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Research Article

Use of carbon dioxide in zebra mussel (*Dreissena polymorpha*) control and safety to a native freshwater mussel (Fatmucket, *Lampsilis siliquoidea*)

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This study was contributed in relation to the 20th International Conference on Aquatic Invasive Species held in Fort Lauderdale, Florida, USA, October 22–26, 2017 (http://www.icais.org/html/previous20.html). This conference has provided a venue for the exchange of information on various aspects of aquatic invasive species since its inception in 1990. The conference continues to provide an opportunity for dialog between academia, industry and environmental regulators.

Abstract

Control technology for dreissenid mussels (*Dreissena polymorpha* and *D. bugensis*) currently relies heavily on chemical molluscicides that can be both costly and ecologically harmful. There is a need for more environmentally neutral tools to manage dreissenid mussels, particularly in cooler water. Carbon dioxide (CO_2) has been shown to be lethal to several species of invasive bivalves, including zebra mussels and Asian clams (*Corbicula fluminea*). We evaluated the effectiveness of unpressurized infusion of CO_2 for 24 to 96 h (100 000–300 000 µatm PCO_2) at a water temperature of 12 °C on mortality, byssal thread formation, and attachment of zebra mussels. The safety of elevated CO_2 to a nontarget native freshwater mussel (Fatmucket, *Lampsilis siliquoidea*) was also determined. Elevated PCO_2 exposure induced narcotization and reduced attachment of zebra mussels within 24 h. Mortality increased with exposure duration and PCO_2. An estimated LT50 (lethal time to produce 50% mortality) for fixed PCO_2 ranged from 24 h at 275 000 µatm to ~ 96 h at 100 000 µatm. Exposure of zebra mussels to CO_2 for 96 h caused 80–100% mortality at all treatment levels. Fatmucket juveniles survived all PCO_2 treatments but burial and byssal thread production were adversely affected during exposure. Our results demonstrate that CO_2 is a viable option for management of zebra mussels in cool water and may have less adverse effect for native lampsiline mussels than current-use molluscicides.

Key words: invasive species, dreissenid, unionid, elevated PCO_2, toxicity, sublethal effects

Introduction

Dreissenid mussels, zebra mussel (*Dreissena polymorpha* Pallas, 1771) and quagga (*D. bugensis* Andrusov, 1897) continue to expand their range across the United States and into Canada (Benson et al. 2018) causing significant ecological and economic consequences where they have established (Nalepa and Schloesser 2014). For example, the estimated economic costs of dreissenid mussels to United States and Canadian power plants and water treatment facilities from 1989 to 2004 was $267 million (Connelly et al. 2007). Westward expansion of dreissenids has the potential to cost tens to hundreds of millions of dollars annually for protection of major generators of hydropower and drinking water supplies (IEAB 2013). The adverse ecological consequences of dreissenids include shifts in trophic structure and food web dynamics (Strayer et al. 1998; Vanderploeg et al. 2002; Zhu et al. 2006; Madenjian et al. 2015), loss of important fish and

zooplankton species (Colvin et al. 2015; Madenjian et al. 2015) and decline of native mussels (e.g., Schloesser et al. 1996; Strayer 1999; Martel et al. 2001). Additionally, dreissenids are linked with the occurrence of harmful algal blooms in several systems (Raikow et al. 2004; Fernald et al. 2007; De Stasio et al. 2014).

Early control efforts for biofouling focused on protection of industrial and municipal water intakes and thus, relied on chemical control such as sodium hypochlorite, copper compounds, and quaternary ammoniums (Claudi and Mackie 1994; Mackie and Claudi 2010; Glomski 2015). These treatments are effective but can be expensive and generate unwanted byproducts. Eradication of dreissenids in open water is more problematic, particularly when nontarget native species are a concern. Registered molluscicides for use in open water include the biopesticide Zequanox® (Marrone BioInnovations, Davis, CA) and copper-based EarthTec QZ® (Earth Science Laboratories, Inc., Bentonville, AR). Zequanox is relatively selective to dreissenids and safe to a range of nontarget organisms (Molloy et al. 2013a, b; Meehan et al. 2014; Luoma et al. 2015; Waller et al. 2016; Waller and Luoma 2017) but can be costly and impractical for application in large systems. Potassium chloride (KCl) or potash, is not registered as a molluscicide but can be used with emergency exemption from the United States Environmental Protection Agency (USEPA). Copper-based compounds (Eisler 1998; OAFB 2009) and potassium chloride (Imlay 1973; Fisher et al. 1991; Waller et al. 1993) can adversely affect nontarget species. Native mussels are of special concern during dreissenid control treatments because of their tenuous status (Lydeard et al. 2004; RéGnier et al. 2009) and immobility. Since their arrival, dreissenids have caused significant decline, and in some cases complete extirpation, of native mussels (Schloesser et al. 1996; Strayer 1999; Martel et al. 2001). Ideally, control efforts should avoid further harm to native fauna when feasible.

Carbon dioxide was first tested as a molluscicide in 1995 and found to effectively reduce attachment of zebra mussels (McMahon et al. 1995; Payne et al. 1998) and cause significant mortality of zebra mussels and Asian clams (*Corbicula fluminea* Müller, 1774) (Elzinga and Butzlaff 1994; McMahon et al. 1995; Payne et al. 1998). Despite promising results from these earlier studies, CO_2 was not pursued as an aquatic invasive species (AIS) control tool until recent studies on its use to deter fish (Kates et al. 2012; Cupp et al. 2016) and as a biocide for bullfrogs (Abbey-Lambertz et al. 2014), New Zealand mud snails (*Potamopyrgus antipodarum* Gray, 1853) (Nielson et al. 2012), and nuisance fish (Cupp et al. 2017). Carbon dioxide offers several advantages over other molluscicides because it does not persist in the environment and can be readily off-gassed when a treatment is complete. If repurposed from an industrial source, CO_2 can be relatively inexpensive to use as an aquatic invasive species control tool.

New infestations of dreissenids are often discovered in the fall when boats and docks are removed from lakes for winterizing. Thus, rapid response control efforts may be conducted when water temperatures are 12 °C or less (Fieldseth and Sweet 2016; Lund et al. 2017). The efficacy of current chemical control tools, EarthTec QZ and potassium (KCl) is significantly reduced in cool water (J. Luoma, USGS, La Crosse, WI, personal communication). The toxicity of the biopesticide Zequanox, depends on ingestion to cause necrosis of the digestive epithelium (Molloy et al. 2013a). Thus, it may be less effective in cool water when zebra mussel feeding and metabolic rate are reduced (Marrone Bio Innovations 2012; Molloy et al. 2013c; J. Luoma, USGS, La Crosse, WI, pers. comm.). We assessed application of unpressurized CO_2 in cool water (12 °C) in a simple infusion system to determine the effects of elevated PCO_2 (100 000–300 000 µatm PCO_2) on survival, attachment, and byssal thread formation of zebra mussels. Similar responses (i.e., survival, burial, and byssal thread presence) were measured on the juvenile stage of a native mussel, Fatmucket (*Lampsilis siliquoidea* Barnes, 1823). Fatmucket has been routinely used in toxicity tests, including several with CO_2 (Hannan et al. 2016a; Waller et al. 2017; Waller et al. 2018), and can serve as a surrogate for other lampsiline species (Bringolf et al. 2007; Wang et al. 2007; Jorge et al. 2013).

Materials and methods

Test system

Tests were conducted in two proportional constant-flow diluter systems in two consecutive trials. A diluter included a mixing box that delivered water to a serial dilution box, partitioned into 10 chambers (Figure 1). The first chamber received CO_2 gas at a predetermined flow rate to produce the highest targeted CO_2 concentration. Carbon dioxide concentration was diluted by ~ 20% in each subsequent chamber. Clean, untreated water from the mixing box outflowed to a control tank. The diluter system delivered a continuous supply of 12 °C well water from the mixing chamber to each test tank at a rate of 360 mL/min (1 tank exchange per hour) throughout the test period. Test tanks (20 W × 60 L × 40 H cm;

glass aquarium) were filled to a volume of 21.6 L. Treatments were assigned to each test tank within a diluter system using a randomized block design. Five CO_2 treatments and a control were tested in duplicate in two trials. Trials were conducted consecutively with each trial lasting 2 weeks.

Food grade CO_2 gas was supplied from 50-lb compressed gas CO_2 cylinders (Airgas Inc., La Crosse, WI, USA) to two flow regulators, through airline (I.D. 6.35 mm, O.D. 4.76 mm) and into an airstone (74 L × 37 W × 37 H mm) that was submerged in the first chamber of the dilution box (Figure 1). Two CO_2 cylinders were connected to a pressure differential automatic manifold (Precise Equipment Co., Denton, TX) so that gas was drawn from one of the cylinders at a time. When one tank emptied a minimum threshold pressure triggered an automatic regulation valve (Smith Equipment, Watertown SD) to switch to the other full CO_2 tank. Outflow from the CO_2 regulator was adjusted to each diluter with an airflow regulator. Carbon dioxide was infused into the test system for 96 h after which the CO_2 cylinders were closed and the test tanks were partially drained and allowed to refill with untreated well water.

Water quality was measured once a day in each tank. Dissolved oxygen and pH were measured with a Hach LDO IntelliCAL probe and Hach pH probe, respectively, attached to a Hach HQ40d Water Chemistry Multimeter (Hach, Loveland, CO). Temperature was measured with a digital thermometer. Conductivity and hardness were measured on a sample of water from each head box at the beginning and the end of each trial. Conductivity was measured with a Fisher Accumet conductivity meter (Fisher Scientific, Waltham, MA) calibrated against a standard solution (APHA 2012). Total hardness (mg/L $CaCO_3$) was determined by titrimetric method with Manver Red indicator (USEPA 1983). Alkalinity was measured on samples from each diluter head box at the beginning and end of each trial. Additionally, alkalinity was measured from one randomly selected tank per diluter on each day of CO_2 infusion. Total alkalinity (mg/L $CaCO_3$) was determined by titrimetric method to a pH endpoint of 4.5 (APHA 2012).

Carbon dioxide was measured daily from each test tank. Free carbon dioxide (mg/L) concentration was determined by a modified HACH® Method 8205 digital titration method using sodium hydroxide (NaOH) and PCO_2 was determined indirectly by calculation. The titrimetric method consisted of collecting a 100-mL sample of water from the test tank and, while slowly stirring, immediately titrating with 3.636 N NaOH to a pH endpoint of 8.3. Partial pressures of CO_2 were calculated from pH, temperature, and alkalinity using USGS CO2calc program (Robbins et al. 2010). A modified infrared probe (Vaisala BMP220 and GMT221, St. Louis, MO) was also used to verify PCO_2 in each test tank once during the exposure period.

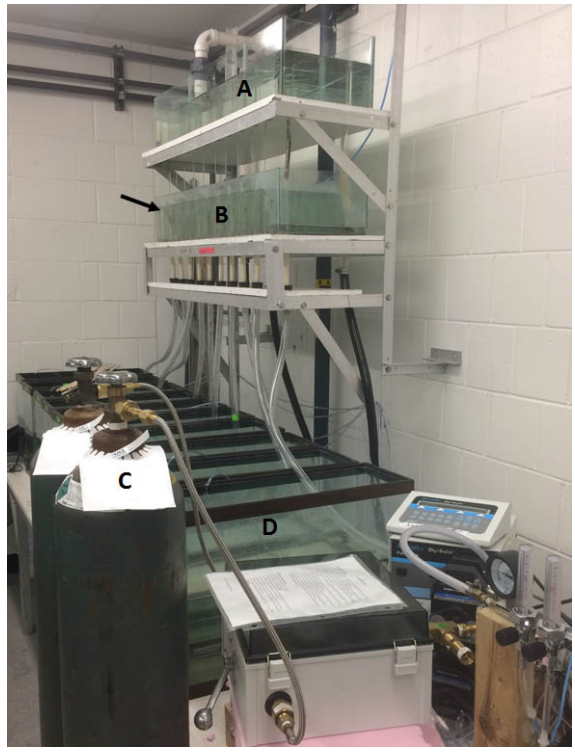

Figure 1. Diluter system and test tanks for exposure of zebra mussels and Fatmucket juveniles to CO_2.(A) Mixing box delivers water to a serial dilution box (B) that is partitioned into 10 chambers. Carbon dioxide is delivered from 50-lb gas cylinders (C), connected to two flow regulators, through airline and an airstone into the first dilution chamber (arrow). Carbon dioxide concentration is reduced by ~ 20% in each dilution chamber and delivered to the test tanks (D) by gravity flow. Each diluter system utilized five CO_2 concentrations and a control. Photograph by D. Waller, USGS.

Zebra mussels

Zebra mussels were hand-collected from Lake Minnetonka, MN in October 2015 (mean shell length 15.6 mm, standard deviation, (SD) 2.4 mm, range 6.8–25.0 mm, n = 943) and transported to the Upper Midwest Environmental Sciences Center (UMESC), La Crosse, WI, U.S.A. Mussels were maintained in flow-through tanks at 12 °C and fed daily with a suspension of mixed algae (Nanno 3600, Shellfish diet 1800, TW1200, TP1800, Reed Mariculture, Campbell, CA) to provide up to 6 mg/L, based on dry weight.

The algal stock was delivered continuously by a peristaltic pump to each holding tank at a rate of about 100 mL/h. About 10 d before trial initiation, 80 acrylic tiles (Plastikote® 12 L × 12 W, cm) and 20 glass petri plates (100 diameter × 60 depth, mm) were placed on the bottom of a raceway to develop a biofilm. One week before trial initiation, zebra mussels were transferred from holding tanks to the raceway and placed onto tiles and plates (n = 25 per tile and plate) for attachment. Mussels that failed to attach before trial initiation were not used in the test.

One day before the onset of CO_2 exposure, a tile with 20 attached zebra mussels was indiscriminately removed from the raceway, placed into a semi-rigid plastic mesh bag (14 L × 14 H × 2 W cm, 3.0 mm, I.D.), and transferred to a randomly selected treatment tank. Bags were suspended vertically in the water column in each tank. The process was repeated until each treatment tank contained 3 tiles of zebra mussels, each in a separate bag. During CO_2 infusion, one tile was removed from each test tank at 24-, 48-, and 96-h of exposure. Immediately after removal, we counted the number of zebra mussels that were narcotized (valves open and or foot extended with no response to probing) and attached. Attachment was scored as 1 = 1 or more threads remain attached when light pressure was applied, 0 = no byssal plaque and/or detached with light pressure or movement of the plate. We did not count the number of byssal attachments per mussel. The tile of mussels was placed back into the mesh bag and transferred to the raceway with untreated water for a 7-d postexposure (PE) period. Algal feed was supplied to zebra mussels in the raceway during the PE period, as described above. Attachment and narcotization were reassessed at 24 h PE; mortality and attachment were assessed at 7-d PE. Zebra mussel mortality was defined as lack of resistance when valves were gently pulled apart. At the conclusion of each trial, a representative sample of zebra mussels from each tank was retained for size measurement. Shell length (longest axis) was measured to the nearest 0.1 mm with a digital caliper.

Simultaneously, we compared the effects of CO_2 on byssal plaque attachment versus byssal thread formation by placing groups of mussels into separate petri plates in each test tank. One plate of mussels (n = 15) was placed into a test tank without disturbance of byssal attachments (Treatment = "Intact"). Mussels on a second plate were detached by cutting the byssal threads near the ventral shell margin (Treatment = "Cut"), returned to the plate, and placed into the test tank. Mussels in both treatments were exposed to CO_2 for 96 h alongside the 3 tiles of mussels. Attachment was scored at 96 h exposure, as described above.

Juvenile Fatmuckets

About 300 juvenile Fatmucket (*L. siliquoidea*), (mean shell length 16.1 mm, SD 0.71 mm, range 15.2–17.6 mm, n = 10,) were supplied by the U.S. Fish and Wildlife Service, Genoa National Fish Hatchery, Genoa, WI, U.S.A. Juvenile mussels were from the same cohort, ~ 9 months-old, and propagated in the same conditions at the facility. After arrival at UMESC, juveniles were transferred to a 20-L tank that contained sand substrate (Mastercraft® playground sand) and continuous water flow at 12 °C. Juveniles were fed daily as described in the *Zebra mussel* section. One week before trial initiation, juveniles (n = 11–13) were transferred from the holding tank to the raceway with zebra mussels and placed side-lying into trays (10.0 L × 11.0 W × 3.5 H cm) that contained a 2–4 cm layer of sand. The day before the onset of CO_2 infusion, juveniles that were unburied were removed from the trial. The remaining juveniles were randomly assigned to a test tank (n = 11–12). Before placement into a tank, juveniles were examined under a stereomicroscope for the presence of a byssal thread. When present, the byssal thread was cut near the ventral margin and then juveniles were placed (side-lying) into a tray of sand. Trays were placed into test tanks with the tiles and plates of zebra mussels. Fatmuckets were exposed to CO_2 for 96 h. Daily observations were recorded of the number of juveniles unburied (> 90% of shell above substrate, side-lying, or on umbo), and gaping (valves open, foot extended, and unresponsive) in each tank. At the conclusion of the exposure period, the test tanks were partially drained and refilled with untreated water. Juveniles were removed from the trays, examined for the presence of a byssal thread and then returned to trays in the test tanks for 7-d PE. Juveniles were fed continuously with the same algal mixture as described in the *Zebra mussel* section. Mortality and byssal thread presence in juveniles were assessed at 7-d PE.

Statistical analysis

For all statistical analyses, differences were considered significant if $P < 0.05$. The Statistical Analysis Software package (SAS Version 9.4, Cary, NC) was used for all analyses. A logistic model was fit using proc glimmix for response variables (i.e., mortality, attachment, byssal thread presence). Zebra mussel mortality was modeled with exposure duration and PCO_2 as numeric predictor variables, and trial (1 or 2) as a random effect. Our estimates of lethal time to 50% mortality (LT50) were based on fixed effects alone. We did not make predictions for a particular trial (random effect), but instead for any trial.

Table 1. Mean (standard deviation) water quality parameters (pH, dissolved oxygen, temperature) and partial pressure (PCO_2) and concentration (mg/L) of carbon dioxide in each trial. n = 5.

Relative CO_2 treatment	pH		Dissolved oxygen (mg/L)		Temperature (ºC)		PCO_2 (µatm) CO_2 (mg/L)	
	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2	Trial 1	Trial 2
Control	7.55 (0.22)	7.70 (0.10)	7.44 (0.46)	7.47 (0.33)	11.6 (0.20)	12.1 (0.15)	4 401 (1 115) 1.8 (0.7)	2 849 (58) 2.6 (0.7)
Low	6.11 (0.04)	6.07 (0.04)	7.41 (0.36)	7.27 (0.16)	11.6 (0.19)	12.1 (0.16)	108 438 (3 515) 157.3 (22.1)	118 053 (5 349) 197.8 (10.3)
Med Low	6.03 (0.03)	5.97 (0.03)	7.13 (0.40)	7.06 (0.21)	11.6 (0.19)	12.1 (0.19)	129 940 (2 771) 239.9 (44.7)	150 363 (10 378) 247.8 (14.6)
Medium	5.92 (0.04)	5.87 (0.04)	6.91 (0.39)	6.70 (0.15)	11.6 (0.20)	12.1 (0.14)	167 932 (13 590) 257.6 (34.1)	188 723 (9 336) 324.2 (15.4)
Med High	5.83 (0.03)	5.76 (0.03)	6.64 (0.42)	6.46 (0.20)	11.8 (0.20)	12.1 (0.15)	205 815 (5 666) 330.5 (42.8)	241 287 (18 681) 402.3 (24.9)
High	5.74 (0.04)	5.69 (0.03)	6.15 (0.42)	6.00 (0.33)	11.7 (0.18)	12.1 (0.18)	253 664 (19 391) 433.0 (37.9)	287 670 (23 317) 477.5 (48.8)

The 95% limits were constructed to include all values of exposure duration for which the associated 95% confidence interval for mortality rate included 0.5. A generalized linear model (proc glm) was used to examine the relationship between shell length and survival. Two separate models were fit for zebra mussel attachment: (1) by byssal thread treatment (cut or intact) and (2) exposure duration (24, 48, and 96 h). In the first, attachment was modeled with thread treatment as a categorical predictor variable, PCO_2 as a numeric predictor variable, and trial as a random effect. The second was similarly modeled with sample time (Time 0, immediately after exposure, Time 24-h PE) and exposure duration as categorical predictor variables and least square means (Tukey's adjustment) for pairwise comparison. Byssal thread presence in Fatmucket was modeled with sample time (Time 0, 7-d PE) as a categorical predictor variable, PCO_2 as a numeric predictor variable, and trial as a random effect. Descriptive statistics (mean, standard deviation) were used to summarize water quality (temperature, dissolved oxygen, pH), water chemistry (alkalinity, hardness, conductivity), CO_2 concentration (mg/L), and PCO_2.

Results

Water chemistry and CO_2

Carbon dioxide levels in treatment tanks ranged from a mean of 108 438 µatm (157.3 mg/L) to 287 670 µatm (477.5 mg/L, Table 1). Levels of CO_2 in replicate tanks were 9 to 15% lower in trial 1 than in trial 2 (Table 1). Water temperature was about 0.5 °C lower in trial 1 compared to trial 2, whereas, dissolved oxygen concentration and pH values were lower in replicate tanks in trial 2 (Table 1). Dissolved oxygen decreased as PCO_2 increased but remained ≥ 6.0 mg in all treatment tanks (Table 1). Water chemistry parameters (alkalinity, hardness, conductivity) were similar between trials (Table 2).

Table 2. Mean (standard deviation) water chemistry parameters in each trial during exposure to carbon dioxide.

Trial	Alkalinity[a] (mg/L as $CaCO_3$)	Hardness[b] (mg/L as $CaCO_3$)	Conductivity[b] (µS/cm)
1	139.6 (4.8)	188.5 (1.0)	379.2 (10.4)
2	141.5 (2.4)	195.7 (3.2)	387.5 (21.4)

[a] n = 12
[b] n = 8

Table 3. Estimated lethal time to 50% mortality (LT50, 95% confidence interval) of zebra mussel at fixed partial pressure of carbon dioxide (PCO_2).

PCO_2 (µatm)	LT50 (h)
75 000	98.9 (87.2 – > 100.0)
100 000	86.4 (77.0 – 97.8)
125 000	75.0 (66.8 – 84.1)
150 000	64.6 (57.0 – 72.7)
175 000	55.1 (48.0 – 62.5)
200 000	46.3 (39.7 – 53.2)
225 000	38.2 (31.6 – 44.7)
250 000	30.7 (23.3 – 37.3)
275 000	23.7 (< 24 – 31.0)

Zebra mussel response

Zebra mussels in all treatments showed signs of narcotization at 24-h exposure. These signs included gaping valves and extended foot with no response to probing. Zebra mussels that survived the treatment generally recovered from narcotization after several hours in untreated water. Zebra mussel mortality

increased with PCO_2 ($F_{1, 67} = 19.94$, $P < 0.001$) and with exposure duration ($F_{1, 67} = 15.63$, $P < 0.001$). The interaction of exposure duration and PCO_2 treatment was not significant ($F_{1, 67} = 2.75$, $P = 0.102$). Exposure of zebra mussels to CO_2 for 24 h did not cause 100% mortality at any treatment level (Figure 2), although > 80% died in the highest treatment level. Within the same treatment, mussel mortality increased from 10% to 40% when exposure duration increased from 24 h to 48 h. Complete mortality occurred in only one tank (High treatment) after 48-h exposure to CO_2. Exposure of zebra mussels to CO_2 for 96 h caused 80–100% at all treatment levels (Figure 2). An estimated LT50 (lethal time to produce 50% mortality) for fixed PCO_2 ranged from ~96 h at 100 000 µatm to 24 h at 275 000 µatm (Table 3). Shell length was not a significant variable in survival of zebra mussels ($F = 0.27$, $P > 0.606$).

Zebra mussels that had a cut byssal thread before the onset of CO_2 infusion did not reform threads during exposure in any treatment tank with the exception of one mussel (Figure 3). In contrast, 75–100% of control mussels were reattached at 96 h. Zebra mussels with intact byssal threads at the onset of CO_2 infusion detached during the 96-h exposure (Figure 3). Significantly more of the "intact thread" mussels (30.3%, SD 16.9) were attached than the "cut thread" mussels ($F_{1, 37} = 25.25$, $P < 0.001$). The level of PCO_2 did not have a significant effect ($F_{1, 35} = 0.15$, $P = 0.700$) on attachment at 96 h.

The effects of exposure duration on attachment at Time 0 and 24 h PE were also modeled (Figures 4A and B). Attachment was significantly affected by PCO_2 ($F_{1, 133} = 41.71$, $P = 0.001$) and exposure duration × sample time ($F_{2, 133} = 3.22$, $P = 0.043$). Attachment was similar among exposure durations at Time = 0 (Figure 4A). In contrast, attachment at 24 h PE was significantly lower ($t = 4.48$, $P = 0.001$) in the 96-h exposure compared to 24-h group (Figure 4B). Mussels exposed for 24 and 48 h in low PCO_2 recovered after 24-h PE and were attached in greater numbers than at Time 0 (Figures 4A and B). Mussels exposed for 96 h lost attachment from Time 0 to 24-h PE (Figures 4A and B), suggesting that latent mortality, rather than recovery, was occurring.

Juvenile Fatmucket response

Overall Fatmucket survival was 99%. Two mussels died during the course of the study – one each in the Medium (167 932 µatm) and High (253 664 µatm) PCO_2 treatments. Carbon dioxide elicited sublethal signs of stress in Fatmuckets, similar to those observed in zebra mussels. Before the onset of CO_2 infusion, 2.6% ($n = 4$) mussels were unburied (not shown).

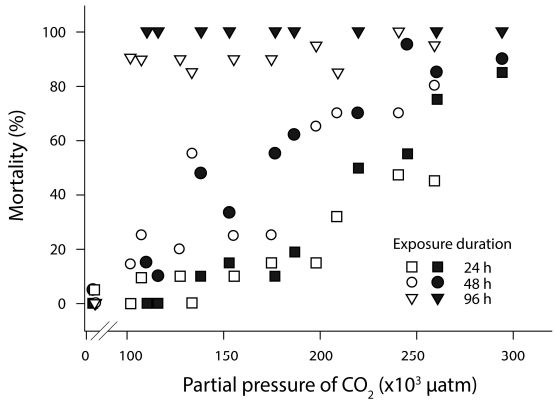

Figure 2. Mortality (7-d PE) of zebra mussels after exposure to CO_2 for 24, 48 and 96 h. Open symbols = Trial 1; closed symbols = Trial 2.

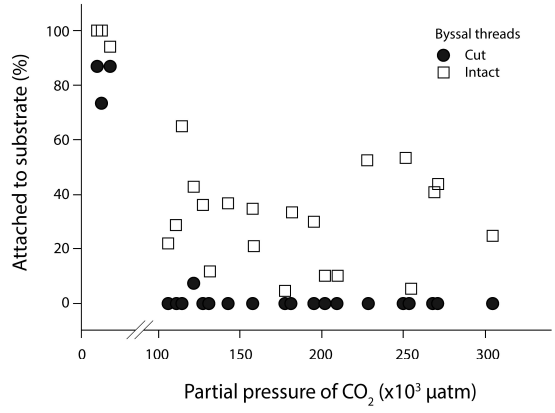

Figure 3. Zebra mussel attachment: Effect of PCO_2 on attachment versus reattachment after 96 h exposure.

Carbon dioxide triggered mussels to move to the substrate surface, unbury, and in some cases, gape and extend the foot. On average, the percent of unburied mussels increased daily throughout the exposure except for a decrease from days 3 to 4 in Low and MedLow treatments (Figure 5A). PCO_2 had a significant effect on burial at 96-h exposure ($F_{1, 21} = 4.91$, $P = 0.038$), but mussels in lower CO_2 treatments unburied at a higher rate than those in the High treatment (Figure 5A). At 7-d PE, mean burial ranged from 9.7% to 30.3% in CO_2 treatment groups (Figure 5A), but differences were not fully explained by PCO_2 ($F_{1, 11} = 4.48$, $P = 0.058$). Byssal thread production was inhibited by CO_2 ($F_{1, 21} = 15.42$, $P < 0.001$; Figure 5B), but recovery was evident at 7-d PE ($F_{1, 9} = 3.15$, $P = 0.110$; Figure 5B).

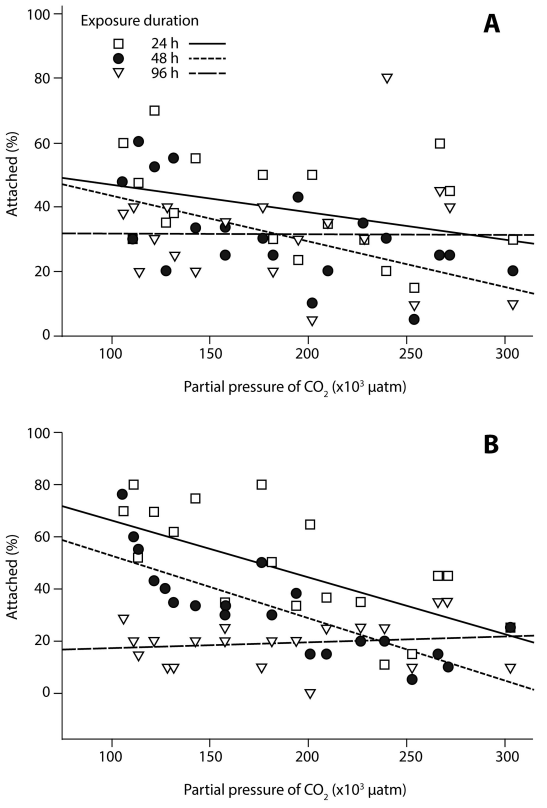

Figure 4. Zebra mussel attachment by PCO_2 and exposure duration at (A) Time 0, immediately after exposure, and (B) 24 h postexposure (PE).

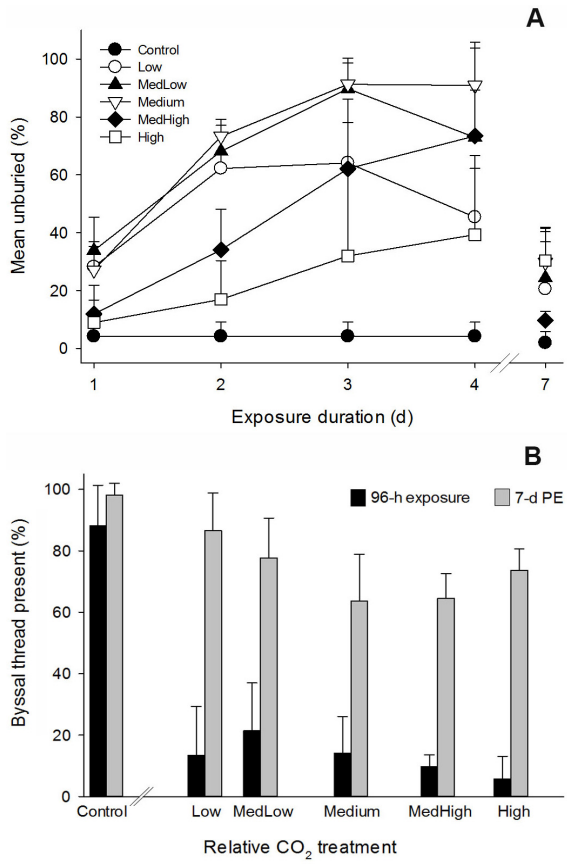

Figure 5. Sublethal responses of Fatmucket to CO_2 treatment: (A) Daily percent unburied mussels (n = 11 or 12 mussels/tank) during 96-h (4 day) exposure and at 7-d PE. (B) Byssal threads present at 96-h exposure and 7-d PE. Refer to Table 1 for the range of CO_2 levels for each treatment.

Discussion

The flow-through diluter systems used in our study reduced CO_2 levels in serial chambers by ~ 20%, as expected. Within a trial, the variability in CO_2 levels between replicates (i.e., diluters) was highest in High CO_2 tanks, but there was no other consistent pattern among treatment levels (Table 1). Variation in CO_2 between replicate tanks could be attributed to differences in water flow rates, mixing patterns, and water temperature, all factors that affect CO_2 retention. The primary source of between-trial variability in CO_2 levels was diluter 1 of trial 1. Carbon dioxide levels in tanks of this diluter were 12.8–19.0% lower than levels in the same tanks in trial 2. In contrast, CO_2 levels in diluter 2 varied by 1.5–5.0% between trials. Lower CO_2 levels in diluter 1 of trial 1 are likely due to reduced CO_2 infusion to the headbox. We accounted for variability between trials and replicate test tanks by using measured CO_2 levels in individual tanks in our analyses.

Carbon dioxide is relatively soluble in water, but the concentration of free CO_2 in water is dependent on temperature and buffering capacity of the water. We expect a positive relationship between CO_2 toxicity and water temperature, similar to that determined for New Zealand mud snails (Nielson et al. 2012). However, there are few comparable data on temperature-dependent toxicity of CO_2 and dreissenids. McMahon et al. (1995) exposed zebra mussels at 25 °C to 100% CO_2 (PCO_2 = 760 Torr) and reported an LT50 of 40.2 h and mean time to 100% mortality of 72 h. McMahon et al. (1995) did not report measured PCO_2 levels in their study, so direct comparisons cannot be made with our results. We estimated comparable LT50 value and mortality percentage of zebra mussels in PCO_2 of ~ 200 000–

250 000 µatm at 12 °C (Table 2; Figure 2). Current studies in our lab indicate that effective exposure duration to CO_2 will increase as water temperature decreases (Waller and Bartsch, USGS, La Crosse, WI, unpublished), but additional tests are needed to establish temperature-dependent toxicity equations. In colder water (< 12 °C), extended application periods (i.e., 7–14 d) of CO_2 may be impractical in open water and limit its use to closed systems. On the other hand, an advantage of CO_2 use in cooler water is the capacity to keep it in solution without off-gassing (Wiebe and Gaddy 1940). It may be easier to maintain CO_2 at a target concentration for an extended period without "bump" injections. For example, Cupp et al. (2017) demonstrated the use of CO_2 as an under-the-ice piscicide in outdoor ponds. Carbon dioxide was injected one time from compressed tanks and a concentration range of 25–100 mg/L was maintained for 2 weeks.

The sensitivity of different life stages and sizes of zebra mussels to CO_2 has not been well studied. McMahon et al. (1995) found a positive relationship between time to death and shell length of zebra mussels exposed to hypercapnic conditions and suggested that CO_2 would be more effective against larger mussels. We found no significant relationship between size and survival of mussels. Although the absolute size range of mussels in our study and McMahon et al. (1995) was similar (6.8–25.0 mm and 10–30 mm, respectively), mussels larger than 20.0 mm represented less than 1% of our test population. Tests are needed across a broad range of sizes, representing discrete life stages (e.g., veligers, newly settled juveniles, 1- and 2-year-old adults), to determine efficacious CO_2 levels. For example, Nielson et al. (2012) tested three life stages of New Zealand mudsnail and found similar survival responses between adults and juveniles but significantly greater sensitivity in neonates. It is likely the zebra mussel veligers are also more sensitive than adults to CO_2, but minimum effective concentrations have not been determined.

The effects of CO_2 on zebra mussel attachment appear to be two-fold: (1) CO_2 and the production of carbonic acid can cause weakening of the byssal threads and plaque (O'Donnell et al. 2013) and (2) CO_2 inhibits production of new byssal threads. Both effects occurred at the lowest CO_2 levels that we tested (Figures 3 and 4A, B); moreover, detachment began within 24 h of exposure. We did not quantify the number of byssal threads, but noted that most mussels were attached by a single thread after 96 h and were easily dislodged with gentle agitation. McMahon et al. (1995) reported similar effects on byssal attachment when zebra mussels were exposed to a gas mixture of 5% CO_2:19% O_2:76% N_2. Zebra mussels produced 60% fewer byssal threads after 5 d, relative to controls, and completely stopped producing threads at 7 d. These results indicate that CO_2 infusion is a viable option to reduce fouling and prevent settlement of dreissenids on infrastructures and could be applied intermittently or continuously at low levels in closed systems.

Zebra mussels avoid a variety of noxious substances, such as chlorine, organic compounds, metals (Sprecher and Getsinger 2000; Borcherding and Wolf 2001; Borcherding 2006), and electrical current (Luoma et al. 2017) by valve closure. Consistent with other studies, we found that CO_2 has the opposite effect on mussels and induces narcotization within hours of exposure (Elzinga and Butzlaff 1994) at relatively low PCO_2 levels (McMahon et al. 1995). Mussels that are narcotized are widely agape, do not respond to touch, and often have the foot extended. It is generally recommended that mussels be held for a recovery period (e.g., 96-h PE) to avoid overestimating mortality of mussels in a narcotized state (Wildridge et al. 1998; Pucherelli et al. 2014; Davis et al. 2018). We extended the PE period to 7 d to account for the reduced metabolic rate of mussels at 12 °C; however, we saw signs of recovery at 24 h PE by the increased attachment of mussels in low dose and duration treatments (Figure 4B). In contrast, mussel attachment decreased in 96-h and high dose treatments (i.e., PCO_2 > 200 000 µatm) over the same time period, an indication of eventual mortality from the treatment (Fig. 4B).

Several studies suggest that narcotization or inhibiting valve closure can reduce the dose and exposure duration of a biocide. Potassium had a synergistic effect on toxicity of polydiallyldimethyl ammonium chloride (polyDADMAC) to adult mussels (Costa et al. 2011). Pretreatment with CO_2 increased the mortality rate in mussels treated with chlorine (Elzinga and Butzlaff 1994; Payne et al. 1998) and was suggested as a strategy to reduce chlorine use. Electrical current had limited effect on zebra mussels because of valve closure and the low conductivity of the shell (Luoma et al. 2017). Pretreatment with CO_2 for several hours could be used to induce gaping and exposure of soft tissues to electrical current and reduce effective exposure duration time to achieve mortality.

Native mussels are often the substrate for zebra mussel colonization and, unlike fish, are unable to move out of a treatment zone. Therefore, the effects of a dreissenid control tool on native mussels, by direct exposure or discharge from treated waters, is an important consideration for resource managers. Zequanox is the only biocide currently registered for use in open water for dreissend control that is safe to

native mussels (Molloy et al. 2013b; Meehan et al. 2014; Luoma et al. 2015). However, it is most effective in water temperatures > 13 °C when mussel feeding and metabolic activity are high (Marrone Bio Innovations 2012; J. Luoma, USGS, La Crosse, WI, pers. comm.). The postexposure mortality period can extend for 1–2 months if Zequanox application occurs in colder water (Molloy et al. 2013c). Copper-based compounds, such as EarthTec QZ, are toxic to a variety of aquatic organisms (Eisler 1998; OAFB 2009; USEPA 2015). The specific toxicity of EarthTec QZ to native mussels has not been reported, but native mussel and fish mortalities were observed following treatments with EarthTec QZ at target copper concentration of 0.3 to 0.5 mg/L in Lake Minnewashta, MN (Fieldseth and Sweet 2016). Potassium has been used effectively and safely to kill dreissenid veligers during fish stocking activity at 750 mg/L as a 1-h pretreatment to formalin (Edwards et al. 2000; Edwards et al. 2002; Pucherelli et al. 2014). It has been applied as potash in several open water control projects at a target concentration of ~ 100 mg/L (Fernald and Watson 2014; Lund et al. 2017; Janusz 2016). However, the target concentration of K^+ for adult dreissenid control (i.e., 50–100 mg/L) far exceeds levels (i.e., 4–10 mg/L) that are safe for native mussels (Imlay 1973; Fisher et al. 1991). Both copper-based compounds and potassium persist in the environment after treatment (Eisler 1998; Fernald and Watson 2014) and may pose a long-term risk to nontarget organisms.

Our results suggest that Fatmucket juveniles can survive acute exposure to relatively high PCO_2 at 12 °C, but the maximum safe dose and duration of exposure remains to be determined. Long-term exposure (28-d) of juvenile lampsiline mussels to CO_2 has significant lethal and sublethal effects. In previous studies, CO_2 was lethal to juvenile Fatmucket (Waller et al. 2017) and Higgins eye (*L. higginsii*) in a 28-d exposure at 21 °C (Waller et al. 2018). The latter study reported 28-d LC20 (lethal concentration to 20% of mussels) values of 58 200 and 31 800 µatm for Fatmucket and Higgins eye, respectively (Waller et al. 2018). Shell growth of both species was significantly reduced in PCO_2 ~ 28 600 µatm. In other studies, adult unionid mussels of several species (i.e., *L. siliquoidea*, *Fusconaia flava*, *Amblema plicata*) survived short- and long-term exposure to elevated PCO_2 in laboratory exposures, although physiological and metabolic responses were altered to maintain acid-base and ionic balance (Hannan et al. 2016a, b; Jeffrey et al. 2017).

The sublethal effects of CO_2 on juvenile unionid mussel behavior and byssal thread production may indirectly affect survival. The byssus of a juvenile mussel, which consists of a single hyaline thread, is used to maintain position in the substrate and as a mechanism for drift and dispersal in the water column (Lasee 1991; Bradley 2011). Juveniles without a byssal thread may have a greater risk for displacement. The effects of CO_2 on burial could expose juveniles to predation, as well as displacement. Mobile species, such as fish (Clingerman et al. 2007; Kates et al. 2012; Cupp et al. 2017) and crayfish (Bierbower and Cooper 2010), can avoid areas of elevated PCO_2. Although more subtle, the response of infaunal bivalves to CO_2 is similar and includes reduced burrowing and increased dispersal (Clements and Hunt 2015; Waller et al. 2017). Juvenile Fatmucket mussels exposed to sublethal levels of CO_2 unburied and moved more times in a grid system than those in control and lethal PCO_2 treatments (Waller et al. 2017). In the present study, we found that more mussels unburied in PCO_2 < 200 000 µatm. Higher CO_2 levels narcotized and inhibited mussel movement, instead of triggering avoidance behavior. Native mussels recovered rapidly after removal of CO_2 and showed no difference in byssal thread production at 7-d PE. Most mussels buried within 24 h after placement in untreated water, but up to 30% remained unburied at 7 d PE (Figure 5A). We are uncertain whether failure to bury was due to the latent effects of CO_2 or cool water temperature or both. Mussel behavior (righting, burial, movement) is highly dependent on water temperature (Waller et al. 1999; Lurman et al. 2014a, b) and a longer PE period may be necessary to determine whether mussels in the higher PCO_2 fully recovered.

Our laboratory test conditions represent a worst case scenario for juvenile mussels during a CO_2 treatment in cool water. Burial depth of juveniles was limited to ~ 2–4 cm in the test tanks. In field conditions, native mussels can burrow well below the surface and remain buried for months. Decreasing water temperatures trigger burial (Amyot and Downing 1997; Watters et al. 2001), extended periods of valve closure (Lurman et al. 2014a, b), and lower metabolic rate in unionid mussels (Huebner 1982; Polhill and Dimock 1996; Lurman et al. 2014 a, b). These behaviors could reduce exposure to CO_2 and the potential toxicity of CO_2 to native mussels.

Conclusion

We demonstrated that unpressurized infusion of CO_2 into 12 °C water effectively reduced attachment and caused mortality of adult zebra mussels. Large-scale, open-water application of CO_2 to eradicate dreissenids is likely not feasible given the large volume of gas that would be required to achieve lethal

concentrations. However, CO_2-delivery systems have been tested to control movement of invasive fish (Cupp et al 2016; Donaldson et al. 2016) and as a piscicide (Cupp et al. 2017) in small water bodies or isolated bays and channels. A CO_2 source can be scaled for the project size, e.g., compressed gas cylinders for small scale projects to a tanker truck for larger treatment areas. Our results indicate that low-level infusion of CO_2 into closed water systems could effectively reduce and prevent attachment of zebra mussels. It is likely that even lower CO_2 levels would prevent settlement of metamorphosed veligers in intake systems. Overall, CO_2 offers several advantages for use as a molluscicide in either closed- or small-scale open-water application. It is relatively inexpensive, safe to apply, does not persist, and can be easily neutralized or off-gassed. Low levels of CO_2 could be initially injected to move fish out of a treatment zone to minimize fish mortality. As a rapid response tool in open water, CO_2 is efficacious in cool water and may be safer to native mussels than several current-use biocides.

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