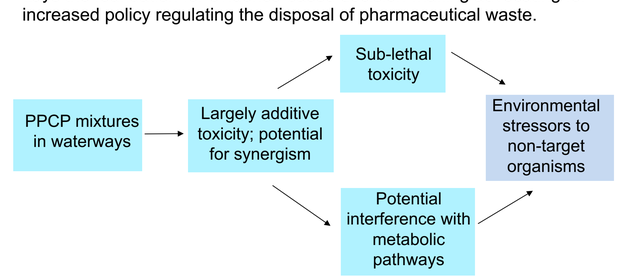
Conclusions/Discussion

- Results indicate that **gemfibrozil and triamterene induce sub-lethal toxic effects** in zebrafish during embryogenesis.
- Increased observed heart physiological abnormalities suggest that **triamterene is toxic to the developing fish heart**, with pericardial blood pooling being a sensitive measure of cardiotoxicity.
- Gemfibrozil induced a similar dose-dependent increase in cardiac defects. The gemfibrozil-induced injury phenotype, however, also included extracardial abnormalities, with a small eye phenotype (microphthalmia), enlarged yolk sac, and a reduced whole-body length.
  - As eye and whole-body length growth are highly linked to embryonic development processes, results suggest that **gemfibrozil induces a developmental delay during zebrafish embryogenesis.**
- **Observed mixture toxicity was consistent with additive toxicity** as determined by RA model for all toxicological endpoints.
  - However, with yolk sac area, observed toxicity is consistently above predicted values for all mixture concentrations—while observed values are not significantly different from predicted ones, this trend in the data suggests that the **drugs may be interacting to produce greater than additive toxicity in terms of yolk sac enlargement.**
- As lipid levels are not altered following triamterene exposure, results suggest that triamterene does not interfere with lipid metabolism.
- As total percent lipid values are significantly elevated following gemfibrozil exposure, results indicate that **gemfibrozil blocks lipid metabolism**.
- Further interpretation suggests that gemfibrozil may be acting through the same metabolic pathway in fish as it does in humans but may be having a converse effect: instead of increasing lipase activity, it may be inhibiting lipase, thereby blocking triglyceride catabolism.
- Due to distinctive lipid makeup in mixtures-exposed embryos, results suggest that **triamterene and gemfibrozil may be interacting to produce toxicity through a different metabolic pathway than single-chemical exposures**.



Implications

- While experimental concentrations were not consistent with environmentally detected levels, results indicate that PPCPs pose as environmental stressors for non-target organisms.
- Morphometric analysis revealed sub-lethal toxic effects in early life stage fish which cannot be detected by crude mortality assays.
- Highlights the need for toxicity assays to take into account the effect of complex PPCP mixtures in order to more accurately predict environmental effects.
- Mixture prediction results using RA model indicate that PPCPs with different mechanisms of action could induce additive toxic effects and, for certain endpoints, potentially metabolically interact to produce synergistic toxicity.
- TLC-FID method allowed for novel examination of potential molecular mechanisms underlying PPCP toxicity.
- Lipid analysis suggests that PPCPs could be inducing similar metabolic pathways in fish as they do in humans.
- Lays the foundation for evidence-based decision-making advocating for increased policy regulating the disposal of pharmaceutical waste.



Future Research Directions

- Identifying and characterizing which specific metabolic pathway is induced by triamterene and gemfibrozil mixtures.
- Conducting gene expression analysis of exposed embryos using qPCR to determine potential genetic markers of toxicity.
- Identifying triamterene mechanism of toxicity in fish by specifically examining sodium channel function in exposed embryos.
- Exposure-linked biomarkers in early life stage fish are next generation tools for ecotoxicological assessment—however, they have not yet been applied to this specific class of contaminants. Therefore, identifying novel molecular biomarkers of toxicity is a viable and promising next step.