AMMONIA AS A TOOL FOR REMOVAL OF INVASIVE CRAYFISH

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ABSTRACT

AMMONIA AS A TOOL FOR REMOVAL OF INVASIVE CRAYFISH

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Several species of invasive crayfish have been introduced globally, impacting ecosystem functioning and biodiversity. The opportunistic feeding habits of nonnative crayfish have negatively affected native aquatic species in the Southwestern United States, and their ability to travel between isolated aquatic systems make them difficult to control once they become established. Efforts to manually remove crayfish from invaded habitats have met with limited success and few chemical tools are available. With increasing numbers of invasive species in aquatic systems comes a growing need for additional management methods. The use of ammonia has shown promise as a removal tool for introduced fish and has several advantages over other chemical removal methods. An ammonia-based tool could be a cost-effective way to eradicate invasive crayfish and support conservation of native aquatic species while utilizing the natural nitrogen cycle to remove the ammonia from the environment and return an ecosystem to baseline conditions. I used laboratory experiments to develop and test lethal concentrations of ammonia for Northern crayfish (*Faxonius virilis)* and Red Swamp crayfish (*Procambarus clarkii*). I used a formulation of ammonium sulfate to elevate ammonia concentrations to 50mg/l, sodium carbonate to increase pH to 9.5, and sodium sulfite to lower dissolved oxygen to 0mg/l. The formulation achieved 100% mortality of *F. virilis* in 24

hours in a laboratory setting, but only 90% mortality of *P. clarkii.* A field trial with *P. clarkii* achieved mortality of most of the crayfish exposed to the ammonia treatment; however, live crayfish were found around the pond after the treatment. These results suggest that an ammonia-based chemical tool could be utilized for invasive crayfish management. I recommend that additional laboratory and field trials focus on further exploring species differences and the effectiveness of ammonia treatments under diverse field conditions.

ACKNOWLEDGMENTS

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Introduction

Invasion by nonnative species is one of the leading causes of population declines and range reductions in freshwater organisms (Dudgeon et al., 2006). Among the most destructive aquatic invasives are multiple species of crayfish, several of which have been introduced globally (Stebbing et al., 2014). *Procambarus clarkii* (Red swamp crayfish), native to the southeastern United States, is listed among the 100 worst invasive aquatic species (IAS) of Europe and is considered invasive in at least seventeen states in the U.S. (Cecchinelli et al., 2012; Loureiro et al., 2018). In Arizona, a state with no native crayfish, population declines of endangered fish and reptiles have been attributed to both *P. clarkii* and *Faxonius virilis* (Northern crayfish) (Mueller et al., 2006; Stone et al., 2014). Efforts to control invasive crayfish populations have met with little to moderate success, and increasing numbers of invasive species in aquatic systems brings a growing need for additional removal methods.

Many different methods have been used for invasive crayfish control. Mechanical removal, specifically trapping, has been the most commonly employed technique (Gherardi et al., 2011). While intensive trapping can reduce crayfish numbers and benefit native species, it frequently catches mostly larger adult males, reducing competition between juveniles and allowing them to grow and produce dense populations (Lodge et al., 2012). The use of biocides, or chemical substances intentionally used to kill living organisms, can be effective for certain aquatic species but their use is controversial given their lack of specificity and potential impacts on non-target organisms. Unlike fish, crayfish will leave the water when exposed to the adverse conditions of a biocide treatment, which requires the use of higher concentrations for biocides to be effective (Bierbower & Cooper, 2010; Peay et al., 2019). Chemical methods are one of the few tools that have the potential to eradicate entire invasive populations but there are currently no crayfish-specific biocides (Peay et al., 2019).

One potential chemical removal tool for aquatic invasive fish and invertebrates could be ammonia, a natural waste product in aquatic systems that many aquatic organisms produce and excrete (Harris et al., 2001; McCahon et al., 1991). Ammonia exists as two forms in aqueous solutions: NH_3 (the unionized form), and NH_4^+ (the ionized form). NH_3 is considered the more toxic form because it readily crosses gills and cell membranes, while NH_4^+ is generally considered to be non-toxic (Firkins, 1993). Crustaceans, including crayfish, excrete the NH_3 they produce by diffusion down its partial pressure gradient, mostly through the gills (Greenaway, 1991; Harris et al., 2001). As ambient NH_3 levels increase, ammonia excretion is inhibited by the lower internal to external gradient, causing ammonia to accumulate in the hemolymph and tissues. (Lin et al., 2022). NH_3 accumulation affects crayfish osmoregulation processes (Jiang et al., 2012), and can impair crayfish growth, reproduction, and survival (Harris et al., 2001).

Ammonia as a chemical tool works by increasing ammonia levels in the water and inhibiting the NH_3 excretion process in all aquatic organisms that have gills, including both fish and crayfish. Crayfish have some of the highest acute toxicity values of aquatic organisms, meaning that the ammonia concentrations that cause crayfish mortality are much higher than the concentrations that are toxic to other organisms (USEPA, 2013). Ammonia may have broader impacts beyond its effects on gilled organisms. Water with high ammonia levels can damage the intestinal lining and alter the microbiome of Red-eared sliders (*Trachemys scripta elegans*) and Chinese striped-necked turtles (*Mauremys sinensis)* (Ding et al., 2021; Khan et al., 2021), indicating that ammonia may also be harmful to aquatic reptiles.

Despite its broad impacts on aquatic animals, ammonia has several characteristics that are advantageous from a chemical treatment perspective. The ratio of NH_3 to NH_4^+ is influenced by temperature and pH, with NH_3 concentrations rising lightly with increasing temperature and steeply with increasing pH (Firkins, 1993; Hargreaves & Tucker, 2004). This relationship enables ammonia toxicity to be manipulated, and moderate amounts of ammonia in alkaline water can cause high and rapid mortality of crayfish (Foster & Turner, 1993). Furthermore, because ammonia is naturally occurring in aquatic systems, these systems have mechanisms to degrade and prevent the buildup of ammonia. Ammonia is broken down by aquatic bacteria via the nitrogen cycle, and can then be taken up by aquatic plants as their nitrogen source or emitted to the atmosphere in the form of N_2 (Wang et al., 2017; Dodds et al., 2017; Peterson et al., 2001). An ammonia-based removal tool could be a cost-effective way to eradicate invasive crayfish in systems already dominated by these species, utilizing the natural nitrogen cycle to clean up the treatments and return the system to a condition that can again support native species.

My goal in this study was to evaluate whether ammonia could be used as a chemical removal tool for invasive crayfish. I asked three main questions:

1. What is the lowest concentration of ammonia and additives such as a pH increaser and oxygen scavenger that will cause 100% mortality of crayfish in a laboratory setting?

I conducted a series of experiments with *F. virilis* to refine the ammonia concentrations from preliminary trials, and repeated the final formulation experiments with *P. clarkii.* I attempted to use concentrations that would achieve mortality, but also prevent crayfish from leaving the water if given a means to escape. The amounts of ammonia that are toxic to crayfish are much higher than the doses that will kill fish (Arthur et al., 1987), however I predicted that the use of sodium carbonate to increase pH and sodium sulfite to lower dissolved oxygen could increase the efficacy of ammonia as a tool.

2. Is there a difference in ammonia sensitivity between Arizona's two invasive species, *Faxonius virilis* and *Procambarus clarkii?*

Procambarus clarkii relies more heavily on burrows than *F. virilis* and may regularly be exposed to higher concentrations of ammonia; therefore, I predicted that there may be different ammonia sensitivities among these two species of crayfish.

3. Will effective laboratory doses cause mortality in a field setting?

After establishing effective concentrations in the laboratory, I conducted a pond trial with *P. clarkii*. I predicted that conditions in the field would necessitate higher concentrations than the doses that are effective in a laboratory setting.

Methods

Collection of crayfish for laboratory trials

Wild northern crayfish (*Faxonius virilis*) used in laboratory trials were caught in hoopnets (0.6 m diameter, 1.0 m length, 6 mm (¼ inch) mesh with a single 0.1 m throat) baited with hatchery-grade fish food Aquamax 600 catfish pellets from Upper Lake Mary near Flagstaff, Coconino County, Arizona. Collected crayfish were kept in 150-gallon fiberglass holding tanks with biofiltration at the US Forest Service, Rocky Mountain Research Station greenhouse facility (Flagstaff, Arizona), allowing for at least 3 days of acclimation before use in experiments. All treatments in each trial used crayfish from the same collection site and collection event to minimize effects caused by differences in physical site characteristics, water chemistry and variations in stress caused by capture and transport.

Preliminary laboratory trials with *F. virilis*

I conducted a series of preliminary trials in the fall of 2021 to determine if ammonia could be used to kill crayfish and to begin refining the dosage of ammonia and additives for maximum toxicity and effectiveness (Appendix A). Ammonia can be introduced into water as a liquid or as a salt. Ammonia salts release less noxious fumes than liquid ammonia and are much easier and safer to work. Ammonium sulfate was selected as the ammonia salt because it is far less expensive than ammonium chloride and appeared to be equally effective. To maximize ammonia toxicity, I used a sodium carbonate dose 210 mg/L to raise the pH to approximately 9.5, and 260mg/L to raise the pH to approximately 10 (Table 1). A pH of 9.5 achieved crayfish mortality from ammonia in previous studies with signal crayfish, and the lower dose of 210 mg/L was chosen for the formulation (Hiley & Peay, 2006; Hyatt, 2004). To reduce dissolved oxygen and further increase crayfish stress and ammonia absorption, I added sodium sulfite as an oxygen scavenger. I began our experiments with low amounts of sodium sulfite, increasing it to 260 mg/L with an ammonia treatment. A trial with only sodium sulfite yielded no mortality of crayfish (Table 1). Thus, I used a treatment with all three components (ammonium sulfate, sodium carbonate and sodium sulfite) in all future trials.

Table 1. Summary of treatment, water chemistry, and results from three preliminary laboratory trials with *Faxonius virilis* exploring different toxin concentrations and combinations: 1. adding low concentrations of sodium sulfite; 2. increasing concentrations of ammonium sulfate, sodium carbonate and sodium sulfite; 3. sodium sulfite with no other chemicals. Trials were conducted from September to November, 2021. Water chemistry parameters (total ammonia nitrogen (TAN), unionized ammonia (NH_3), and pH) are listed as a range over 24 hours.

Trial	Treatment			TAN mg/L	NH_3 mg/L	pH	Proportion crayfish dead in 24 hours
	Ammonium Sulfate (mg/L)	Sodium Carbonate (mg/L)	Sodium Sulfite (mg/L)				
1	160 160 160	210 210 210	0 26 50	40-48 39-50 40-49	16-26 17-25 17-26	9.1-9.5 9.1-9.4 9.2-9.4	20% 20% 40%
2	160 330 330	260 260 260	260 260 0	38-52 79-99 80-99	34-48 59-82 56-69	9.9-10.4 9.7-10.0 9.6-10.0	100% 100% 29%
3	-	-	530 400 260 210	-	-	8.5-8.8 8.6-8.8 8.5-8.7 8.5-8.7	0 0 0 0

Final laboratory trials for *F. virilis*

Aquatic ecosystems have mechanisms to break down ammonia, however high levels of ammonia can persist for several months before returning to baseline conditions (Frye, 2021).

To find the minimum lethal concentration, I used three lab trials in spring and summer of 2022 to further refine dosages of ammonium sulfate identified in the preliminary trials. I used three doses of ammonium sulfate: 160 mg/l (the lowest dose used in preliminary trials) and two lower doses of 130 and 110 mg/l to test a range of ammonia doses. I added 210 mg/l of sodium carbonate (to increase pH to 9.5) and 160 mg/l of sodium sulfite (to lower dissolved oxygen levels to approximately 0 mg/l for 24 hours) to each tank with the ammonium sulfate. Each trial had two replicates for each treatment (n=6 replicates per treatment across the three trials). Because of temperature differences between tank locations, treatments were randomly assigned to tanks for each trial.

I filled experimental tanks with 378.5 Liters (100-gallons) of tap water at least three days before each trial to allow off-gassing of chlorine. I added the chemicals one hour before the trial began and thoroughly stirred the tanks to ensure mixing and dissolving of chemicals. Each trial commenced when ten crayfish were added to each tank for a total of 60 crayfish per trial. Crayfish carapace lengths (CL) were between 30-45 mm. Tanks were checked for crayfish mortality every six hours for 24 hours and crayfish were recorded as dead when there was no response to stimuli and no movement of legs, antennae or pleopods. I removed dead crayfish from the tanks to prevent effects of decomposition on ammonia levels. The CL of each crayfish was measured with calipers and recorded. Crayfish sex was determined and recorded.

I measured and recorded temperature, pH, total ammonia concentrations and dissolved oxygen at the beginning of each trial, and every six hours until the end of each trial at 24 hours. I used a Hannah instruments pH meter (HI98128 pHep®5) and high-range ammonia colorimeter (H1733 Checker®HC). I measured dissolved oxygen and temperature with a YSI multiparameter water quality meter (ProDSS). I calculated total NH_3 concentrations using the Emerson ammonia equation which estimates NH_3 (the toxic form of ammonia) from pH, temperature, and total ammonia (Emerson et al., 1975).

Laboratory escape trial

Freshwater crayfish species can survive out of water for some time, and certain species, especially *P. clarkii*, may exit a water body in response to adverse conditions or hypoxia (Holdich et al., 1999; Ramalho & Anastácio, 2015). This potential for escape is important to assess because it could substantially reduce mortality. I conducted additional laboratory trials in the Rocky Mountain Research Station with the same three dosages (160, 130, 110 mg/l) of ammonium sulfate to quantify escape attempts made by *F. virilis*. I used four 100-gallon tanks for each escape trial (n=3); 1 tank per trial for each ammonia concentration and one control tank per trial with no chemicals added. Each tank had an “escape ramp” (a piece of coarse plywood that crayfish would not slide off) that led to a 5-gallon bucket filled with tap water. Crayfish could use the ramp to exit the tank and subsequently fall into a bucket. The escape trial was left undisturbed for 24 hours so as not to interfere with crayfish behavior; i.e., potentially startle crayfish back into the water from the ramp. I recorded mortality and escape, as well as sex and CL for each crayfish at the end of the trial. Water temperature, pH, total ammonia nitrogen and dissolved oxygen were recorded at the beginning of the trial, and at the end of the trial after 24 hours. It was important to conduct this trial separately from the mortality trial because escape attempts would confound mortality estimates. Crayfish that exit the ammonia-treated water will no longer be affected by ammonia toxicity, and the lethal effects of the ammonia will be delayed if not negated.

Laboratory trial for *P. clarkii*

Red swamp crayfish (*Procambarus clarkii*), although common in southern Arizona, are not established in the northern part of the state and are illegal to transport within the state of Arizona. For this reason, I captured and tested *P. clarkii* in a location other than our research laboratory in Arizona. Rancho Jamul Ecological Reserve near San Diego (San Diego County) in southern California contains a large population of *P. clarkii,* and so *P. clarkii* were caught via seining from Jamul Creek and used in laboratory trials on site at Rancho Jamul. Crayfish were transported in 5-gallon buckets and acclimated in a holding tank filled with oxygenated tap water for one hour before trial.

In order to test for species differences in ammonia sensitivity, I conducted one trial with *P. clarkii* in Rancho Jamul, California in September 2022. I used the same methods as the *F. virilis* trials, with the following exceptions. I conducted only one trial over a 24-hour period because of time constraints. Crayfish (n=120) were added to each of 6 treatment tanks, with 20 crayfish in each tank, for a total of 2 tanks per ammonia treatment. Tanks were filled with tap water approximately 18 hours before the trial. I tested water for chlorine with an aquarium chlorine test strip kit, and chlorine concentrations were negligible. Crayfish were captured from Jamul Creek the same day as the treatment and allowed to acclimate in a tank containing oxygenated, untreated tap water for one hour before trial. Forty crayfish were added to two control tanks (n=20/tank) to assess whether stress of capture, handling and transport would cause mortality.

Field Trial

Previous investigations of biocides have determined that field settings require higher concentrations than those that effectively cause mortality in a laboratory setting (Lidova et al., 2019; Peay et al., 2006; Peay et al., 2019). To investigate whether our laboratory ammonia concentrations would also cause mortality of crayfish in a field setting, I conducted a field test in a crayfish-free stock pond at the Rancho Jamul Ecological reserve on September 8th, 2022. The Environmental Protection Agency (EPA) requires an experimental use permit for experimental biocide treatments; however, treatments can be exempt from this permit if they are less than 1 acre in size, not connected to other water bodies, and do not contain water or organisms used for drinking water or consumption, respectively. Rancho Jamul Ecological reserve has a stock pond that meets these requirements, thus this pond was chosen for the treatment and subsequently *P. clarkii* were the target species. The pond is a seasonal water source for the reserve managers and grazing use, and is filled with rainwater, as well as inflow from a well pump. Rancho Jamul and employees erected silt fencing around the pond perimeter 15-30cm from the water's edge 3 days before the pond trial. Two days before the planned treatment of the pond, personnel dug 10 plastic, bottomless garbage cans (50 gallons) into the mud substrate of the pond to serve as controls. The areas enclosed by these cans had the same substrate and water chemistry as the surrounding pond, but were protected from chemical treatment.

The treatment pond does not contain any crayfish, thus *P. clarkii* were captured and transported from Jamul Creek. Crayfish were placed in treatment containers, Gee steel wire mesh (¼-inch) minnow traps with entrances zip-tied shut or stuffed with Styrofoam to prevent escape. Each control can contained one minnow trap with 6 crayfish, and 6 crayfish loose in the can to assess if burrowing would occur in 24 hours. Each control can had an adjacent minnow trap, also containing 6 crayfish, that was subjected to the treatment (Figure 1). I released the remaining crayfish (n=480) loose into the pond to quantify attempts to escape out of the pond. I caught the *P. clarkii* to use in the pond trial using minnow traps and via seining (n=660) in Jamul Creek. Minnow traps were set at 16:00 hrs the day before pond trial and collected at 09:00 the morning of the trial. I caught additional crayfish with seines in Jamul Creek the morning of the pond trial. Crayfish were transported to the pond site and placed directly into treatment containers or were released into the treatment pond. Water quality parameters of pH, temperature, dissolved oxygen and total ammonia nitrogen (TAN) were taken at 11am before crayfish or chemicals were added (Appendix B).

I estimated the pond size to be approximately 560,241 liters (148,000 gallons), and chemical amounts were calculated based on this estimate and a concentration goal of 50mg/l total ammonia nitrogen (TAN). The application of chemicals began once all crayfish were in the pond. I first applied six 50 lb bags of ammonium sulfate, then six 50 lb bags of sodium sulfite. I added eight 50lb bags of sodium carbonate last, as previous applications have found that elevated pH levels can begin decreasing before the other additives depending on soil characteristics (Frye, 2021). I achieved mixing and stirring of chemicals by walking throughout the pond as chemicals were dispersed.

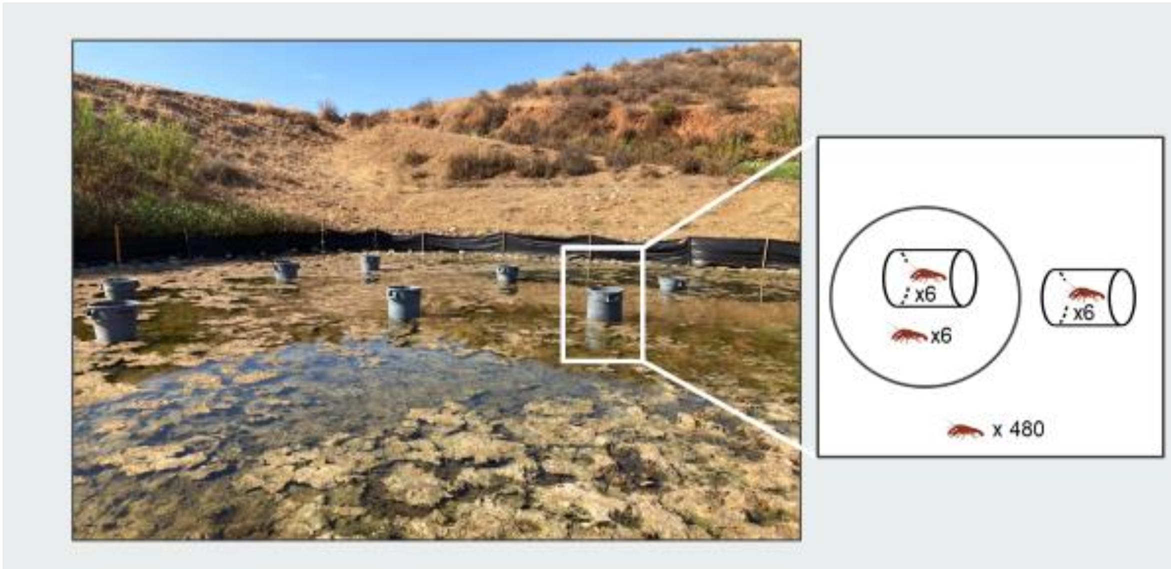

Figure 1. Photograph and schematic representation of ammonia treatment pond trial and placement of crayfish (*Procambarus clarkii*) at Rancho Jamul Ecological Reserve (San Diego County, California) on September 8th, 2022. Treatment setup contained control cans that were not affected by the treatment (n=10). Each control can contained one minnow trap with crayfish (n=6) and loose crayfish (n=6). One minnow trap was placed next to each can (n=10) that contained 6 crayfish that were exposed to the treatment (n=60). We placed loose crayfish in the pond that were also exposed to the treatment (n=480).

Data Analysis

To assess survival of crayfish after ammonia treatments in the laboratory trials, I conducted a survival analysis using the Kaplan-Meier method; a non-parametric time-to-event analysis. One difficulty of survival analyses is that there are often individuals that do not experience the event, in this case death, and so survival time and probability is underestimated. The Kaplan-Meier method uses survival times of uncensored and censored individuals (those that experience, and do not experience the event, respectively) to estimate survival probabilities over period of time (Clark et al., 2003; Zabor, 2022). I tested for differences in survival over 24 hours due to species (*F. virilis* vs. *P. clarkii*); size (carapace lengths of 25-30mm, 31-35 mm, 36-40mm); sex (male vs. female), and ammonia dose (110, 130, 160 mg/L). All survival analyses were performed using the survival package in R (R version 4.1.3, 2022). Differences in survival between groups were assessed using a non-parametric log-rank test with the survdiff function. I created time-to-event Kaplan-Meier plots with the ggsurvfit package (Sjoberg et al., 2022).

Results

Mortality rates in the laboratory

Faxonius virilis

The Northern crayfish (*Faxonius virilis*) laboratory trials resulted in nearly 100% mortality of *F. virilis* over 24 hours (Figure 2a). The two lower doses of 110 mg/L and 130 mg/L were sufficient to cause 100% mortality in all trials, and I found no significant differences in survival between the three doses (Log-rank difference test, p=0.2). In the first six hours, the 160mg/L dose appeared to have slightly higher mortality (about 98% vs. 80 % for the two lower doses), but this difference was not statistically significant (p=0.3).

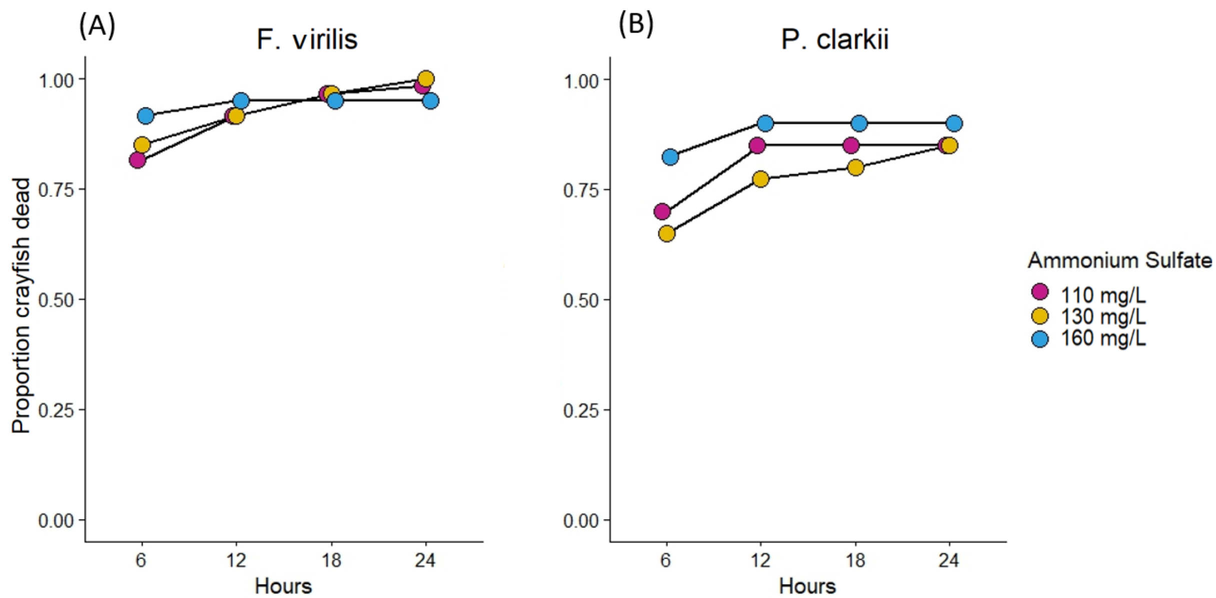

Figure 2. *Faxonius virilis* (Figure 2a) and *Procambarus clarkii* (Figure 2b) mortality during 24-hour laboratory ammonia dose refinement trial with three doses of ammonia (110, 130, and 160 mg/l). All treatments contained 210 mg/l sodium carbonate and 160 mg/l sodium sulfite. *Faxonius virilis* trials conducted in Flagstaff, Arizona in spring and summer 2022. *Procambarus clarkii* trials conducted in Rancho Jamul Ecological Reserve, San Diego, California in Septermber, 2022.

Procambarus clarkii

None of the formulations in the Red swamp crayfish (*P. clarkii)* laboratory trial were able to achieve 100% mortality in 24 hours (Figure 2b). The higher dose of ammonia appeared to achieve slightly higher mortality (90% vs. 85% for the two lower doses); however, the differences between ammonia treatments were not significant (Log-rank difference test, p=0.4). I observed multiple *P. clarkii* at the surface of the water, either attempting to climb out of the tank or place their gills out of the water to intake oxygen from the air. At times there were as many as 9 crayfish at the surface of the water during the *P. clarkii trial,* whereas no more than one crayfish was seen near the surface during the *F. virilis* trials. There was no mortality of *P. clarkii* in either of the two control tanks during the trial.

The *F. virilis* and *P. clarkii* trials occurred in different locations, and the water chemistry parameters of pH and unionized ammonia (NH_3) varied between the two species' trials despite using the same ammonia formulation (Figure 3). The realized pH in the *P. clarkii* trial was consistently lower by about 0.5 throughout the 24 hours. Therefore, although total ammonia nitrogen (TAN) levels and temperatures were comparable across, the NH_3 values were lower in the *P. clarkii* trial because of the relationship between pH and unionized ammonia (Hargreaves & Tucker, 2004).

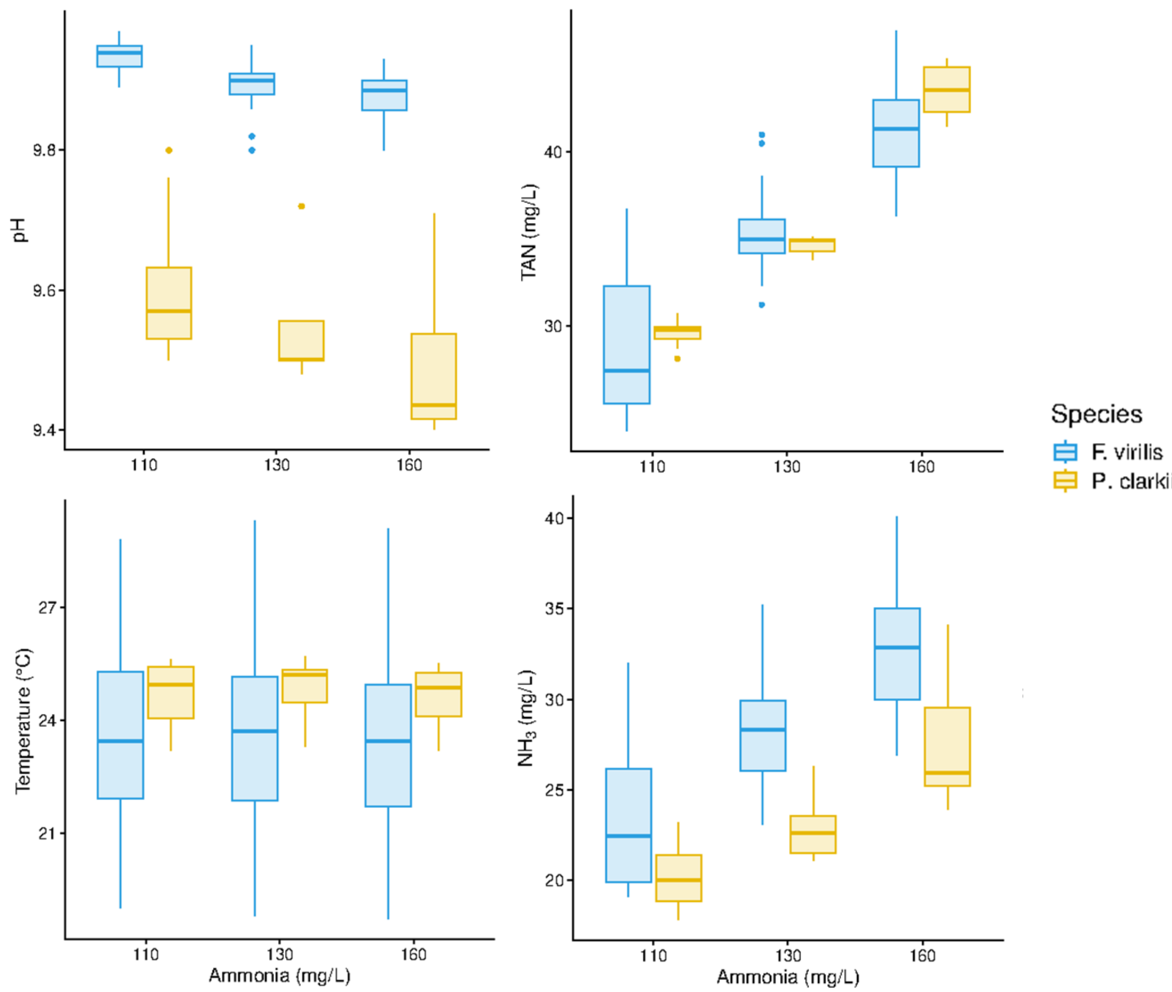

Figure 3. Box and whisker plots of water chemistry parameter averages during 24-hour refinement trials for three doses of ammonium sulfate (110, 130, 160 mg/L). Dark lines within each box indicate median; upper and lower boxes represent 1st and 3rd quartiles (25th and 75th percentiles); whiskers represent variability outside Q1 and Q3; dots represent outliers. *Faxonius virilis* trials (blue) were conducted in Flagstaff, Arizona during spring/summer, 2022 and *Procambarus clarkii* (yellow) was conducted in Rancho Jamul, California in September, 2022. Parameters were measured at the beginning of each trial and every six hours for 24 hours.

Survival analysis

The overall probability of survival for *F. virilis* after 24 hours was 1.1% (Table 2). The survival curve showed a steep drop within the first 6 hours to almost 0 probability at 24 hours

(Figure 5). Female *F. virilis* had a slightly but not significantly higher survival probability than male *F. virilis* (p=0.2) (Appendix C).There was also no significant difference in survival between the size classes (p=0.1). The highest ammonia dose treatment (160mg/l) appeared to have lower survival in the first six hours, but this difference was not significant (p=0.3).

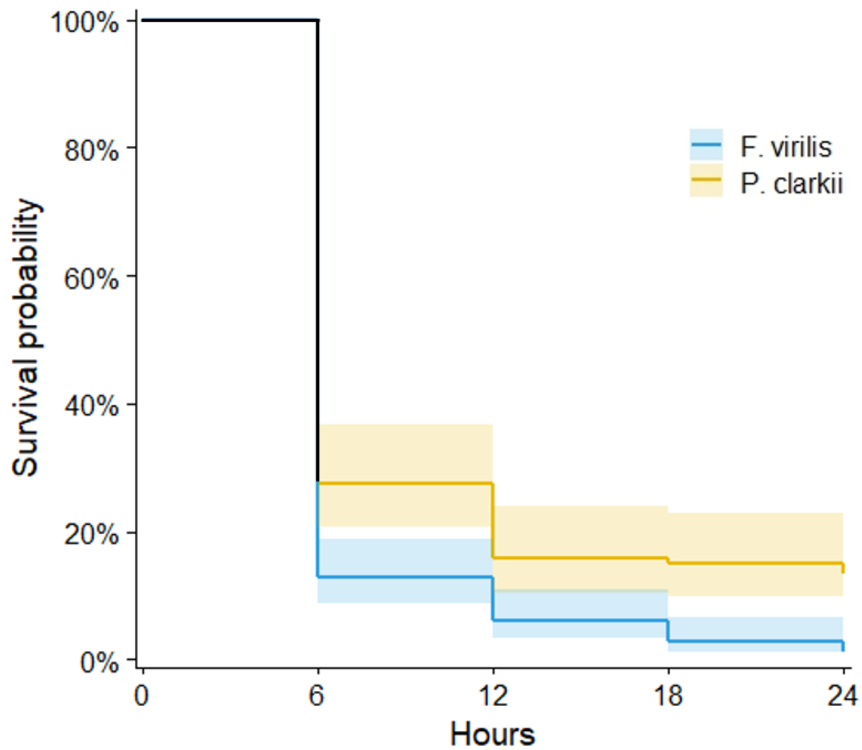

Figure 4. Kaplan-Meier plot of survival probability over 24 hours for *Faxonius virilis* (blue) and *Procambarus clarkii* (yellow) with 95% confidence intervals. Black line is the survival curve for both species. *Faxonius virilis* trials (blue) were conducted in Flagstaff, Arizona during spring/summer, 2022 and *Procambarus clarkii* trial (yellow) was conducted in Rancho Jamul, California in September, 2022.

Table 2. Crayfish Survival Probability of laboratory ammonia refinement trials. Probabilities given for crayfish overall and for each species. Crayfish probability of survival after 6 hours and after 24 hours with 95% confidence intervals in parentheses. *Faxonius virilis* trials were conducted in Flagstaff, Arizona during spring/summer, 2022 and *Procambarus clarkii* trial was conducted in Rancho Jamul, California in September, 2022.

	6 hours	**24 hours**
Overall	60% (3.8%, 9.4%)	6% (3.8%, 9.4%)
F. virilis	57% (52%, 62%)	1.1% (0.28%, 4.4%)
P. clarkii	64% (58%, 70%)	13.3% (8.5%, 21%)

The probability of survival for *P. clarkii* after 24 hours was 13.3%, 11% higher than the survival probability of *F. virilis* (Table 2). *Procambarus clarkii* female survival probability was 7% (but not significantly) higher than males (p>0.1) (Appendix C). There was no effect of size (p=0.5) or ammonia dose (p=0.4) on *P. clarkii* survival (Appendix C). Comparing the two species, I did find a significantly higher survival probability for *P. clarkii* at 24 hours (Log-rank test of survival, p<0.0005, Table 3). *F. virilis* had consistently lower survival than *P. clarkii* throughout the 24-hour trial. For both species overall, female crayfish had a 6% higher survival than males, but these differences were not significant (p=0.05 at 24 hours and p=0.07 at 6 hours).

Table 3. Results of survival analysis log-rank test for differences between groups (species, ammonia dose, size, and sex) at 24 hours and 6 hours. *Faxonius virilis* trials were conducted in Flagstaff, Arizona during spring/summer, 2022 and *Procambarus clarkii* trial was conducted in Rancho Jamul, California in September, 2022.

24 Hours	**Model**	**n**	**$\chi2$**	**df**	**p value**
	Species	120, 180	18.2	1	0.00005***
	Ammonia Dose	100, 100, 100	3.3	2	0.2
	Size	94, 139, 67	5.4	2	0.07
	Sex	101, 198	3.7	1	0.05
6 Hours	**Model**	**n**	**$\chi2$**	**df**	**p value**
	Species	120, 180	8.5	1	0.004**
	Ammonia Dose	100, 100, 100	5.2	2	0.08
	Size	94, 139, 67	4.6	2	0.1
	Sex	101, 198	3.2	1	0.07

Escape Trial

I found that ammonia treatment did not trigger northern crayfish to escape their tanks. Whereas most crayfish in the control tanks (63%) did escape into the freshwater bucket during the escape trial, no crayfish in treatment tanks escaped. All Crayfish in the two higher dose treatments (130 mg/l and 160 mg/l) were dead after 24 hours in treatment tanks, which is similar to our findings in the mortality trials (Figure 4). In the lowest dose treatment (110 mg/l), 11% of crayfish were still alive at the end of 24-hour escape trial.

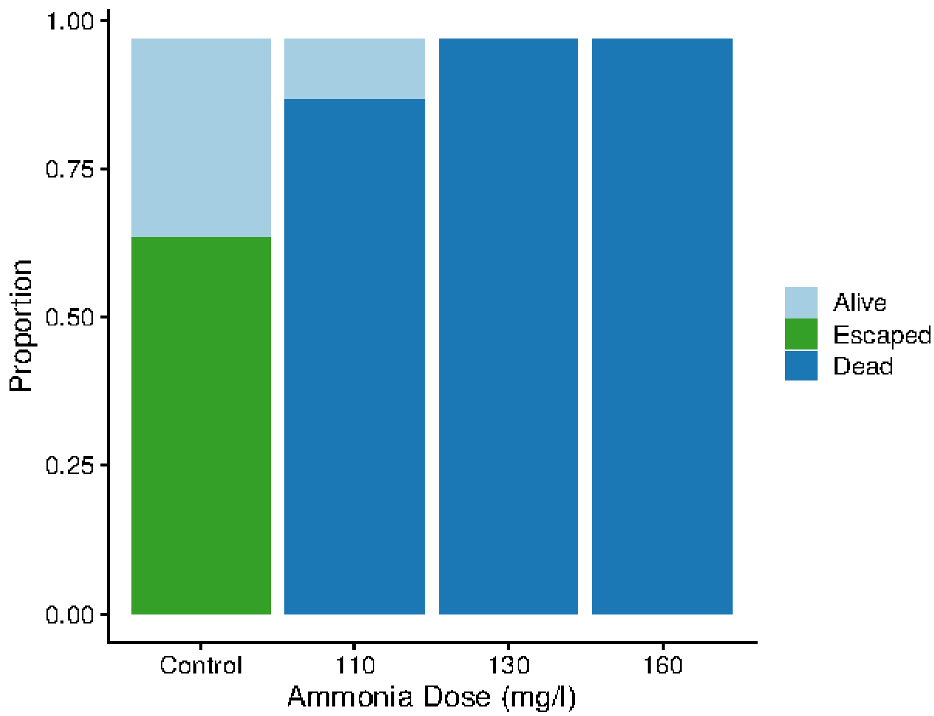

Figure 5. Proportions of crayfish (*Faxonius virilis;* n=30 crayfish per treatment, including control) that escaped, survived or died during 24-hour escape trials in Flagstaff, Arizona during summer 2022. I used the same concentrations as for the mortality trials; in addition to the ammonia dose, treatment tanks had 160mg/l sodium sulfite and 210 mg/l sodium carbonate. Control tanks had no chemicals added.

Field Trial

In the pond trial, all crayfish (*P. clarkii)* placed in minnow traps and exposed to ammonia treatment were dead within the first 6 hours of the trial. There was no mortality of crayfish in control cans over 24 hours, except for two crayfish that were crushed when minnow traps were replaced after first mortality check. Throughout the first 6 hours, I observed several crayfish crawling on top of algal mats and out of the water. Over the 24-hour trial, a number of crayfish (n=27) were partially submerged in shallow water at the edges of the pond, or completely out of the water on the pond banks. The treatment killed the majority of loose crayfish, although not all 480 crayfish were accounted for because of difficulty recovering them from the water. Two live crayfish were found around the pond: the first at 6 days after treatment, and the second at 14 days.

Water chemistry parameters were recorded before treatment, right after beginning of trial and for every 6 hours for 24 hours. Based on the calculated TAN values, the water volume of the pond was likely much less than what was assumed when calculating necessary dosages (Appendix 2). Total ammonia levels approached levels of approximately 200 mg/l. Thus the *P. clarkii* in the pond trial were exposed to ammonia levels nearly 4 times higher than the concentration used in laboratory trials.

Discussion

Invasive crayfish have become a global concern in the conservation of aquatic ecosystems. Many studies have investigated techniques for management and control of nonnative crayfish, but few have examined ammonia as a potential biocide for crayfish removal. The experiments completed in this study have shown that a combination of ammonia, a pH increaser (sodium carbonate) and an oxygen scavenger (sodium sulfite) can cause crayfish mortality in the laboratory and in a field setting. Although these are promising preliminary results, a better understanding of the formulation components, species differences in sensitivity, effectiveness under field conditions, and the impacts on non-target organisms and aquatic ecosystems is still needed.

Formulating minimum lethal doses of ammonia and adjuvants

Ammonia is the natural waste product of aquatic organisms, but it is also a pollutant from industry and agriculture, as well as wastewater treatment effluent and sewage (Jiang et al., 2012). Adding ammonia into the environment is prohibited, and the ammonia concentrations that are acceptable in natural water bodies systems are highly regulated. The environmental protection agency (EPA) recommends ammonia levels should never exceed 17 mg/l at a neutral pH and temperature of 20 °C. This is the suggested dose for protecting freshwater organisms from the potential effects of acute ammonia toxicity (USEPA, 2013). Under Section 303(c)(1) of the clean water act (CWA), states and Tribes are responsible for developing and maintaining water quality standards. Therefore, laws and regulations for adding ammonia to bodies of water may differ by state and agency (USEPA, 2013).

Ammonia is naturally broken down via the nitrogen cycle in aquatic systems, however the length of time for systems to return to baseline conditions will depend on temperature, sediment, and water chemistry parameters of the system. For ammonia to be used as a potential biocide, the time it would take for higher doses that are lethal to crayfish to naturally break down via the nitrogen cycle must be considered. Previous field applications of ammonia for removal of invasive fish in Arizona found that it could take 3 months or longer to return to baseline conditions (Frye, 2021). A 2011 application took 45 days for ammonia to drop from 38 mg/l to baseline conditions, with water temperatures averaging between 10 and 15 °C (Ward, Morton-Starner, & Hedwall, 2013). The authors of this study surmised that the colder water temperatures slowed nitrification bacteria growth, causing ammonia to persist longer. This ammonia application also induced an algal bloom which appeared 10 days after the treatment. Ammonia is broken down into nitrates which are taken up by plants (Rabalais, 2002), and the possible eutrophication after adding ammonia to an aquatic system should be taken into account as a potential risk of an ammonia treatment.

The environmental impacts and possible accumulation of the other two adjuvants in this ammonia formulation must also be considered. My preliminary laboratory trials found that both sodium carbonate and sodium sulfite were necessary to achieve 100% mortality of *F. virilis,* increasing the efficiency of ammonia and enabling less ammonium sulfate to be used. Hiley & Peay (2006) found that a pH increaser was necessary to raise the percentage of NH_3 and induce mortality of *Pacifastacus leniusculus* (Signal crayfish) using ammonia. Lloyd (1961) found that lowering dissolved oxygen with a substance like sodium sulfite also lowers excreted carbon dioxide at the gill surface, thus raising pH of the water around the gills and increasing ammonia toxicity. Sodium carbonate is labeled as a safer chemical of low concern by the EPA, and because of its natural occurrence, is not expected to adversely affect wildlife or water resources (National Center for Biotechnology Information, 2023; USEPA, 2006). Sodium sulfite is widely used as a food additive and preservative and because it is listed as a 'food-grade substance', there is little information about its environmental impacts and breakdown. Although sodium sulfite and sodium carbonate are both considered low-risk chemicals by the EPA, persistence of higher concentrations and how they may affect aquatic wildlife would need to be thoroughly researched and considered before this formulation could be more widely applied.

Factors affecting crayfish sensitivity to ammonia

I found clear differences in survival between the two species of crayfish, with *P. clarkii* having higher survival than *F. virilis*. I hypothesized that *P. clarkii*'s heavier reliance on burrows, and subsequent exposure to higher ammonia concentrations when confined in burrows, would make them less sensitive to the ammonia treatment. However, the higher mortality of *F. virilis that* I observed in the laboratory trials may not be entirely due to differing ammonia sensitivities. The *P. clarkii* laboratory trial was conducted in a different location with different capture methods, acclimation times, and water conditions. The water used in the *P. clarkii* trials was consistently a lower pH than the water used in the *F. virilis* trials. The pH of water is an important determinant of NH_3 percentages, and the lower pH values in the *P. clarkii* trial resulted in lower amounts of NH_3, or lower amounts of ammonia's toxic form. There may be an ammonia toxicity threshold for *P. clarkii* that the lower pH levels in the trial did not enable us to reach. Future investigations of ammonia treatments for different crayfish species would require the same NH_3 values to confirm whether there are interspecific differences or not.

Previous studies have documented differences in toxicity and deoxygenation sensitivities among crayfish species (Firkins, 1993; Banha & Anastacio, 2014; Lidova et al., 2019; Wisniewski et al., 2020). In a review of acute toxicity trials of different toxicants' effects on crayfish, Eversole & Seller (1997) concluded that the *Procambarus* genus was less sensitive to toxicants than Orconectes (now classified as Faxonius for surface-dwelling species). An investigation of the efficacy of different potential biocides on *P. leniusculus* found that 20 mg/L of ammonia at pH of 9.5 could achieve 100% mortality in 24 hours (Hiley & Peay, 2006), about 2-3 times less than what was needed for *F. virilis* in our experiment. In this same study, Hiley and Peay were able to achieve 100% mortality of *P. leniusculus* after 20-24 hours at temperatures of 20-30 °C by reducing the dissolved oxygen to 0.67 mg/l saturation. Alternatively, Cowan & Storey (2001) found 100% survival of *F. virilis* after 20 hours in anoxic conditions, and only 10% mortality after 48 hours. When manipulating only oxygen in our preliminary trials, I failed to achieve any mortality of *F. virilis* even after 72 hours in 0% oxygen (Table 1), which suggests clear species differences in deoxygenation sensitivity. I recommend further experimentation on multiple crayfish species to better quantify appropriate concentrations of ammonia and additives such as sodium sulfite.

Sensitivity to ammonia may have been confounded by differing sensitivities to deoxygenation. More *P. clarkii* were observed at the surface breathing air during the trial, sometimes as many as nine crayfish at once. This would cause them to take in less ammonia through their gills and thus be less affected by the treatment. Previous studies have found that when dissolved oxygen levels drop to less than 3mg/l, *P. clarkii* will climb to the surface or leave the water to obtain atmospheric oxygen (Souty-Grosset et al., 2006). Lowering the dissolved oxygen to 3mg/l as opposed to 0mg/l may not elicit an escape response in *P. clarkii*, allowing the ammonia treatment to be more effective. Therefore, although sodium sulfite was a necessary component for achieving 100% mortality of *F. virilis* in the laboratory, I recommend more research to attain a better understanding of sodium sulfite and deoxygenation's role in increasing effectiveness of ammonia-based formulations for crayfish control.

My experiments found marginally significant survival differences between males and females of both species over the 24-hour trial period. Many previous studies on crayfish sensitivities to toxicants such as pyrethroids and carbon dioxide have not found sex differences in sensitivity (Cechinelli et al., 2012; Fredricks et al., 2020). However, berried females of certain species might spend more time in burrows, and thus might be exposed to higher ammonia concentrations and slightly more tolerant, as I hypothesized might happen with *P. clarkii.* I did not find survival differences by size, but all of our crayfish were adults, and similar in size. Smaller crayfish in early life stages and juveniles tend to be much more susceptible to toxicants (Eversole & Seller, 1997; Morolli et al., 2006). Additional trials focusing on differences in sensitivity and survival by size and sex may help to inform applications and how to use crayfish life history to maximize treatment effectiveness.

Effectiveness of field treatments

Studies testing other chemical control methods have cautioned that field conditions produce unpredictable effects for biocides and chemicals, and that laboratory toxicity values do not transfer over to field conditions (Eversole & Seller, 1997). Peay et al. (2006) found it took almost 4 times as much time to kill crayfish in the field with a pyrethroid concentration that was lethal in 24 hours in a laboratory setting. My pond treatment at Rancho Jamul Ecological achieved 100% mortality of crayfish in treatment minnow traps and was able to kill the majority of loose crayfish in the pond (Figure 1). However, due to uncertainty in the volume of water to be treated, the realized treatment concentration was roughly four times the target laboratory concentrations. The higher concentration of sodium carbonate kept the pH at 9.5 for 24 hours, which can start to drop quickly at lower concentrations in field settings

(Frye, 2021). Therefore, I cannot be certain if the ammonia concentrations used in the laboratory trials would have been as effective as the higher dose.

One of the additional challenges with invasive crayfish control in the field is their ability to leave the water body and survive for some time outside of it. Previous biocide treatments have documented crayfish emergence out of the water as well as into burrows where the treatment often does not reach (Ramalho & Anastácio, 2015; Holdich et al., 1999; Gherardi et al., 2011). An effective biocide treatment would have to debilitate crayfish quickly enough to prevent escape or would need to include a way to capture or treat escaping crayfish. Studies with pyrethroids have found that spraying the biocide on the surrounding shoreline can effectively kill any crayfish attempting to leave the water body (Sandodden & Johnsen, 2010; Ballantyne et al., 2019). This method would not work with ammonia, as its mode of entry is through the gills. Thus, concentrations would have to be high enough to prevent escape, or alternative methods to address escaping crayfish would have to be used.

In my laboratory escape trial with *F. virilis,* none of the crayfish in the treatment tanks escaped into the freshwater bucket, whereas the majority of crayfish in the control tank with no chemicals added were found in the escape bucket after 24 hours (Figure 4). Thus, my ammonia treatments seemed to be strong enough to prevent *F. virilis* from being able to find the ramp and leave the water. During my laboratory trials, I observed many *P. clarkii* breathing air at the surface. There were also a number of crayfish that crawled out of the water onto algal mats, or were partially submerged at the pond edges during the field trial. Because of these observations, and previous studies documenting this behavior in *P. clarkii,* more trials investigating escape potential of *P. clarkii* should be conducted. Understanding the escape behavior of *P. clarkii* in response to ammonia and water temperature and chemistry would be valuable for determining an appropriate ammonia concentration for that species. The formulation concentrations investigated in this study should be applied in more field settings to better understand how factors such as sediment, water chemistry, aquatic vegetation, and microbial communities, would impact the effectiveness of an ammonia treatment.

Impacts on non-target organisms and ecosystems

Use of biocides for control of undesirable species remains controversial due to impacts on non-target organisms. The toxic form of ammonia, NH_3, enters the body through the gills and will thus affect any aquatic organisms with gills, including but not limited to fish, invertebrates, tadpoles, and snails. In a study of five fish and nine invertebrates, the crayfish *Faxonius immunes* was the least sensitive to the ammonia treatment, requiring concentrations over thirty times what was lethal to the most sensitive fish species (Arthur et al., 1987). The acute toxicity values of crayfish species in the *Faxonius* genus are three times higher than the acute toxicity values for Northern Leopard Frog embryos and tadpoles (*Rana pipiens*)

(USEPA, 2013). Water containing high ammonia concentrations can cause mortality in adult frogs (Oldham et al., 1996), and can damage the intestines of aquatic turtles (Ding et al., 2021). Thus, ammonia treatments have the potential to harm non-target aquatic organisms that do not have gills and terrestrial organisms that use treated water sources.

In the desert southwest, small waterbodies are important sources of biodiversity and water sources for terrestrial organisms. These include ponds and lotic systems that form perennial pools during dry periods, and they are becoming more threatened because of long-term drought and lowering of water tables with increased water demand of a growing human population (Biggs et al., 2017). Natural and anthropogenic water sources, such as stockponds, can be important refuges of biodiversity in an arid landscape, but can also harbor invasive species such as crayfish and American bullfrogs (*Lithobates catesbeiana)* that can lower invertebrate richness and diversity (Hale et al., 2015). The application of biocides to sensitive water bodies should be carefully considered, however small water bodies that are dominated by invasive species with few native species could be ideal localities for developing chemical tools such as ammonia.

Integration of ammonia with other control tools

Integrated management using a range of control and removal techniques is most likely to yield the best management results for invasive crayfish (Freeman et al., 2010; Fredricks et al., 2020). Crayfish occupy different habitats and adopt different life history traits in their invaded territories (Jackson et al., 2014), and one single method would not be effective in all invasive crayfish situations. Eradication of invasive populations becomes increasingly difficult once a species is established, either due to expense constraints or unacceptable environmental impacts (Simberloff et al., 2013). To date, only two methods have achieved eradication of invasive crayfish; infilling of ponds, which is not often feasible, and the application of biocides, which can be expensive and harmful to non-target organisms (Peay et al., 2019). The difficulty of eradication presents a need to seriously consider the costs and benefits of using harmful, non-specific treatments like biocides. Some combination of biocides such as ammonia and other removal techniques may be the best option, limiting the harm of chemical additives while complementing the less effective aspects of other methods. Continuing to refine the effective concentrations of ammonia and accompanying adjuvants that are toxic to crayfish will help establish it as a tool. Ammonia can then potentially be used with other management techniques such as trapping or a form of biological control. I recommend that future studies investigating ammonia as a removal tool consider this combinative option.

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Appendix A

Preliminary trials Methods

In the fall of 2021, I conducted a series of preliminary trials to begin refining the dosage of ammonia and additives for maximum toxicity and effectiveness (Table 1). I determined that there was no difference in crayfish mortality between ammonium sulfate and ammonium chloride, thus all trials used ammonium sulfate as the ammonia salt, as ammonium sulfate is far less expensive than ammonium chloride.

Trial 1 September 20 2021: Adding sodium sulfite

Crayfish collected via hoopnetting and seining methods on 9/19/2021 from Upper Lake Mary. Kept in holding tank with biofiltration for 48 hours before beginning of trial. On 9/18/2021, 6 tanks were filled with 100 gallons of water. Tanks were filled 2 days before trial to allow off-gassing of chlorine to occur.

On morning of trial, 3 different treatments of sodium sulfite were added (n=2 tanks per treatment); 0 mg/l sodium sulfite, 26 mg/l sodium sulfite, 50 mg/l sodium sulfite. All tanks also received 160 mg/l ammonium sulfate and 210 mg/l sodium carbonate. Chemicals were thoroughly mixed and dissolved before adding crayfish. Trial commenced at 10:30 when 10 crayfish were added to each tank (n=60). Crayfish were checked for mortality and water chemistry tested and recorded every 6 hours for 48 hours.

Trial 2 November 3 2021: Increasing chemical amounts

Crayfish collected via seining methods on 10/25/2021 from Upper Lake Mary. Kept in holding tank and allowed to acclimate with biofiltration for 8 days before beginning of trial. Crayfish were fed hatchery fish food 3 days before trial. 9 experimental tanks were filled with 100 gallons of water 3 days before beginning of trial.

On morning of trial, chemicals for three different treatments were added to tanks: (1) Low ammonium sulfate (160 mg/L), high sodium carbonate (260 mg/L), high sodium sulfite (260 mg/L); (2) High Ammonium sulfate (330 mg/L), high sodium carbonate (260 mg/L), high sodium sulfite (260 mg/L); (3) High ammonium sulfate (330 mg/L), High sodium carbonate (260 mg/L), No sodium sulfite (0 mg/L). Chemicals were thoroughly mixed and dissolved before adding crayfish. Trial commenced at 09:00 when 10 crayfish were added to each tank (n=90). Crayfish were checked for mortality and water chemistry tested and recorded every 6 hours for 48 hours.

Trial 3 November 22 2021: Sodium Sulfite and no other chemicals

Crayfish were collected via seining methods on 11/20/2021 from a stockpond located on 89A south of flagstaff. Crayfish were kept in biofiltrated holding tank for 48 hours before start of trial. 9 experimental tanks were filled with 100 gallons of tap water water four days before trial on 11/18/2021.

On morning of trial, 4 different treatments of sodium sulfite were added to 8 tanks (n=2 tanks per treatment): 210 mg/L, 260 mg/L, 400 mg/L, 530 mg/L. One control tank had no chemicals added. Chemicals were thoroughly mixed and dissolved before adding crayfish. Trial commenced at 08:00 when 10 crayfish were added to each tank (n=90). Crayfish were checked for mortality and water chemistry tested and recorded every 6 hours for 24 hours, then every 12 hours for 5 more days.

Appendix B

Pond trial water chemistry parameters

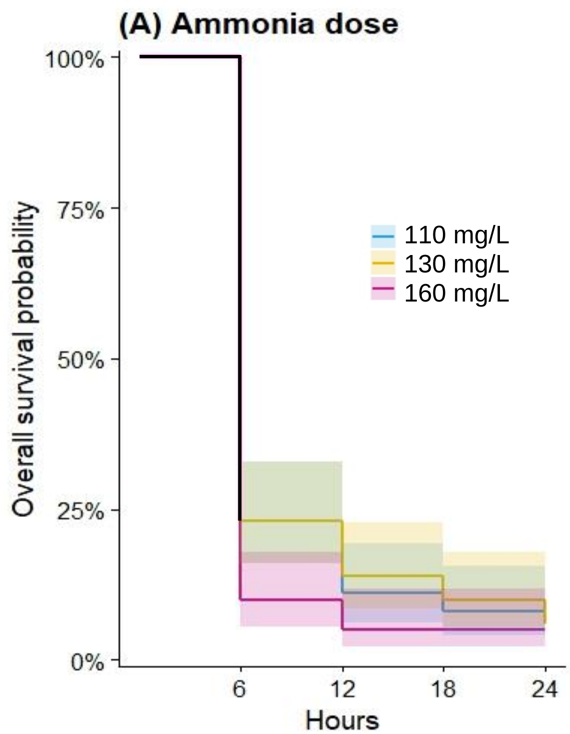
Table 4. Water chemistry parameters of treatment pond in Rancho Jamul Ecological Reserve, San Diego, California in Septermber, 2022 a) before ammonia treatment and b) after treatment. Pre-treatment total ammonia nitrogen (TAN) levels were at 0 or too low to register on Aquarium ammonia test kit. Parameters were taken several times around pond and averaged. Chemicals were added between 13:45 and 14:15. Parameters from control cans were taken in three different cans at random for each time, and averaged.

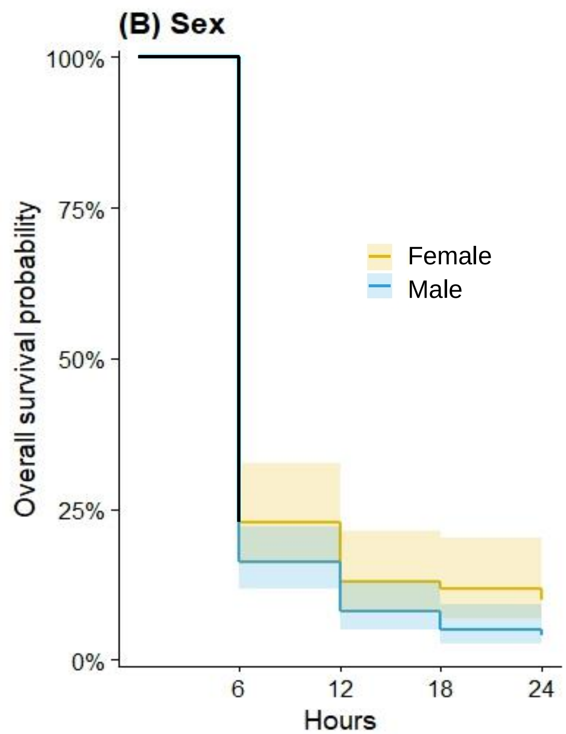
*TAN values were above 99.9 (highest value on our ammonia reading) values were extrapolated from watered down readings.

	Time		**Temperature (˚C)**	**pH**	**DO (Mg/l)**	**TAN (Mg/l)**
a)	12:50	Pond	26.3	8.9	4.2	-
		Cans	27	8.62	4	-
b)	14:30	Pond	27.7	9.91	0.26	200*
		Cans	28	8.6	4	-
	19:45	Pond	27.6	9.88	0.2	200*
		cans	26	8.5	4	-
	01:45	Pond	24.7	9.92	0.35	200*
		Cans	23.1	8.4	3.8	0.25
	07:45	Pond	23.8	9.8	0.5	192*
		Cans	23.8	8.4	3.5	0.75
	13:45	Pond	24.5	9.5	0.3	190*
		Cans	25.4	8.5	3	4

Appendix C

Supplemental Survival Plots





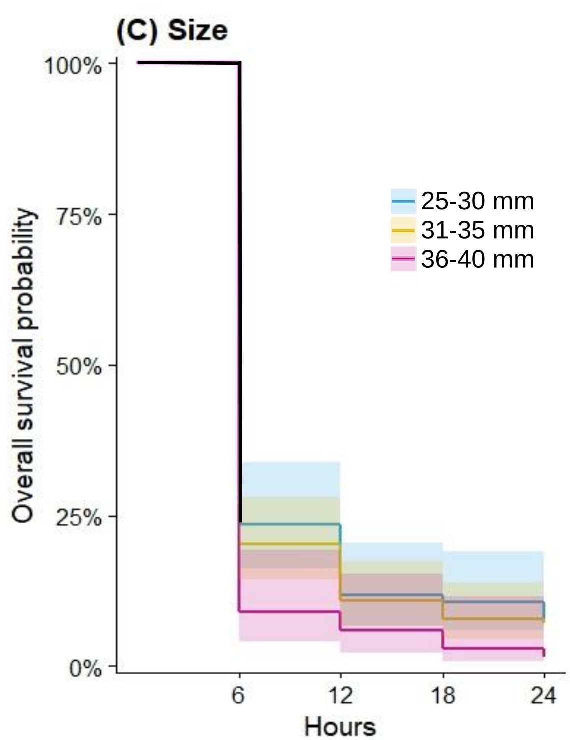
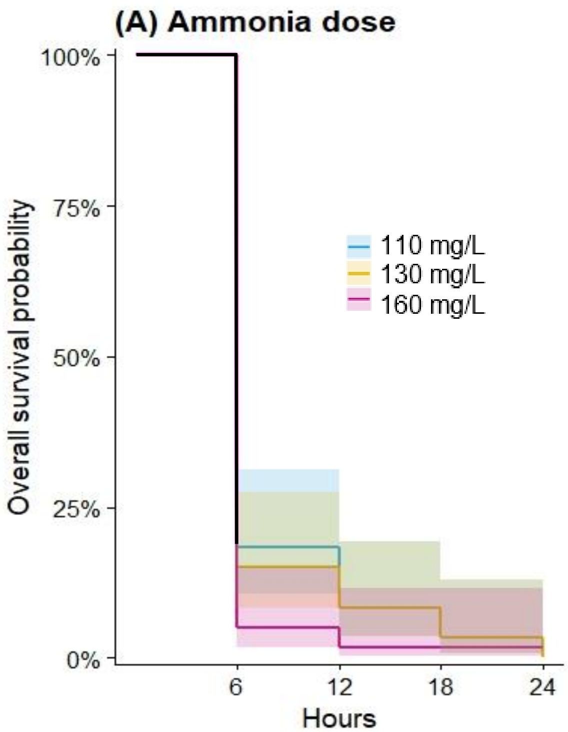
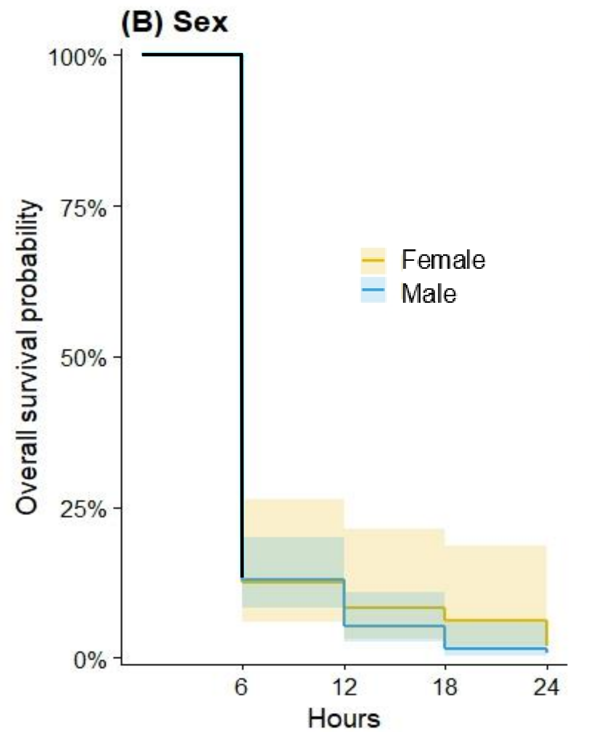


Figure C(1). Kaplan-Meier Plots of overall crayfish survival for *Faxonius virilis* and *Procambarus clarkii* with different curves for (A) Ammonia Dose; (B) Sex; (C) Size (carapace length). Survival data was collected from trials conducted in spring and summer, 2022, in Flagstaff, Arizona (Coconino County) for *F. virilis;* and September 2022, Rancho Jamul Ecological Reserve, California (San Diego County) for *Procambarus clarkii.*





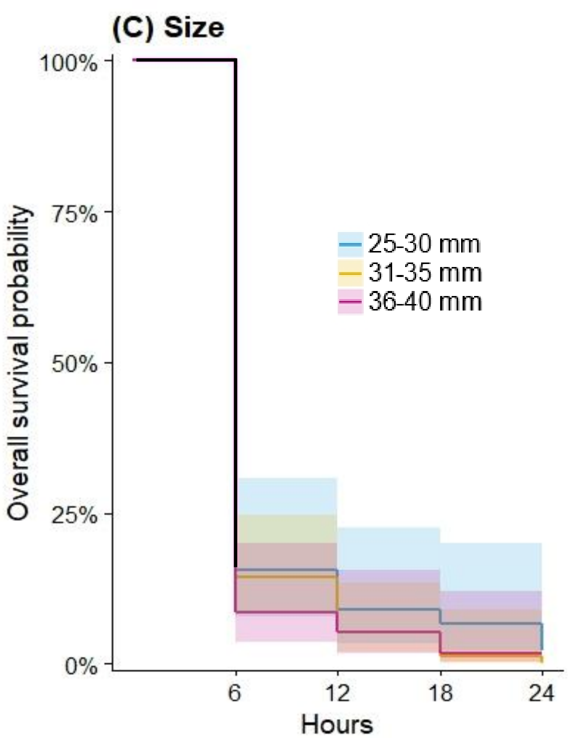
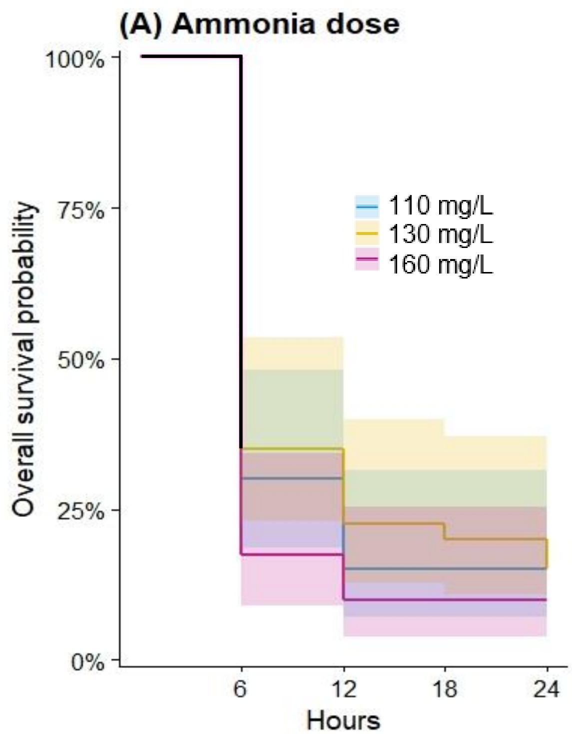
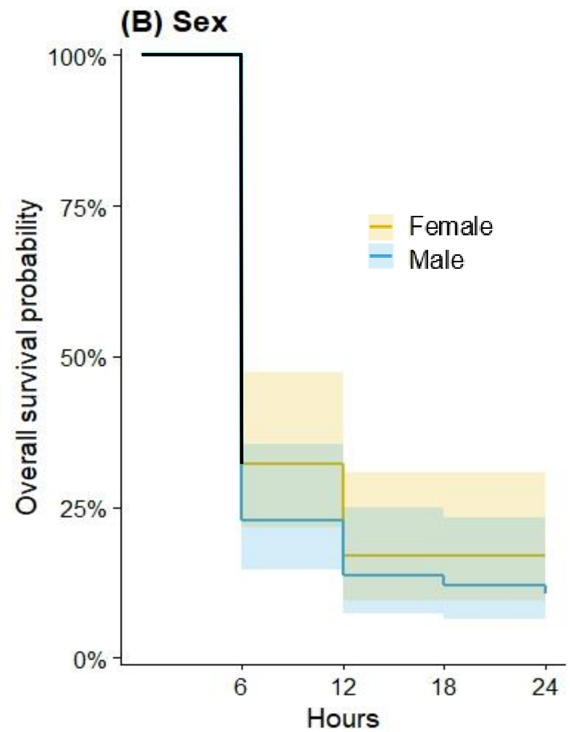


Figure C(2). Kaplan-Meier Plots of overall crayfish survival for *Faxonius virilis* with different curves for (A) Ammonia Dose; (B) Sex; (C) Size (carapace length). Survival data was collected from trials conducted in Flagstaff, Arizona (Coconino County) during spring and summer 2022.





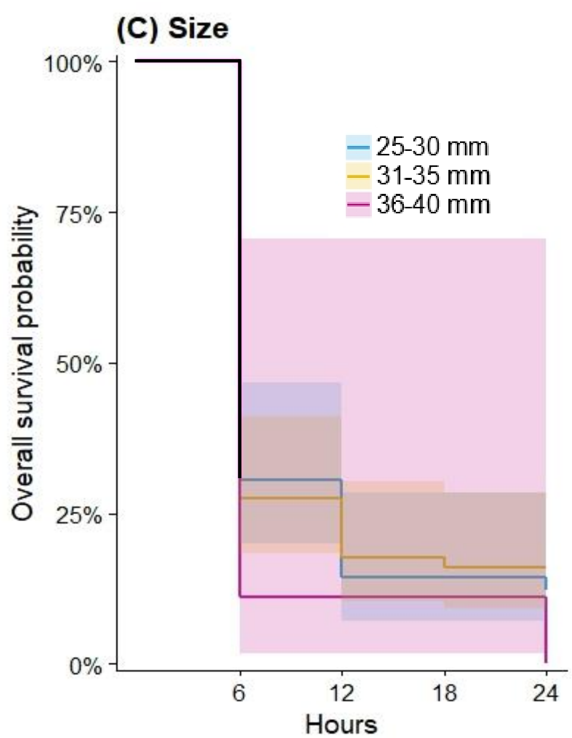


Figure C(3). Kaplan-Meier Plots of overall crayfish survival for *Procambarus clarkii* with different curves for (A) Ammonia Dose; (B) Sex; (C) Size (carapace length). Survival data was collected from trials conducted in Rancho Jamul, California (San Diego County) during September, 2022.