A messy situation: effects of treated human wastewater on aquatic biota

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Abstract

Removal of personal care products (PCPs), pharmaceuticals and other substances from human wastewater is often unsuccessful and released back in to receiving water. This includes anti-psychotics and cosmetic additives, and inorganic nitrogen. These substances are capable of producing unintended biological changes in aquatic biota located downstream from wastewater treatment plants. The present study examined whether wastewater would cause adverse effects in aquatic biota. Medaka (*Oryzias latipes)* and the zooplankton (*Daphnia magna)* were used in a controlled laboratory study. Medaka and Daphnia were exposed to 0 %, 50 % and 90 % wastewater. Mortality, development, and reproduction (only in Daphnia) were observed. Daphnia were exposed as neonates and the medaka were exposed as eggs and hatched larvae. There was a significant beneficial effect of wastewater on Daphnia mortality, growth and reproduction ($p < 0.001$). While in contrast, wastewater had a significant negative effect on the medaka rate of hatching and embryo mortality ($p < 0.05$). These outcomes suggest that there are ecologically important changes occurring as a result of the release of wastewater into natural water bodies. In addition, this study also iterates the significance of using multiple organisms in environmental toxicological studies.

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Introduction

The accumulation of wastewater is an unavoidable consequence of growing populations within developed communities. Most cities have combined sewer systems that are designed to collect both wastewater and general run-off that is then treated within conventional wastewater treatment plants (WWTPs) (Canadian Council of Ministers of the Environment, 2006; City of Winnipeg, August 2018). Water treatment occurs over three steps in which large debris, suspended materials, and elemental contaminants are removed (City of Winnipeg, February 2018). However, with increasing rates of drug-use in urban areas and fertilizer-use within agriculture lands, it is becoming more difficult to treat wastewater to ensure 'clean' effluent (Joss, *et al.,* 2005; Yamashita and Yamamota-Ikemoto, 2014; Karelid, *et al.,* 2017). For example, current methods of wastewater clarification may not be sufficient to remove harmful contaminants (Radjenovic *et al.,* 2007). With many regions using treated wastewater as a source of drinking water, the quality of treated wastewater is of concern (Joss, *et al.,* 2005). In addition, when there are conditions for high volumes of wastewater (i.e., heavy rain fall events), the combined sewer systems are designed to discharge untreated wastewater into local water bodies to avoid backflow at the WWTP (City of Winnipeg, August 2018).

An emerging concern of municipal wastewater is the volume discharged into receiving waters (Bernet, *et al.,* 2000; Joss, *et al.,* 2005; "Municipal wastewater effluent in Canada", 2006; Kocbas, *et al.,* 2015; Karelid, *et al.,* 2017). It is estimated that over three trillion litres of treated wastewater are discharged into Canadian surface waters annually (Canadian Council of Ministers of the Environment, 2006). The level to which the water is treated varies from none to fully treated (Canadian Council of Ministers of

the Environment, 2006; Kocbas, *et al.,* 2015). Treated wastewater may have high levels of nitrogen and phosphorus, which can have a cascade of effects on local aquatic ecosystems (Yamashita and Yamamota-Ikemoto, 2014; City of Winnipeg, August 2018). Other contaminants may also be present including an array of pharmaceuticals and personal-care products (PCPs) (Joss, *et al.,* 2005; Muir, *et al.,* 2017 Simmons, *et al.,* 2017). Though, Health Canada works closely with the World Health Organization and other organizations, such as the United States Environmental Protection Agency, to establish guidelines and laws for the quality of effluent water to protect Canadians (Government of Canada, 2018).

Pharmaceuticals and PCPs are designed to produce specific biological changes in humans and it is possible that these will cause similar effects in aquatic environments (Bernet, *et al.,* 2000; Joss, *et al.,* 2005; Escapa, *et al.,* 2016). For example, remnants of pharmaceuticals and PCPs have been found worldwide within treated effluent (Movahedian, *et al.,* 2005; Quinn *et al.,* 2008; Kocbas, *et al.,* 2015; Ryeo-Ok *et al.,* 2017). These contaminants appear in small concentrations often as low ng/L in effluents, and were generally considered not to be acutely toxic to aquatic biota (Quinn *et al.,* 2008). However, recent studies have demonstrated that treated effluent can produce adverse effects in aquatic biota. A 2017 study completed by Simmons, *et al.* (2017), showed that fish caged downstream from WWTPs experienced metabolic changes. It was found that there were significant changes in the fish's ability to metabolize fatty acid proteins, in addition to other metabolites (Simmons, *et al.,* 2017). This finding was further confirmed by Muir, *et al.,* 2017 who found bioaccumulation of 15 various PCPs within fish blood plasma. Overall, it appears that effluent with remnants of

pharmaceuticals and PCPs may have negative consequences for aquatic biota. However, there is still a knowledge gap.

In this study, two model freshwater organisms were used to examine the effects of treated wastewater on organismal biology. *Daphnia magna* (Daphnia) were used as an invertebrate model, and medaka (*Oryzias latipes*) as a freshwater fish model. Daphnia have been used in literature as standards for evaluating the toxicity of municipal effluents and their use has become endorsed by national organizations (Persoone, *et al.,* 2009). Daphnia are small fresh water, filter-feeding, zooplankton that reproduce asexually via parthenogenesis (TheEbertGroup, 2013). Wastewater may produce adverse effects on Daphnia life history- e.g., several studies have been able to identify a 50 % lethal concentration (LC_{50}) for Daphnia exposed to treated effluent ranging from 29 % to 85 % (Movahedian, *et al.,* 2005; Kocbas, *et al.,* 2015). In addition, slowed growth and reproduction have also been seen in wastewater exposed Daphnia (Cao, *et al.,* 2009; Movahedian, *et al.,* 2005; Kocbas, *et al.,* 2015), and thus they are a useful model for investigating the toxicity of local treated effluent. Medaka are also sensitive to environmental contaminants especially in early stages of development (Zha and Wang, 2006). Medaka are small freshwater, visual predators that feed on zooplankton (Chen, *et al.,* 2016). The toxic effects of wastewater on medaka have been well documented in literature. Chen, *et al.* (2016) found that medaka fish exposed to treated effluent showed increased expression of genes associated with drug metabolism. In addition, other studies have found wastewater effluent to cause changes in the rate of hatching and growth (Zha and Wang, 2006), and reduced reproductive success of medaka (Ma, *et al.,* 2005). Because these two organisms are native to freshwater, have many well-studied life

history traits, and have well established protocol, they will be useful models within the present study.

The objective of this study was to examine whether local wastewater would cause adverse effects in medaka and Daphnia. A controlled laboratory study where mortality, growth, reproduction (only in Daphnia) and development (only in medaka) could be observed was conducted. It was expected that Daphnia exposed to wastewater would show adverse effects including increased mortality, reduced size and reduced reproductive activity. Similar results were also expected in the medaka, with increased mortality, reduced size, and delayed or improper development. These hypotheses were based on trends seen in previous studies, where Daphnia and medaka showed negative effects from wastewater exposure.

Materials and Methods

Collection and storage of treated wastewater

Wastewater was collected from UV treated, filtrate ponds at the City of Winnipeg West End Sewage Treatment Plant, site C, mid- October, 2018. Site C marks the final point at which treated wastewater is tested before it is released back into receiving waters. The wastewater was stored at 7 °C and was filtered twice using linen cotton sheets to remove solid contaminants before being used in assays. The presence of pharmaceutical contaminants was determined using mass spectrometry. The presence of non-pharmaceuticals and inorganic contaminants were not quantified for this study.

Animal husbandry

Protocols for care and maintenance of *Daphnia magna* (Daphnia) were adopted from previous work completed at the University of Winnipeg (Sorokopud-Jones, 2017). Neonates (Daphnia < 24 h) were obtained from stock Daphnia reared in 4-gallon tanks of Artificial Daphnia Medium (ADaM) (Appendix I). The stock tanks were kept on a 16 h light: 8 h dark cycle using daylight fluorescent bulbs, and water temperature was maintained between 22-24 ˚C. The stock was fed *Chlorella vulgaris* (*C. vulgaris*) suspension (Appendix II), and were transferred to new tanks as needed due to *C. vulgaris* accumulation. Individual Daphnia were fed approximately five million cells of *C. vulgaris* every day. The feeding regimen ensured the survival and reproduction of Daphnia, while in keeping with a routine maintained for generations of Daphnia. All experiments were completed under similar conditions.

Protocols for the care of medaka were also adopted from previous work completed at the University of Winnipeg (Sorokopud-Jones, 2017), and were completed

in in compliance with the animal care committee (Protocol #12415). Medaka eggs (<24 h) were obtained from stock medaka, which were reared in 2 gallon tanks of water treated for the removal of chlorine (aquatic water). The stock tanks were also kept on a 14 h light: 10 h dark cycle, and the water temperature was maintained 27-30 ˚C. The stock medaka were fed Zeigler Adult Zebrafish food which is prepared with Spirolina, Cyclop-Eeze® and Golden Pearls larval diet (5-50 Microns). Stock tanks were maintained in a Aquaneering© Zebrafish housing system with fresh, constantly circulating aquatic water. 20 % of the water in the aquarium system is changed out with fresh aquatic water every day and is then circulated through the stock tank housing system. Experiments followed the same variables for temperature and light. The feeding protocol for the present study followed the University of Winnipeg's protocol on feeding medaka larvae, where larvae are fed a combination of live paramecium and larval diet four times daily.

Experiment 1: Daphnia exposure to treated wastewater

To collect neonates to be used in the study, a matward was set up with 30 adult Daphnia. The purpose of the matward was to breed Daphnia for the study, to ensure that all neonates selected were of the same age. The Daphnia were collected from stock tanks and placed individually in 80 mL vials containing ADaM. The matward was fed approximately 7 million cells of *C. vulgaris* per day to promote reproduction. The number of broods produced by each individual was logged and the neonates were removed daily. Neonates from the second to fifth brood were used in the current study, and randomly assigned to their study treatment.

Neonates in individual 80 mL vials were exposed to one of five conditions: 0 % wastewater (100 % ADaM control), 50 % wastewater and 50 % ADaM, 90 % wastewater and 10 % ADaM , 50 % artificial pondwater and 50 % ADaM, and 90 % artificial pondwater and 10 % ADaM. The artificial pondwater (Appendix III) served as a control to ensure any adverse effects seen in wastewater treatments could be attributed to wastewater exposure and were not due to the reduction of ADaM media. Every three days individuals were transferred to new vials with clean medium, and their assigned wastewater or pondwater exposure concentration. Each trial lasted 21d. There were 10 replicates per treatment and two trials were completed.

Over the course of the trials, Daphnia lengths were measured every three days. Five individuals were chosen at random from each experimental condition. Individuals were removed from their vials with a pipette and placed onto a petri dish. Excess fluid was removed and a flatbed scanner was used to measure length using Delphi software (Embacadero RAD Studio XE2). The length was measured from the eye of the Daphnia to the caudal curve of their digestive tract. The five random individuals were then placed back into their respective vials. Mortality and reproductive activity (i.e., number of neonates produced) were also observed over the course of the trials. Mortality was determined by the lack of activity and non-avoidance of the pipette. In addition, the exoskeleton would appear opaque rather than translucent. The number of neonates produced was determined by the number of neonate individuals within the vial on the day of observation. Neonates were counted and removed every day.

Experiement 2: Exposure of medaka eggs and larvae to wastewater

Medaka eggs (<24 hours post-fertilization) were put into petri dishes of E2 embryonic development medium (prepared by U of W animal care technicians) (Appendix IV) with the exposure conditions of 0 %, 50 % or 90 % wastewater. The 0 % concentration was assigned 27 eggs, the 50 % concentration was assigned 32 eggs, and the 90 % concentration was assigned 33 eggs. The difference in egg sample sizes among the treatments was due to egg mortality at the time of harvesting. The medaka eggs were monitored daily during incubation (approximately over a 10 d period), and the hatching rate was calculated. Eggs were incubated at 28 ˚C until hatching, and a daily 50 % media change was completed. To determine that the medaka eggs were dead, several methods were used depending on the developmental stage of the egg. For medaka eggs stages < 21 (up to ~ 35 h) (Appendix V), dead eggs would appear white and opaque (Iwamatsu, 2004). For eggs development stages > 22 (up to ~ 8 d), death was determined by the lack of a heartbeat, and the egg would also become opaque (Iwamatsu, 2004).

Larval medaka were observed following egg hatching (for a total 15 d experimental period). The larvae were exposed to the same concentrations of wastewater and aquatic water that they were treated with during the egg phase. Larvae were held in 2 L tanks and the temperature was maintained at 28 °C. To reduce the accumulation of nitrates and nitrites, 50 % of the water in each tank was changed every 2 d. Following the 15 d period, the larvae were viewed under a dissecting microscope and then euthanized with MS-222 (Appendix VI). Parameters measured included mortality, total growth, morphological deformities, and startle response. The startle response for the larvae was recorded by tapping the petri dish containing larvae and monitoring the

response of the larvae using an overhead camera (Colwill and Creton, 2011). The overhead camera was also used to identify presence of morphological deformities (i.e., no swim bladder inflation and spinal deformities) (Iwamatsu, 2004). Larvae total growth was recorded by placing the larvae cadavers on a petri plate, and utilizing a flatbed scanner and Delphi software (Embacadero RAD Studio XE2).

Statistical Analysis

All statistical analyses were completed using R (R Core Team, 2018; Wickham, 2017; Bates, 2015; Kuznetsova, 2017) and tested at the significance level $\alpha = 0.05$. Daphnia growth reproduction and mortality data were analyzed using ANOVAs. The medaka egg and larval mortality, and hatching rate were also analyzed using ANOVAs. Startle response and swim bladder presence were analyzed using binomial logistic regression models, and total overall growth of medaka larvae was analyzed using linear regression.

Results

Experiment 1: Daphnia exposure to treated wastewater

One trial was terminated prior to completion due to unusually high mortality within one control group (0 % - 100 % ADaM). In both trials, Daphnia growth and reproduction were greater in wastewater treatments than in controls. Daphnia exposed to wastewater were consistently larger in average size ($p < 0.001$; Figure 1A and 1B; Appendix VII). Daphnia exposed to treated wastewater were on average 13 % (0.35 mm ± 0.04, n=20) larger than control treatments at the end of the trial. Daphnia exposed to pondwater treatments in trial #1 also grew larger than controls ($p < 0.01$; Figure 1A).

Daphnia exposed to wastewater treatments were found to have a higher mean number of neonates produced per day per individual for trial 1 ($p < 0.001$; Figure 2; VIII). Daphnia exposed to 90 % wastewater had significantly higher reproductive activity than controls in both trials ($p < 0.001$); 50 % pondwater exposed daphnia also showed improved reproductive activity in both trials ($p= 0.002$, $p=0.005$). Neonates produced in wastewater treatments also appeared healthier and larger.

The wastewater exposed Daphnia had similar survival to controls and pond water treatments ($p > 0.1$; Figure 3A and 3B; Appendix IX). Overall, there was no significant difference in the survival among treatments. However, 50 % wastewater exposed Daphnia in trial #1 had better survival than other treatments ($p=0.334$; Figure 3A). Lab data showed that wastewater exposed Daphnia did have a ~30 % greater survival than the controls, however this not statistically significant.

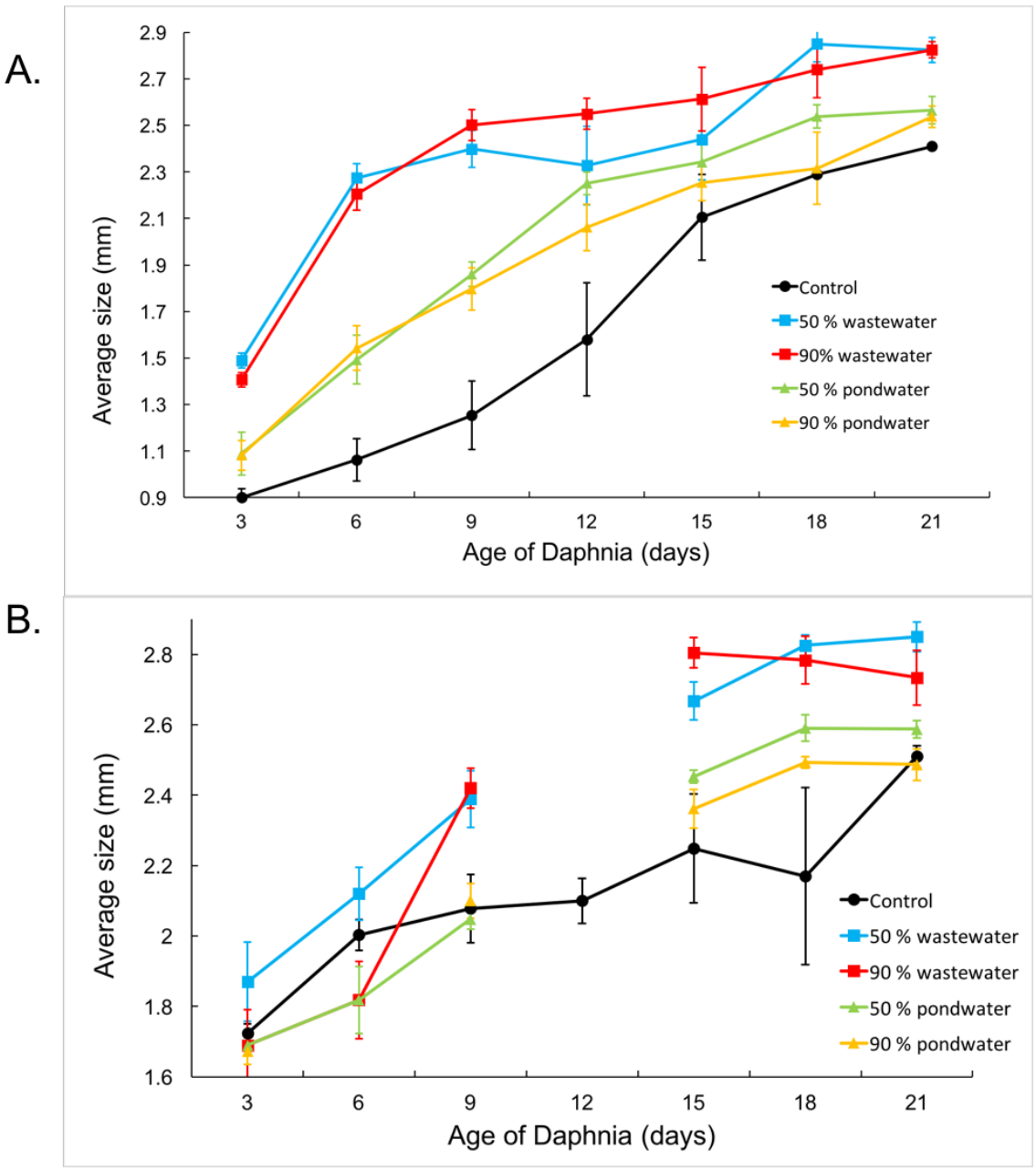

Figure 1. The average size of Daphnia for trials # 1 (A) and # 2 (B) over 21 d when exposed to wastewater treatments (controls, 50 % and 90 %) and pondwater treatments (50 % and 90 %). Five randomly selected Daphnia from each treatment were scanned and measured every three days (using Embacadero RAD Studio XE2 Delphi software). Measurements of the Daphnia were taken in millimeters (mm), and the average size was calculated for each treatment. Standard error was also calculated for both trials, which is represented by error bars. The data gap for trial # 2 day 12 is due to loss of data.

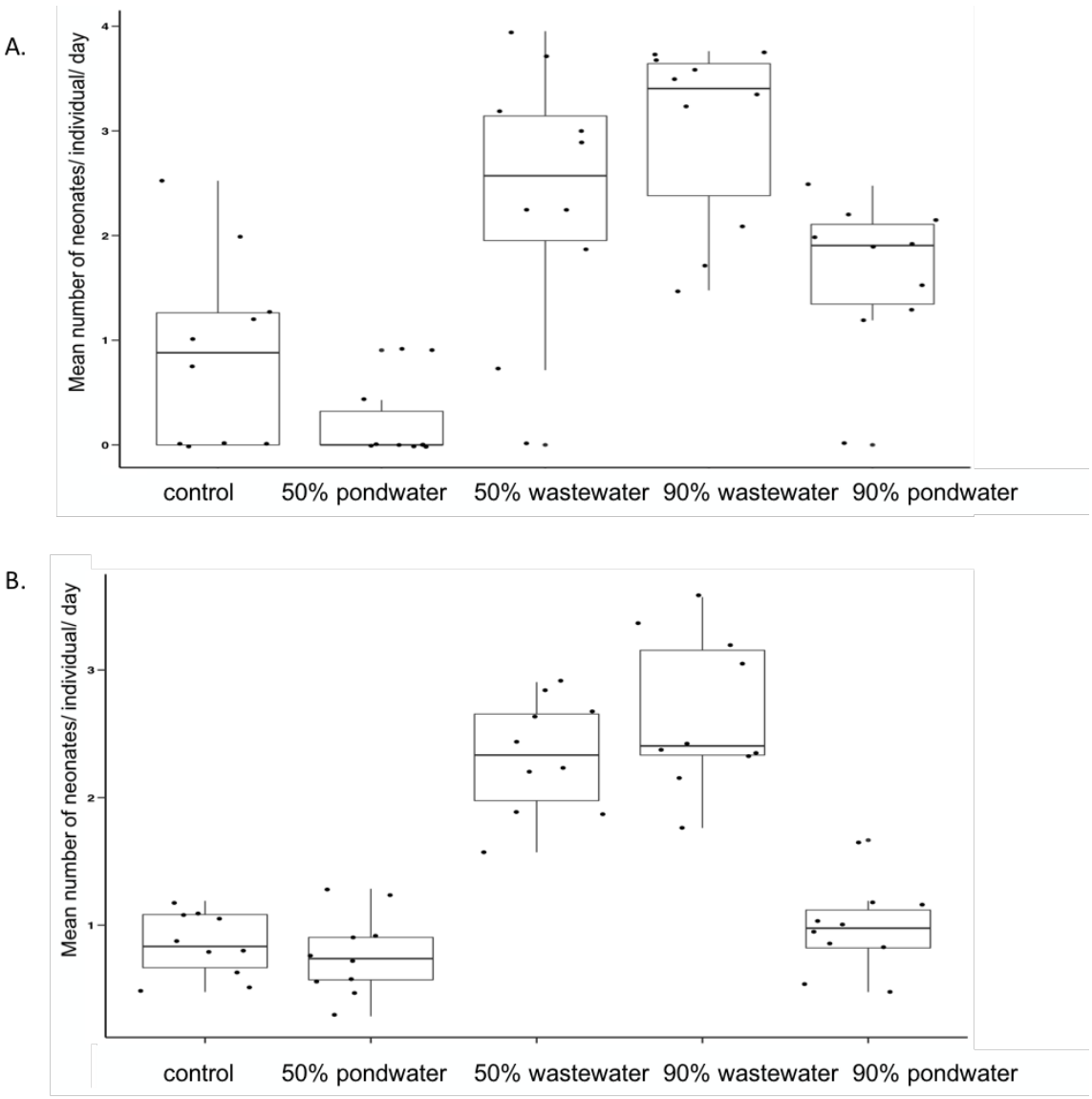

Figure 2. The mean number of neonate Daphnia produced per day per individual for trial # 1 (A) and trial # 2 (B). The Daphnia were exposed to wastewater treatments (controls, 50 % and 90 %) and pondwater treatments (50 % and 90 %). The neonates were collected every 24 h over the course of the trial. The center line of each box represents the mean number of neonates produced per day per individual. The upper and lower lines on the box represent the upper and lower 25 % quartile. Standard deviation was also calculated for each treatment, and is represented by the upper and lower box whiskers. The mean number of neonates produced per day per individual was plotted using Rstudio®.

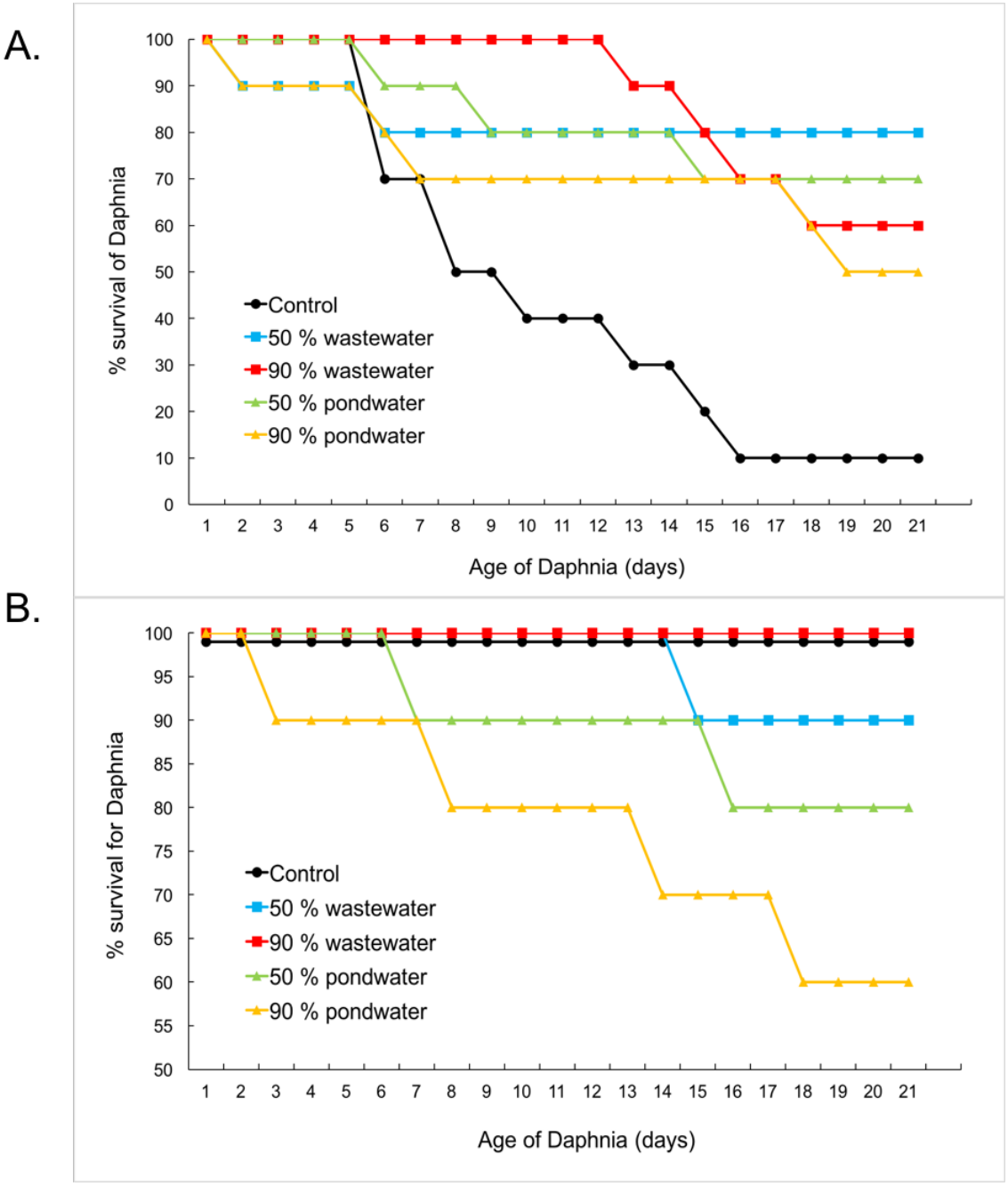

Figure 3. The percent survival of Daphnia for trials # 1 (A) and # 2 (B), over 21 days when exposed to wastewater treatments (controls, 50 % and 90 %) and pondwater treatments (50 % and 90 %). Daphnia mortalities were tracked for each treatment, and the % survival was calculated.

2: Exposure of medaka eggs and larvae to wastewater

Medaka eggs exposed to wastewater treatments took longer to hatch ($p < 0.05$; Figure 4; Appendix X). The average time to hatching for 90 % wastewater was longer than controls (0.998 d ± 1.61, n=19). There were 35 % fewer larval births from the 90 % wastewater treated eggs than controls, and 5% fewer larval births for medaka eggs from the 50 % exposure ($p= 0.042$).

Wastewater exposure had a significant negative effect on survival of medaka eggs ($p < 0.05$; Figure 5A; Appendix XI). The 90 % wastewater exposed eggs survival was 12 % lower than controls ($p= 0.017$). In addition, approximately 70 % of the egg mortalities occurred after six days of incubation. In contrast, there was no significant effect of the wastewater exposure on the larval survival ($p > 0.05$; Figure 5B; Appendix X). The survival of 50 % and 90 % wastewater exposed larvae did not vary from control exposures ($p= 0.556$, $p=1.00$).

The length of the medaka larvae exposed to 90 % wastewater was significantly less than that of the controls ($p=.001$; Figure 6; Appendix XII). The overall total size 50 % wastewater exposed larvae did not differ from controls ($p= 0.065$).

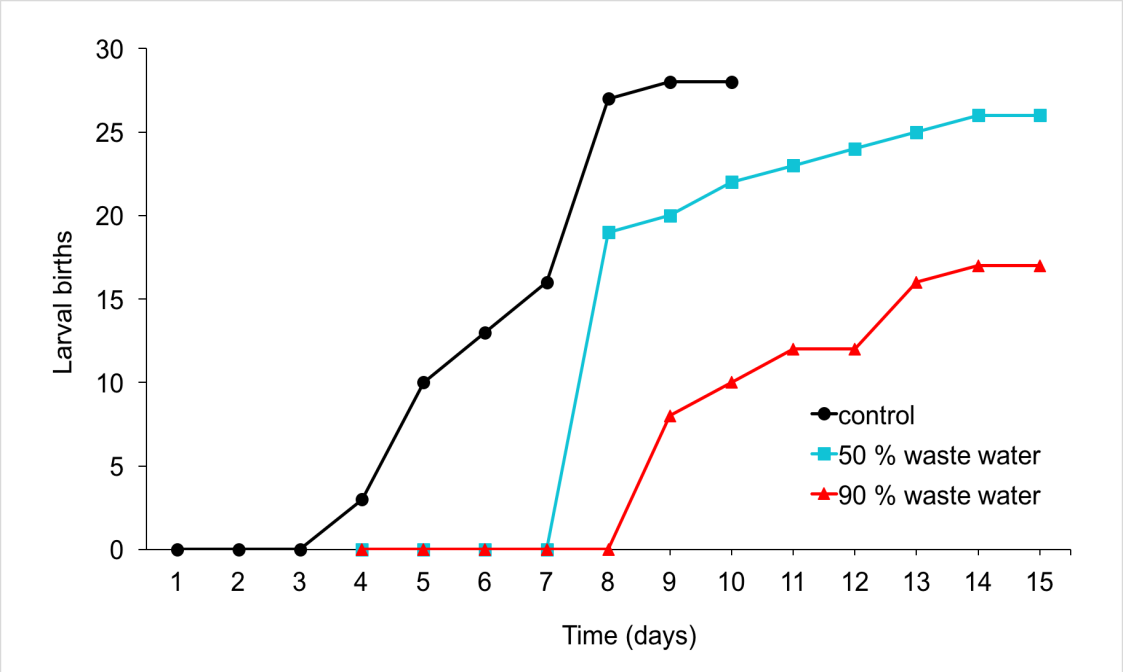

Figure 4. The cumulative number of eggs hatched ($n_{control}$= 27, $n_{50\ \%}$= 32, $n_{90\ \%}$= 33) over 21d when exposed to wastewater concentrations (0 %, 50 % and 90 %). Eggs were checked 4- times daily to ensure that all larval births were recorded for the 24-hour period. Larvae were removed from incubation upon hatching and moved into 2 L tanks.

The last parameters looked at for the medaka was the presence of morphological deformities and the presence of an appropriate startle response. No spinal deformities were seen in any of the treatments. Proportions of individuals that had a swim bladder and startle response are summarized in Table 1. Binomial logistic regression analysis showed that there was no significant difference in startle response among treatments (p= 0.433, p= 0.939; Appendix XIII). Since the startle response occurs so quickly, full and partial capture of a startle responses were considered positive for the reflex. Additionally, no there was no significant difference in swim bladder inflation between wastewater and control treatments (p=0.727, p=0.501).

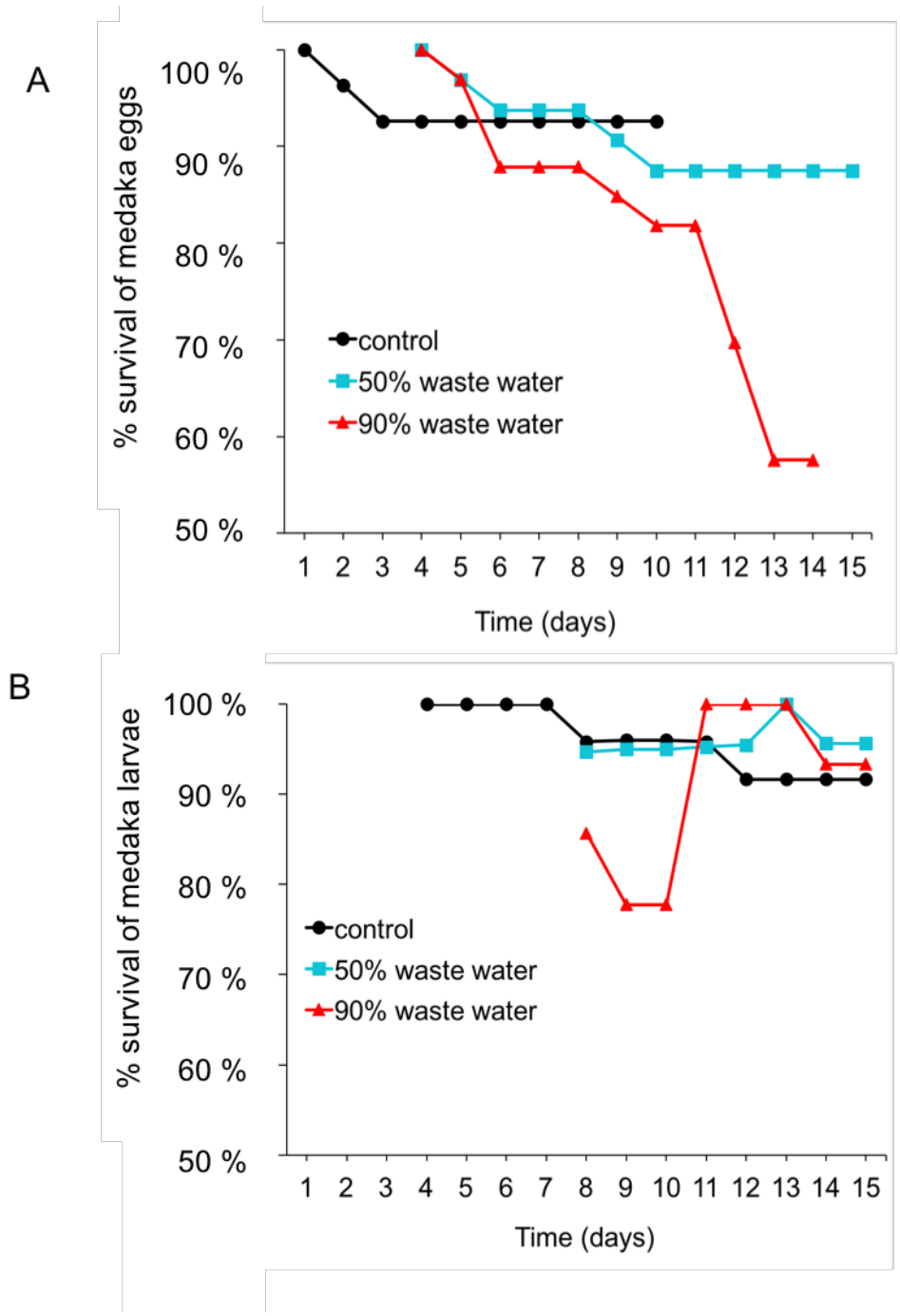

Figure 5. The survival of medaka eggs (A) and medaka larvae (B) over 15 d when exposed to wastewater concentrations (0 %, 50 % and 90 %). Medaka eggs were monitored 4- times daily to ensure that all egg deaths were recorded and removed for the 24-hour period. Larvae were removed from incubation upon hatching and moved into 2 L tanks, where they were monitored 4- times daily to ensure that all larval deaths were recorded and removed for the 24-hour period. Increased % survival between days 10 and 11 was due to an increased amount of hatched larvae.

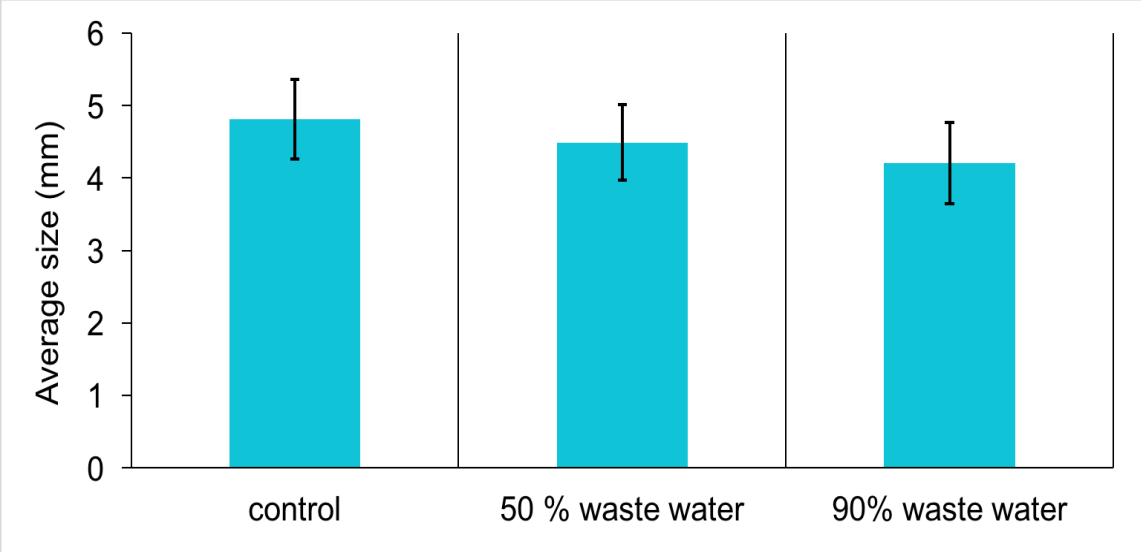

Figure 6. The average total size of medaka larvae after exposure of wastewater concentrations (0 %, 50 % and 90 %) after the trial period (15 days). Medaka larvae were euthanized, scanned, and measured (using Embacadero RAD Studio XE2 Delphi software). Measurements of the larvae were taken in millimeters (mm), and the average size was calculated for the specific treatment. Standard error was also calculated for each treatment, which is represented by error bars.

Table 1. Percentage of medaka larvae exposed to wastewater concentrations (0 %, 50 % and 90 %) for the trial period (15 days) with an inflated swim bladder and startle response.

Treatment	Sample size (n)	Proportion with swim bladder	Proportion with startle response
Control	22	82%	73%
50% waste water	20	80%	80%
90% waste water	15	93%	73%

Discussion

Daphnia

Daphnia were not negatively affected by exposure to treated wastewater, but rather benefitted. Daphnia exposed to wastewater had an mean total size 13 % larger than controls (Figure 1). The growth trend of the Daphnia was likely due to the extra traces of algae and bacteria within the water in addition to the feeding regimen of *C. vulgaris*. The number of neonates produced per individual was also higher in wastewater exposed individuals than controls (Figure 2). These results are likely not independent, as the number of offspring produced is often proportional to body size. Traces of bacteria and algae within the wastewater would have supplied the Daphnia with more energy to devote to processes such as growth and reproduction.

Mortality in Daphnia was 30 % lower in wastewater exposures than controls. However, this was not found to be statistically significant. This result was unexpected as several studies have found treated effluent exposure to increase mortality in Daphnia (Movahedian, *et al.,* 2005; Cao, *et al.,* 2009; Kocbas, *et al.,* 2015). Again, the Daphnia within the current trial likely benefitted due to algae and bacteria still within the water. The adverse results in the studies mentioned may be because they did not follow the same feeding regimen as the current study, substituting the food source for yeast (Movahedian, *et al.,* 2005) or no additional feeding (Kocbas, *et al,* 2015). The Kocbas, *et al.,* study was short-term (96 h), and may not have required feeding, while the current study did feed the Daphnia during that period of time. Thus, it is possible that Daphnia in the current study responded better to the combination of *C. vulgaris* and diluted wastewater, than in conditions from other studies.

Medaka

In contrast medaka, a vertebrate, were negatively affected by the exposure to treated municipal wastewater. High egg mortality was observed when eggs were exposed to wastewater. Specifically, medaka egg survival was 12 % lower for eggs exposed to 90 % wastewater when compared to eggs exposed to 0 % wastewater. Early stages of medaka development are known to be sensitive indicators of toxicity (Zha and Wang, 2005; Cao, *et al.,* 2009; Maya, *et al.,* 2017). Egg survival was likely affected by higher amounts of inorganic nitrogen compounds within the treated wastewater (Shimura, *et al.,* 2004), as concentrations of less than 25 mg N/L were necessary for regular embryo development and higher concentrations resulted in higher mortality and liver damage (Shimura, *et al.,* 2004). Damage to embryo development may delay hatching, as medaka embryo development is a conservative and sequential process (Appendix V). The current study also found that wastewater exposed medaka eggs had fewer larval births (up to 35 %) and longer hatching times. Delayed or prolonged hatching is common among medaka eggs exposed over early development, and it was suggested that delayed hatching may be due to the inhibition of choriolysis (Maya, *et al.,* 2017). However, the mechanism was not described. Although the inorganic nitrogen was not quantified in this study, wastewater often has high concentrations (Yamashita and Yamamota-Ikemoto, 2014). Thus, it can be speculated that inorganic nitrogen in the wastewater does have adverse effects on medaka eggs.

In contrast to the medaka eggs, wastewater exposed larvae in the current study did not show statistically higher mortality than controls. This suggests that the medaka larvae are less vulnerable to the contaminants in the wastewater than eggs. Ishibashi, *et al.*

(2004), found that medaka larvae had a 2 times greater LC_{50} concentration of triclosan compared to embryos. The semi-permeable characteristics of the chorion layer surrounding the egg before hatching may allow nitrates to enter the egg and result in mortality of the egg (Gonzalez-Doncel, *et al.,* 2003; Ishibashi, *et al.,* 2004).

The growth of larvae can be an index of water quality. Ninety percent wastewater exposed larvae were significantly smaller than controls at the end of the trial (Figure 6). However, the 50 % wastewater exposed larvae did not differ from controls. Ma, *et al.* (2005), found that the growth of medaka fish was affected by exposure to treated effluent. They suggested that growth was inhibited because energy was diverted to other biological processes, such as vitellogenesis (Ma, *et al.,* 2005). And Shimura, *et al.* (2004), suggested that the liver's role in metabolism and growth may be hindered by lesions resulting from nitrate exposure. However, with no physiological parameters measured in the current study, it is difficult to conclude whether energy allocation or impaired hepatic function was a factor or something else. Moreover, with the prolonged hatching time, the 90 % exposed larvae had less time to grow following hatching. The prolonged hatching time is likely the cause of apparent reduced growth within the current study, with prolonged hatching time being the more significant parameter.

The current study also identified no spinal deformities and no significant difference in swim bladder inflation for wastewater exposed medaka larvae. This differs from earlier work where exposure to environmental contaminants resulted in a deflated swim bladder and more spinal deformities (Zha and Wang, 2005; Kupsco and Schlenk, 2016). Last, no significant differences were seen in the development of a startle response in medaka larvae. Simmons, *et al*. (2017), similarly found that fish located up and

downstream from WWTPs did not differ in their startle response. It does not appear that wastewater, at this level of treatment, has an effect on morphological development or behaviour of medaka fish.

Limitations

All the possible contaminants of the wastewater used within the current study are not known. This presents limitations to what can be concluded regarding the observed adverse effects on aquatic biota. Mass spectrophotometry was used to identify pharmaceutical contaminants within the water. Among the most abundant were carbamazepine and sulfamethoxazole, with concentrations of 379 ng/ L and 176 ng/ L respectively, in addition to the unknown concentrations of inorganic nitrogen (Appendix XIV). These concentrations are much lower than what has been used in literature when investigating pharmaceutical toxicity on aquatic biota. Chronic effects of pharmaceuticals and nitrates at low concentrations, similar to those seen in treated effluent, are not well documented. In addition, few studies have investigated whether any pharmaceutical drugs may have agonistic interactions in the presence of other contaminants such as nitrate. It is also important to note that the presence and concentration of pharmaceuticals and other contaminants may vary over regions due to water treatment process, climate and local ecology (Ryeo-Ok, *et al.,* 2017). These differences may make it difficult produce similar results in model organisms exposed to treated wastewater from different locations.

The time at which the medaka eggs were harvested produced some limitations in regards to the parameters that could be studied. Medaka development occurs over a rigid time line, in which different developmental stages may occur every 30 minutes (Iwamatsu, 2004). The medaka eggs for the current study were gathered within a 24 h

period, meaning that the eggs collected may have been at varying developmental stages. Previous studies have found that later stages of development (>24 h) (Appendix V) were most susceptible to environmental toxicants (Gonzalez-Doncel, *et al.,* 2003; Kupsco and Schlenk, 2016). These studies suggest that specific stages of medaka embryo development differ in their vulnerability to the effects of the treated wastewater, which could affect the severity of adverse effects. Identifying the susceptibility of medaka eggs at different stages of development, when exposed to treated wastewater, will be an essential future study.

Conclusions

Current water treatment methods are not always effective at removing harmful contaminants from municipal wastewater. Possible contaminants include inorganic nitrogen, pharmaceuticals and PCPs, effects of which are still being studied on aquatic biota. In this study, local treated municipal wastewater was used to investigate possible toxicological effects on local aquatic biota. Exposure to treated wastewater negatively affected medaka fish, but benefitted Daphnia. Significant reduced survival and rate of hatching were seen in medaka eggs exposed to treated wastewater, specifically at a 90 % concentration. Medaka larvae were not as vulnerable to the wastewater exposure as the eggs. These results suggest that wastewater may have contaminants that limit egg survival and proper egg development. Further studies are needed to fully understand the susceptibility of medaka egg development when exposed to treated wastewater. Exposure to wastewater did not have a negative effect on Daphnia, but rather resulted increased survival, growth and reproductive activity. These results are likely due to additional nutrients in the wastewater. The opposing results found in these two organisms demonstrate the necessity of using multiple model organisms in environmental aquatic studies.

The current study provided preliminary data with small sample sizes. While the data suggests that treated municipal wastewater does have an effect on aquatic biota, future studies with larger sample sizes need to be completed to validate these conclusions. Specifically, studies investigating the susceptibility of medaka egg developmental stages when exposed to wastewater and chronic exposure of Daphnia to wastewater would be significant contributions.

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Appendix I

ADaM (Aachener Daphnien Medium)

Mix the following in RO water.

Sea salt: .333g/L
$CaCl_2$ [117.6g/l]:2.3ml/l
$NaHCO_3$ [25.2g/l]:2.2ml/l
SeO_2: 1.0 ml/l

Appendix II

WC algae growth medium

MAJOR ELEMENTS:

	Stock (100nM) g/100ml	[final] mg/l	Add ml/l
$NaNO_3$	0.850	17.0	2.0
KH_2PO_4	1.361	1.4	0.4
KCl	0.746	3.0	0.4
$MgSO_4$ $7H_2O$	2.465	37.0	1.5
$CaCl_2$ $2H_2O$	1.471	36.8	2.5
$NaHCO_3$	0.840	12.6	1.5
Na_2SiO_2 $9H_2O$	2.842	56.8	2.0

TRACE ELEMENTS:

Combine compounds in order listed in 1 liter of water. Add 2.5ml to 1 liter of medium.

Na_2EDTA
$FeCl_3\ 6H_2O$
H_3Bo_3
$MnCl_2\ 4H_2O$
$Na_2MoO_4\ 2H_2O$
$ZnSO_4\ 7H_2O$
$CoCl_2\ 6H_2O$
$CuSO_4\ 5H_2O$

BUFFER:

Add 16.32mg of bicine to 1 liter of RO water. Adjust the pH to 7.0. Add 10ml/l to the medium. Adjust the pH of the medium to be between 7.3 and 7.7 using HCl or NaOH/KOH.

VITAMINS:

3 stock solutions prepared:
- A: Cynocobolamine 10.0mg/100ml H_2O
- B: Biotin 10.0mg/100ml H_2O
- Thiamine 10mg + .5ml of A and .5ml of B in 99ml of H_2O

Add 1ml of thiamine solution per liter of medium.

Appendix III- artificial pondwater

Mix the following (for 20L carboy)

Solution A: dilute salts in 500 mL distilled H_2O. Then add to 15 L of distilled H_2O.
- [1.3mM]: 1.04 g NaCl
- [0.8mM]: 2.35g $CaCl_2 \bullet 2(H_2O)$
- [0.1mM]: 0.15g KCl

Solution B: dilute salt in 500mL H_2O. Add to container with solution A. Fill to 20L.
- [0.2mM]: 0.34g $NaHCO_3$

Appendix IV

Embryonic E2 Medium

BUFFER MIX
Dissolve below reagents in 1L of water. Filter sterilize.

Reagent	Desired concentration (mM)	Weight of Salt (g)
KH_2PO_4	750	102.1
Na_2HPO_4	250	67.0

E2A
Dissolve reagent list below in 2L of water. Add 40mL of E2A buffer mix. Filter sterilize.

Reagent	Desired concentration	Weight of Salt (g)
NaCl	1.5 M	175
KCl	50 mM	7.5
$MgSO_4 \bullet 7(H_2O)$	100 mM	49.3
KH_2PO_4	15 mM	4.08
Na_2HPO_4	5 mM	1.42

E2B
Dissolve reagent list below in 1L of water. Filter sterilize.

Reagent	Desired concentration (mM)	Weight of Salt (g)
$CaCl_2 \bullet 5(H_2O)$	500	73.5

E2C
Dissolve reagent list below in 1L of water. Filter sterilize.

Reagent	Desired concentration (mM)	Weight of Salt (g)
$NaHCO_3$	300	14.7

FINAL E2
Fill 20L carboy to 19L of water, and aerate until ready to mix. Add the below solutions list. Adjust volume to 20L. Adjust pH to 7.2-7.6. Store at 28° C. Good for one week.

Reagent	Volume (mL)
E2A	100
E2B	20
E2 C	20
0.1 % methylene blue	10

Appendix V

Medaka significant developmental stages (adopted from Iwamatsu, 2004).

Stage interval	Key development
1-7 (3.5 hours)	Cell stage: egg is fertilized, 5 cleavage planes develop
8-9 (5.25 hours)	Morula stage
10-16 (21 hours)	Gastrula stage: yolk sphere develops, embryonic shield, and rudimentary brain
17-18 (24 hours)	Neurula stage
19-32 (4 days 5 hours)	Auditory vesicles, optical vesicles, tubular heart, blood circulation, retinal pigmentation, swim bladder
35 (5 days 12 hours)	Visceral blood vessles
36 (6 days)	Heart development
39 (9 days)	Hatching

Appendix VI

Ms-222 anesthetic
Dissolve reagents below in 500mL of aquatic water. Store securely.

Reagent	Weight (g)
Ms-222	1.0
$NaHCO_3$	0.5

Appendix VII

P-value significance: 0 $^{(***)}$, 0.001 $^{(**)}$, 0.01 $^{(*)}$, 0.05, > 0.05

ANOVA table for Daphnia growth trial #1

Treatment	Intercept	Estimated Standard Error	Degrees of Freedom (n_{groups}-1, $n_{observations}$-1)	F statistic	P-value and significance
Control	1.65748	0.19890	4, 6	8.333	6.88e-5***
50 % wastewater	0.71480	0.08707	4,6	8.210	1.99e-5***
90 % wastewater	0.74848	0.08707	4,6	8.596	8.64e-9***
50 % pondwater	0.36229	0.08707	4,6	4.161	3.51e-4***
90 % pondwater	0.28383	0.08707	4,6	3.260	3.32e-3**

ANOVA table for Daphnia growth trial #2

Treatment	Intercept	Estimated Standard Error	Degrees of Freedom (n_{groups}-1, $n_{observations}$-1)	F statistic	P-value and significance
Control	2.11895	0.13719	4, 6	15.445	7.68e-7***
50 % wastewater	0.33169	0.07284	4,6	4.554	2.05e-4***
90 % wastewater	0.25318	0.07284	4,6	3.476	0.003**
50 % pondwater	0.07608	0.07284	4,6	1.044	0.309
90 % pondwater	0.03419	0.07727	4,6	0.442	0.663

Appendix VIII

P-value significance: 0 (***), 0.001 (**), 0.01 (*), 0.05, > 0.05

ANOVA table for Daphnia reproductive activity trial #1

Treatment	Intercept	Estimated Standard Error	Degrees of Freedom (n_{groups}-1, $n_{observations}$-1)	F statistic	P-value and significance
Control	0.2639	0.2971	4, 20	19.217	0.385
50 % wastewater	2.0055	0.4201	4, 20	19.996	1.16e-4***
90 % wastewater	2.6089	0.4197	4, 20	22.669	2.58e-6***
50 % pondwater	1.3266	0.4202	4, 20	19.217	0.005**
90 % pondwater	0.6167	0.4201	4, 20	19.610	0.158

ANOVA table for Daphnia reproductive activity trial #2

Treatment	Intercept	Estimated Standard Error	Degrees of Freedom (n_{groups}-1, $n_{observations}$-1)	F statistic	P-value and significance
Control	7.364e-1	2.363e-1	4, 20	3.116	0.002**
50 % wastewater	1.691	3.342e-1	4, 20	5.060	4.91e-7***
90 % wastewater	1.950	3.342e-1	4, 20	5.836	7.05e-9***
50 % pondwater	1.864e-1	3.342e-1	4, 20	0.558	0.577
90 % pondwater	7.727e-2	3.342e-1	4, 20	0.231	0.817

Appendix IX

P-value significance: 0 $^{(***)}$, 0.001 $^{(**)}$, 0.01 $^{(*)}$, 0.05, > 0.05

ANOVA table for Daphnia % survival trial # 1

Treatment	Intercept	Estimated Standard Error	Degrees of Freedom (n_{groups}-1, $n_{observations}$-1)	F statistic	P-value and significance
Control	0.4286	0.1139	4, 20	3.763	2.87e-4***
50 % wastewater	-0.3333	0.1540	4, 20	-2.164	0.033*
90 % wastewater	-0.2381	0.1540	4, 20	-1.546	0.126
50 % pondwater	-0.2381	0.1540	4, 20	-1.546	0.126
90 % pondwater	-0.1905	0.1540	4, 20	-1.237	0.220

ANOVA table for Daphnia % survival trial # 2

Treatment	Intercept	Estimated Standard Error	Degrees of Freedom (n_{groups}-1, $n_{observations}$-1)	F statistic	P-value and significance
Control	-2.2e-17	4.949e-2	4, 20	0.000	1.000
50 % wastewater	4.762e-2	6.999e-2	4, 20	0.680	0.498
90 % wastewater	4.229e-2	6.999e-2	4, 20	0.000	1.000
50 % pondwater	4.762e-2	6.999e-2	4, 20	0.680	0.498
90 % pondwater	1.905e-1	6.999e-2	4, 20	2.722	0.008**

Appendix X

P-value significance: 0 $^{(***)}$, 0.001 $^{(**)}$, 0.01 $^{(*)}$, 0.05, > 0.05

ANOVA table for medaka time to hatching

Treatment	Intercept	Estimated Standard Error	Degrees of Freedom (n_{groups}-1, $n_{observations}$-1)	F statistic	P-value and significance
Control	1.9333	0.9195	2, 14	2.103	0.042*
50 % wastewater	-0.1333	1.1485	2, 14	-0.116	0.908
90 % wastewater	-0.6000	1.1485	2, 14	-0.522	0.605

Appendix XI

P-value significance: 0 $^{(***)}$, 0.001 $^{(**)}$, 0.01 $^{(*)}$, 0.05, > 0.05

ANOVA table for medaka egg % survival

Treatment	Intercept	Estimated Standard Error	Degrees of Freedom (n_{groups}-1, $n_{observations}$-1)	F statistic	P-value and significance
Control	0.1333	0.2390	2, 14	0.558	0.580
50 % wastewater	0.2000	0.3147	2, 14	0.635	0.530
90 % wastewater	0.8000	0.3147	2, 14	2.542	0.017*

ANOVA table for medaka larvae % survival

Treatment	Intercept	Estimated Standard Error	Degrees of Freedom (n_{groups}-1, $n_{observations}$-1)	F statistic	P-value and significance
Control	2.667e-1	1.333e-1	2, 14	2.000	0.058
50 % wastewater	6.667e-2	1.117e-1	2, 14	0.597	0.556
90 % wastewater	-1.2e-16	1.117e-1	2, 14	0.000	1.000

Appendix XII

P-value significance: 0 $^{(***)}$, 0.001 $^{(**)}$, 0.01 $^{(*)}$, 0.05, > 0.05

Linear regression table for medaka total average size

Treatment	Intercept	Estimated Standard Error	Degrees of Freedom (n_{groups}-1, $n_{observations}$-1)	F statistic	P-value and significance
Control	4.8120	0.1207	2, 20	39.863	2.0e-16***
50 % wastewater	-0.3220	0.1707	2, 20	-1.886	0.065
90 % wastewater	-0.6108	0.1811	2, 15	-3.373	0.001**

Appendix XIII

P-value significance: 0 $^{(***)}$, 0.001 $^{(**)}$, 0.01 $^{(*)}$, 0.05, > 0.05

Bionomial logistic regression table for medaka startle response proportion

Treatment	Intercept	Estimated Standard Error	Degrees of Freedom (n_{groups}-1, $n_{observations}$-1)	Z statistic	P-value and significance
Control	1.04145	0.47486	2, 20	2.193	0.028*
50 % wastewater	0.56798	0.72491	2, 20	0.784	0.433
90 % wastewater	0.05716	0.74754	2, 15	0.760	0.939

Binomial logistic regression table for medaka swim bladder proportion

Treatment	Intercept	Estimated Standard Error	Degrees of Freedom (n_{groups}-1, $n_{observations}$-1)	Z statistic	P-value and significance
Control	1.8971	0.6191	2, 20	3.064	0.002**
50 % wastewater	-0.2877	0.8266	2, 20	-0.348	0.728
90 % wastewater	0.8109	1.2042	2, 15	0.673	0.501

Appendix XIV

Pharmaceutical Present	Concentration (ng/L)
Atenolol	11.36
Atrazine	1.50
Carbamazepine	392.00
Metoprolol	9.88
Propanolol	182.40
Sulfamethoxazole	182.40
Sulfapyridine	60.20
Trimethoprim	7.16
Diclofenac	51.80