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Life History and Metabolic Responses of Daphnia magna Exposed to Effluents of Urban Origin Treated by Advanced Oxidation

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An Abstract of a Thesis

In

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Submitted in Partial Fulfillment

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Abstract

Modern sewage treatment processes do not remove many chemicals that pass through the sewer system, including pharmaceuticals and personal care products that have been shown to have negative effects in ecosystems. New processes have been developed to remove these chemicals, known as advanced oxidation processes (AOPs). In this study, *Daphnia magna* are used to examine the effects of wastewater effluent treated conventionally compared to effluent treated with AOPs (Peracetic Acid/Ultraviolet Light and Hydrogen Peroxide/Ultraviolet Light). For this purpose, two sets of experiments were preformed: a five-day survivorship trial, and a 14-day life history experiment. Growth, survival, and reproduction of *Daphnia magna* raised in the different effluents were examined. Furthermore, *Daphnia* metabolites were quantified using metabolomics. Results from survivorship trials show an increase in survival of animals exposed to effluent treated with concentrations of 6 mg of peracetic acid/UV. In the 14-day life history experiment it was found that the 3 mg peracetic acid/UV treatment had a negative effect on *Daphnia*'s body growth, lipid production, and reproduction as well as the intrinsic rate of population growth, or the potential for the population to exist overtime, more so than the conventional secondary effluent. In contrast, the effects of hydrogen peroxide/UV treatment showed the lowest toxic effects, comparable to the control animals. These experiments offer insights into how wastewater treated with AOPs and discharged into a natural body of water could affect zooplankton and sensitive early life stages of other aquatic organisms, which together represent the primary consumer level of aquatic food webs.

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A Thesis in Biology

By

Benjamin Szczygiel

Submitted in Partial Fulfillment of the Requirements
for the Degree of

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Introduction

Sewage Effluent and Pharmaceutical and Personal Care Products (PPCPs)

Wastewater treatment plants (WWTPs) are vital for the existence of cities and do an adequate job of recycling used water back into the environment. However, much of the technology and many of the techniques used in treating wastewater are dated and do not remove modern waste additions to sewage (Bilińska et al., 2016). As a result, treatment facilities do not remove some dangerous chemicals from the wastewater before it is released back into the environment, specifically, high levels of pharmaceutical and personal care products (PPCPs) (Blair et al., 2013).

Pharmaceutical and personal care products include a variety of substances such as residual medicines that have either been poured down a drain or excreted (Arnnok et al., 2017). These chemicals are often stable, long lasting, and have a variety of negative effects on the environment. PPCPs tend to be lipophilic and are easily bioaccumulated within the food chain, often with physiological consequences. For instance, in *Daphnia magna* PPCPs have been known to alter sex ratios, decreasing the production of males when water conditions are poor, and decreasing overall vitality (Baer et al., 2009). High exposure to PPCP-laced sewage effluent tends to cause organ damage in exposed aquatic organisms, such as kidney structure alteration in the zebrafish (*Danio rerio*) (Galus et al., 2013), as well as an alteration of gene regulation and neurotransmitter activity in various fish and zooplankton (Arnnok et al., 2017, Yamamoto et al., 2011). Recent examinations have found that the levels of antidepressants and other PPCPs in fish are substantially higher than previously thought, indicating a much more serious problem

(Arnnok et al., 2017, Blair et al., 2013) that requires additional cleansing of the final effluent at WWTPs.

Advanced Oxidation Processes

Advanced oxidation processes (AOPs) are chemical treatments that are designed to break down and remove organic and inorganic materials, including many PPCPs, through oxidation with radicals, especially hydroxyl radicals. Hydroxyl radicals are neutral OH molecules that are highly reactive and short-lived. Their high energy and reactivity allow them to break apart chemicals in the sewage effluent, creating less toxic and more manageable byproducts for the most part (Gligorovski et al., 2015). While radicals can be useful, excess amounts can damage biotic systems in a variety of ways, including breaking down amino acids and damaging digestive capabilities (Tang et al., 2006). To prevent this situation, excess radicals are removed through the addition of thiosulfates before sewage effluent is released.

There are a variety of AOP treatments that range in quality and convenience. A main benefit of AOPs compared to other advanced effluent treatments is that many can be installed at the end of the usual wastewater treatment process without having to overhaul the entire plant. A basic AOP that can be implemented with minimal change to the WWTP is ultraviolet light (UV) exposure at the current chlorine disinfection end stage (Qin et al., 2014, Zhang et al., 2015). Another simple but effective treatment involves mixing peracetic acid (PAA) into the effluent and exposing the mixture to UV light (Cai et al., 2017). Ultraviolet light is a common tool in many AOPs as it provides the energy needed to break chemical bonds. These two treatments are relatively inexpensive to implement but are not the most effective. Some of the better treatments dealing with removal of pharmaceuticals include bubbling ozone through the effluent

(ozonation) or adding hydrogen peroxide (H_2O_2) and exposing the mixture to UV light. Hydrogen peroxide has been shown to be one of the most effective AOPs in terms of removing PPCPs (Wols et al., 2013). In addition, all AOPs are designed to break down and leave no obvious negative residuals in the released sewage effluent that could harm aquatic organisms (Qin et al., 2014).

Although effective, the main problem preventing the implementation of AOP methods relates to the cost of running these processes (UV light and chemical addition) and the changes it would require to a treatment facility (Bilińska et al., 2016). Another problem is that while much research has been done on the general chemical changes these AOPs cause, little work has been done on the environmental impacts of AOP-treated effluent. The absence of studies clarifying the environmental impact of the treatment itself is likely another factor contributing to delays.

Daphnia magna *as Model Organism to Test Advanced Oxidation Processes (AOPs)*

Many studies of the environmental impact of treated effluent in general have been done using species such as *Daphnia magna*. The zooplankter *D. magna* is a large species of freshwater water fleas (Crustacea) with adults measuring between 1.5-5 mm, making them easy to observe and monitor. In addition, they tend to reproduce asexually, allowing for replication with genetically identical individuals. These features, as well as their low trophic level, easy culturing, and general sensitivity to changes in the environment, make *Daphnia magna* an ideal test organism (Baer et al., 2009). The ecological relevance of using *Daphnia* in experimental work resides in their importance as primary consumers in the aquatic food web and their role as conduits of basal energy (from algae) to upper trophic levels (i.e., various species of fishes, fishing birds, etc.). Any deterioration at the zooplankton level carries the impact through the food

web, causing declines and shifts in all the interconnected species, including those valued by humans such as sport fish. To further understand the effects of PPCPs in *Daphnia*, in this study I briefly introduce the production of distinct groups of metabolites by the animals exposed to the different experimental treatments (Taylor et al., 2018).

Objective

The purpose of this project was to evaluate *Daphnia magna*'s survivorship, condition, and changes in life history events when reared in effluents that have been AOP-treated. Further, I intended to gain an understanding of the level of molecular markers produced by the animals when exposed to the various treatments by using metabolomic analysis. Metabolomic analysis involves examining the levels of different metabolites in the body in order to identify changes in body processes that would produce the metabolites. This metabolomics research is part of a collaborative study with the University at Buffalo, and only preliminary results are available at the time of writing this thesis. However, I have included a snapshot of those results to show how the different treatments produced different sets of molecules in the *Daphnia* in each of the treatments.

Hypotheses

1. Because hydrogen peroxide is very effective at removing PPCPs and breaks down easily (Wols et al., 2013), *Daphnia* exposed to effluent treated with H_2O_2 will not produce many distinct metabolites compared to a control of *Daphnia* maintained in clean water that has not been exposed to any kind of effluent.
2. Because peracetic acid (PAA) and ultraviolet light are less effective at removing PPCPs than H_2O_2 (Cai et al., 2017), *Daphnia* exposed to effluent treated this way will express a similar number of unique metabolites, but to a lesser degree, as *Daphnia* that have been directly exposed to the WWTP secondary effluent, which is the effluent before chlorination and release into the environment.
3. *Daphnia* will show effects of exposure to the various effluents compared to the control as reflected in changes in development and life history events in all AOP and secondary effluent treatments. These effects will include whether they reproduce successfully, whether the timing and number of molts is altered, and their nutritional status as measured by lipid content. Survivorship and reproduction will be greater in the H_2O_2 treatment than in the peracetic acid treatments.

Methods

Effluent Collection and Treatment

The effluent for this study was collected by students from the University at Buffalo (Wei Lab). Effluent was collected from a Western New York wastewater treatment plant in the Southtowns and treated with the various AOPs used in this study. At the treatment plant, a bucket (roughly 5 L in size) was used to collect sample effluent from the secondary clarifier. For each experimental treatment, the effluent was chemically treated as indicated below and ran through a loop under UV lights. The effluent ran through this loop until appropriate amounts of energy were added to each treatment. This process ensured proper mixing of chemicals and an even distribution of energy. A thiosulfate solution (6 g/L $Na_2S_2O_3$) was added to the AOP-treated effluent (amount differed between treatments, Table 1) in the amounts necessary in order to quench residual hydroxyl radicals. To evaluate the PPCPs' concentrations in the experimental treatments' water, samples were analyzed using a mass spectrometer to determine the decrease in PPCPs using AOPs (Figure 1). The AOP-treated effluents were then filtered using a 0.7 µm glass fiber filter (pre-combusted) and frozen at -80^{o}C until use in the *Daphnia* experiments.

The AOP treatments for the following experiments were: 3 mg/L peracetic acid with exposure to UV light at 3000 mJ/cm^{2}A; 6 mg/L peracetic acid with exposure to UV light at 3000 mJ/cm^2; and 6 mg/L hydrogen peroxide with exposure to UV light at 3000 mJ/cm^2. The fourth treatment was the secondary effluent without any additions, and I used two controls: filtered tap water control and a control with filtered tap water and the addition of 1 mL/L thiosulfate (for the survivorship experiment only) (Table 1).

Daphnia*'s Toxicity Tests*

To examine the effects of AOP-treated effluent on aquatic life, I used *Daphnia magna* in a series of toxicity tests. The *Daphnia* used for these tests were raised in an incubator in filtered Buffalo city tap water (source: Lake Erie, filter: redox KDF/carbon). These *Daphnia* were fed a diet of fresh *Ankistrodesmus sp*. (alga cultured in the laboratory) and kept at 20°C with a photoperiod of 16:8h L:D. A clonal population of *Daphnia* was created by separating one individual and allowing it and its progeny to reproduce asexually until a sustainable population was achieved. The *Daphnia* used for the experiments were born from the second clutch of eggs. Neonates released by the mother in a window period of 6 h were isolated for the experiment to ensure that all animals used in the experiments had similar age and size. From this pool, 50 individuals were randomly selected and measured to ensure similar lengths for each trial and were expected to have initial similar metabolite composition (Đức et al. 2016).

Toxicity tests were run using the following six treatments (experimental effluents) and will be referred to as follows (abbreviations in parenthesis):

- 3 mg/L peracetic acid with exposure to UV light at 3000 mJ/cm^2. (3 mg/L PAA).
- 6 mg/L peracetic acid with exposure to UV light at 3000 mJ/cm^2. (6 mg/L PAA).
- 6 mg/L hydrogen peroxide with exposure to UV light at 3000 mJ/cm^2. (H_2O_2)
- Secondary effluent (non-AOP treated effluent). (Secondary effluent).
- Control of filtered tap water. (Control).
- Control of filtered tap water with thiosulfate. (Control+thiosulfate).

Analysis of PPCPs in Treatment Effluents

The analysis of PPCPs in the effluents used for this study was performed at the University at Buffalo (Ph. D. student Laura Brunelle, Chemistry Dept., Aga Lab). Briefly, a sample of 500 ml from each experimental effluent was filtered and the pH brought to 2 ± 0.5. Surrogate standards to the PPCPs of interest were spiked into the sample (100 ppb) and 500 ml of spiked sample was run through a conditioned Hydrophilic-Lipophilic-Balanced Solid Phase Extraction cartridge. The cartridge was dried and later eluted twice with 4 ml portions of Acetonitrile, dried under Nitrogen, resuspended and analyzed using an LC/MS/MS instrument.

Two experiments were performed for this study separately in an incubator at a constant temperature of 20^{o}C and 16:8 h L:D photoperiod. The vials were placed randomly in the incubator and moved daily to ensure no bias related to location in the incubator.

Survival Rates Experiment

In order to determine which of the experimental effluents had an effect on *Daphnia*'s metabolism and survival, I performed tests of survivorship. These tests consisted of exposing *Daphnia* to different concentrations of effluent and monitoring survivorship. Each test lasted five days. Five replicates of 16 individuals each were used for each treatment and the controls. These individuals were placed in 150 mL of treatment solution. The effluent was diluted with filtered tap water to attain the desired concentrations. The test concentrations for each experimental effluent were: 6.25%, 12.5%, 25%, 50% and 100% (Villegas-Navarro et al., 1997, Ra et al., 2007). For the survivorship tests, each of the treatments was tested in separate runs (i.e., one experimental effluent at a time) with controls. This procedure allowed for each treatment to be run in sets of 30 vials (five replicates × six treatments, including the controls). A thiosulfate

control was run for this experiment, as indicated above, to examine if the thiosulfates used to quench excessive hydroxyl radicals had any effect on *Daphnia* mortality. This control had the highest thiosulfate concentration used in the AOP-treated effluents (Table 1). The surviving *Daphnia* were frozen at -80^{o}C for metabolomic analyses.

Table 1: Concentrations of thiosulfates present in the different AOP treatments.

Treatment	$Na_2S_2O_3$ Quenching after UV (mL/liter of effluent)	pH
6 mg/L PAA + 3000 mJ/cm^2 UV	1	7.13
6 mg/L H_2O_2 + 3000 mJ/cm^2 UV	0.47	7.65
3 mg/L PAA + 3000 mJ/cm^2 UV	0.5	7.26
Control	N/A	7.12
Control+Thiosulfate	1	7.46
Secondary Effluent	N/A	7.49

Daphnia*'s Extraction Procedure for Metabolomics*

A preliminary exploration of the differences in metabolic composition between the *Daphnia* that died during the survivorship trials and those who survived revealed there were no significant differences; therefore, all the *Daphnia* from each treatment were pooled together. The *Daphnia* were freeze-dried, weighted, and placed in a Dounce homogenizer with 3 ml methanol:water (80:20) and homogenized for 30 strokes. The resulting solution was centrifuged at 500 RCF for ten minutes at 4^{o}C. From the supernatant, 2.7 ml were removed and dried under

nitrogen. The samples were resuspended in methanol:water (80:20) with 25 ppb D_{10}-carbamazepine and filtered. Samples were then run through TSQ Quantum Ultra Orbitrap (Thermo Fisher, Waltham, MA) and Compound Discoverer™ 3.0 (Thermo Fisher, Waltham, MA) for untargeted analysis of metabolites.

Daphnia*'s Life History Experiment*

In order to study the effects of AOP-treated effluent on the life history of *Daphnia*, a longer exposure test was performed. For each treatment, ten replicates of 20-mL vials were filled with the experimental treatments (undiluted) and in each vial was placed only one *Daphnia* neonate (<10 h. old). As the thiosulfate control showed no significant difference from the tap water control in the survivorship trial results, it was not used for this experiment (Figure 2, $p \geq 0.05$). All treatments were run concurrently for 14 days, close to the life span of the *Daphnia*. *Daphnia* were examined daily on the same schedule and moved to new vials with fresh media and food (*Ankistrodesmus sp.* 5.6×10^5 cells/ml per animal/day). The visual examinations of the *Daphnia* included: survivorship, presence of molts, presence and number of eggs and neonates born. Neonates and dead *Daphnia* were removed from the vials and frozen at -80°C. At the end of the experiment, each surviving *Daphnia* was measured (top of head to base of spine) and lipids and ovaries were assessed using a scoring system between 0 and 3, using defined pictorial scales (Tessier and Goulden 1982). A lower score on these scales indicated an individual was low in lipid stores and had poor reproduction, while a high score represented an individual with lipid reserves that was reproducing well. After these assessments at the end of 14 days, all *Daphnia* were frozen and stored at -80°C.

Statistical Analysis

For the survivorship trials, all data was tested for normality (Kolmogorov-Smirnov) and analyzed using ANOVA. Survivorship, measured as the number of days individuals were alive during treatment period, was compared between treatment concentrations and the controls.

For the life history experiment, the intrinsic rate of population increase, *r*, was calculated using a computer program developed by Dr. Javier Peñalosa (SUNY Buffalo State) that uses Jackknife as a measure of accuracy to sample the distribution of *r*. The equation used was:

$$\sum e^{-rx} l_x m_x = 1$$

where l_x is the proportion of individuals surviving at time x, and m_x is the mean number of eggs produced per surviving individual at time x. For *r*, positive values indicate growth in a population and negative values indicate that there are more individuals dying than being born in that population.

To analyze life history parameters measured in this study (reproduction, survivorship, molting, etc.), parametric and non-parametric statistics were used to compare *Daphnia*'s responses to the treatments. All data was tested for normality using Kolmogorov- Smirnov tests. When the data were not normal, they were transformed using (ln+1). If transformations did not improve normality, non-parametric statistics were used (Kruskal-Wallis test). The variables that were analyzed with ANOVA included total number of molts, body length, total number of neonates and total number of eggs. The variables analyzed using non-parametric tests included the intrinsic rate of population increase, *r*, and the lipid and ovary indices. For all data sets, *post-hoc* tests of pairwise multiple comparisons were performed using the Bonferroni correction at $p<0.01$ (for five comparisons) for significance.

Results

Pharmaceuticals and Personal Care Products (PPCPs) removal

Water samples were taken from the experimental effluents for each treatment and examined with a mass spectrometer to quantify PPCPs. The decrease in pharmaceuticals in the AOP treatments can be seen in Figure 1. The most prevalent PPCP in the effluent, Iopamidol, was also the most reduced by the treatments. Iopamidol is a contrast agent used for computerized tomography (CT) scans and other radiologic examinations. Figure 1 highlights the different abilities of the AOPs to decrease PPCPs. The control tap water was found to contain trace amounts of caffeine.

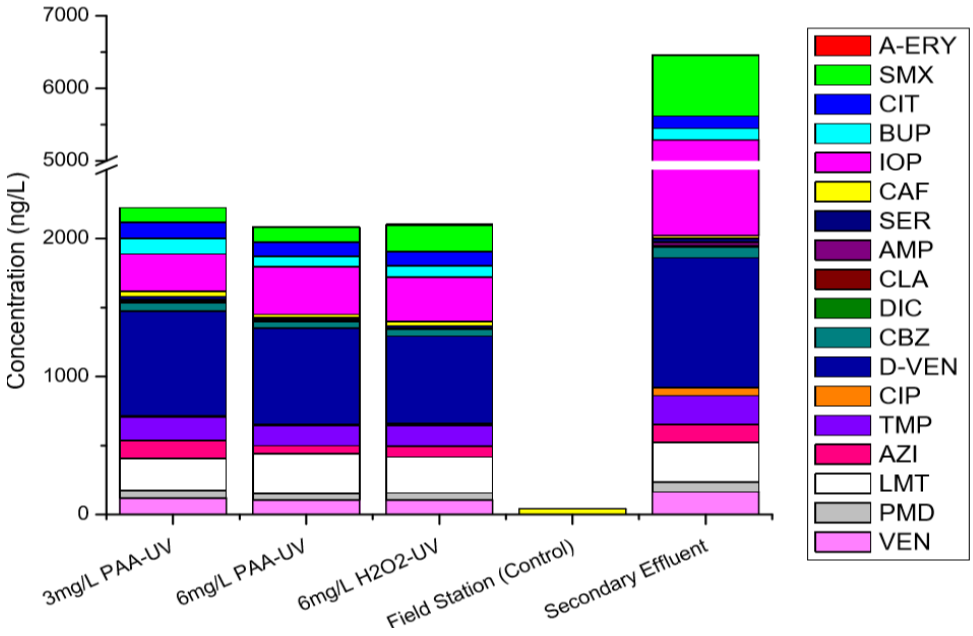

Figure 1: Mass spectrometer analysis of treatments for PPCP presence. Concentration (ng/L) of 18 targeted PPCPs. The Field Station Control reflects Buffalo's tap water. The abbreviations are A-ERY: Anhydro-Erythromycin, SMX: Sulfamethoxazole, CIT: Citalopram, BUP: Bupropion, IOP: Iopamidol, CAF: Caffeine, SER: Sertraline, AMP: Amitriptyline, CLA: Clarithromycin, DIC: Diclofenac, CBZ: Carbamazepine, D-VEN: Desvenlafaxine, CIP: Ciprofloxacin, TMP: Trimethoprim, AZI: Azithromycin, LMT: Lamotrigine, PMD: Primidone, VEN: Venlafaxine. The PPCPs categories and values are listed in Appendix A, Table 1.

Survivorship Experiment Survival Rates and Metabolomics Analysis

The survivorship of the *Daphnia* in the survivorship trials was measured as the number of days they were alive during the study period. Survival was significantly increased in the 6 mg/L PAA treatments, with *Daphnia* raised in 100% and 50% concentrations having higher survival rates (ANOVA, p= 0.00003, Figure 2, Table 3). No other treatment had significant differences in survival from the controls. In addition, in all cases there was no difference in survival between the filtered water control and the control + thiosulfate (Bonferroni, $p \geq 0.05$, Table 2).

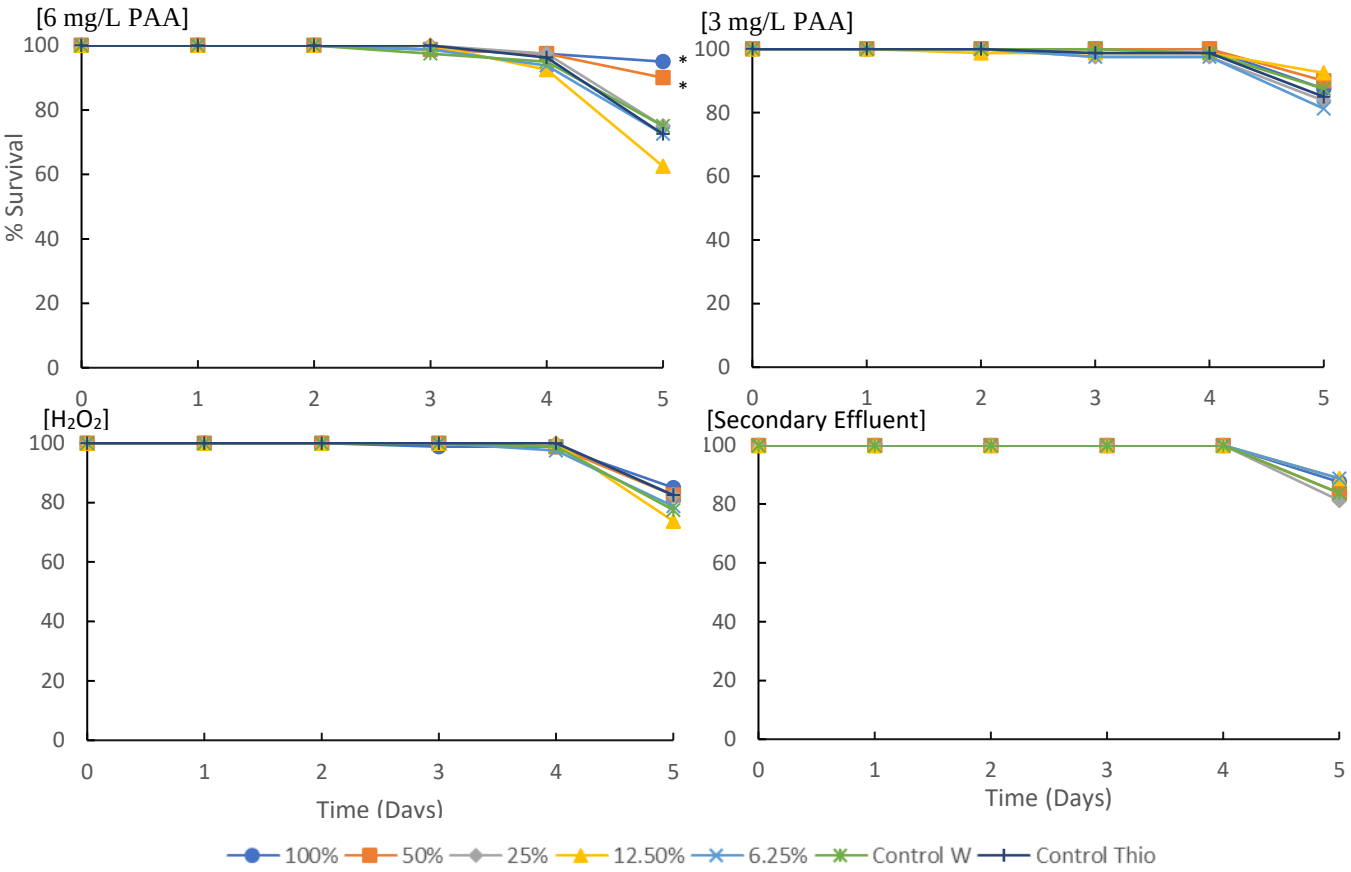

Figure 2: Percent survival of *Daphnia* in the Survivorship Experiment. Legend indicates percent concentration of each treatment, and the two controls filtered tap water (Control W) and filtered tap water plus thiosulfate (Control Thio). Asterisk indicates significant difference from Control (tap water).

Table 2: Total number of deaths at the end of 5 days for each concentration of each treatment for the survivorship experiment (mean ± SE). N=16. Each treatment was run separately and had its own controls. Asterisk denotes significance**.**

Treatment	**100%**	**50%**	**25%**	**12.5%**	**6.25%**	**Control**	**Control+ Thiosulfates**
6 mg/L PAA	0.8±.4*	1.6±.79*	4.0±.5	6.0±.7	4.4±1.0	4.0±.4	4.4±.4
3mg/L PAA	2.0±.3	1.6±.5	2.6±.7	1.2±.6	3.0±.5	2.0±.4	2.4±.7
H_2O_2	2.4±.2	2.8±.9	2.8±.7	4.2±1.1	3.4±.9	3.6±.4	2.8±.8
Secondary Effluent	2.0±.5	2.6±.5	3.0±.5	1.8±.5	1.8±.6	2.6±.7	NA

Table 3: One-way ANOVA for the average number of deaths of experimental *Daphnia* in the survivorship experiment. Factor: Water treatment

Survivorship Experiment ANOVA						
		Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**
6 mg/L PAA	Between Groups	96	6	16	8.235	3.45E-05
	Within Groups	54.4	28	1.943		
	Total	150.4	34			
3 mg/L PAA	Between Groups	11.143	6	1.857	1.171	0.350
	Within Groups	44.4	28	1.586		
	Total	55.543	34			
H_2O_2	Between Groups	11.486	6	1.914	0.632	0.703
	Within Groups	84.8	28	3.029		
	Total	96.286	34			
Secondary Effluent	Between Groups	6.3	5	1.26	0.796	0.563
	Within Groups	38	24	1.583		
	Total	44.3	29			

The metabolomics results (Figure 3) reflect the comparison of each experimental effluent against the filtered tap water control. The number of different metabolites between the control and a treatment are shown as those that are not shared in the "rose" graph. The control+thiosulfate treatment produced 56 unique metabolites, the secondary effluent treatment had 43 unique metabolites, the H_2O_2 treatment produced 32 unique metabolites and the PAA treatments produced 34 unique metabolites (3 mg/L treatment) and 38 unique metabolites (6 mg/L treatment) but they also shared 9 metabolites. The control+thiosulfate treatment produced the largest number of unique metabolites, followed by the secondary effluent. The three AOP treatments produced similar numbers of unique metabolites. Because the metabolite detection was untargeted, the identity of these metabolites is currently unknown. Further research beyond the scope of this thesis will determine the molecules involved.

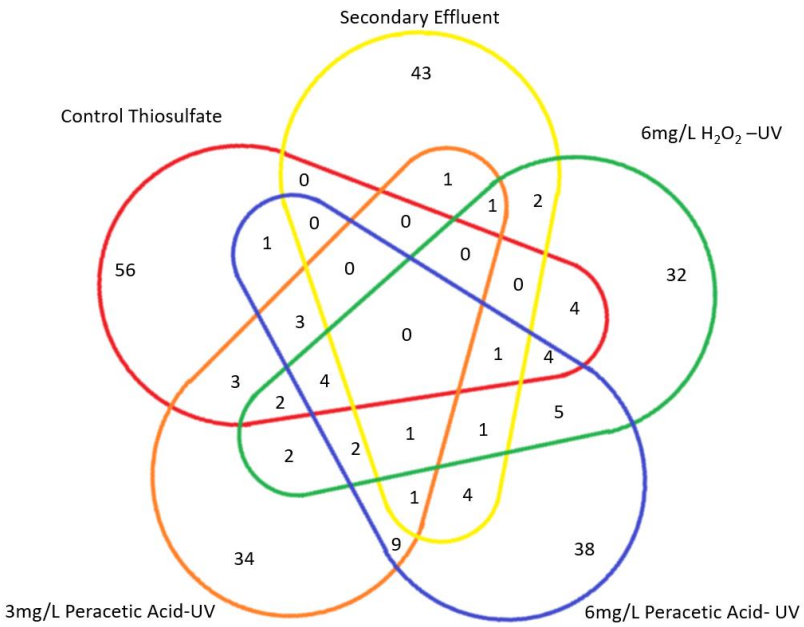

Figure 3: Rose graphic of the metabolomics of *Daphnia magna* from the survivorship experiment. Treatments are compared to the filtered tap water control. Number of shared metabolites appear in the center and unique metabolites for each treatment appear in the edges of the petals.

Daphnia *Life History Experiment*

Through the course of the 14-day life history experiment, one *Daphnia* died in each treatment including the control. As mentioned earlier, because there were no significant differences in *Daphnia* mortality rates between the tap water control and the tap water + thiosulfate control in the survivorship experiment, only the tap water control was used in this experiment. All other treatments remained the same.

Table 4: Statistical results for life history experiment. A is one-way ANOVA for the average length as well as molts, eggs and neonates produced by experimental *Daphnia*. B is Kruskal-Wallis test for the lipid and ovary indices as well as intrinsic rate of population increase, *r*, for experimental *Daphnia*. Factor: Water Treatment.

A. Life History ANOVA						
		Sum of Squares	**df**	**Mean Square**	**F**	**Sig.**
Length (mm)	Between Groups	5.896	4	1.474	2.999	.030
	Within Groups	19.656	40	.491		
	Total	25.551	44			
# Total Molts	Between Groups	9.911	4	2.478	1.533	.211
	Within Groups	64.667	40	1.617		
	Total	74.578	44			
# Total Eggs	Between Groups	346.311	4	86.578	1.142	.351
	Within Groups	3032.000	40	75.800		
	Total	3378.311	44			
# Total Neonates	Between Groups	60.089	4	15.022	2.578	.044
	Within Groups	233.111	40	5.828		
	Total	293.200	44			

B. Life History Kruskal-Wallis			
	Ovary	**Lipid**	***r***
Kruskal-Wallis H	8.204	9.731	14.255
df	4	4	4
Asymp. Sig.	.084	.045	.007

Body Length

The final body length of the *Daphnia* measured at the end of the 14-day life history experiment was significantly affected by the AOP treatment (ANOVA, p=0.03, Figure 4, Table 4). *Daphnia* raised in effluent treated with 3 mg/L PAA were significantly smaller than those raised in the control (Bonferroni method, p=0.023, Figure 4). No other treatment significantly affected body length (Figure 4), however, *Daphnia* in the control had the largest animal sizes, followed by the secondary effluent and the hydrogen peroxide treatment. Both PAA treatments had, on average, smaller animals at the end of the experiment.

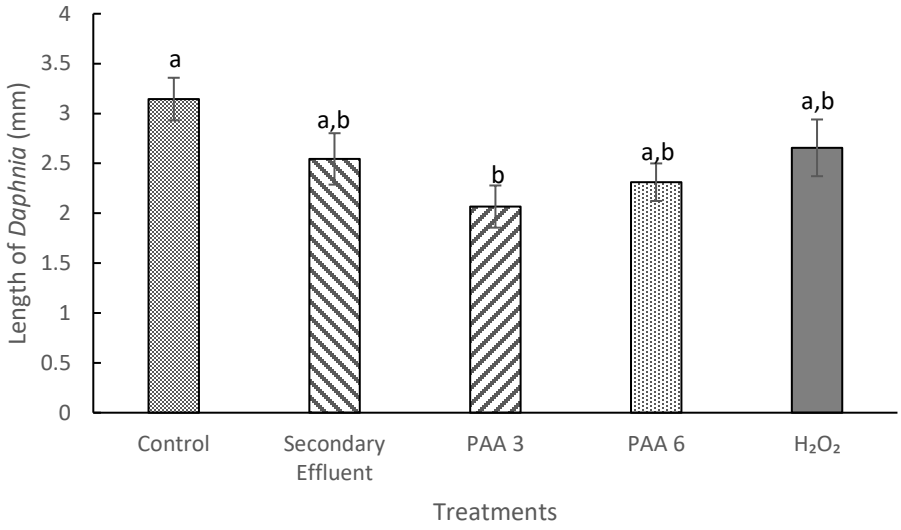

Figure 4: Body length of *Daphnia* at end of the life history experiment (Mean ± SE). Different letters above columns indicate significant differences. Control is filtered tap water, secondary effluent is non-AOP treated, PAA 3 is the 3 mg/L PAA treatment, PAA 6 is the 6 mg/L PAA treatment, H_2O_2 is the 6 mg/L H_2O_2 treatment.

Molting

The number of molts produced by *Daphnia* as they grew throughout the 14-day experiment did not differ statistically in any of the treatments (ANOVA, $p > 0.05$, Figure 5, Table 4) but, on average, the three AOP treatments seemed to induce an increase in molting events.

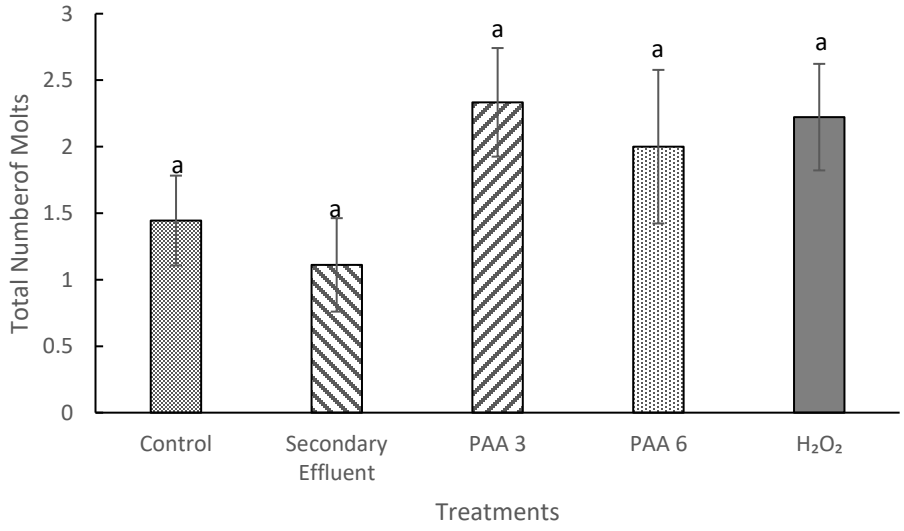

Figure 5: Total number of molts produced by *Daphnia* during the 14-day life history experiment (Mean ± SE). Same letter above columns indicates lack of statistical differences. Abbreviations as in Figure 4.

Number of Eggs in the Carapace

The number of eggs produced by *Daphnia* during the 14-day experiment did not differ significantly in any of the treatments (ANOVA, $p > 0.05$, Figure 6, Table 4), however, the 3 mg/L PAA treatment produced on average the lowest number of eggs, followed by secondary effluent, 6 mg/L PAA, H_2O_2 and the control.

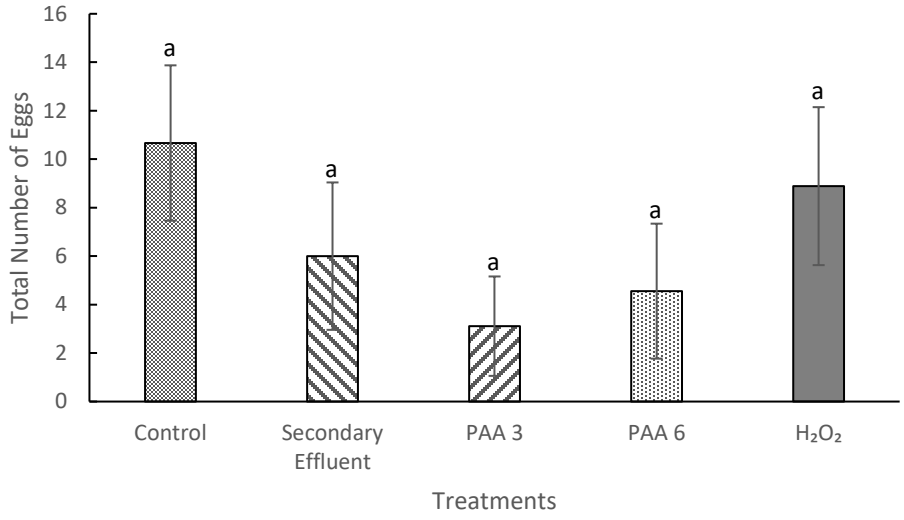

Figure 6: Total number of eggs produced by *Daphnia* during the 14-day life history experiment (Mean ± SE). Same letter above columns indicates lack of statistical differences. Abbreviations as in Figure 4.

Number of Neonates Produced

The number of neonates produced by *Daphnia* in the 14-day life history experiment was significantly different between treatments (ANOVA, p=0.04, Figure 7, Table 4). *Daphnia* raised in the secondary effluent had significantly fewer neonates (none produced in the secondary effluent) compared to those raised in the control (Bonferroni, p=0.021, Figure 7), or those raised in the H_2O_2 treatment (Bonferroni, p=0.022, Figure 7). In addition, those raised in the 6 mg/L PAA treatment produced significantly fewer neonates then the control (Bonferroni method, p=0.008, Figure 7).

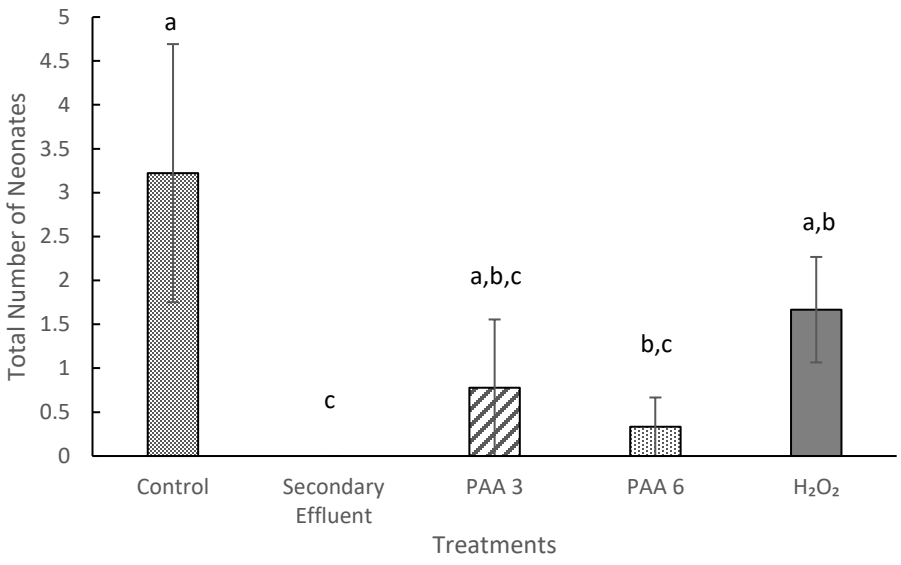

Figure 7: Total number of neonates counted during the 14-day individual exposure experiment (Mean ± SE). Different letters above columns indicate significant differences. Abbreviations as in Figure 4.

Lipid and Ovary Indices

The lipid and ovary indices were measured on individuals that survived to the end of the experiment. The ovary index did not differ significantly between the treatments (Kruskal-Wallis, $p > 0.05$, Figure 8A, Table 4). The lipid index, on the other hand, did differ significantly (Kruskal-Wallis, $p = 0.045$, Figure 8B, Table 4). *Daphnia* raised in the 3 mg/L PAA treatment had significantly less observable lipid droplets compared to the control (Bonferroni, $p = 0.049$, Figure 8).

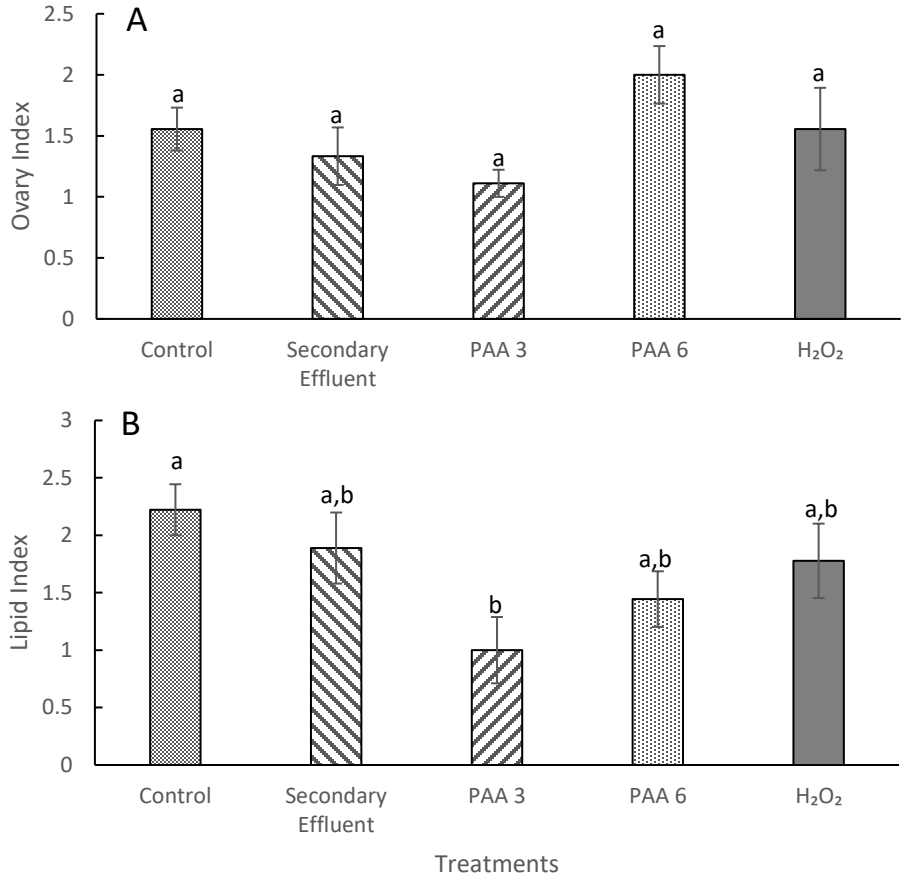

Figure 8: Ovary (A) and lipid (B) average indices' rank of individual *Daphnia* that survived the different treatments (Mean ± SE). Different letters above columns indicate significant differences. Abbreviations as in Figure 4.

Intrinsic Rate of Population Increase (r)

The intrinsic rate of population increase, *r*, was calculated using data on survivorship and number of eggs produced by the experimental *Daphnia* (for *r* based on average neonates, see Appendix B, Figure 1). This *r* did change significantly between the treatments (Kruskal-Wallis, p = 0.007, Figure 9, Table 4). *Daphnia* raised in the 3 mg/L PAA treatment had a significantly lower intrinsic rate of population increase compared to those raised in the control (Bonferroni, p = 0.01, Figure 9) as well as those raised in the H_2O_2 treatment (Bonferroni, p = 0.045, Figure 9).

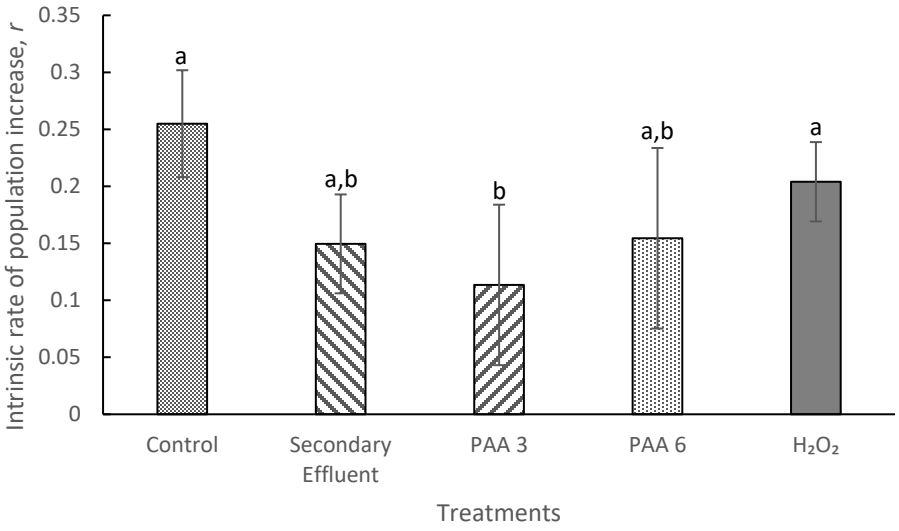

Figure 9: Intrinsic rate of population increase (*r*) calculated for each treatment (Mean ± SE) using number of eggs in the carapace. Abbreviations as in Figure 4.

Table 5: Summary of results from the life history experiment (Mean ± SE). Asterisk represents values that differed significantly from the control.

	Control	**Secondary Effluent**	**3 mg/L PAA**	**6 mg/L PAA**	**H_2O_2**
Length (mm)	3.14±0.21	2.54±0.26	2.07±0.21 *	2.31±0.19	2.66±0.28
# Molts	1.444±0.34	1.111±0.35	2.333±0.41	2±0.58	2.222±0.40
# Eggs	10.667±3.21	6±3.04	3.111±2.05	4.556±2.78	8.889±3.26
# Neonates	3.222±1.47	0±0 *	0.778±0.778	0.333±0.33*	1.667±0.60
Ovary Index	1.556±0.18	1.333±0.24	1.111±0.11	2±0.24	1.556±0.34
Lipid Index	2.222±0.22	1.889±0.31	1±0.29 *	1.444±0.24	1.778±0.32
r	0.25±0.05	0.15±0.04	0.11±0.07 *	0.15±0.08	0.20±0.03

Discussion

Daphnia are often used as a test organism to examine water quality. This is due to the fact that many physiological processes in *Daphnia*, such as reproduction and allocation of resources (lipids), are sensitive to nutrient amounts and presence of toxins. My experiments were designed to use this sensitivity to examine the effects of different AOP treatments of wastewater effluent on these organisms.

Survivorship and Metabolomics

The results of the survivorship experiments show that none of the treatments caused an increase in mortality during the five days of the test, or even during the 14-day life history experiment. The (non-AOP treated) secondary effluent served as a baseline to compare if the AOP treatments improved the quality of the effluent for *Daphnia* and similar zooplankton. One would expect the secondary effluent to have the most negative impact given the highest presence of PPCPs and other dissolved pollutants. However, results from the life history experiment indicated that the treatment of 3 mg/L PAA negatively impacted the quality of the effluent for *Daphnia*, followed by the 6 mg/L PAA, which had impacts as negative as those of the secondary effluent. The other AOP treatment, H_2O_2, showed no detrimental effects in the life history responses of *Daphnia*.

The number of unique metabolites (compared to the filtered tap water control) expressed by *Daphnia* exposed to the various treatments, indicates that they expressed a lower number of them in the AOP treatments than in the secondary effluent. Thus, the reduction of some of the PPCPs through the AOP processes resulted in *Daphnia* producing less unique metabolites, which

could be interpreted as a positive effect of the AOP treatments (except for the impact of the AOPs on *Daphnia*'s population growth). Interestingly, the *Daphnia* in the control + thiosulfate produced the highest number of unique metabolites, even higher than in the secondary effluent, which had high levels of PPCPs. The explanation for this result may be in the fact that the thiosulfate in the filtered control water did not react with organic and chemical compounds as it did in the other treatments, thus more of the chemical activity was present. Although changes in pH have been reported by the addition of thiosulfates to water, in this study the pH in all the treatments and the control were neutral (Table 1) (Basu et al., 2011).

Growth

Molting is an essential process for growth in *Daphnia*. The shedding and regrowth of the carapace is an energy intensive process that allows for body growth and repair. In this experiment, *Daphnia* in the AOP treatments tended to have more frequent molting than those in the control or the secondary effluent. This strategy, which *Daphnia* exhibits when under stress (Pérez-Fuentetaja et al., 2016), impacts the energy supplies of the animals and, ultimately, their ability to reproduce. Therefore, the AOP treatments had a tendency to induce increased molting and the extra energy spent in somatic maintenance, such as building the carapace, could have taxed their ability to contribute to population growth.

The adult *Daphnia* at the conclusion of the experiment was significantly smaller in the 3 mg/L PAA treatment (mean = 2.07 mm) than in the control (mean = 3.14 mm). Previous research has shown that the larger the *Daphnia* is, the higher the cost of molting (Hessen and Rukke,, 2000). Therefore, the molts produced by the smaller *Daphnia* in the 3 mg/L PAA took less

energy compared to the larger *Daphnia* in the controls. One explanation could be that *Daphnia* raised in the 3 mg/L PAA treatment were either impaired for energy uptake or had to redirect energy that would be used for growth and ovary development to more survival-based processes, such as detoxification and somatic maintenance. Studies have shown that the higher the initial concentration of peracetic acid, the more effective it is at breaking down PPCPs and the less residual peracetic acid is present in the resulting effluent (Rizzo et al., 2018). A second explanation could be that higher levels of residual peracetic acid in the 3 mg/L PAA treatment could have impaired *Daphnia*'s growth and cause more stress in these animals compared to those in the other treatments. Yet, another explanation could involve the level of OH radicals present. OH radicals are generated through the AOP treatment process and are responsible for the breakdown of PPCPs. Effluents containing OH radicals can potentially enhance sublethal toxicity by disturbing *Daphnia*'s natural antioxidant protection (Oropesa et al., 2017). AOP treatments tend to use thiosulfates to remove any excess hydroxyl radicals before the release of the effluent (Basu et al., 2011, Luukkonenm et al., 2014). However, if residual peracetic acid was present, it is possible for the residue to create unquenched OH radicals that could have damaged *Daphnia*. *Daphnia* from both the 3 and 6 mg/L PAA treatments tended to be smaller than those in the secondary effluent or the H_2O_2 treatment, which indicate that some aspect of the peracetic acid treatments was interfering with growth.

The overall negative effect of the 3 mg/L PAA treatment can be further seen in the results from the lipid index. The lipid index ranks how many resources an individual has stored within the body. There were significantly fewer observable lipids in the *Daphnia* raised in the 3 mg/L PAA treatment compared to the control *Daphnia,* despite food quality and quantity being equal among treatments. Additionally, the amount of lipids in the 3 mg/L PAA was also lower than in

the *Daphnia* raised in the 6 mg/L PAA treatment, though not significantly. Lipids are the most susceptible molecules to be damaged by oxygen radicals, which cause deterioration of the lipid and damage to systems, so the low levels of lipids could be due to exposure to unquenched oxygen reactive molecules (Oropesa et al., 2017). Whichever the molecular cause, *Daphnia* in the 3 mg/L PAA treatment was not able to accumulate enough energy as lipid droplets, which affected their growth (Riessen et al., 2012).

Reproduction

Although *Daphnia* suffered reproductive disadvantages across the AOP treatments compared to the control, those raised in the secondary effluent did not produce any neonates. The presence of eggs but no neonates in the secondary effluent treatment could be explained as a response to metabolic stress due to the presence of high levels of PPCPs (Figure 1) and other contaminants in the water. Although these stressed animals started to produce eggs, these eggs might not have been supplied with sufficient resources for their full development into neonates, or the eggs may have been killed in the brood chamber (Baird et al., 1991). Alternatively, they might have been reabsorbed by the parent to conserve resources and energy (Ebert, 1992) as the environment was inauspicious. This pattern was also seen in the 3 and 6 mg/L PAA treatments, but in these two treatments rates of 0.7 and 0.3 neonates (respectively) were produced. Therefore, the interplay of lower dissolved PPCPs and chemical interference from the peracetic acid resulted in decreased neonate production, but above non-AOP treated secondary effluent.

Daphnia in the 6 mg/L PAA treatment tended to perform better than those in the 3 mg/L PAA treatment in all categories except for neonate production. This would indicate that any

negative byproduct from the 3 mg/L PAA treatment could still be present in the 6 mg/L PAA treatment, but at much lower levels. In contrast, *Daphnia* in the control and in the H_2O_2 treatments were the only ones who showed little to no egg reabsorption or loss. In fact, the levels of eggs and neonates in the H_2O_2 treatment were the closest to the control (1.6 and 3.2 neonates, respectively), indicating that that the H_2O_2 treatment was the least stressful for *Daphnia*.

Various other strategies are available to *Daphnia* to overcome stressful situations. *Daphnia* from the 3 mg/L PAA treatment showed signs of stress, such as small size, high molting rates, and the lowest lipid and ovary indices but, despite their disadvantageous condition, they still focused their efforts on reproduction. In order to gain a minimum body size to support reproduction, these *Daphnia* may have allocated more energy to attain the necessary growth and used their energy reserves to support reproduction (Pérez-Fuentetaja et al., 2016). The strain of producing eggs at a small body size can also explain the lower amount of lipids present in the 3 mg/L PAA *Daphnia*. The ovary index was not substantially different among treatments, indicating that despite the stresses of their environments, reproduction was a clear priority for energy allocation.

The development of eggs and neonates can further be analyzed by considering how much each individual *Daphnia* was contributing to the growth of the overall population. This can be done through the examination of life tables and the value of *r*, the intrinsic rate of population growth. In this experiment, the AOP treatments did cause a significant change in *r*, both when calculated using the number of eggs present in the carapace and when using the number of born neonates (see Appendix B for those results). For the calculation using the number of eggs, the 3 mg/L PAA treatment produced *Daphnia* that had a significantly lower *r* ($r = 0.11$) compared to the *Daphnia* in the control ($r = 0.25$) and in the H_2O_2 ($r = 0.20$) treatment, and was non-

significantly lower than the secondary effluent (r = 0.15). This last piece of information indicates that if effluent is treated with 3 mg/L PAA and discharged into a natural body of water, it could have population-wide impacts by lowering the ability of aquatic crustaceans at the lower rungs of the food web to reproduce, potentially reducing their ability to rebound from population crashes and heavy predation. While the population would still be able to slowly increase, the individuals would be smaller and in worse condition. This outcome was also seen in other treatments to some extent, as the secondary effluent, 6 mg/L PAA and H_2O_2 treatments all had *r* values lower than the control, though not significantly (see also results using born neonates, as many eggs did not produce neonates, Appendix B).

Therefore, a 3 mg/L peracetic acid/UV treatment of wastewater-processed secondary effluent is not suitable for crustacean populations as it causes chronic toxicity in *Daphnia magna* and it impacts their reproduction negatively.

Conclusion

While the effects of PPCPs in sewage effluent can have a variety of negative effects including reduced reproduction, it is important to make sure that the fix is not worse than the problem. The growth and reproduction of *Daphnia* can be affected by many different factors and a tradeoff between growth and reproduction exists under natural stressful conditions (Tessier et al., 2000). In wastewater secondary effluent treated with 3 mg/L PAA, *Daphnia* had a lower reproductive output and a low intrinsic rate of population growth (*r*) compared to the control, which would result in reduced increases in population size. Were further stresses to be applied to

these *Daphnia*, such as low food or nutrients, the offspring may not have the reserves necessary to survive. This could have disastrous effects on the local food web as *Daphnia* make up the basal level of many freshwater ecosystems.

The reduced body size and lipid content of *Daphnia* under stressful conditions could have further consequences when considering whole ecosystems. The small size would be vulnerable to higher rates of predation from copepods, while the low lipid content would decrease their quality as food for larval and planktivorous fish (Voronov et al., 2008). As *Daphnia* species make up the lower links in the aquatic food web, a change in quality and size of these cladocerans could have reverberating effects across the whole food web. In addition, the low population growth rate could lead to *Daphnia* becoming extirpated from certain areas, shifting the base of the food web to copepods (Voronov et al., 2008), with the possibility of completely restructuring species composition in that area.

Daphnia raised in the H_2O_2 treatment, on the other hand, exhibited life history responses that were most similar to the control. These results indicate that H_2O_2, while already known to be one of the best AOP treatments in terms of eliminating PPCPs (Wols et al., 2013), may also have the least negative impacts on aquatic life.

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Appendix A

Table 1. Full name of PPCPs listed in Figure 1 as well as Concentration in ng/L (parts per trillion) in the different treatments.

	Name	Function	3 mg/L PAA-UV	6 mg/L PAA-UV	6 mg/L H2O2-UV	Field Station (Control)	Secondary Effluent
VEN	Venlafaxine	Antidepressant	119.40	104.86	105.22	0	164.31
PMD	Primidone	Anticonvulsant	52.20	50.08	51.78	0	70.30
LMT	Lamotrigine	Anticonvulsant	234.19	284.12	260.41	0	288.04
AZI	Azithromycin	Antibiotic	131.21	57.43	78.52	0	129.21
TMP	Trimethoprim	Antibiotic	169.19	149.85	150.25	0	209.34
CIP	Ciprofloxacin	Antibiotic	10.30	9.07	12.66	0	56.36
DES	Desvenlafaxine	Antidepressant	757.36	696.45	632.99	0	944.33
CBZ	Carbamazepine	Anticonvulsant	60.76	50.96	48.60	0	76.26
DIC	Diclofenac	Anti-inflammatory	0.00	0.00	0.00	0	5.72
CLA	Clarithromycin	Antibiotic	7.21	2.69	4.52	0	7.56
AMI	Amitriptyline	Antidepressant	15.46	8.82	10.18	0	22.53
SER	Sertraline	Antidepressant	23.74	10.96	13.07	0	27.63
CAF	Caffeine	Stimulant	35.83	24.41	30.80	40.81	22.09
IOP	Iopamidol	Contrast agent	273.09	345.93	320.86	0	3264.02
BUP	Bupropion	Antidepressant	111.04	76.44	83.86	0	160.63
CIT	Citalopram	Antidepressant	117.03	101.56	99.93	0	167.43
SMX	Sulfamethoxazole	Antibiotic	106.90	109.56	193.83	0	836.42
A-ERY	Anhydro-Erythromycin	Reagent	0.00	0.00	4.63	0	6.18

Appendix B

Intrinsic rate of population growth, *r*, calculated using neonates instead of eggs. This *r* did change significantly between the treatments (Kruskal-Wallis, p = 0.00, Figure 1). *Daphnia* raised in the 3 mg/L PAA treatment had a significantly lower intrinsic rate of population increase compared to those raised in the control (Bonferroni, p = 0.015, Figure 1). *Daphnia* in the 6 mg/L PAA treatment had a significantly smaller *r* than those raised in the control (Bonferroni, p = 0.000, Figure 1) as well as those raised in the H_2O_2 treatment (Bonferroni, p = 0.000, Figure 1). The neonate production was more variable between individuals, resulting in large error bars. These results indicate that the 3 mg/L PAA and 6 mg/L PAA treatment populations would crash without outside pressure, given time.

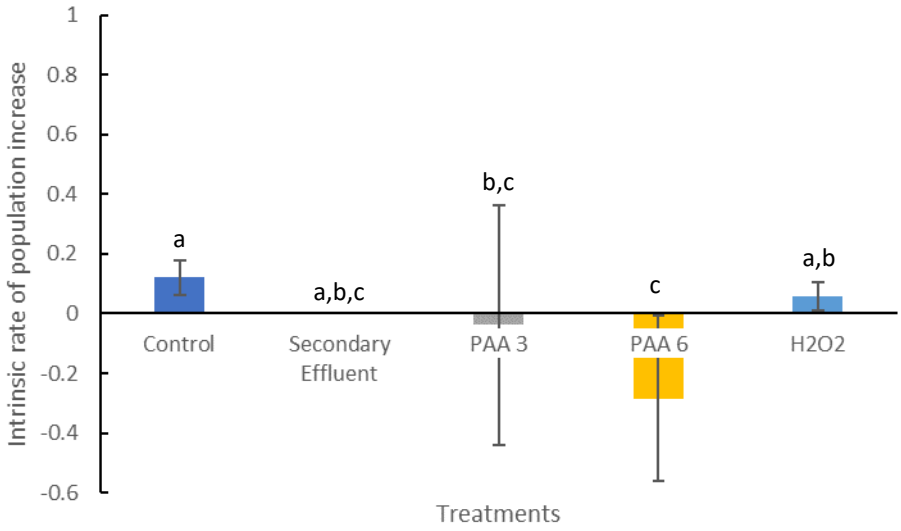

Figure 1: Intrinsic Rate of Population Increase (*r*) calculated using number of neonates produced for each treatment (Mean ± SE). Abbreviations as in Figure 4.