Determining the toxicity of antifreeze to quagga mussels

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Abstract

Recreational boats are considered high risk vehicles for transporting invasive mussels. There are spread prevention programs in place to minimize the risk of transporting invasive mussels during the active boating season. In fall and early winter many boats are removed from the water and winterized. The winterization process of recreational boats includes the use of propylene glycol antifreeze. This study investigated the toxicity of three common formulations of commercially available antifreeze to veliger and juvenile quagga mussels. The antifreeze formulations had three ratings: -50, -100, and -200ºF and these formulations were tested at three different temperatures (18.4, 3.7, and -18.6ºC) over a 28-day exposure period on veliger and juvenile quagga mussels in static open containers. We found that all veligers exposed to antifreeze died within 48 hours. However, juvenile mussels survived the antifreeze treatments. The -100ºF antifreeze treatment was the most toxic to the juvenile mussels, independent of treatment temperature, but there was high variability in response and complete mortality did not occur at all temperatures tested. The -200ºF antifreeze treatment offered the most protection to juvenile mussels and survival increased with time of exposure at all temperatures tested. The mussels were observed closing their shell in response to the antifreeze and they recovered after removal into clean water. We recommend that managers consider winterized boats as potential risk vehicles that should be decontaminated.

Introduction

Recreational boats are considered high risk vehicles for spreading quagga and zebra mussels (*Dreissenia sp*.) to naïve lakes and rivers (Johnson et al. 2001; Rothlisberger et al. 2010; 100th Meridian Initiative 2011). Federal, state and provincial managers have developed programs and protocols to decontaminate boats coming out of infested areas and to recheck those boats when transported to uninfested areas to reduce the risk of spread (e.g. Forest Service 2007; 100th Meridian Initiative 2011; Elwell and Phillips 2016). Managers have worked together to construct uniform protocols and standards for watercraft inspection and decontamination (Elwell and Phillips 2016). These standards recommend that in the fall to winter boats obtain a final inspection and decontamination before being winterized. Winterizing a boat, includes removal of water, flushing systems, sanitizing and cleaning, and charging the lines with antifreeze (Forest Service 2007; CDEEP 2013). An antifreeze charge is used to prevent residual water remaining in the boat from freezing and damaging boat components. In the spring, boat owners flush out the antifreeze from the boat system before re-entry into the water.

Automobile antifreeze often uses solutions with ethylene glycol but recreational vehicles and boats are treated with antifreeze containing propylene glycol. The ethylene glycol containing antifreeze is strongly discouraged from use in boats because it is toxic to mammals (Jobson et al. 2015). Propylene glycol containing antifreeze is recommended for boat winterization as it is not toxic to humans (OECD 2001; West et al. 2014) and typically used in tubing that supplies water for consumption.

Few studies have been conducted to determine the survival of mollusks in antifreeze solutions and none on zebra or quagga mussels. Hartwell et al. (1995) and Pillard (1995) investigated effects of airplane deicers on fish and aquatic invertebrates to find that additives in these were more toxic than pure propylene or ethylene glycol. Propylene and ethylene glycol have been shown to undergo rapid biodegradation and were not toxic to fish and aquatic invertebrates (Staples et al. 2001; OECD 2001). The mollusk aquaculture industry uses glycol products to cryopreserve sperm, oocytes, embryos, trochophore and shelled larvae (Paniagua-Chavez and Tiersch 2001; Wang et al. 2011; Paredes et al. 2013; Liu and Li 2015). These studies show that small life stages of mollusks, especially mussels can survive in pure propylene and ethylene glycol substances.

In a survey of several marinas and associated mechanics in various regions of the US, key staff reported that only propylene glycol containing antifreeze was used in winterization protocols (Lake Mead Marina, Boulder City, Nevada, personal communication, June 2, 2016; Best Marine Service; Wheat Ridge, Colorado, personal communications, June 2, 2016; Boat Doctor Marine; Montgomery, Minnesota, personal communication, June 2, 2016). Ethylene glycol formulated antifreeze was not used in boats because it was harmful to the environment and not industry standard. Nevada operators typically used an antifreeze formulation with a rating of -50°F or -100°F. The antifreeze formulation that was used in Colorado had a rating of -100°F and in Minnesota, the formulation used had a rating of -200°F. Mechanics reported that small juvenile mussels and veligers were of concern because they could be in the remaining water or in hard to clean and flush areas of the boat. They indicated house boats were of the highest concern and hardest to decontaminate.

This study examined three formulations of propylene glycol antifreeze as used in boat winterization procedures on quagga mussel veligers and juveniles at three exposure temperatures for up to 4-weeks.

Methods

Study Location, Source Water Collection, and Test Organism Preparation

Tests were conducted at Willow Beach National Fish Hatchery, Willow Beach, AZ (WBNFH) (35º52′N, 114º39′W), in February to March 2017. Filtered water for use in the trials was obtained by filtering pumped hatchery water through a 35 µm-mesh filter and kept at room temperature. Juvenile quagga mussels (*Dreissena bugensis*) were collected from dock structures in the Colorado River adjacent to the hatchery. Mussels were washed and sorted to remove dead (empty shells, floating engorged mussels, and broken shelled) mussels. Quagga mussel veligers were collected with a 53 µm-mesh plankton net from the pumped hatchery inflow water. Contents of the cod end of the plankton net were poured through a 300 µm filter into a 0.9 L glass container. Three to four collections were combined into the glass container as a stock solution of veligers, and retained in a refrigerator for habituation overnight. The next morning the plankton tows were removed from the refrigerator and poured onto a 53 µm-mesh filter, which was backwashed into a beaker with 20 to 50 mL of filtered water. Three 1 mL aliquots

were removed from the well mixed beaker and number of live veligers was assessed for density and the counts combined to estimate an average number of veligers per mL.

Test Chemicals

Three commercially available freeze protection products with distinct ratings were assessed in this study: West Marine Pure Oceans -50° (-46°C) Marine Antifreeze, Pure Oceans -100° (-73°C) Marine Antifreeze, and Pure Oceans -200°F (-129°C) Super Concentrated Antifreeze manufactured by Star brite Distribution Inc. Ft. Lauderdale, FL labeled in this report as -50ºF antifreeze, -100ºF antifreeze, and -200ºF antifreeze. In all trials, controls with filtered water were included.

Experimental Design

Test containers were 50 mL plastic centrifuge tubes for tests of juvenile and veliger quagga mussels. For tests of juveniles, approximately 30 live and apparently heathy juvenile sized mussels were placed into each of the 50 mL plastic centrifuge tubes with 35 mL of filtered water. The juvenile mussels were habituated to the tubes (uncapped) and held at room temperature or refrigerated temperatures overnight. Juvenile quagga mussels held in refrigerated temperatures were subsequently tested at refrigerated or frozen temperature, whereas the tubes and mussels held at room temperatures were tested at room temperature. The following morning the juvenile quagga mussels from each tube were poured into a container and assessed for health. Any dead or damaged juvenile mussels were removed and replaced with fresh live mussels (from the same habituation temperature). The mussels were returned to the centrifuge tubes and 8 mL of filtered water was added. Trial start times for each exposure period were randomized and staggered over a 4-day period; the exposure duration Day 4 and 28 were started on collection day 1, exposure duration Day 14 and 21 were started on collection day 2, exposure duration Day 1 and 6 were started on collection day 3, and exposure duration Day 2 were started on collection day 4.

For tests of veligers, at least 200 veligers (determined from density estimations) were pipetted from the refrigerated stock solution into each of several 50 mL plastic centrifuge tubes. Filtered water was then added to bring the volume to 8 mL. Trial start times for each exposure period and testing temperature were staggered over a 3-week period with one to two sets were started each day, where a set was 12 tubes consisting of 3 replicates for one exposure duration and testing temperature for each antifreeze formulation and control. The longer durations were started first and the shorter durations were conducted later in the 3-week period.

For tests of both veliger and juveniles, three replicate tubes were used to assess the response to the selected antifreeze treatment for each of the juvenile and veliger trials. The tubes were removed from each testing temperature after the exposure duration of 1, 2, 4, 6, 14, 21, or 28 days. A trial was initiated by adding 32 mL of antifreeze, or filtered water for the control, to the 50 mL centrifuge tube containing the quagga mussels. Trials were conducted with an 80% dilution of antifreeze to mimic any potential water left in the boat where mussels would thrive. Tubes were placed at room (18.4°C), refrigerator (3.7°C) or freezer (-18.6°C) temperatures, which were monitored with a temperature data logger (Onset HOBO UA-001-08, Onset Computer Corporation Bourne, MA) (Figure 1).

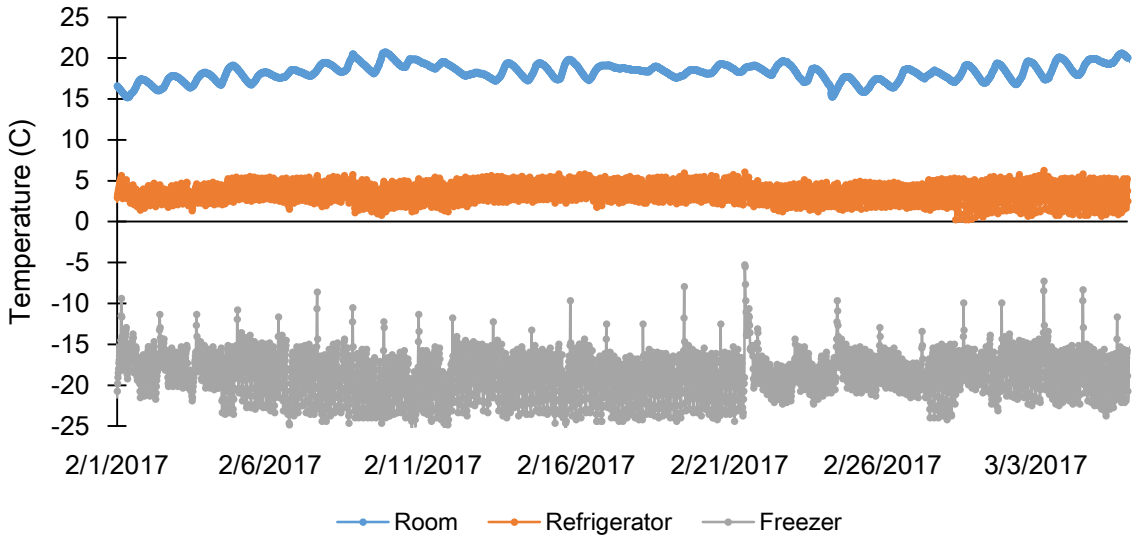

Figure 1. Temperatures logged for each treatment location, room, refrigerator, or freezer over the study period.

Water Quality, Mussel Processing and Analysis

At the end of the exposure duration, three replicate tubes were removed from the testing temperature for analysis. Tubes from the freezer temperatures were thawed at room temperature for 10 min to 1 h (-200ºF antifreeze solutions took the shortest time to thaw and the controls took the longest) to at least 4°C prior to analysis. Water quality parameters of dissolved oxygen (DO), pH, and conductivity were measured at the start of each exposure and at the end of each exposure time for both veliger and juvenile tests with a Hach HQ40d with LDO 101, CDC401, and PHC705 probes (Hach, Loveland, CO). To obtain specific conductivity from the Hach conductivity measurements, the conductivity readings were adjusted as follows:

$$\frac{C}{1+(0.0191(T-25))};$$

where C is conductivity (mS/cm) and T is temperature (°C), as described in Carlson (2015). Water quality parameters were also taken of the test substances prior to use (Table 1).

Table 1. Water quality parameters of the filtered river water and the three antifreeze solutions prior to treatment of juvenile and veliger quagga mussels.

Test Substance	Temperature ºC	DO (mg/L)	pH	Specific Conductivity (mS/cm)
Filtered water	15.9	9.70	8.26	1.227
-50ºF Antifreeze	15.8	9.81	8.33	2.593
-100ºF Antifreeze	15.8	9.42	9.01	1.019
-200ºF Antifreeze	16.1	9.74	8.78	0.044

Juvenile trials – After water quality measurements, the juvenile mussels of each tube were poured out onto a 53 µm sieve and rinsed with at least 100 mL of filtered water until antifreeze was visibly removed. Antifreeze waste was collected and disposed of according to hazardous waste guidelines. The juveniles were then placed into a 30 mm petri dish with 50 mL fresh filtered water and examined for mortality. Juvenile mussels that were dead (open and agape) were removed, and the remaining individuals were placed in fresh filtered water in the rinsed out treatment tube and maintained at room temperature overnight. The juvenile mussels were reassessed after 24 hours. Live mussels were closed or moving, and dead mussels were open, agape or when prodded decomposed tissue was expelled. Counts of live and dead were recorded and representative mussels were preserved with 70% isopropanol for later measurements of length, height and width of preserved mussels were measured as described in Beggel et al. (2015).

Veliger trials – Tubes containing veligers were poured onto a 53 µm sieve after test solutions were evaluated for water quality. The veligers were rinsed with approximately 100 mL of filtered water until antifreeze was visibly removed. Veligers on the filters were placed into ~5 mL of 0.4% aqueous solution of fast green FCF (Sigma-Aldrich, St. Louis, MO, lot MKBV0354V) for 20 min to assess mortality using procedures of Stockton-Fiti and Claudi (2017). The filters were then washed with filtered water until dye was visibly removed and then the contents were back flushed from the filter into a recovery beaker with <5 mL filtered water. A 2 mL sample of veliger concentrate was removed from each recovery beaker with a disposable pipet and visually evaluated using a gridded Sedgewick-Rafter counting cell on a compound microscope and were scored. Scoring levels included live (non-stained with mantle intact), dead (stained mantle), and open shells (non-stained but with no mantle intact).

Statistical Analysis

Juvenile response was evaluated by comparing the proportion of live to dead mussels in replicate treatments. Chi-square tests of independence were used to determine significant differences among the replicates at each sampling interval. The proportion of mortality in replicated containers was evaluated for test temperature, antifreeze formulation, and duration of exposure with a generalized linear model (GLM). Control treatments were separated from antifreeze treatments to determine variable significance among the antifreeze treatments. To illustrate the difference in response between treatment and controls at each time interval, we calculated a relative mortality by subtracting the average treatment mortality from that of the controls at each time of exposure. The size of juvenile mussels over the four days of collection were compared with a GLM.

Veliger response was assessed using the proportion of live, dead, and empty veligers in each of the studies. Empty shells were included in the assessment of dead veligers due to the longer exposure times having only empty shells due to the decomposition rate of dead tissues observed during testing.

Measures of temperature, dissolved oxygen, pH, and specific conductivity were summarized and evaluated with a GLM to evaluate the relationships between replicates, treatment formulations,

testing temperatures, and exposure times for each of the measured water quality parameters to determine differences for both veliger and juvenile tests.

Frequency tests were conducted using SAS 9.4 (SAS Institute, Cary, NC). The GLM procedure was conducted in R 3.1.3 (R Development Core Team, 2015). Tukey's HSD was used to determine how the variables related to each other if significant differences were found using package *multcompView* (Graves et al. 2012).

Results

Juvenile Mussel Trials

No antifreeze formulation was 100% effective in causing mortality of the juvenile mussels for any of the three temperatures tested (Figure 2), except the -100ºF antifreeze treatment at freezer temperature that had multiple time points at 100% mortality but also had variation among the replicates at Day 21. The -100ºF antifreeze treatment was the most toxic to the mussels over the entire exposure duration at all testing temperatures and the -200ºF antifreeze treatment was the least toxic to the juvenile mussels. Freezing temperatures (-18.6ºC) showed the highest mortality within the antifreeze formulations and mortality was similar in the room (18.4ºC) and refrigerated (3.7ºC) temperature treatments. In the full model to predict mortality testing temperature ($F_{2, 174}$ =400.87; P<0.001), formulation of antifreeze ($F_{3, 174}$ =717.62; P<0.001), and exposure duration ($F_{6, 174}$ =6.07; P<0.001) were significant variables, along with the interaction terms. Removal of the control showed that the antifreeze formulation, testing temperature and exposure duration with their interaction terms were significant (Table 2).

Control mortality for the freezing temperature resulted in 100% mortality for all exposure durations. Mortality of the juvenile mussels differed significantly by testing temperature ($F_{2, 60}$ =14.99; P<0.001), where freezing (-18.6ºC) had the highest mortality and refrigerated (3.7ºC) had the lowest mortality. Control mortality increased with exposure duration at both room (18.4ºC) and refrigerated temperatures, resulting in exposure duration as a significant variable ($F_{2, 60}$ =5.21; P<0.001). There were some significant differences among the replicates of controls held in the room temperature at exposure Day 2, 4 and 6 (Day 2: χ^2=10.9 P=0.004; Day 4: χ^2=7.8 P=0.02; Day 6: χ^2=6.2 P=0.05; Figure 2). No significant differences were detected among the replicates of controls held in refrigerated or freezer treatments. Mussels in the refrigerated control treatments had the lowest mortality for each exposure duration and noticeable mortality started after 14-days compared to mortality observed after 4-days in room temperatures.

Mortality of juveniles held in the -50ºF antifreeze was highly variable over exposure duration at all testing temperatures (Figure 2). In the freezer temperature, 100% mortality was achieved at some of the early exposure durations, but after 28-days of exposure some juvenile mussels survived. No differences were detected in mortality over time in tests of juveniles held in room and refrigerated temperatures, and no treatment showed 100% mortality. There were some treatments at Day 1 (room and refrigerated) and room temperature at Day 28 that had significant differences among the replicates (Figure 2).

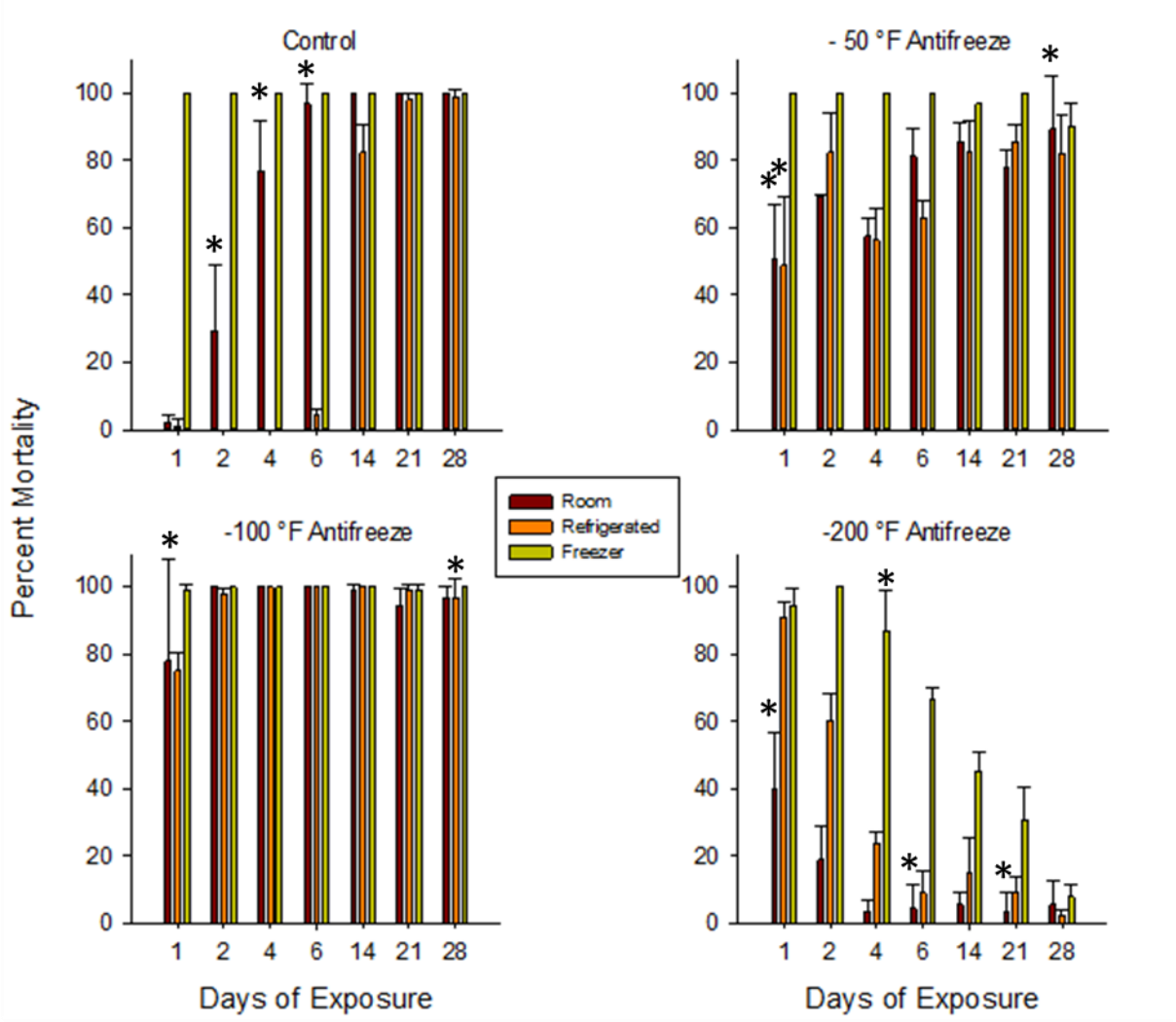

Figure 2. Average percent mortality and standard deviation of juvenile quagga mussels over days of exposure to one of three antifreeze formulations or held as controls in trials at the three temperatures tested (Room: 18.4, Refrigerated: 3.7, Freezer: -18.6ºC). *Above the treatment indicates mortality among replicates were significantly different.

Table 2. The ANOVA table for the full model predicting juvenile mortality of quagga mussels to antifreeze treatments without control.

Variable	DF	Mean Sq.	F value	P-value
Temperature	2	2.558	197.24	<0.001
Concentration	2	12.459	960.60	<0.001
Exposure Time	6	0.812	20.86	<0.001
Temperature*Concentration	4	1.178	45.41	<0.001
Temperature*Exposure Time	12	0.753	9.67	<0.001
Concentration*Exposure Time	12	4.242	54.51	<0.001
Temperature*Concentration*Exposure Time	24	0.919	5.90	<0.001
Residuals	132	0.856		

Juveniles held in the -100ºF antifreeze treatment had the highest mortality over all exposure durations. No treatment resulted in complete mortality at the end of 28-days. Considerable variability in the mussel response was observed among replicates, especially in Day 1 room and Day 28 refrigerated treatments (Figure 2). After 28-days, one replicate held in the refrigerated temperature had 3 surviving juveniles, but the other two replicates resulted in 100% mortality (90% mortality in replicate 3; χ^2=6.21 *P*=0.045). We did not observe differences in water chemistry parameters measured in the solutions removed from replicates. Investigation of water chemistry did not reveal differences between the replicates. The surviving mussels were about the same size and were about 7 mm in length. The room temperature juvenile mussels after 28-days of exposure to the -100ºF antifreeze had one replicate with one surviving mussel. Juveniles held for 28-days in freezing temperatures were all dead.

Mortality of the quagga mussel juveniles decreased over duration of exposure in the -200ºF antifreeze treatment at all testing temperatures (Figure 2). Juveniles held in freezer temperatures showed 100% mortality after 2-days, but showed increased survival over time of exposure. Survival at room and refrigerated testing temperatures also increased after 2-days. By the end of the study the mortality was similar across all testing temperatures. The significant differences were detected for Day 1, 6 and 21 at room temperature trials and Day 4 at freezer temperature trials (Room Day 1: χ^2=15.38 *P*=0.01; Day 6: χ^2=7.84 *P*=0.02; Day 21: χ^2=6.21 *P*=0.045; Freezer Day 4: χ^2=7.50 *P*=0.02).

The differences in survival were clarified when relative mortality metrics were examined. The antifreeze solutions provided some protection to the mussels at all temperatures tested (Figure 3). This protection was most apparent with the -200ºF antifreeze treatment as relative mortality from the control was negative. In the -200ºF antifreeze treatment, survival compared to the control increased as exposure duration increased at all testing temperatures. At room temperature, the mortality of the antifreeze treatments was higher than the control mortality in the first two days, but as exposure time increased the mortality of the juvenile mussels held in antifreeze was lower than that of the controls. It took longer at refrigerated temperatures for the antifreeze to show protection of the mussels. The mussels held in controls at freezing temperatures were all dead by Day 2, so when the mussels survived in the antifreeze past Day 2, then protection occurred with the antifreeze solutions.

Juvenile mussels selected on Day 1 were significantly smaller than those selected on Day 2, 3, or 4 ($F_{3,505}$=12.1, *P*=<0.001; Figure 4). These mussels were selected for exposure duration Day 4 and 28. Mortality of the treated mussels from these exposure days were comparable to the surrounding exposure days; there were no outliers in response from the Day 1 selection.

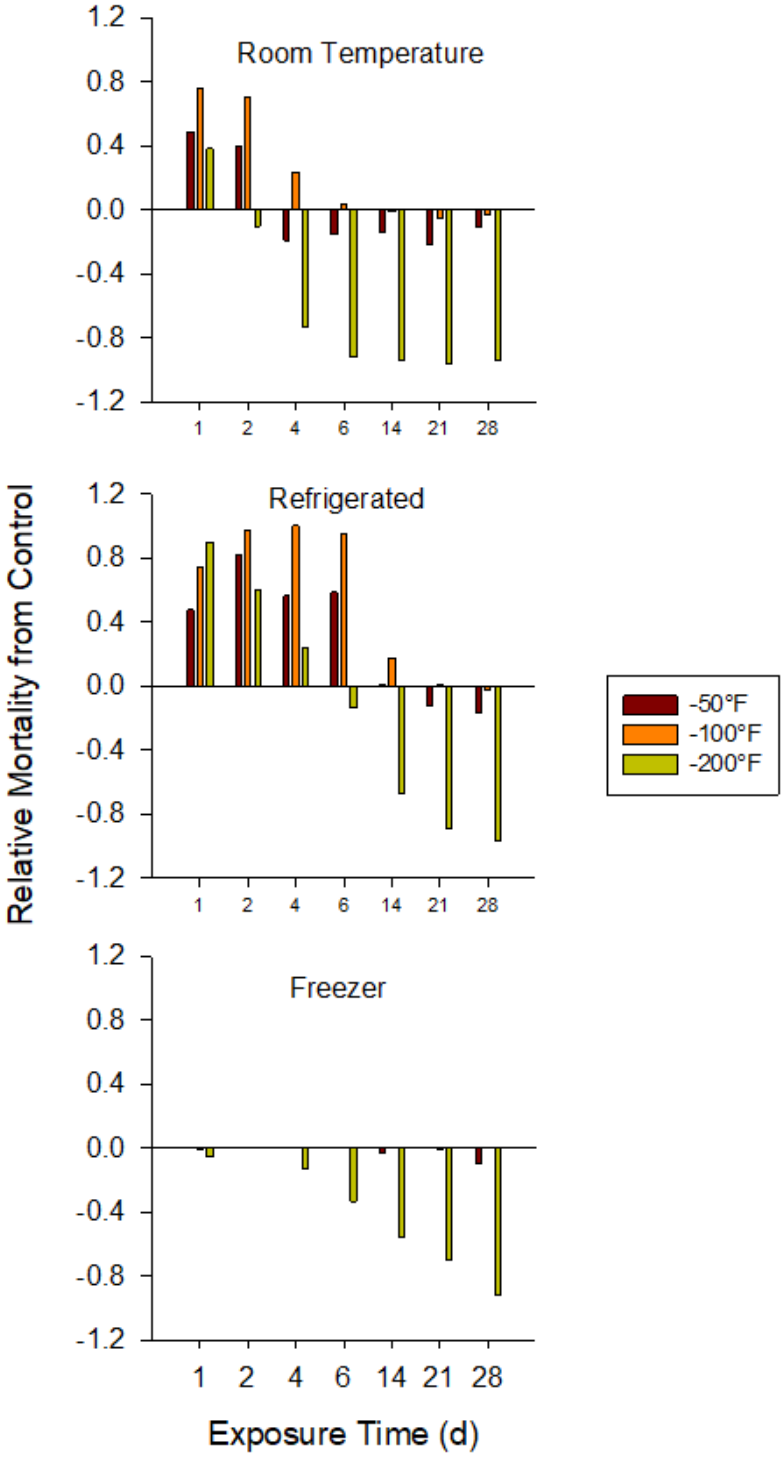

Figure 3. Relative mortality from control of juvenile quagga mussels for each treatment at each temperature tested (Room: 18.4, Refrigerated: 3.7, Freezer: -18.6ºC).

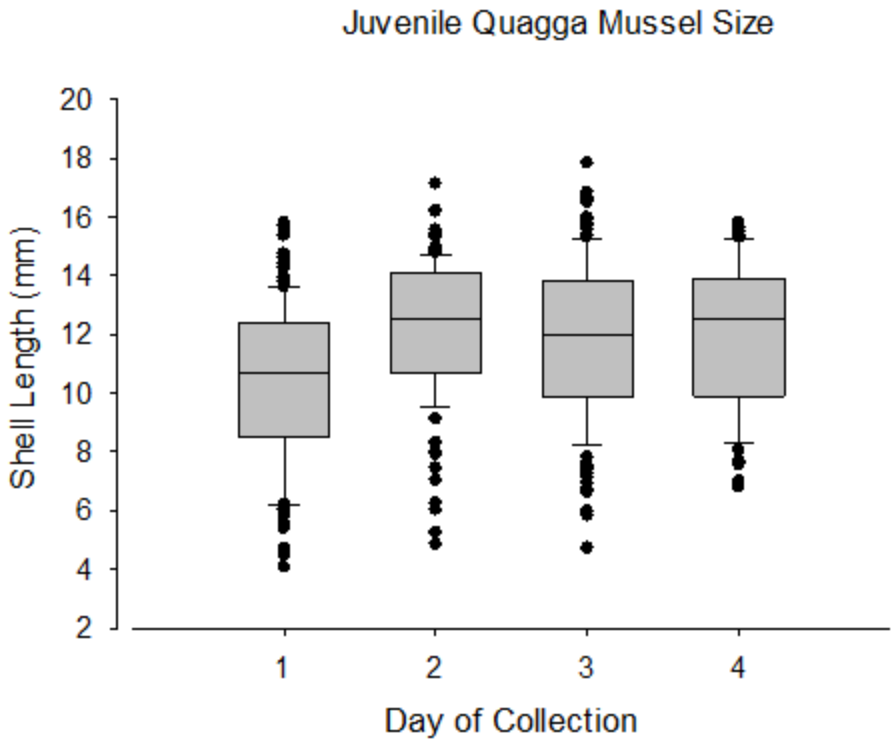

Figure 4. Boxplot of the length of juvenile mussels selected for testing the three types of antifreeze and control at the three different temperatures.

No significant differences were detected in the measurements of temperature, dissolved oxygen (DO), pH, and specific conductivity over exposure duration for each of the antifreeze treatments at each testing temperature for juvenile tests (Table 3). The initial readings for each treatment were significantly different in many of the parameters (DO and specific conductivity in the control; specific conductivity in the -50ºF antifreeze, DO and pH in the -100ºF antifreeze, and DO in the -200ºF antifreeze treatments) compared to the longer exposure durations. Measured temperature of the freezer treatments was not significantly different from the refrigerated treatments, which was expected as the freezer treatments were thawed at room temperature and readings were made when probes could be put into the solution. In the control treatments, the DO dropped at all temperatures, with room temperature treatments near zero. In the antifreeze treatments, the DO also decreased at all testing temperatures, but not below 5.0 mg/L, which was needed for mussel survival. DO levels were highest in the -200ºF antifreeze treatments (Table 3).

Table 3. Mean, standard deviation and range (in parentheses) of water quality measures in the juvenile mussel tests by treatment and testing temperature (Room: 18.4, Refrigerated: 3.7, Freezer: -18.6ºC) for the start of treatment and for the 7 exposure duration tested with the replicates combined. Freezer treatments were measured when thawed.

	Temp C		DO mg/L		pH		Sp. Cond. mS/cm	
	Start	**Day 1-28**	**Start**	**Day 1-28**	**Start**	**Day 1-28**	**Start**	**Day 1-28**
				Control				
Room	16.8 ±0.1 (16.7-16.9)	17.4 ±1.0 (15.6-19.4)	7.53 ±0.79 (6.63-8.08)	0.57 ±0.37 (0.18-1.56)	8.40 ±0.03 (8.37-8.43)	7.45 ±0.42 (6.76-8.19)	1.196 ±0.001 (1.195-1.196)	2.479 ±1.221 (1.326-4.704)
Refrigerated	15.4 ±0.1 (15.3-15.5)	6.3 ±0.8 (4.7-7.8)	9.61 ±0.05 (9.57-9.67)	6.20 ±2.41 (1.48-9.29)	8.40 ±0.03 (8.37-8.42)	7.69 ±0.399 (6.85-8.23)	1.231 ±0.006 (1.224-1.235)	1.863 ±0.468 (1.356-2.851)
Freezer	15.6 ±0.3 (15.3-15.8)	5.2 ±2.5 (1.4-10.0)	9.11 ±0.55 (8.48-9.51)	6.01 ±1.13 (4.27-9.00)	8.34 ±0.01 (8.33-8.34)	8.13 ±0.13 (7.84-8.40)	1.238 ±0.009 (1.229-1.247)	0.776 ±0.176 (0.501-2.851)
				-50ºF Antifreeze				
Room	16.9 ±0.1 (16.9-17.0)	17.4 ±1.1 (15.1-19.0)	8.89 ±0.08 (8.80-8.94)	7.58 ±1.07 (5.50-9.40)	8.22 ±0.01 (8.22-8.23)	8.16 ±0.50 (7.77-9.93)	2.503 ±0.003 (2.501-2.506)	2.617 ±0.318 (2.465-3.985)
Refrigerated	15.3 ±0.1 (15.3-15.4)	5.8 ±0.6 (4.8-7.0)	9.40 ±0.10 (9.39-9.49)	10.73 ±0.67 (9.58-11.95)	8.23 ±0.02 (8.22-8.25)	8.02 ±0.09 (7.75-8.14)	2.559 ±0.006 (2.552-2.564)	3.093 ±0.124 (2.871-3.261)
Freezer	15.5 ±0.2 (15.4-15.7)	7.7 ±5.2 (-3.7-13.2)	9.10 ±0.07 (9.04-9.17)	6.56 ±0.59 (5.12-7.43)	8.22 ±0.00 (8.22-8.22)	8.25 ±0.03 (8.19-8.32)	2.560 ±0.014 (2.549-2.576)	2.667 ±0.406 (2.229-3.655)
				-100ºF Antifreeze				
Room	17.6 ±0.1 (17.5-17.7)	17.3 ±1.5 (12.4-19.0)	9.56 ±0.32 (9.35-9.93)	6.59 ±1.48 (3.43-9.70)	8.36 ±0.01 (8.35-8.37)	8.03 ±0.10 (7.84-8.16)	1.443 ±0.028 (1.410-1.462)	1.476 ±0.044 (1.388-1.536)
Refrigerated	15.9 ±0.1 (15.8-15.9)	6.5 ±0.7 (5.5-8.0)	9.60 ±0.03 (9.57-9.63)	9.34 ±1.05 (7.00-11.19)	8.33 ±0.02 (8.32-8.35)	8.17 ±0.08 (8.03-8.28)	1.480 ±0.019 (1.463-1.501)	1.530 ±0.098 (1.325-1.645)
Freezer	16.2 ±0.1 (16.1-16.3)	11.8 ±2.6 (6.3-14.8)	9.30 ±0.10 (9.21-9.41)	7.96 ±0.70 (6.28-9.07)	8.36 ±0.02 (8.34-8.38)	8.32 ±0.03 (8.27-8.4)	1.437 ±0.013 (1.422-1.448)	1.489 ±0.067 (1.345-1.608)
				-200ºF Antifreeze				
Room	19.0 ±0.4 (18.5-19.3)	17.4 ±2.0 (10.9-19.1)	11.61 ±0.15 (11.46-11.76)	9.37 ±0.45 (8.59-10.30)	8.54 ±0.07 (8.46-8.60)	8.56 ±0.06 (8.43-8.69)	0.150 ±0.063 (0.080-0.201)	0.326 ±0.099 (0.146-0.553)
Refrigerated	17.0 ±0.1 (16.9-17.1)	7.4 ±0.8 (5.9-8.9)	11.97 ±0.13 (11.87-12.12)	10.72 ±0.62 (9.51-12.10)	8.55 ±0.09 (8.46-8.63)	8.58 ±0.06 (8.47-8.73)	0.382 ±0.109 (0.307-0.508)	0.270 ±0.124 (0.102-0.593)
Freezer	17.0 ±0.0 (17.0-17.0)	13.8 ±2.4 (7.6-16.0)	12.29 ±0.27 (11.99-12.51)	10.81 ±0.55 (9.65-12.41)	8.68 ±0.01 (8.68-8.69)	8.67 ±0.07 (8.52-8.80)	0.099 ±0.035 (0.069-0.138)	0.236 ±0.128 (0.081-0.512)

The pH of the antifreeze treatments did not change significantly over exposure duration and in the different temperature treatments. Different antifreeze formulations had different mean pH readings. The -200ºF antifreeze formulation was the most basic pH; the -100ºF and the -50ºF antifreeze formulations were a neutral pH (Table 3). Specific conductivity was very different among the formulations of antifreeze, the -50ºF antifreeze had the highest specific conductivity and the -200ºF antifreeze had the lowest specific conductivity throughout testing (Table 3). In the control, specific conductivity increased over exposure duration where measurements over the first week were significantly lower than measurements from Day 14, 21 and 28; it increased the most in room temperature treatments followed by refrigerator treatments. The specific conductivity of solutions in controls held at freezer temperatures was much lower than the start of trials (Table 3), and differences remained consistent over all testing durations. Specific conductivity did not increase significantly over exposure time in the -50ºF antifreeze, -100ºF antifreeze, or -200ºF antifreeze treatments.

Veliger Trials

After 1-day of exposure to all formulations of antifreeze mortality of quagga mussel veligers exceeded 99% at all temperatures. One pediveliger was considered alive in each of the replicate treatments of veligers held for 1-day in the -200ºF antifreeze solution at the refrigerated temperatures. After 2-days of exposure all veligers in antifreeze treatments at all temperatures were dead. Longer exposure periods consistently resulted in 100% mortality of the veligers.

The control mortality of the quagga mussel veligers increased as exposure time increased (Table 4) in the room and refrigerated temperatures; the freezer temperature had 100% mortality. Mortality of veligers held at room temperature was similar to mortality in refrigerated temperatures for the first 2-days, and at Day 4 room temperature mortality increased to >85% mortality. A few veligers in control treatments were alive in the refrigerated temperatures after 28-days of exposure (Table 4).

Table 4. Percent mortality with standard deviation of quagga mussel veligers exposed to different formulations of antifreeze over time at three testing temperatures (Room: 18.4, Refrigerated: 3.7, Freezer: -18.6ºC).

	Day 1	**Day 2**	**Day 4**	**Day 6**	**Day 14**	**Day 21**	**Day 28**
				Control			
Room	23.0±2.2	32.4±6.8	85.3±10.7	97.9±0.4	99.7±0.5	100	100
Refrigerated	26.3±3.4	34.9±5.9	30.7±4.6	61.9±6.3	82.3±5.0	94.7±0.3	98.0±1.2
Freezer	100	100	100	100	100	100	100

Water quality measurements throughout the testing period stayed consistent over the exposure time. There were no significant differences between replicates, and data were combined (Table 5). Temperature was significantly different among the testing temperatures ($F_{2, 261}$ =472.3; P<0.001). Freezer vials were removed and thawed at room temperature before analysis was conducted and thus measures of water quality were likely affected. The refrigerated and freezer temperatures were not significantly different from each other in the -100 and -200ºF antifreeze

treatments. Dissolved oxygen levels were highest in the -100 and -200ºF antifreeze treatments and were significantly different from the control and the -50ºF antifreeze dissolved oxygen measurements ($F_{3, 248}$ =25.11; P<0.001). Dissolved oxygen was significantly different over testing temperature for control ($F_{2, 51}$ =126.3; P<0.001), -50ºF antifreeze ($F_{2, 63}$ =379.2; P<0.001), -100ºF antifreeze ($F_{2, 63}$ =42.0; P<0.001) and -200ºF antifreeze ($F_{2, 63}$ =27.4; P<0.001). The room temperature dissolved oxygen levels were the lowest (Table 5), except for in the -50ºF antifreeze treatments.

Measurements of pH stayed consistent over exposure duration and testing temperature, but pH varied by antifreeze treatments. The -200ºF antifreeze had the highest pH and the -50ºF antifreeze treatments had the lowest pH. In the veliger treatments, specific conductivity was significantly different among the different antifreeze formulations ($F_{3, 245}$ =2351; P<0.001). Specific conductivity measures were less than 0.2 mS/cm in the -200ºF antifreeze treatments and greater than 2 mS/cm in the -50ºF antifreeze treatments; the control and the -100ºF antifreeze treatments did not differ and were around 1 mS/cm (Table 5). Specific conductivity was highest in the refrigerated temperature treatments in the control, -50ºF antifreeze and -100ºF antifreeze treatments. There were no significant differences in the specific conductivity readings of the -200ºF antifreeze treatments between the start and end of trials.

Table 5. Mean with standard deviation of temperature, dissolved oxygen, pH and specific conductivity measured over the 7 exposure durations for each treatment by temperature tested (Room: 18.4, Refrigerated: 3.7, Freezer: -18.6ºC). Freezer treatments were measured when thawed.

	Temp ºC	DO mg/L	pH	Sp. Cond. mS/cm
Control				
Room	18.3±0.7	8.30±0.65	8.34±0.09	1.209±0.056
Refrigerated	6.9±2.2	11.43±0.71	8.33±0.11	1.490±0.132
Freezer	3.6±1.9	8.40±0.82	8.34±0.29	1.072±0.196
-50ºF Antifreeze				
Room	18.3±0.7	9.74±0.22	8.29±0.03	2.479±0.027
Refrigerated	6.2±1.9	12.45±0.53	8.33±0.04	3.047±0.125
Freezer	8.0±3.8	7.85±0.77	8.35±0.03	2.690±0.217
-100ºF Antifreeze				
Room	18.4±0.7	9.96±0.24	8.46±0.04	1.316±0.051
Refrigerated	6.7±1.8	12.24±0.64	8.44±0.03	1.386±0.101
Freezer	6.8±5.5	12.39±1.65	8.49±0.11	1.377±0.053
-200ºF Antifreeze				
Room	18.7±0.6	10.55±0.52	8.78±0.06	0.191±0.083
Refrigerated	7.8±1.9	12.47±0.86	8.70±0.09	0.170±0.050
Freezer	9.5±5.0	11.83±1.20	8.81±0.11	0.151±0.071

Discussion

Quagga mussel veligers were highly susceptible to the antifreeze formulations and short exposures resulted in complete mortality in all test formulations. The juvenile mussel response to the formulations and temperatures tested was variable, but antifreeze acted as a protectant to the mussels as juvenile mussels survived longer in the antifreeze than in the control waters. Details about the formulations were not available to us, but factors such as conductivity and concentration of propylene glycol appeared to be correlated with responses.

Juvenile mussels exposed to the -200ºF antifreeze solutions had less mortality over the exposure durations compared to the other antifreeze solutions and control. The -200ºF antifreeze solution had the lowest conductivity measurements and highest amount of propylene glycol (≤96% propylene glycol) (Star brite Distributing Inc. 2013). When we factored the response of controls to assessment of survival using relative mortality, the -200ºF antifreeze solution offered the most protection to the juvenile quagga mussels at all three testing temperatures.

The juvenile mussel's response to the -50ºF antifreeze formulation was highly variable; mortality increased over exposure duration, but variability increased too. This formulation had the highest specific conductivity and the lowest amount of propylene glycol (<30% propylene glycol) (Star brite Distributing Inc. 2005a). Juvenile mussel mortality was the highest in the -100ºF antifreeze formulation. In the short exposure durations (less than 1 week) with the -100ºF antifreeze treatments 100% mortality was achieved at all three testing temperatures, but as exposure time increased, variability increased and 100% mortality was not achieved. The -100ºF antifreeze formulation had similar conductivity to the source water and consisted of 60% propylene glycol (Star brite Distributing Inc. 2005b).

The only other ingredient in the antifreeze was dipotassium phosphate at <2% in each antifreeze formulation, which is a salt. Monobasic potassium phosphate was toxic to mussels at low concentrations (Fisher et al. 1991) and potassium has been found to be toxic to both zebra and quagga mussels and related to the concentrations of sodium in the water (Moffitt et al. 2016). According to the specific conductivity readings of each antifreeze formulation, the -50ºF antifreeze had the highest amount of potassium salts and the -200ºF antifreeze had the least amount of salts. This does not explain why the -100ºF antifreeze formulation had the highest mortality. There could be a proprietary ingredient in the antifreeze formulation that was toxic to the quagga mussels (Pillard 1995).

Variability among the replicates that was observed in the juvenile mussel mortality could not be attributed to mussel size or water quality. There was a variety of mussel sizes used in testing and surviving mussels were from all sizes both large and small. The juvenile mussels that were selected for this study came from one population and were sorted and used for testing over a 4-day period, but the exposure duration assigned each day was randomized and no trend of survival by exposure duration was observed. The variability in water quality measures among replicates, could not explain variability in mortality. Antifreeze solutions had pH and DO measurements that were within ranges tolerable for survival for both juvenile and veliger quagga mussels

(Sprung 1987; Sprung 1993). Specific conductivity of the test solution in the antifreeze trials did not increase with exposure time.

It was expected that the -50ºF antifreeze would show the least protection and the -200ºF antifreeze would show the most protection as hypothesized related to the increasing concentration of propylene glycol (Paniagua-Chavez and Tiersch 2001; Wang et al. 2011; Paredes et al. 2013; Liu and Li 2015). It was observed that the mussels were closed during the duration of the exposure period. Adult mussels can detect toxicants and will close tightly to avoid exposure to toxicants whereas veliger shells are not developed enough to close and avoid toxicants (Fisher et al. 1994; Kilgour and Baker 1994). Kilgour and Baker (1994) indicated that mussels which did not close continue to syphon and were exposed to the toxicant. Since the -100ºF had higher than expected mortality, perhaps the mussels were continually siphoning the antifreeze product due to inability to close or detect the product.

The control treatments responded as expected. In the refrigerated untreated controls for both the veliger and juvenile trials, mussels survived the longest and 0% survival did not occur. In room temperature treatments, 100% mortality did occur by about 14 days for both the veligers and juveniles. Temperature that mussels were exposed to was found to be closely associated with the metabolism of mussels; as exposure temperature decreased, their metabolism decreased, which decreased the amount of toxic byproducts released into the water, which increased survival time of the mussels (Schneider 1992; Karatayev et al. 1998). Specific conductivity of the controls in the room and refrigerated treatment temperatures increased with exposure duration, potentially indicating that there were more byproducts in the water as exposure time increased. Juveniles and veligers did not survive the freezing temperatures even after a one day exposure, and this was consistent with previous studies that showed mussels were intolerant of aerial freezing temperatures and water temperatures below 0ºC (McMahon 1996; Mills et al. 1996). The specific conductivity in the juvenile mussel control treatments in the freezer temperatures decreased from the initial readings. Conductivity measurements were taken prior to complete thaw, which resulted in lower conductivity of the ice water as ions were forced out of solution by the ice crystals thus lowering the conductivity of the measured water. Antifreeze treatments were completely thawed prior to conductivity measures as they thawed more quickly or were not frozen. Water changes were not conducted in this study due to the need to mimic conditions in a winterized boat system where water would be stagnant and exposed to the air and not replaced with fresh water.

The three antifreeze formulations used in this study provided some protection from mortality at most of the temperatures tested. However, due to the increase in variability observed over the testing period, further testing is recommended to be certain that the results are reliable. We recommend that further testing occur with a longer exposure duration with juvenile mussels at the three testing temperatures as boats can be stored and winterized for up to 6 months in cold regions and that the additives in addition to the propylene glycol solutions be explored. Different responses may be measured with other manufactured products as the exact formulations were not available.

Quagga mussels were removed from the Colorado River at the end of January through February, which typically is the coldest time point for this region. The organisms in this study should be representative of mussel condition during the fall to winter season and provide representative outcomes. However, further investigation of different water sources during the fall to winter with lower concentrations of sodium should be investigated to determine if the antifreeze solutions are more toxic in lower conductivity waters. Mussels in this study were separated from each other and placed into solution, and not allowed to adhere to the testing tubes. An additional study, to increase the certainty of these results, should be conducted with attached mussels as this could result in less mortality to the antifreeze through more resilience (Rajagopal et al. 2002).

This study used an 80% dilution of antifreeze product to mimic water left in the boat, but further testing with more and less dilution should be investigated. It is hypothesized based on these results that the less diluted product could result in higher toxicity as there would be more dipotassium phosphate and less sodium, but the less diluted product could also offer more protection because of the increased amount of propylene glycol. This hypothesis could also apply to lower conductivity waters. If there are additional toxic additives in the antifreeze formulations, then less dilute treatments could be more toxic. Different densities of mussels should also be investigated to increase the certainty of the results, as more mussels are exposed to the antifreeze the amount of toxic product may decrease and limit the toxic effects.

Recreational boats are a vehicle of concern to transport dreissenid mussels from one watershed to another naïve area (Johnson et al. 2001; Rothlisberger et al. 2010). Many state and local watershed managers have implemented boat inspections to prevent the spread of the dreissenid mussel and other invasive species. However, in the spring winterized boats are usually not inspected due to the assumption that the long period out of water with the use of antifreeze has killed everything and that these boats are free of dreissenid mussels. This study showed that the assumption that overwintering storage is 100% effective in causing mortality may not be correct. We recommend that managers utilize these results in their inspection programs when assessing recreational boats that have been winterized with antifreeze.

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