The Negative Effects of Gibberellic Acid (GA_3) on Freshwater Daphnids' Mortality and Reproduction

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ABSTRACT: Gibberellic acid (GA_3) is a plant hormone commonly used in the agricultural industry to increase crop yield. Gibberellic acid is considered a crop additive and thus has been tested for acute toxicity by the US EPA as required by law. The EPA concluded that it was not hazardous to aquatic invertebrates because the LC_{50} concentration was above environmentally relevant levels. However, their conclusion came from an acute LC_{50} concentration of one aquatic invertebrate species (*Daphnia magna*). The purpose of this study is to expand upon their research due to the lack of data their report provides. This study used a modified EPA protocol for acute testing of crop additives on aquatic invertebrates. Buffered and unbuffered gibberellic acid solutions were used to determine if acidity was causing toxicity. *Daphnia pulex* were used alongside *D. magna* as a comparison to determine if they have different sensitivities to gibberellic acid. A chronic study was done as well using only *D. magna*. There was no statistical difference in LC_{50} concentration between the buffered and unbuffered solution for *D. magna*. This suggests that the toxicity of gibberellic acid comes from the structure itself and not from its acidity. There is also no statistical difference between the two species tested; although, the data suggest that *D. pulex* may be more tolerant to the compound. The mean number of offspring per replicate decreased in the three highest concentrations in the buffered acute *D. pulex*. Sub-lethal effects could possibly be seen at environmentally relevant levels.

Introduction

With the industrialization of agriculture, companies have been testing various methods to increase efficiency and quantity of production (Curry, 2013). One particular method is to decrease the amount of time needed for the desired food product to grow to a harvestable quality. Another method is to increase the size of the food product. This can be done by using chemicals and plant hormones to cause plants to grow faster than normal. Examples of a commonly used plant hormones are Gibberellins, which have been used in the agricultural industry for decades (Palmer, 1974; Sharma et al., 2014).

Gibberellins are a group of naturally occurring hormones that are involved in plant growth (Dar et al., 2015). Gibberellic acid (GA_3) is a gibberellin that can be found in most crop plants as it regulates ripening of fruit and shoot growth (Bhattacharyya and Jha, 2012; Narula, et al., 2006). However, agricultural companies provide more than the natural occurring amount of gibberellic acid to their crops. When gibberellic acid is sprayed in the fields, it can contaminate bodies of water through run-off or off target spraying, which occurs with conventional air-assisted spraying (Wei et al., 2016).

The Federal Insecticide, Fungicide and Rodenticide Act (7 USCS 136, et seq.) and the Toxic Substance Control Act (15 USCS 2601) require the use a model organism to test the effects of a crop additive. Daphnids are a typical model species used for aquatic toxicity studies (Jaafarzadeh et al., 2013). They tend to produce asexually and thus genetic variation amongst test subjects is reduced (Baird et al., 1991). Daphnids are a vital part of the aquatic food chain as they are primary consumers; therefore, any disruption to their livelihood could have a dramatic effect on the ecosystem as they are a key food source for their predators (Miner et al., 2012).

Gibberellic acid acute toxicity has been reported by the US Environmental Protection Agency (EPA). Their acute toxicity tests determined environmentally relevant concentrations of gibberellic acid was not lethal as it was above 100 mg/L. The results concluded that gibberellic acid had a LC_{50} of 143 mg/L (US Environmental Protection Agency, 1992). The US EPA later indicated that gibberellic acid is practically non-toxic to aquatic invertebrates (US Environmental Protection Agency, 1995). However, the non-toxic classification comes solely from an acute experiment LC_{50} of a single species: *Daphnia magna*. Using multiple aquatic species is valuable to toxicity classification because while a species may be commonly used such as *D. magna*, it may not be the most sensitive species (Cairns Jr., 1986). Therefore, it would be logical to use multiple aquatic invertebrates to determine the toxicity of gibberellic acid.

The data reported by the EPA leaves out other desired information such as whether the concentrations were buffered or not. This is desired because a low pH can be detrimental to the health of aquatic invertebrates. When Sillanpaa et al. (2002) did their acute toxicity study with organic acids, they buffered the solutions and used multiple aquatic invertebrates. The EPA further did not report sub-lethal effects on *D. magna* caused by gibberellic acid. Wollenberger et al. (2000) conducted a study using multiple growth promoting compounds commonly used in the farming industry and discovered they had caused *D. magna* to experience a decrease in clutch size during reproduction.

The purpose of this research is to expand upon the previous research on the effects that gibberellic acid has on the environment. We designed an experiment that would compare the effects of buffered and unbuffered gibberellic acid solutions. They were compared by recording the pH and the conductivity of the experimental environment and mortality of daphnids. The data provided by the US EPA only used *Daphnia magna*; therefore, to expand the scope of the effects gibberellic acid has on aquatic invertebrates, *Daphnia pulex* was used as well. *D. magna* and *D. pulex* have different sensitivities to toxins (Kuster and Elert, 2013). We also do not know what kind of sub-lethal effects (e.g., reproduction) gibberellic acid may have on aquatic species. For these reasons, we investigated the of gibberellic acid on reproduction in acute and chronic experiments. We hypothesize that the unbuffered gibberellic acid solution will be lethal at a lower concentration as compared to the buffered gibberellic acid solution due to the acidity of the compound. Gibberellic acid will also decrease the number of offspring in both short term and long term exposures.

Methods and Materials

Subject Organisms

For this experiment, we used *Daphnia magna* and *Daphnia pulex* that were cultured from stocks from Aquatic Bio Systems Inc. (Fort Collins, CO). Each species was cultured individually in 900 mL glass jars filled with moderately hard water. We created moderately hard water using the recipe as follows: 0.473 g $CaSO_4$, 0.959 g $NaHCO_3$, 1.223 g $MgSO_4 \cdot 7H_2O$, and 0.039 g KCl per 10 L of deionized water. All references to water for the remainder of the paper refers to moderately hard water unless otherwise stated. These daphnid cultures were fed 2 mL of algae (*Selenastrum*

capricornutum) containing 3.0 x 10^7 cells per mL on Monday, Wednesday, and Friday. The water was changed every Friday. Once a month, 1 mL of a yeast and trout chow mixture (YCT) was given to each jar. This was done for approximately eight months before the beginning of the experiment.

To prepare organisms for use in experiments, we used a variation of the US EPA's (1996) protocol for daphnid culturing. Eight to ten individuals from the stock culture of each species were put into separate 1 L glass beakers (i.e. *D. magna* in one beaker and *D. pulex* in another). These culture beakers followed the same feeding and water changing protocols previously mentioned; however, YCT was not given. After the individuals had reproduced (about two to three weeks), the original daphnids (F_0) placed in the beakers were taken out to ensure they are not used for this experiment. About five to seven days after the original daphnids were removed, the F_1 daphnids were old enough to be used for acute and chronic toxicity experiments.

Acute Toxicity Study

We carried out four 48-hour acute toxicity studies. We examined the effects of buffered and unbuffered gibberellic acid on *Daphnia magna* and *Daphnia pulex*. We used six different concentrations: 0 mg/L (control), 1 mg/L, 5 mg/L, 25 mg/L, 125 mg/L, and 625 mg/L. Gibberellic acid (99% purity, Chemservice, West Chester, PA) was dissolved in deionized water as it ionized into gibberellin and H^+ ions. Buffered and unbuffered gibberellic acid 5000 mg/L stock solutions were used for spiking exposure jars. To buffer the stock, we added sodium bicarbonate until the solution reached a neutral pH. Glass jars were filled with moderately hard water and spiked with the appropriate amount of the gibberellic acid 5000 mg/L stock solution to total 100 mL. As an example, the 625 mg/L concentration had 12.5 mL of gibberellic acid 5000 mg/L stock solution and 87.5 mL of moderately hard water. The jars were allowed to equilibrate after being spiked. Control jars were set up similar to the 625 mg/L concentration except deionized water was added instead of the gibberellic acid 5000 mg/L stock solutions.

Prior to placing the daphnids in the jars, pH and conductivity were recorded in two randomly chosen replicates from the highest concentration and the control in every acute toxicity study. For each experiment, there were three replicates for every concentration and every replicate had four daphnids of the same species. The daphnids of the chosen species were haphazardly placed into the glass jars. Across both species, there were 36 glass jars that were spiked with the buffered stock solution and 36 glass jars that were spiked with the unbuffered stock solution. We placed the glass jars in a Thermo Scientific Precision incubator (Fisher Scientific, Hampton, NH) at a holding temperature of 20˚ C and on a 12:12 light: dark cycle. To limit the rate of evaporation, a translucent sheet of plastic was positioned on top of the jars. We recorded mortality in each jar every 24 hours.

Chronic Toxicity Study

We carried out a 14-day chronic toxicity study on *D. magna* using a buffered gibberellic acid 5000 mg/L stock solution; however, due to high control mortality, the experiment only lasted six days. Three different concentrations of buffered gibberellic acid were used: 0 mg/L, 1 mg/L, and 100 mg/L. The lowest concentration, 1 mg/L, was chosen because the acute study showed sublethal effects could be seen between 1 and 100 mg/L. The highest concentration, 100 mg/L, was chosen because the negative effects of gibberellic acid were anticipated at this concentration. The glass jars were filled in the same manner as the jars in the acute experiment. As an example, a 100 mg/L replicate would contain 2 mL of the buffered gibberellic acid 5000 mg/L stock solution and 98 mL of moderately hard water. In a similar manner as the acute experiment, the pH and the conductivity were recorded before the daphnids were placed in the jars. The daphnids were placed in the jars, after the jars were spiked and allowed to

equilibrate. There were four replicates for every concentration with every replicate having three daphnids.

We changed the water every four days. We recorded pH and conductivity before and after each water change. We recorded mortality, the number of offspring, and fed the daphnids 0.2 mL of algae every two days. When the water was changed, a new set of jars was made that followed the same set up procedure as the initial set up. The jars were respiked with every water changed. The adult daphnids were transferred to their new respective jars with care so that the least amount of water from the previous jar was transferred with them to the new jar. The offspring from the previous jars were quantified and not transferred to the new jars. The jars were kept in the same incubator as the acute experiment and were under the same conditions.

Statistical analysis

We analyzed all data collected using R statistical software (Ver 3.3.1). We used a log-logit model to estimate the LC_{50} of gibberellic acid on the daphnid species in the acute studies by using the "glm" function from the "MASS" package in R (Venables & Ripley, 2002). The standard error was obtained through the "glm" coding and we calculated the confidence intervals from the standard error. The effects of gibberellic acid on the number of daphnid offspring from the buffered *D. pulex* acute study was evaluated using analysis of variance. Significance within the analysis of variance was determined by using a posthoc Tukey's Highly Significant Difference Test. Due to high control mortality, low offspring count in all concentrations in the chronic experiment, and the early termination of the chronic experiment, the data collected was not statistically analyzed. For all tests, each individual jar was the unit of replication and the alpha value used was 0.05.

Results

From the acute experiment, the LC_{50} concentrations for the unbuffered and buffered gibberellic acid solutions were not statistically different (Table 1). The unbuffered and buffered solutions suggested similar LC_{50} concentrations for *D. magna* (LC_{50} ± 95% CI = 256.9 mg/L ± 68 mg/L and 239.9 mg/L ± 68.3 mg/L, respectively). The buffered gibberellic solution was lethal to *D. pulex* at a higher concentration (LC_{50} ± 95% CI = 957.6 mg/L ± 256.9 mg/L); however, due to the high standard error, it is not statistically different from the concentrations that were lethal to *D. magna*. Due to the high control mortality (25%) in the unbuffered *D. pulex* experiment, the LC_{50} concentration was not calculated.

Group	Concentration (mg/L)	48-h mortality (%)	LC_{50} (mg/L)	SE	Lower CI	Higher CI
Buffered *D. magna*	0	0	239.9	69.3	103.9	375.7
	1	33.3				
	5	0				
	25	0				
	125	16.7				
	625	100				
Unbuffered *D. magna*	0	0	256.9	68	123.62	390.18
	1	16.7				
	5	16.7				
	25	8.3				
	125	8.3				
	625	100				
Buffered *D. pulex*	0	0	957.6	337	297.08	1618.12
	1	0				
	5	8.3				
	25	8.3				
	125	8.3				
	625	25				
Unbuffered *D. pulex*	0	25	NC	NC-	NC	NC
	1	33.3				
	5	16.7				
	25	16.7				
	125	33.3				
	625	91.7				

Table 1 – Acute toxicity of buffered and unbuffered gibberellic acid. SE = Standard Error. 95% confidence levels were used in determining statistical differences. No statistical differences were shown. Unbuffered D. pulex data was not calculated due to high control (0 mg/L) mortality.

The analysis of variance of the number of offspring from the buffered *D. pulex* acute study showed a statistical difference shown in the treatment data ($F_{5,12} = 4.076$, $p = 0.0215$; Figure 1). A posthoc Tukey's Highly Significant Difference Test revealed the control jars were statistically different from 25, 125, and 625 mg/L jars ($p < 0.05$); however, the control jars were not statistically different from the 1 and 5 mg/L jars ($p = 0.128$). The control jars (0 mg/L) had the highest mean number of offspring per jar (mean ± SE = 10 ± 2.65) while the next highest means were in the 1 mg/L and 5 mg/L jars (mean ± SE = 5 ± 1.72; 5 ± 2.65, respectively). The highest concentration of the buffered gibberellic acid solution, 625 mg/L, had the lowest mean number of offspring per jar (mean ± SE = 2.33 ± 2.52).

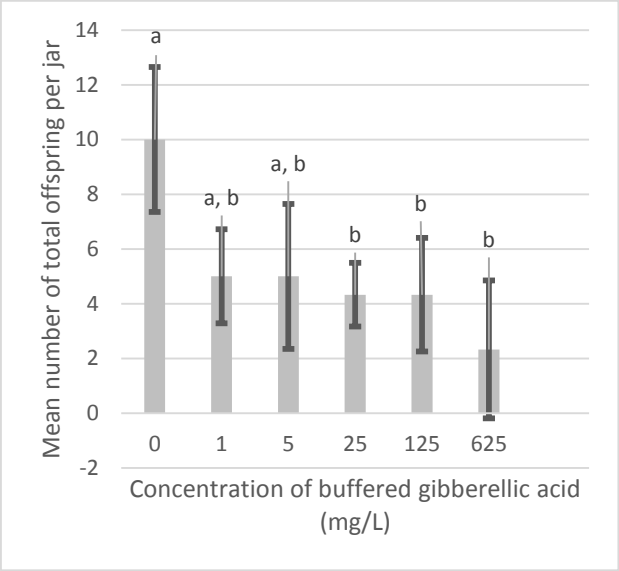

Figure 1 – Mean number of offspring from the buffered D. pulex acute toxicity experiment. Error bars are the standard error. There is a statistical difference in the mean number of offspring within the groups ($F_{5, 12} = 4.076$, $p = 0.0215$). Means that have different letters represent statistical differences as determined by a posthoc Tukey's Highly Significant Difference Test.

Results from the chronic experiment are not shown due to high control mortality. The planned 14-day study was stopped at day 6. Mortality from the control group at day 6 was 75%. Mortality from the 1 mg/L and 100 mg/L groups were 92% and 100% respectively.

The pH and conductivity values are shown for each experiment in table 2. In the acute studies, values for the replicates exposed to the buffered solution were all within an acceptable range: pH of 6.9 to 7.1, and conductivity of 253 µS/m to 270 µS/m. The replicates exposed to the unbuffered solution had a pH range of 4.1 – 4.5 and conductivity of 227 µS/m to 233 µS/m. In the chronic study, values for the replicates were within an acceptable range: pH of 6.7 – 7.1, and conductivity of 260 µS/m to 276 µS/m. After each water change in the chromic study, the pH and conductivity were consistently within the ranges recorded before the start of the experiment.

Species	Study	Type of Solution	Concentration (mg/L)	pH	Conductivity (µS/m)
D. magna	Acute	Buffered	0	7.4	270
			625	7.1	253
		Unbuffered	0	6.9	257
			625	4.5	233
D. pulex	Acute	Buffered	0	7.2	258
			625	6.9	264
		Unbuffered	0	6.5	255
			625	4.1	227
D. magna	Chronic	Buffered	0	6.7	260
			100	7.1	276

Table 2 – The recorded pH and conductivity values from the acute and chronic studies. The values were from one randomly selected replicate of select concentrations from each study.

Discussion

Acute data analysis

Due to a lack of significant difference in the buffered and unbuffered *D. magna* acute studies, our hypothesis of the unbuffered concentration having a lower LC_{50} was not

supported. However, this might suggest that the toxicity comes from the structure of the compound and not the fact that it is acidic. The pH in the buffered 625 mg/L was essentially neutral and yet it had caused the same mortality as its unbuffered counterpart.

While there may not have been a statistical difference in the LC_{50}s between the daphnid species, *D. pulex* did have a much higher LC_{50} estimate. This may suggest *D. pulex* is more tolerant to gibberellic acid than *D. magna*. However, a limitation of the study was the mortality being less than 50% in any concentration in the buffered *D. pulex* acute study. This caused the LC_{50} concentration to be extrapolated beyond the experimental scope. This also explains the high standard error associated with that study. We also cannot compare the buffered study to the unbuffered study as we did for the *D. magna* because of the high control mortality in the unbuffered study.

Even though the buffered *D. pulex* study was the only acute study where offspring were counted, it does provide valuable insight into the sub-lethal effects of gibberellic acid. The mean number of offspring in the control and 1 mg/L may not be statistically different. The mean offspring count in 1 mg/L was half of the control's offspring count. This could have been due to a redistribution of their energy expenditure. Knops et al. (2001) exposed *D. magna* to toxicants and the *D. magna* displayed a reduced weight and reproduction rate while keeping their metabolic rate constant. Hence in our study, the *D. pulex* may have reproduced less due to expending more energy into breaking down gibberellic acid that entered their body. Therefore, we suggest for future research, more definitive tests on how gibberellic acid affects the number of offspring should be done.

Overall, our data showed that the acute LC_{50} of gibberellic acid is non-toxic. The LC_{50} was double the concentration that was obtained by the EPA, which was 143 mg/L. However, reduced offspring count were statistically seen at the 25, 125, and 625 mg/L concentrations. This means that more tests should be done to determine when sub-lethal effects will start to occur and to determine their severity. Sub-lethal effects, such as malformation and function loss of physical structures, have been found in acute exposures at concentration range of 1.9 mg/L to 130.5 mg/L in *D. magna* neonates (Wang et al., 2011). Gibberellic acid has been shown to reduce fecundity in the terrestrial invertebrate *Locusta migratoria migratoria* at a concentration of 125 mg/L (Abedellaoui et al., 2009). While research has been started in figuring out the sub-lethal effects of gibberellic acid, more evidence is still needed.

Chronic data analysis

The data collected was not statistically analyzed due to the high control mortality and the study was stopped before it had reached its completion. The high mortality could have been due to stressful culture conditions. The daphnids had more than enough food in their beaker; consequently, that could have led to the mortality. *D. magna* has been reported to become increasingly sensitive to stress as their food concentration increases (Smolders et al. 2005). Therefore, it would be plausible to think the test subjects died due to the stress of the chronic test conditions. The beakers the test subjects were raised in could have been stressful due to overcrowding (Preuss et al., 2009) as the beaker they were raised in had 40 to 50 daphnids. Another reason for high control mortality was that the F_0 could have come from a stressful culture jar. It has been shown that when parents were raised in stressful conditions, their offspring can be affected negatively (Cowgill et al., 1984) Stress in one generation can affect generations that come afterwards. However, we can rule out the chemistry of the water was a cause in the mortality because the pH was neutral and the conductivity was similar to the acute buffered studies.

Conclusion

Our hypothesis of the unbuffered gibberellic acid solution being toxic at a lower

concentration as compared to its buffered counterpart was unsupported. This study supports that a high concentration of gibberellic acid is needed to show lethal effects in daphnids when it comes to short term exposure. The data also suggest that sub-lethal effects could occur at environmentally relevant levels. Hence more studies that focus on finding the concentrations where sub-lethal effects begin should be done. Due to the chronic experiment being unsuccessful, no data on the effects of gibberellic acid could be determined. Therefore, we suggest that more chronic studies that focus on the effects of gibberellic acid should be done to expand upon the lack of chronic data available. To conclude, we agree with reservation in regards to the EPA's non-toxic classification of gibberellic acid. However, we believe gibberellic acid's sub-lethal effects should be acknowledged and studied.

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