HIGH SCHOOL EDITION
Journal of Student Research
Volume 12 Issue 2 (2023)

Behavioral and Physiological Impacts of the Hormesis of Chemical Contaminants On Embryonic Zebrafish

Akshay Kumar[1], Leya Joykutty[#] and Juliana Caulkins[#]

[1]American Heritage High School
[#]Advisor

ABSTRACT

Pharmaceutical chemicals are being produced, consumed, and excreted in human civilization at an increasing rate. These chemicals have the capacity to accumulate, especially in environments such as freshwater systems, but there have not been any major responses to this threat yet as the present concentrations of the chemicals is not viewed as dangerous. Previous research has shown that the developing concentrations of chemicals is an issue, supporting that these chemicals, though not present in large doses, have impacts on exposed organisms. However, prior research has not been conducted to examine the specific effects of chemicals at hormetic concentrations on freshwater organisms. "Hormetic concentration" defines the concentrations of chemicals at specific levels where the response to a low dose of chemical differs from the response to the high dose, and these were the ranges of concentration that were tested in this experiment. Zebrafish were acquired at zero days post fertilization, transferred to the medium containing the appropriate concentration of chemicals for the group that they would be a part of, and used as a model for aquatic organisms to show the resulting chemical, neural, and physical response to the chemical concentrations. The zebrafish were euthanized via bleaching and freezing prior to seven days post fertilization. The results of this experiment show that there is an ecological risk associated with the environmental accumulation of pharmaceutical chemical contaminants that is inherent to their use in human civilization, a result which makes it clear that this issue needs to be addressed.

Introduction

Over 600 pharmaceutical substances are present globally in the environment, found in ground, surface, and drinking waters (Küster and Adler, 2014). This number of active pharmaceutical ingredients (APIs) found in environmental media is a result of the inability to keep pharmaceuticals as a closed system locked to humanity, and this has led to consequences like that of that of the increasing trace contamination concentrations in aquatic environments due to how these types of environments are susceptible to accumulating pollutants (Bouzas-Monroy et al., 2022). Human consumption of chemicals leaves some behind in waste, and while water treatment plants are not 100% efficient in the removal of drugs, which contributes to this issue, untreated effluent is also a concern when considering the routes these chemicals take as potential pollutants (Chander et al., 2016). The occurrences of these chemicals in aquatic systems is a concern that has been raised by many in the past, but is even now only growing (Bouzas-Monroy et al., 2022). However, while the concentrations of chemicals in freshwater systems may be relatively lower than what is largely considered an issue, they are still sufficient to trigger hormetic reactions that suggest severe ecological risks and thus should be met with urgency (Birnbaum, 2012).

The principle of hormesis refers to the biphasic dose/response phenomena of a stimulatory low dose of a stressor compared to the inhibitory high dose of the same stressor (Hashmi et al. 2014). The theory of hormesis holds true for all stressors, and one of the most common examples of this is with chemicals (Birnbaum 2012). Observations of hormesis are growing more common due to increasing chemical usage, causing both a greater relevance and a greater number of individuals interested in the topic. The principle of hormesis illustrates the potential dangers of the

increasing numbers and concentrations of APIs in the environment: the danger that these APIs could have an impact on organisms in nature. One chemical may fall under the beneficial end of hormesis for an individual, but the synergistic effects of another chemical compounded with the beneficial one could completely alter this effect (Lee and Jacobs, 2019). Moreover, the impacts of hormesis on a system could be negative when considering the system as a whole as it would shift the natural balance of the system by introducing a new, foreign factor. Studies have shown that the presence of chemicals in low concentrations and in great numbers have connections to negative affects on health (Birnbaum 2012). What is beneficial to one organism could also harm another directly or indirectly, which means that any chemicals at hormetic ranges would make the environment less stable. No form of stress acts in a vacuum, and multi-stressor tests would more accurately represent the organisms as they would be in real life (De Abreu et al., 2021). Hormesis has been linked to increased growth through chemical exposure in concentrations that are incredibly small, with the caveat that even doses such as one µg could be considered too large and prove to be harmful to the organisms in question (Hashmi, et al., 2015). Hashmi (2015) reports that concentrations at 0.42 µg of PCB 31 per gram of water can trigger hormesis in a beneficial way that increases the size of the fish affected. The fish would grow longer and weigh more with exposure of PCB 31 up until exposure to 10-20 µg per gram for 28 days, at which point they would start being affected negatively. The chemical concentrations in lakes often far exceeds this number, with common chemicals like caffeine, acetaminophen, metformin, carbamazepine, and a long list of others being prevalent in water systems at the range of .1 µg to 129 µg per gram of lake water at a bias towards the lower concentrations (Blair et al., 2013). Quantities within this range are sufficient to induce hormesis, which could have a wide variety of impacts on the growth of the organisms exposed, and as such show that the conditions for this potential issue already exist in the natural environment. The phrase “hormetic responses” describes the effects of stressors on organisms at low, hormetic doses that have known and/or harmful effects at high doses/ Zebrafish (danio rerio) embryos from 1-7 days post fertilization will be used for this experiment because they are a good model organism to see the potentially concerning hormetic responses that could arise as a consequence of increasing APIs in the environment.

Zebrafish are a good organism to use to study hormesis in part because zebrafish show clear indicators of physiological stress. Zebrafish show higher cortisol releases over acute physical stressors as compared to psychological stressors (De Abreu et al., 2021). This makes it simple to map what stresses the zebrafish. Testing cortisol levels gives an image of how stressed out the zebrafish are, which shows what effects the experimental conditions have on their bodies. Stressors that trigger cortisol production aren’t solely limited to more physical forms of stress. Chemical stressors also increase whole body cortisol levels and tend to induce behavior similar to anxiety in zebrafish, showing altered physiology and psychology in the organisms (De Abreu et al., 2021). Zebrafish also show variety in how they cope with stress, which is connected to their behavioral and physiological characteristics (Tudorache et al., 2015). Zebrafish expressing physiological stress in an easily measurable way as well as expressing it in their physical and cognitive development makes them a functional model organism to use in an experiment regarding how certain chemicals can impact the aquatic wildlife of the environments they pollute.

The neurological impacts of stressors on zebrafish at hormetic concentrations could also represent an ecological risk. Experiments conducted show that zebrafish larvae respond to stress in similar ways that adult zebrafish do and that larval zebrafish have a level of mental development that is comparable to adult zebrafish (Tudorache et al., 2015). Another experiment, this one involving zebrafish exposed to stressors early on in their development, showed that chemical stressors may alter the behavior of the zebrafish due to interactions with their brain chemistry, where one of the altered behaviors was a suppressed desire to feed (De Marco et al., 2014). The results of De Marco (2014) show that chemicals have the ability to alter the brain chemistry of zebrafish during their development in a permanent way. Cheng et al. (2011) conducted an experiment that would suggest that data collected from larval and even younger zebrafish would also prove to be accurate for older zebrafish because they have similar levels of cognitive development, which would mean that a majority of the development of the brain of a zebrafish happens very early in their life cycle. Zebrafish afflicted in ways that alter their brain chemistry would be at a severe disadvantage compared to those unaffected and be at a severe disadvantage compared to other zebrafish as they are social animals who rely on their instincts (Suriyampola et al., 2016). The impacts of stressors on the neurology of zebrafish populations could have

dangerous implications. Understanding the extent to which this occurs is as important as understanding how the zebrafish are affected physiologically because both are integral elements of the survival of these animals in nature.

The drugs used in this experiment will be carbamazepine, sulfamethoxazole, and metformin hydrochloride. These drugs have a recorded history of contaminating freshwater systems, which is why they have been chosen, as well as their prevalence in everyday life (Bouzas-Monroy et al., 2022). The chemicals will be tested at three concentrations: one with a possible impact on the zebrafish, one with a probable impact on the zebrafish, and one with a projected impact on the zebrafish. The three concentrations will be in the hormetic ranges of the chemical, falling under the idea of a "low dose". Bouzas-Monroy (2022) helped to predict that the no effect concentrations of the chemicals are carbamazepine at 2.5 µg per liter, that of metformin hydrochloride is 100 µg per liter, and that of sulfamethoxazole is 0.6 µg per liter. The higher dose concentrations will be 5 µg per liter for carbamazepine, metformin hydrochloride will be 200 µg per liter, and sulfamethoxazole will be 1.2 µg per liter. The highest dosage of the chemicals will be 25 µg of carbamazepine per liter, 1000 µg of metformin hydrochloride per liter, and 6 µg of sulfamethoxazole per liter. The zebrafish were euthanized via freezing and bleaching.

The pharmaceuticals chosen were picked for their prevalence and common use as well as the already existing concentrations of them in areas such as Lake Michigan and lakes in Minnesota (Blair et al., 2013). These locations are not unique and many lakes around the world are in similar states of contamination. Bouzas-Monroy's (2022) study of 137 sampling campaigns over 104 countries monitoring 61 API had 23 APIs in levels deemed as ecotoxicological risks in 34.1% of the locations tested. This issue is a global one, not limited to any country, continent, or people.

Hypotheses

Hypothesis #1: If the zebrafish are dosed with hormetic concentrations of chemicals at any range of the hormetic scale, then they will show altered behavior compared to zebrafish that were not dosed with these chemicals.

Hypothesis #2: If the zebrafish are dosed with hormetic concentrations of chemicals at any range of the hormetic scale, then they will show altered physiology compared to zebrafish that were not dosed with these chemicals.

Statement of Purpose

Chemicals are becoming more ubiquitous in nature and are accumulating in concentration for a multitude of reasons. This is not seen as an issue because the scale seems to be too small to impact the ecosystems present, but this could be untrue. The purpose of this experiment is to determine whether chemicals in hormetic ranges of concentration have an effect on the development and/or function of aquatic organisms, which were modeled here using zebrafish (*danio rerio*) embryos.

Matrix

	No Chemical	Sulfamethoxazole (SMO)	Carbamazepine (CMP)	Metformin Hydrochloride (met)
No Concentration	Control	-	-	-
Concentration 1	-	0.6 µg/L	2.5 µg/L	100 µg/L

	No Chemical	Sulfameth-oxazole (SMO)	Carbamazepine (CMP)	Metformin Hydro-chloride (met)
No Concentration	Control	-	-	-
Concentration 2	-	1.2 µg/L	5.0 µg/L	200 µg/L
Concentration 3	-	6.0 µg/L	25 µg/L	1000 µg/L

Figure 1. Chemical concentration and chemical matrix

Materials

- Wild type *danio rerio* embryos
- Sulfamethoxazole (SMO)
- Carbamazepine (CMP)
- Metformin Hydrochloride (met)
- Centrifuge
- Spectrophotometer
- Vortexer
- Homogenizer
- Microscope with camera
- 100% ethanol
- PBS buffer
- 12-well plates
- Cortisol ELISA Kit

Procedures (Research Methods)

Plating The Zebrafish Embryos

First, create the 1L 100x stock solutions for later dilutions (60 µg SMO, 10mg met, 2.5 mg CMP) in accordance with proper safety precautions. This includes proper eye protection, a lab coat, gloves, and proceeding in a clean, sterile environment with emergency equipment nearby. For SMO and CMP, first dissolve the drug in ethanol. If necessary, place the ethanol in a beaker on a hot plate. If not, place a beaker filled with distilled water on the hot plate instead. Carefully place a stir bar in the solutions and turn the stirring on. Stop the stirring to slowly add distilled water to the solutions until the solution reaches a liter. Pour the fully dissolved solutions into bottles using funnels and label solutions. Migrate zebrafish from the vials they arrived in to large petri dishes and acquire a separate beaker. Take the quantity of solution calculated that will be required for a diluted solution of the concentration required out of the solution and place it in the beaker. Finish doing the dilutions in the new beaker and set aside carefully. Continue dilutions in this manner. Label well plates with the solutions that they will be filled with. Fill the well-plates correctly and accurately. Place zebrafish in their solutions using a pipet. Conduct Spontaneous Tail Coiling (STC) assay at one day post-fertilization. Conduct Cardiac Rate Assessment assay at five days post fertilization. Conduct Cortisol Enzyme Linked Immunoassay (ELISA) assay at 6 days post fertilization. Perform statistical analysis on recorded data.

Assays

Spontaneous Tail Coiling (STC)

Transport zebrafish to the microscope in their 12 well plates. Acclimate zebrafish away from their well plates for five minutes to account for disturbances in transportation to microscope. Select five embryos, pipette them onto a microscope slide, and group them together. Record grouped zebrafish embryos using the camera mounted on the microscope. Take zebrafish, put them back in their well-plates of medium, and repeat for all organisms in the groups and their repetitions. Analyze data manually.

Cortisol Enzyme Linked Immunoassay (ELISA) Kit (Canavello et al., 2010)

Complete behavioral studies and then freeze sample zebrafish embryos. Partially thaw the zebrafish, weigh them, and homogenize the organisms with 500 µL of 1X PBS buffer. Wash the homogenizer with another 500 µL of PBS buffer solution and contain the resulting material in a tube. Sterilize and clean the blade in 100% ethanol and deionized water after homogenization to prevent cross-contamination after each sample. Add 750µL of toluene to each sample. Vortex samples for one minute and centrifuge samples at 3500 rpm for 5 minutes. Remove the resulting cortisol level from the centrifuge tube and place in a separate test tube for storage after repeating thrice. Place samples in the fume hood overnight for evaporation. Reconstitute the sample in 1mL of 1X PBS the next day, incubating overnight at 4 degrees C. Prepare Wash Buffer from 20 mL of 25x solution and 480 mL distilled water. Prepare Cortisol Standard with 1.05 mL of deionized water. Remove the strips that are unneeded from the 96-well-plate. Prepare cortisol standard dilutions within sixty minutes of use. Add 150 µL of calibrator diluent into the Non-Specific Binding Wells. Add 100 µL of calibrator diluent into the wells meant for the blanks, the standards, and the samples. Add 50 µL of cortisol conjugate to each well. Add 50 µL of Primary Antibody Solution to the non NSB wells. Incubate for 2 hours on a horizontal microplate shaker at 500 RPM. Prepare substrate solution (50/50 Color Reagents A and B) at 1:45 through the incubation and protect from light. Begin to work in the dark. Aspirate each well and wash with the wash buffer 3 times. Add 200 µL of substrate solution to each well. After waiting thirty minutes, add 50 µL of stop solution. Read the final plate at 450 nm using a spectrophotometer.

Cardiac Rate Assessment Assay

Take the zebrafish embryos out of their well-plates at 72 hours. Place them in microscope slides that have been wet to accommodate the zebrafish. Take a video of five zebrafish at a time for ten seconds. Manually count the heartbeats of each zebrafish. Graph the heartbeats as related to the variable groups.

Results

(*) Represents statistically significant data.

Each trial had three replicates with ten organisms being tested each trial.

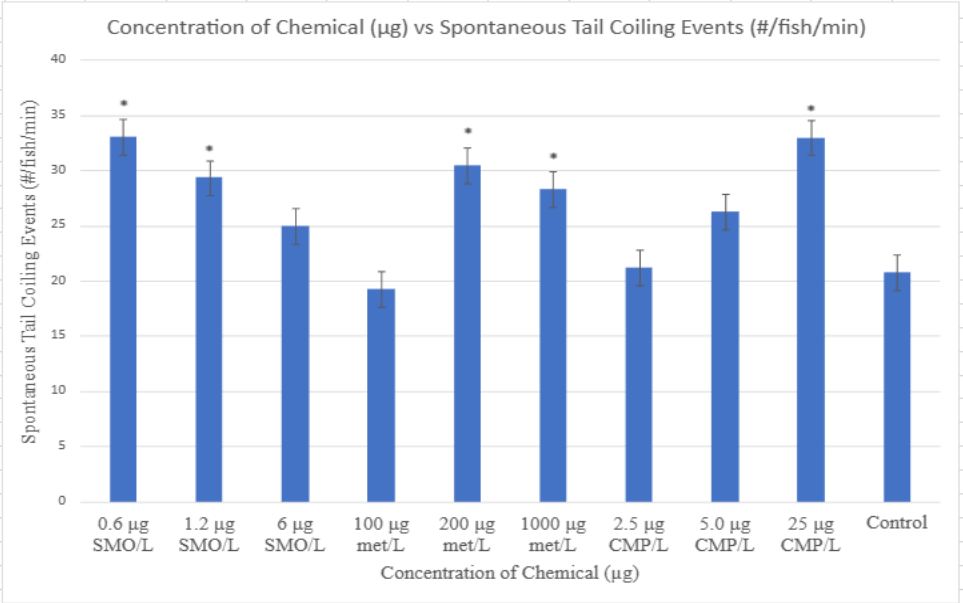

Figure 2. Spontaneous Tail Coiling assay graph

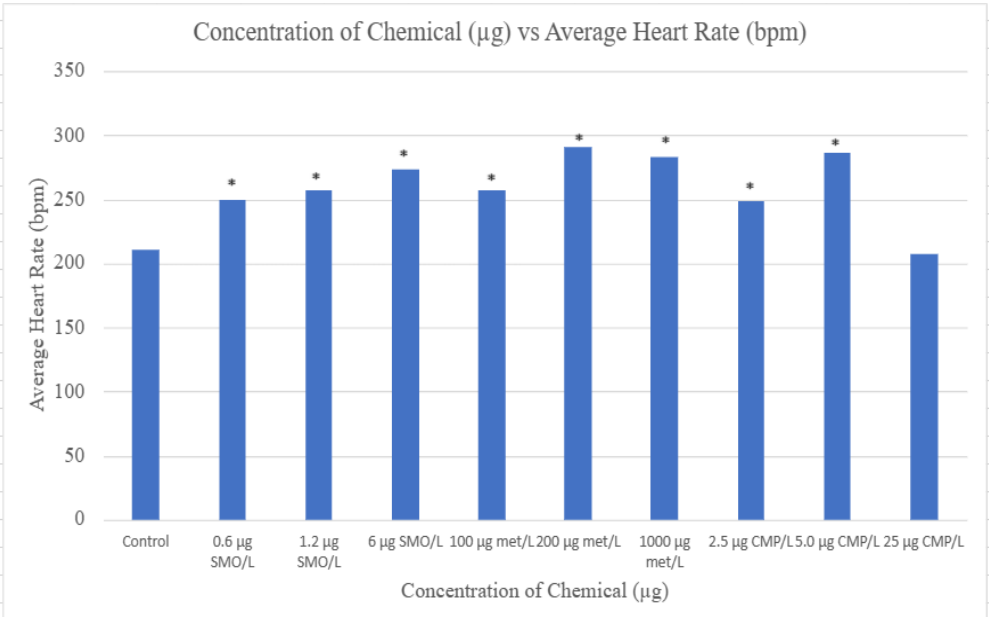

Figure 3. Cardiac rate assessment assay graph

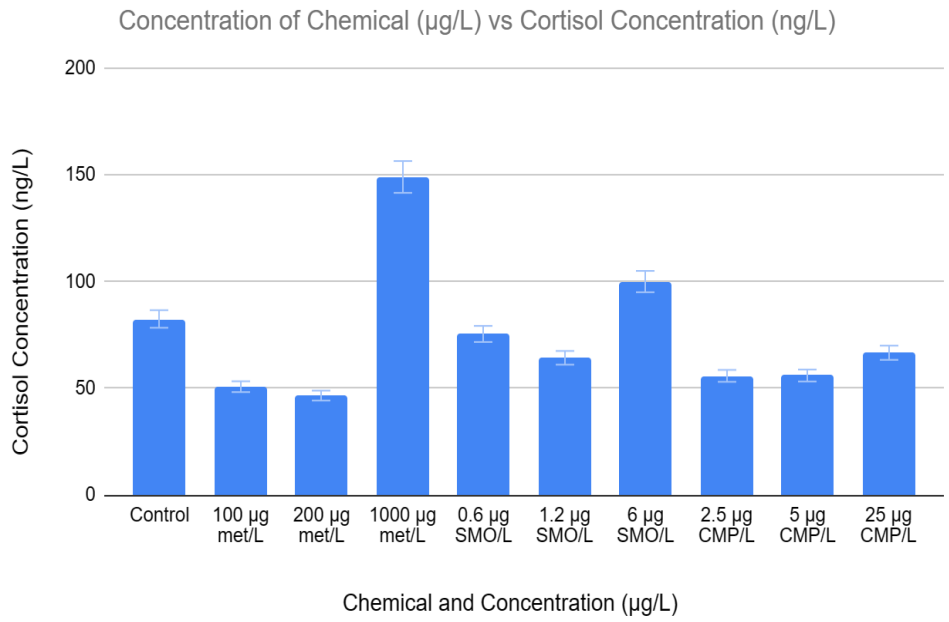

Figure 4. Cortisol ELISA assay graph

The groups with an asterisk are the ones which represent statistically significant data according to ANOVA testing. Nearly every group was statistically significant under the cardiac rate assessment assay, over half the groups were for the spontaneous tail coiling assay, and no groups were for the cortisol ELISA assay. Figure 2 represents the spontaneous tail coiling assay, and it shows that there were changes to the neurological development of the zebrafish. The zebrafish representing statistically significant data showed a change to their behavior that would not have been likely without the interference of hormesis, so there was an impact on the zebrafish's behavior and its development. Figure 3 represents the cardiac rate assessment assay, showing the vast majority of the groups to be statistically significant: the chemicals did, then, impact the physiology of the zebrafish embryos. Figure 4 represents the Cortisol ELISA assay with no statistically significant groups. The issue of increasing chemical concentrations in nature was known, but the impacts of this were thought to be not concerning. However, the results of this experiment suggest quite the opposite, where freshwater organisms - modeled using zebrafish embryos - were impacted by chemicals in these low concentrations in their living environment. They showed physiological and behavioral changes, and the fact that they were impacted means that these chemicals even in low concentrations serve a disruptive presence in freshwater systems. As a result of the doses, on average, the zebrafish were more likely to undergo events of spontaneous tail coiling - a behavior that is indicative of early brain development - and also on average had higher heart rates. The results of this experiment show that this is a pressing issue that should be addressed as soon as possible.

Discussion

Based on the experimental hypothesis and completed assays, the project's hypotheses failed to be rejected, meaning that the null hypothesis for both was rejected and thus that chemical contaminants in hormetic concentrations impacted the behavior and physiology of the zebrafish. The drugs and their concentrations tested in hormetic doses were shown to generally have an impact of some kind on the zebrafish in the tested categories. It affected their behavior, as shown in the STC assay, and their physiology, as shown in the cardiac rate assessment assay. While the ELISA assay did not represent statistically significant data, this only means that the chemicals tested did not alter cortisol excretion in the

zebrafish embryos, and does not signify that the dangers of chemical hormesis in aquatic systems is non-existent. The data collected from the experiment means that the hormetic concentrations did have impacts on the zebrafish. The nature of the trends in data was odd, with results shifting from concentration in either direction. Generally, however, the data had correlations between the chemical concentrations as was expected, and when it didn't, it was similar enough to something expected. It is possible that some chemicals passed the hormetic threshold for some impacts, resulting in the variances in results. Overall, hormesis of chemical contaminants was shown to impact aquatic life.

Applications

This experiment supports that change needs to be made in the approach to chemicals in the natural environment. The experiment showed how chemical concentrations in the environment have quantifiable impacts on the organisms living in it even at a lower scale of concentration. This supports those who would attempt to pass greater restrictions on the use of chemicals and the disposal of chemicals. However, due to the ubiquitous nature of chemical use in human societies this solution would be less than ideal. It could be met with a great deal of controversy, and restriction of the use of something so integral to human societies could be potentially dangerous if these restrictions are too heavy-handed. A more widespread distribution of this knowledge could help solve this problem on a more individual, citizen level as well, pushing part of the responsibility into these general populace.

Limitations/Error Analysis

There were a couple uncontrollable limitations of this experiment, like the high mortality rate of the model organisms that rendered the experiment paused for weeks at a time. Limitations with being a student researcher were also present, including the inability to work on the project during school hours. A final limitation was the inability to work with multiple stressors: all arranged multiple stressor groups had incredibly high mortality rates to the point where no assays could be performed. Errors may have occurred due to variances in the different lab equipment and in the modification of the ELISA protocol to use the organic solvent toluene instead of diethyl ether.

Future Research

Future research with a more expansive list of chemicals, with a greater range of concentrations, on a larger variety of aquatic organisms could be done to widen the breadth of knowledge regarding the breadth of this project. More work on cross-stressor interactions would also further this field of research as it is a prominent potential issue. Future research could also potentially be done on the effects of chemical hormesis on the expression of genes, on the social behaviors of zebrafish, and on other elements of the zebrafish physiology. This would give a greater comprehensive view of the impacts left on the organisms by the stressors beyond the idea that they exist.

Acknowledgments

I would like to thank my advisor for the valuable insight provided to me on this topic.

References

Birnbaum, L. S. (2012). Environmental chemicals: Evaluating low-dose effects. *Environmental Health Perspectives*, *120*(4). https://doi.org/10.1289/ehp.1205179

Blair, B. D., Crago, J. P., Hedman, C. J., & Klaper, R. D. (2013). Pharmaceuticals and personal care products found in the great lakes above concentrations of environmental concern. *Chemosphere*, *93*(9), 2116-2123. https://doi.org/10.1016/j.chemosphere.2013.07.057

Bouzas?monroy, A., Wilkinson, J. L., Melling, M., & Boxall, A. B. A. (2022). Assessment of the potential ecotoxicological effects of pharmaceuticals in the world's rivers. *Environmental Toxicology and Chemistry*, *41*(8), 2008-2020. https://doi.org/10.1002/etc.5355

Canavello, P. R., Cachat, J. M., Beeson, E. C., Laffoon, A. L., Grimes, C., Haymore, W. A.m., Elegante, M. F., Bartels, B. K., Hart, P. C., Elkhayat, S. I., Tien, D. H., Mohnot, S., Amri, H., & Kalueff, A. V. (2010). Measuring endocrine (Cortisol) responses of zebrafish to stress. *Neuromethods*, 135-142. https://doi.org/10.1007/978-1-60761-953-6_11.

Chander, V., Sharma, B., Negi, V., Aswal, R. S., Singh, P., Singh, R., & Dobhal, R. (2016). Pharmaceutical Compounds in Drinking Water. *Journal of xenobiotics*, *6*(1), 5774. https://doi.org/10.4081/xeno.2016.5774

Cheng, R. K., Jesuthasan, S., & Penney, T. B. (2011). Time for zebrafish. *Frontiers in integrative neuroscience*, *5*, 40. https://doi.org/10.3389/fnint.2011.00040

De Abreu, M. S., Demin, K. A., Giacomini, A. C., Amstislavskaya, T. G., Strekalova, T., Maslov, G. O., Kositsin, Y., Petersen, E. V., & Kalueff, A. V. (2021). Understanding how stress responses and stress-related behaviors have evolved in zebrafish and mammals. *Neurobiology of Stress*, *15*, 100405. https://doi.org/10.1016/j.ynstr.2021.100405

Hashmi, M. Z., Naveedullah, Shen, C., & Yu, C. (2014). Hormetic responses of food-supplied PCB 31 to zebrafish (Danio rerio) Growth. *Dose-Response*, *1*(-1), 1-14. https://doi.org/10.2203/dose-response.14-013.Chaofeng

Küster, A., & Adler, N. (2014). Pharmaceuticals in the environment: scientific evidence of risks and its regulation. *Philosophical transactions of the Royal Society of London. Series B, Biological sciences*, *369*(1656), 20130587. https://doi.org/10.1098/rstb.2013.0587

Lee, D. H., & Jacobs Jr, D. R. (2019). New approaches to cope with possible harms of low-dose environmental chemicals. *Journal of Epidemiology and Community Health*, *73*(3), 193?197. https://doi.org/10.1136/jech-2018-210920

National Center for Biotechnology Information (2023). PubChem Compound Summary for CID 5329, Sulfamethoxazole. Retrieved January 6, 2023 from https://pubchem.ncbi.nlm.nih.gov/compound/Sulfamethoxazole.

National Center for Biotechnology Information (2023). PubChem Compound Summary for CID 14219, Metformin hydrochloride. Retrieved January 6, 2023 from https://pubchem.ncbi.nlm.nih.gov/compound/Metformin-hydrochloride.

Suriyampola, P. S., Shelton, D. S., Shukla, R., Roy, T., Bhat, A., & Martins, E. P. (2016). Zebrafish Social Behavior in the Wild. *Zebrafish*, *13*(1), 1?8. https://doi.org/10.1089/zeb.2015.1159

ThermoFisher. (2021, December 25). *Safety Data Sheet - Fisher Scientific*. fisher-sci. Retrieved January 6, 2023, from https://www.fishersci.com/store/msds?partNumber=AC344360050&productDescription=CARBAMAZEPINE%2C+98%25+5GR&vendorId=VN00032119&countryCode=US&language=en

Tudorache, C., ter Braake, A., Tromp, M., Slabbekoorn, H., & Schaaf, M. J. M. (2015). Behavioral and physiological indicators of stress coping styles in larval zebrafish. *Stress*, *18*(1), 121-128. https://doi.org/10.3109/10253890.2014.989205