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exposure duration to CO_2 will increase as water temperature decreases (Waller and Bartsch, USGS, La Crosse, WI, unpublished), but additional tests are needed to establish temperature-dependent toxicity equations.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.

Acknowledgements

The study was funded in part by the Great Lakes Restoration Initiative and the U.S. Geological Survey Ecosystem Mission Area Invasive Species Program. We thank UMESC staff - Matt Barbour, Jeremy Wise, Todd Severson, and Riley Buley for assistance in the laboratory. Barb Benny, University of Wisconsin-La Crosse, provided valuable assistance with statistical analysis. The U.S. Fish and Wildlife Service, Genoa National Fish Hatchery provided juvenile Fatmucket mussels.

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